Stony Brook University

Contributions of the RON Receptor Tyrosine Kinase to the Progression of Breast Cancer

A Dissertation Presented

by

Kimberly Joy Feres

to

The Graduate School

in Partial fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular Genetics and Microbiology

Stony Brook University

August 2009

Stony Brook University

The Graduate School

Kimberly Joy Feres

We, the dissertation committee for the above candidate for the degree of

Doctor of Philosophy

Hereby recommend acceptance of this dissertation

Dr. Michael J. Hayman Ph.D. Dissertation Advisor
Professor
Department of Molecular Genetics and Microbiology

Dr. Janet Hearing Ph.D. Chairperson of Defense
Associate Professor
Department of Molecular Genetics and Microbiology

Dr. Patrick Hearing Ph.D.
Professor
Department of Molecular Genetics and Microbiology

Dr. Howard Crawford Ph.D.
Associate Professor
Departments of Pharmacological Sciences and
Molecular Genetics and Microbiology

Dr. Holly Colognato Ph.D.
Assistant Professor
Department of Pharmacological Sciences

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

Contributions of the RON Receptor Tyrosine Kinase to the Progression of Breast Cancer

by

Kimberly Joy Feres

Doctor of Philosophy

In

Molecular Genetics and Microbiology

Stony Brook University

2009

Breast cancer is frequently associated with the deregulation of receptor tyrosine kinase (RTK) signaling that normally exhibits tight control over cellular processes involved in cell growth, death, differentiation, metabolism, angiogenesis, motility and invasion. The RTK known as recepteur d'origine nantais (RON) exhibits increased expression in at least 47% of human breast cancers. Furthermore, increased expression of RON and its ligand, the macrophage stimulating protein (MSP), has been correlated with a poor patient prognosis and a reduced 5-year survival rate.

It is currently not clear how RON-mediated signaling contributes to the progression of breast cancer. To address the signaling mechanisms that RON utilizes to promote oncogenic behavior in breast cancer, we generated RON-expressing MCF-10A non-transformed breast epithelial cells. In MCF-10A cells, RON signals via two

alternative pathways: one that requires MSP, and one that occurs in the absence of MSP. Although MSP was necessary to promote RON-mediated proliferation and migration, RON-mediated cell adhesion, spreading, 3-D morphological changes and survival was not dependent on MSP. The intracellular tyrosine kinase Src was necessary for MSP-independent phosphorylation of RON in MCF-10A cells, and chemical inhibition of Src completely abrogated both cell spreading and survival. In addition, we found that MCF-10A/RON cells exhibited increased expression of the extracellular matrix protein hyaluronan on their surface compared to MCF-10A control cells. These data imply that MSP-independent activation of RON is potentially explained by interactions of RON with hyaluronan-activated cell surface receptors, some of which are known to influence RON signaling in other cell systems.

Taken together, the results from this study suggest that RON signaling may contribute to the development and progression of human breast cancers, even the absence of MSP.

Dedication

This dissertation is dedicated to my great-grandmother,

Leona M. Johnson,

who inspired me to trust that anything is possible

with an optimistic attitude

and the courage to believe in oneself.

Table of Contents

List of Tables and Figures

Abbreviations

CSPG	Chondroitin Sulfate Proteoglycan
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-Regulated Kinase
FAK	Focal Adhesion Kinase
HGF	Hepatocyte Growth Factor
HA	Hyaluronan
HAase	Hyaluronidase (general types)
HYAL 2	Hyaluronidase 2
MAPK	Mitogen-Activated Protein Kinase
MSP	Macrophage Stimulating Protein
MT-SP1	Membrane-type Serine Protease-1
PI3K	Phosphatidyl Inositol 3-Kinase
PTB	Phospho-Tyrosine Binding (domain)
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
RHAMM	Receptor for Hyaluronan-Mediated Motility
RON	Recepteur d'origine nantais
RTK	Receptor Tyrosine Kinase
SH-2	Src-homology-2 (domain)
TGF-α/β	Transforming Growth Factor-α/β
TKI	Tyrosine Kinase Inhibitor
WFA	*Wisteria floribunda* agglutinin

Acknowledgements

I would like express my gratitude to Dr. S. John Gatley who offered his laboratory at BNL to provide me with my first scientific research experience. Without encouragement from Dr. Gatley, I may have never pursued a doctoral degree. I would especially like thank Dr. Michael J. Hayman for being a supportive mentor throughout my graduate school years. Mike bestowed on me a great love for science, and had the patience to teach me to become a productive member of the scientific community. Mike's lab is a wonderful environment to work in, especially due to its friendly and thoughtful lab members, Nobuhide Ueki, Hongling Zhao and Irene Ischenko. I would not have been successful in graduate school without all of the good advice, expert technical help, and many good laughs that I have shared with Irene over the years.

I would like to thank the Molecular Genetics and Microbiology Department for giving me the opportunity to participate in such an innovative and exciting graduate program. Specifically, I would like to thank my committee members: Janet Hearing, Patrick Hearing, Howard Crawford and Holly Colognato, for donating their time to offer many important suggestions that have improved my scientific work.

I would like to thank members of my family: Mom, Dad, Missy, Gary, Cora, Leona, Eleanor and Jim. I thank my friends: Lesley H., Anna L., Gus and Ann F., Betty P., Victor M., Teresa H., Joe M., Christina B., Danielle T., and so many more for encouraging me to persevere to the end of graduate school and making life more enjoyable along the way. And lastly, I would like to thank my husband and best friend, Brandon, for being a pillar of strength for me throughout these years. Without Brandon's relentless patience, I would not have accomplished so many things in my life.

Publications

The majority of the work contained in Chapters 1-3 was originally published in the following citation:

Feres, KJ, Ischenko, I and Hayman, MJ. (2008). The RON receptor tyrosine kinase promotes MSP-independent cell spreading and survival in breast epithelial cells. *Oncogene*, 28, 279-288.

Introduction

Cancer Overview

Cancer is a devastating disease both in terms of mortality as well as financial burden. In the United States, cancer accounts for approximately 23% of all annual deaths, with nearly 565,000 people expected to succumb to the disease in 2008 (ACS, 2008). Economically, the cost of cancer in the United States was estimated to be over $200 billion in 2006 (ACS, 2008). Compared to the other major causes of death, such as heart disease, cerebrovascular disease and infectious diseases, the number of deaths from cancer has not substantially dropped since 1950 (ACS, 2008), suggesting that the scientific efforts needed to understand the causes of cancer, as well as to identify successful treatments for cancer need to be ongoing.

On a basic level, cancer is a disease that stems from deregulation of cellular pathways, which ultimately leads to uncontrolled growth and the potential to spread to other sites within the body. However, cancer is a highly complex disease, with several different types of pathways and classes of molecules involved in the development and progression of the disease. Cancers are adept at thwarting a cell's innate ability to defend itself against the disease by attacking with at least six different strategies. These strategies include "self-sufficiency in growth signals,

insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (Hanahan & Weinberg, 2000).

With these strategies, cancer exploits the normal functions and responses of non-cancerous cells. Normal cells require exogenous growth factor stimulation in order to proliferate, yet cancer cells have devised several ways in which to generate proliferative signals in the absence of growth factors, or in some cases, cancer cells create an ectopic synthesis of growth factors to which the cells respond. Cancerous cells can continue to grow without control because they have learned how to block the ability of normal cells to shut-off growth signals, and have learned to switch cellular programming in order to become unresponsive to anti-growth signals. Furthermore, these tumor-promoting cells are no longer susceptible to apoptosis, which is often used as a mechanism for normal cells to prevent the spread of abnormal cells (Hanahan & Weinberg, 2000). In later stages of cancer progression, deregulated cells can undergo/induce blood vessel development, connecting the tumor to the circulatory system. Angiogenesis enables the tumors to further increase in size, as well as develop a means for tumor cells to escape the primary site and move to a secondary site for metastatic growth. Finally, those tumor cells that do escape the primary tumor have acquired additional mutations that allow them to lose normally tight cellular adhesion and inappropriately digest extracellular matrix in order to move into the vasculature (Chiang & Massague, 2008; Fidler, 2003). The ability of a

cancer cell to gain entry into the vascular system is a key step in the formation of metastatic disease, which is the main cause of cancer-related deaths.

Receptor tyrosine kinases

The above strategies are achieved in part by mutations in both oncogenes and tumor suppressors. As oncoproteins, receptor tyrosine kinases (RTKs) are frequently found to play a role in many of the events in cancer progression, including uncontrolled proliferation, evasion of apoptosis, angiogenesis and migration/invasion (Levitzki & Gazit, 1995). Structurally, RTKs are cell surface receptors that contain an extracellular ligand-binding domain, an intracellular catalytic domain and cytoplasmically-located tyrosines that can be phosphorylated to recruit downstream effector molecules (Ullrich & Schlessinger, 1990). RTKs phosphorylate substrates reactions by transferring the γ-phosphate of ATP to the hydroxyl group on tyrosines of the target protein (Hunter, 2000). Phosphorylation, whether on tyrosine, serine or threonine, is an essential mechanism for cells to transduce intracellular signals and ultimately promote target gene expression (Hunter, 2000; Hunter & Eckhart, 2004).

Monomeric RTKs become activated when their ligand binds to the extracellular domain, thereby leading to receptor dimerization and auto-phosphorylation of the cytoplasmic domains (Schlessinger, 2000). Receptors can either homo-dimerize or hetero-dimerize with other receptors in the same or different family (Schlessinger, 2000). For example, the RTK known as c-Met can auto-phosphorylate via homodimerization with another c-Met monomer (Wickramasinghe

& Kong-Beltran, 2005), which leads to its biological effects. Activation of c-Met also leads to cross-activation of other RTKs, such as EGFR, which is thought to be a potential mechanism of drug resistance in cancer treatment (Guo *et al.*, 2008; Puri & Salgia, 2008). C-Met can also bind directly to other surface receptors such as β4-integrins (Trusolino *et al.*, 2001) and Plexins (Giordano *et al.*, 2002) in order to promote cell signaling. Once activated by ligand binding, auto-phosphorylation in the cytoplasmic domain of the RTK serves to switch conformationally the catalytic domain into an open position for ATP and substrate binding, as well as provide binding sites for downstream signal transducers in the C-terminal region (Schlessinger, 2000). Several types of transducers that contain Src-homology-2 (SH-2) and phospho-tyrosine binding (PTB) domains play a role in signaling by RTKs, which include intracellular kinases, such as c-Src, and adaptor molecules, such as Grb 2 or Gabs (Pawson, 1995; Schlessinger, 2000). Heterodimerization and the recruitment of differential downstream effectors are two potential mechanisms for cells to give signal specificity to alter cell responses.

After ligand-stimulation and phosphorylation of its intended target proteins, RTKs must be down-regulated from the cell surface to prevent continuous signaling. RTKs are recycled from the cell surface after signaling through an intracellular transport system. Much work on this process has been described for EGFR, however, it has been shown to apply to other RTKs as well. After ligand-stimulation of EGFR, the EGFR gets mono-ubiquitinylated on several sites by an E3 ligase known as cbl at the cell surface (Marmor & Yarden, 2004; Miranda & Sorkin, 2007). Ubiquitinylation

of the receptor causes it to become enclosed in a clathrin-coated pit, which is formed by the invagination of the cell membrane (Marmor & Yarden, 2004). The pit moves the receptor into endosomes and multivesicular bodies (MVBs), where sorting of receptors occurs. If, in the endosomes or MVBs, the ligand remains bound to its receptor, the pair is moved into the stronger pH of lysosomes where they are degraded. However, if the ligand has dissociated from its receptor, the receptor can be recycled back to the cell surface for a renewed ability to signal (Sorkin & Von Zastrow, 2002). In the case of EGF-stimulation, generally, EGFR is transported to the lysosomes in order to attenuate signaling, whereas signaling induced by an alternative EGFR ligand, the transforming growth factor-α (TGF-α), leads to receptor recycling from the endosomes (Sorkin & Von Zastrow, 2002).

Signaling by RTKs

Once activated, RTKs promote biological events by signaling via pathways that culminate in a change in gene expression. There are several different pathways that can be activated following receptor dimerization which depend on the cell type, the specific receptor being activated, and type of ligand involved. Furthermore, each signaling pathway may lead to differential cellular responses depending on which downstream effectors get recruited to the RTK, the location of the RTK in the cell, the overall signal strength and the signal duration (McKay & Morrison, 2007).

MAPK Signaling

One important pathway that regulates cell proliferation is the Mitogen-Activated Protein Kinase (MAPK) cascade, which promotes the phosphorylation of MAPK-activated transcription factors as well as other cellular targets. The MAPK-activated transcriptional program plays a role in cell cycle progression (Meloche & Pouyssegur, 2007), cell survival (Yujiri *et al.*, 1998) and cell migration (Johnson & Lapadat, 2002; Yoon & Seger, 2006). Although there are three distinct MAPK pathways, it seems to be the extracellular signal-regulated kinases 1 & 2 (ERK 1 & 2) pathway that is primarily implicated in human cancers (Hunter, 1997). Generally, ERK 1/2 activate gene transcription through a series of phosphorylation events that involve the guanine nucleotide exchange factor SOS, the GTPase RAS, and the kinases Raf and MEK 1 & 2 (McKay & Morrison, 2007). Negative regulation of the MAPK kinase pathway can occur via the Sprouty proteins (Hanafusa *et al.*, 2002). Sprouty proteins are phosphorylated after RTK activation, and, in some model systems, act by downregulating various effector molecules, such as Src-Homology domain-containing protein tyrosine Phosphatase 2 (SHP2), which leads to attenuation of activity promoted by members of the Ras family of small GTPases (Kim & Bar-Sagi, 2004).

PI3K Signaling

Another pathway that plays a key role in cellular functions, such as survival and migration, is the Class I phosphatidyl inositol 3-kinase (PI3K) pathway. PI3K has a regulatory subunit for binding specificity and a p110 catalytic subunit. There are several sub-types of regulatory subunits, such as p85α, p85β, and p55α, among others. There are also four types of catalytic subunits: p110α, p110β, p110δ and p110γ, which differ in respect to their preferential regulatory subunit binding and may exhibit distinct cellular functions (Zhao & Vogt, 2008). p110α is commonly found to be mutated in human cancers and seems to play a role in cell growth and p110β may have a role in blood clotting (Zhao & Vogt, 2008). p110δ and p110γ have immune system functions and are generally only expressed in leukocytes, however, there is mounting evidence that they contribute to hematopoietic cancers (Zhao & Vogt, 2008). Once the RTK is activated, the regulatory subunit of PI3K binds to phosphotyrosine residues on the receptor and p110 phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 recruits the serine-threonine kinase Akt and the 3-phosphoinositide-dependent kinase 1 (PDK1) to the membrane. PDK1 phosphorylates Akt on threonine 308, and in turn, Akt further activates other targets in the cytoplasm and nucleus (Franke, 2008; Zhao & Vogt, 2008). Targets of Akt include forkhead O transcription factors (FOXO), which are involved in apoptosis regulation (Rena *et al.*, 1999) and p21-activated kinase-1 (Pak-1), which induces cell migration when phosphorylated by Akt (Zhou, GL *et al.*, 2003). Downregulation of

the PI3K pathway occurs when PIP3 is dephosphorylated to form PIP2 by the lipid phosphatase phosphatase and tensin homolog deleted on chromosome ten (PTEN), which in turn, abrogates PI3K signaling (Blanco-Aparicio *et al.*, 2007).

FAK Signaling

Focal Adhesion Kinase (FAK) signaling links the extracellular matrix (ECM) with the actin cytoskeleton via interactions with ECM-binding β-integrins on the cell surface (Burridge *et al.*, 1988). These interactions influence cell morphology, cell migration, cell survival and cell proliferation (Mitra & Schlaepfer, 2006). When integrins bind to ECM components, FAK becomes activated and gets recruited to the cell surface. In turn, FAK recruits other signaling molecules to the newly formed focal adhesion (FA), including the intracellular Src kinase (Schaller *et al.*, 1994), the adaptor proteins, paxillin (Hildebrand *et al.*, 1995; Schaller, 2001) and p130Cas (Ruest *et al.*, 2001) and the p85 regulatory subunit of PI3K (Chen *et al.*, 1996; Chen & Guan, 1994). Through its interactions with Rho GTPases, FAK plays a role in cell contractibility and may act as a sensor for cell tension, which ultimately regulates many cellular processes (Chen, BH *et al.*, 2002; Tilghman & Parsons, 2008).

RTKs influence signaling via FAK, usually through interactions with integrins, thereby activating the MAPK and PI3K signaling pathways (Borges *et al.*, 2000; Cabodi *et al.*, 2009; Eisinger & Ammer, 2008). Integrin-RTK interactions also provide a mechanism for RTKs to interact with and potentially change the dynamics of the actin cytoskeleton, providing a means to induce migratory and invasive

behaviors (Feldner & Brandt, 2002; Tilghman & Parsons, 2008). There is no evidence that FAK itself directly interacts with RTKs, however, FAK is required for RTK-integrin mediated biological events (Renshaw *et al.*, 1999).

β-catenin Signaling

The β-catenin pathway is tightly linked to the E-cadherin system of cell-to-cell adhesion and it is also activates the T-cell factor (Tcf) and Lymphoid enhancer factor (Lef) transcription factors, which promote activation of a number of cell cycle genes, such as Cyclin D1 and Myc (Morin, 1999). Whether β-catenin functions at the structural level or the transcriptional level depends on several factors, including the phosphorylation of specific tyrosine residues (Lilien & Balsamo, 2005). Prototypically, in the absence of the Wnt ligand, β-catenin is phosphorylated by casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK-3β), leading to its sequestration in the cytoplasm and degradation by the proteasome (Liu *et al.*, 2002; Peters *et al.*, 1999). When Wnt ligands bind to their receptor, Dishevelled (DVL) proteins inhibit the degradation complex so that β-catenin is stabilized and moves into the nucleus to activate Tcf and Lef (Klaus & Birchmeier, 2008). In addition to Wnt-activated β-catenin, there is evidence that other cell surface receptors, including Met and RON, can promote translocation of β-catenin to the nucleus to activate target genes (Danilkovitch-Miagkova *et al.*, 2001; Monga *et al.*, 2002).

Activation of RTKs in Cancer

The deregulation of RTKs in cancer gives rise to alternative mechanisms by which RTKs can be activated, independent of ligand-binding, providing additional opportunities for cancer cells to hijack normal cell signaling. The two general mechanisms for ligand-independent activation are over-expression of RTKs and a variety of different mutations that lead to constitutive activation of RTKs (Zwick *et al.*, 2001). It has been demonstrated that RTKs can gain an increased level of expression in cancers due to gene amplification, contributing to increased cell signaling (Sunpaweravong *et al.*, 2005). In invasive human breast cancers, for example, the EGFR was over-expressed in approximately 20% of tumors, whereas ErbB2 was expressed in approximately 30% of tumors (Abd El-Rehim *et al.*, 2004). Furthermore, it was shown that in the case of ErbB2, the increased expression level was directly correlated with an increased risk of death in breast cancers (Slamon *et al.*, 1987). The c-Met RTK also exhibited increased expression in approximately 55% of invasive breast cancers (Lengyel *et al.*, 2005) which might be due to gene amplification (Park et al., 2005).

Several RTK mutations have been described to contribute to constitutive signaling in human cancers as well. For example, in small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC), point mutations of c-Met have been identified in the juxtamembrane and semaphorin domains (Ma *et al.*, 2005; Ma *et al.*, 2003). Additional activating point mutations of c-Met have also been described in melanomas (Puri *et al.*, 2007) and papillary renal carcinoma (PRC) (Giordano *et al.*,

2000). Some of these c-Met mutants have been shown to transform fibroblasts and promote invasive growth *in vitro* (Jeffers *et al.*, 1998). Interestingly, c-Met was first discovered as part of a 65 kDa fusion protein that contained sequences from the translocated promoter region (tpr) and the cytoplasmic domain of Met (Park *et al.*, 1987). This constitutively active Tpr-Met oncoprotein was formed by a chromosomal translocation and has been shown to be expressed in human gastric carcinomas (Soman *et al.*, 1991) and promotes mammary tumors in transgenically-engineered mice (Liang *et al.*, 1996). Deletion mutants of RTKs can contribute to the progression of cancer, including the active EGFRvIII variant, which lacks exons 2-7 in its extracellular domain (Yamazaki *et al.*, 1988), is expressed in human breast cancer samples (Yu *et al.*, 2008) and is able to transform human breast cancer cells (Tang *et al.*, 2000).

Lastly, in addition to over-activation of RTKs in cancer, there is evidence that the failure of RTKs to be internalized and recycled after signaling may also play a role (Bache *et al.*, 2004; Roepstorff *et al.*, 2008). Some members of the ubiquitinylation and endocytic pathways have been implicated in human malignancies, including c-cbl and Tsg101, a subunit of the ESCRT-1 complex involved in intracellular trafficking (Bache et al., 2004). Tpr-Met promotes its constitutive signaling in part by avoiding receptor recycling because it cannot bind to the ubiquitin-ligase, c-cbl, and is therefore not targeted for lysosomal degradation (Peschard & Park, 2003). Other mutant RTKs use a similar strategy to avoid recycling, including EGFRvV and EGFRvIII found in human glioblastomas and

several retrovirally-transformed oncoproteins such as v-Sea found in avian erythroid cancers and v-FMS found in feline sarcomas (Peschard & Park, 2003).

RTKs as targets for cancer therapy

Due to the frequently high expression levels of RTKs in a variety of tumor types, several RTKs have become chemotherapeutic targets in the treatment of human cancers (Levitzki & Gazit, 1995). The two basic approaches that are being used clinically are monoclonal antibodies directed against the extracellular ligand-binding domain and small-molecule inhibitors which target specific RTKs to block kinase activity (Baselga, 2006; Zwick *et al.*, 2001). Both of these strategies have been clinically successful in the treatment of some cancers that exhibit increased activation of certain RTKs. For example, a monoclonal antibody directed against erbB2 (HER2) known as trastuzumab (Herceptin), has reduced metastatic growth and increased survival in the 38% of breast cancer patients that exhibit increased expression of the erbB2 RTK (DiGiovanna *et al.*, 2005; Romond *et al.*, 2005). ErbB2 is a member of the epidermal growth factor receptor (EGFR) family, which also includes EGFR (erbB1), erbB3 and erbB4. ErbB2 does not have a known ligand itself, but acts by forming heterodimers with erbB1, 3 and 4 to enhance cell signaling (Yarden & Sliwkowski, 2001). Trastuzumab blocks erbB2-mediated signaling by decreasing erbB2 expression at the cell surface and promoting antibody-dependent cell-mediated toxicity, along with other possible mechanisms (Baselga, 2006).

Several other treatments targeting the EGFR family are now in clinical use. Cetuximab is a monoclonal antibody targeting the ligand-binding domain of EGFR and has been clinically successful in treating certain colorectal and head-and-neck cancers (Baselga, 2006). Also targeting EGFR are the small-molecule tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, used in the treatment of non-small-cell lung cancers that have increased EGFR expression, although these drugs seem to have activity against other erbB family members as well (Baselga, 2006). EGFR-targeting TKI's appear to be selective for tumors with high levels of EGFR or ErbB3 expression or EGFR mutations (Baselga, 2006; Helfrich *et al.*, 2006). In particular, gefitinib appears to specifically target mutated forms of EGFR in NSCLCs and may not be active against wild-type EGFR (Chou *et al.*, 2005; Helfrich et al., 2006). Currently, there are several other drugs in development to target other RTKs, such as Platelet-Derived Growth Factor Receptor (PDGFR), Vascular Endothelial cell Growth Factor (VEGFR), Hepatocyte Growth Factor Receptor (HGFR; also called c-MET) and the Recepteur d'Origine Nantais (RON) (Baselga, 2006; Eder *et al.*, 2009; O'Toole *et al.*, 2006; Ren *et al.*, 2007).

RON receptor tyrosine kinase

Since RTKs have become an integral part of cancer treatments, it is important to consider individual RTKs for their potential contribution to the development and progression of cancer. It is also necessary to determine signaling mechanisms and interactions with other signaling molecules in order to attack the relevant pathways in

cancer treatment. The RON receptor is an RTK in the scatter factor family of receptors (Ronsin *et al.*, 1993), which includes the well-studied c-Met (Rubin *et al.*, 1993). Also in the scatter factor family are RON homologs found in other species, including chickens (Sea) (Huff *et al.*, 1993; Smith *et al.*, 1989), cats (Stem-Cell Kinase (STK)) (De Maria *et al.*, 2002), and mice (STK) (Wang *et al.*, 1995). The scatter factor receptors are named for their ability to induce migration, as well as a change in epithelial cell morphology toward a more spindle-like shape compared to the typically tightly adherent, cobblestone appearance of epithelial cells (Medico *et al.*, 1996). The scattering response is thought to be reminiscent of invasive cell behavior found in invasive, metastatic cancer cells (Comoglio *et al.*, 1999; Comoglio & Trusolino, 2002). When cultured in three-dimensional gels, scatter factor receptors induce cells to form branching tubules and cell invasion through collagen in response to their ligands (Medico et al., 1996; Weidner *et al.*, 1993). Interestingly, all known homologs of RON have oncogenic properties. For example, STK was found to be expressed in feline breast carcinomas (De Maria et al., 2002), STK promotes progression of breast carcinomas in mice (Zinser *et al.*, 2006) and a fusion protein consisting of the tyrosine kinase domain of Sea and a viral envelope protein promotes erythroid leukemia in chickens (Larsen *et al.*, 1993; Morimoto & Hayman, 1994).

Structure and function of RON

Structurally, RON is a heterodimer consisting of a 35 kDa α chain and a 150 kDa β chain, which are connected with an extracellular disulfide bond (Gaudino *et*

al., 1994). Figure IN.1 illustrates the general structure of RON. The α chain remains extracellular, whereas the β chain spans the extracellular and transmembrane space into the cytoplasmic domain, which contains the catalytic site as well as several important tyrosine residues (Gaudino et al., 1994; Ponzetto *et al.*, 1994; Xiao *et al.*, 2000). The extracellular domain of RON is divided into a sema domain (a structural domain found in semaphorin proteins on the cell surface), four IPT domains (Immunoglobulin-like regions in Plexins and Transcription factors) and a Met-Related Sequence domain (Artigiani *et al.*, 1999; Bork *et al.*, 1999). Although few studies have documented the roles of these domains in RON function, it has been indicated that the sema domain contains the binding pocket for the Macrophage Stimulating Protein (MSP), which is the only known ligand for RON (Angeloni *et al.*, 2004; Wang *et al.*, 1994b). Much of RON's activity is regulated by several C-terminal tyrosines which bind to a variety of signal-promoting effectors (Ponzetto et al., 1994). Hence, the C-terminus of RON is required for RON-mediated signaling (Lu *et al.*, 2008). In addition to recruitment of downstream effectors, the C-terminal tyrosines are critical for autoinhibition, most likely acting to suppress kinase activity in the absence of MSP-stimulation (Yokoyama *et al.*, 2005).

RON is normally expressed in several different cell types, including macrophages (Iwama *et al.*, 1995), erythroblasts (van den Akker *et al.*, 2004), embryonic neurons (Funakoshi & Nakamura, 2001) and epithelial cells (Gaudino *et al.*, 1995). The expression of RON in normal epithelial cells is generally much lower than the levels found in RON-expressing carcinomas (Chen *et al.*, 2000; Maggiora *et*

al., 1998; Zhou, YQ *et al.*, 2003). It is not clear how the expression levels of RON are regulated, although some evidence suggests that hypermethylation of a particular CpG island in the RON promoter allows for transcription of full-length RON (Angeloni *et al.*, 2007). Recently it has been shown that the transcription factor Sp-1 is required for RON transcription (Thangasamy *et al.*, 2008). Further, the NF-κB transcription factor positively regulates RON expression in breast carcinoma cells, which can be blocked by chemical inhibitors to NF-κB or NF-κB binding-site mutants (Narasimhan & Ammanamanchi, 2008). The RON gene is found on chromosome 3p21 (Angeloni *et al.*, 2000), which is a region that also includes tumor suppressor genes affected in several tumor types (Senchenko *et al.*, 2004; Zabarovsky *et al.*, 2002). The promoter region of RON also contains binding sites for interleukin-6 response elements, AP-2 (Thangasamy *et al.*, 2008) as well as NF-κB and ets-1 (Waltz *et al.*, 1998). Mechanisms involved in the upregulation of RON that is frequently seen in human carcinomas remain elusive.

Macrophage Stimulating Protein

MSP was discovered by its activation of macrophages, which is why it is so named (Leonard & Skeel, 1978; Leonard & Skeel, 1979; Lutz & Correll, 2003). Like RON itself, the gene for MSP is located on chromosome 3p21 (Yoshimura et al., 1993). MSP is synthesized as a pro-protein in hepatocytes, and must be proteolytically cleaved by matripase/ membrane-type serine protease-1 (MT-SP1) on the surface of the target cell (Bhatt *et al.*, 2007; Degen *et al.*, 1991; Wang, MH *et al.*,

1996b). After cleavage by MT-SP1, MSP forms an enzymatically inactive heterodimer containing an 53 kDa α chain and a 39 kDa β chain connected by a disulfide bond (Yoshimura *et al.*, 1993). The cleavage of pro-MSP allows for conformational changes that increase receptor binding (Carafoli *et al.*, 2005). MSP is a member of the kringle family of proteins, which includes plasminogen, apolipoprotein and hepatocyte growth factor (HGF), which is the ligand for c-Met (Donate *et al.*, 1994). MSP is approximately 40% homologous to HGF(Nakamura *et al.*, 1989; Yoshimura et al., 1993) and they utilize different mechanisms to bind to their respective receptors (Okigaki *et al.*, 1992; Wang *et al.*, 1997).

RON Signaling

After MSP binding, a range of effector molecules attach to phosphorylated tyrosine residues in the cytoplasmic domain of RON. Phosphorylated RON recruits c-Src (Danilkovitch-Miagkova *et al.*, 2000; Wei *et al.*, 2005), PI3K (Xiao et al., 2000), Grb2 and Gab adaptor proteins (van den Akker et al., 2004). There are species-specific differences as to whether Gab 1 or Gab 2 is involved in RON signaling. Human RON, much like its family member, c-Met, apparently signals exclusively through Gab 1 (van den Akker et al., 2004), however, in mice (STK) and in chickens (Sea), RON signals primarily via Gab 2 (Ischenko *et al.*, 2003; Teal *et al.*, 2006).

Several downstream pathways are activated after MSP-stimulation, such as the MAPK (Wang *et al.*, 2005; Wei *et al.*, 2005), PI3K/Akt (Danilkovitch *et al.*,

2000; Danilkovitch *et al.*, 1999), jun N-terminal kinase (JNK) (Chen et al., 2000), FAK (Danilkovitch et al., 1999), and β-catenin pathways (Xu *et al.*, 2005). In addition to the cell scattering effect, MSP-stimulation of RON induces proliferation (Wang, MH *et al.*, 1996a), migration (Suzuki *et al.*, 2008; Wang, MH et al., 1996a), cell morphogenesis (Medico et al., 1996) and protection from apoptosis (Danilkovitch et al., 2000; Wang et al., 2005) in some cell systems. Figure IN.2 highlights select signaling pathways activated by RON. Although the majority of RON studies have looked at its role in human cancers, it is becoming clear that RON also functions in the innate immune system through its expression and activation in macrophages (Wilson *et al.*, 2008). RON has additional functions in wound repair (Cantaluppi *et al.*, 2008; Cowin *et al.*, 2001), inflammation (Muraoka *et al.*, 1999; Wang *et al.*, 1994a), phagocytosis (Lutz & Correll, 2003) and hematopoiesis (van den Akker *et al.*, 2004).

MSP-independent signaling of RON

There is strong evidence that RON induces cell signals in the absence of MSP. High expression levels of RON induced receptor phosphorylation and activation of the MAPK pathway in the absence of MSP (Cote *et al.*, 2007). Ligand-independent activation of RON promoted c-Src-mediated MAPK kinase signaling in human embryonic kidney cells (Wei et al., 2005). Some studies have indicated that oncogenic mutants of RON bind to β-catenin and contribute to constitutive β-catenin phosphorylation, nuclear accumulation and increased expression of the β-catenin

targets of c-myc and cyclin D1, even in the absence of MSP (Danilkovitch-Miagkova et al., 2001; Xu *et al.*, 2005). In a transgenic mouse model of breast cancer, both wild-type and oncogenic mutant RON promoted MSP-independent β-catenin activation, and an increase in c-myc and cyclin D1, leading to a 100% incidence of aggressive breast tumors (Zinser *et al.*, 2006). Similarly, mice expressing WT-RON in the lung epithelial cells exhibited RON tyrosine phosphorylation and developed lung adenomas (Chen, YQ *et al.*, 2002a; Chen, YQ *et al.*, 2002b).

Interactions of RON with other receptors

RON may also have alternative means of activation during development, as a complete knockout of RON in mice was embryonically lethal by day E6.5 (Muraoka et al., 1999), however MSP-deficient mice develop normally without any obvious defects (Bezerra *et al.*, 1998). Further, mice engineered to express only the extracellular and juxtamembrane domains of RON, therefore lacking the tyrosine kinase and docking site regions, are healthy, with only minor inflammatory defects (Leonis *et al.*, 2002; Waltz *et al.*, 2001). Interestingly, mice that lack only the extracellular ligand-binding site are also viable (Correll *et al.*, 1997). Taken together, these data suggest that potential interactions of juxtamembrane domain of RON with other cell surface receptors may propagate signaling, which is absent in the WT-RON knock-out mice. However, these potential interactions with other kinases could be specific in mouse embryonic development, since the kinase activity of RON is

required for all known RON-mediated biological effects in cancer development (Danilkovitch-Miagkova et al., 2000; Peace *et al.*, 2005).

In fact, it is known that RON can interact with other cell surface receptors to promote cell signaling and biological effects. Interaction with EGFR was important for cell scattering in human embryonic kidney cells (HEK 293) after MSP stimulation of RON (Peace *et al.*, 2003). Although it is not clear whether the interaction between EGFR and RON is direct, EGFR co-immunoprecipitated with RON in human bladder carcinoma cells both in the presence and absence of MSP (Hsu *et al.*, 2006). Further, in this study, EGFR-inhibitors reduced RON-mediated biological effects, including cell survival and cell migration (Hsu et al., 2006). It has also been demonstrated that RON can cross-talk with c-Met, in that the kinase activity of RON is required for the ability of a c-Met mutant to transform NIH3T3 cells (Follenzi *et al.*, 2000). Data suggests that together, c-Met and RON have the ability to enhance one another's signals in ovarian (Maggiora *et al.*, 2003), colorectal (Lee, CT *et al.*, 2008) and in breast carcinomas (Lee *et al.*, 2005)

Other cell receptors that RON has been shown to interact with include integrins, which bind extracellular matrix proteins (Danilkovitch-Miagkova *et al.*, 2000; Santoro *et al.*, 2003), plexins, which bind to semaphorin receptors on the cell surface (Conrotto *et al.*, 2004), CD44 (Matzke *et al.*, 2005) and the Receptor for Hyaluronan-Mediated Motility (RHAMM) (Manzanares *et al.*, 2007). The latter two receptors are both receptors for hyaluronan. RON also cooperates with TGF-β1 receptors to enhance oncogenic signaling and promote an epithelial-to-mesenchymal

transition (EMT) (Wang *et al.*, 2004; Wang et al., 2005). Although EMT is an important normal process in development, it is also currently believed to be a precursor to metastatic development in human cancers (Thiery, 2002). Mechanistically, RON may reduce claudin-1 expression in the tight junctions of epithelial cells, paving the way for a reduction in E-cadherin, which is a marker for the development of EMT (Zhang, K *et al.*, 2008). Given the significant number of receptor interactions, it is implied that RON may have several means to activate its signaling pathways in the absence of MSP.

Biologically active RON mutants

In addition to receptor interactions, constitutively active mutants of RON generate MSP-independent signaling in some human cancers. These mutant versions of RON have generally been splice variants (Wang *et al.*, 2003). RONΔ165 is a splice variant of RON that lacks exon 11, and functionally, it can promote cell migration and invasion (Collesi *et al.*, 1996). RONΔ165 has been identified in a stomach cancer cell line (KATO III), and both in normal colon tissue and colon carcinomas (Collesi et al., 1996; Okino *et al.*, 1999). Another activating RON mutant, RONΔ160, was isolated from colon carcinoma cell lines (HT29 and SW837) and primary colorectal carcinomas and is missing exons 5 and 6, allowing for abnormal receptor dimerization (Chen et al., 2000; Wang *et al.*, 2000; Zhou, YQ et al., 2003). RONΔ155, with a deletion of exons 5, 6, and 11, was also isolated from colorectal carcinomas and exhibits constitutive activity (Zhou, YQ et al., 2003). In mice,

susceptibility to the erythroleukemia-causing Friend virus (Ruscetti, 1999) requires a truncated version of RON, known as short-form Stk (sf-Stk), which lacks the majority of the extracellular domain (Nishigaki *et al.*, 2001; Persons *et al.*, 1999), implying that constitutively active RON contributes to cell transformation in erythroid leukemias. The short-form of RON has also been identified in human lung cancer cell lines (Angeloni et al., 2000). Lastly, studies have shown that oncogenic point mutants of RON that were engineered based on kinase domain mutations found in the Kit (D1232V) or Ret (M1254T) kinases caused cell transformation and the formation of tumors in nude mice (Santoro *et al.*, 1998). However, no point mutants of RON have been isolated from human tumors as of yet, so the biological relevance of these artificially designed mutants is questionable.

Down-regulation of RON

Similar in mechanism to other RTKs (Haglund *et al.*, 2003), down-regulation of RON can occur after ligand-stimulation by a ubiquitinylation mechanism mediated by the binding of the c-cbl ubiquitin ligase to the cytoplasmic domain (Penengo *et al.*, 2003). Ubiquitinylation of RON leads to endocytosis and lysosomal degradation (Penengo et al., 2003). RON can also be degraded via an alternative pathway involving the CHIP E3 ligase, which targets RON to the proteosome for degradation rather than lysosomes (Germano *et al.*, 2006). In human pancreatic cancer cells, the Smad-4 transcription factor, which acts downstream of TGF-β signaling, plays a role in the suppression of RON expression by blocking RON promoter activity (Zhao *et*

al., 2008). In Smad-4-containing cells, RON protein expression was reduced in the presence of exogenous TGF-β, however, in Smad-4-null cells, the RON protein was upregulated (Zhao et al., 2008). Negative regulation of RON can also be mediated by hyaluronidase 2 (HYAL2), which binds to and suppresses RON signaling in epithelial cells (Danilkovitch-Miagkova *et al.*, 2003). Interestingly, HYAL 2 is the cell surface receptor for jaagsiekte sheep retrovirus (JSRV), which causes lung cancer in sheep (Rai *et al.*, 2001). When JSRV binds to HYAL2, HYAL2 can no longer sequester RON activity, implying that RON may also play a role in the development of this infectious lung cancer.

Clinical significance of RON in human cancer

Clinically, the increased expression of RON has been described in several primary human cancer types, including ovarian, breast, bladder, colorectal, gastric, kidney, pancreatic, and lung carcinomas, among others. Approximately 73% of ovarian (Ferrandina et al., 2008), 33% (Lee *et al.*, 2005) up to 47% of breast (Maggiora et al., 1998), 33% of bladder (Cheng et al., 2005), 42% of thyroid (Wang *et al.*, 2007b) and 59% of colorectal (Zhou, YQ et al., 2003) carcinomas exhibit increased RON levels relative to corresponding normal tissues. Chromophobe renal cell carcinomas exhibited increased RON expression 96% of the time, whereas only 17% of other renal cell carcinomas expressed increased levels of RON (Patton *et al.*, 2004). In patient samples of gastric carcinoma tissues, 56% were positive for RON expression compared to 25.6% of paraneoplastic tissues and normal gastric mucosa

showed no expression of RON (Zhou *et al.*, 2008). Using detection by reverse-transcriptase PCR, one study found that 63% of primary non-small-cell lung carcinomas exhibit detectable RON mRNA levels (Willett et al., 1998). More recent work has demonstrated that up to 93% of late stage neoplasia, primary and metastatic pancreatic cancers exhibit increased RON expression whereas early stage neoplasia and normal tissue expressed significantly less RON (Thomas et al., 2007).

Increased expression of RON in some human cancers is generally associated with a more severe disease. In both bladder and breast cancers, RON is a prognostic marker for reduced patient survival. Specifically, in breast cancers, RON expression is associated with metastatic development, and a reduced 10-year disease-free survival rate of 30% compared to 59% of patients with tumors without RON positivity (Lee et al., 2005). Transitional-cell bladder cancer patients whose tumors expressed RON were more likely to have a higher grade tumor, increased tumor size, muscle invasion and an overall reduction in survival rate (Cheng et al., 2005). There is a correlation between RON expression levels and the depth of tumor invasion in gastric cancers, but RON expression was not correlated to patient survival rate (Zhou et al., 2008). In advanced ovarian cancers, high expression levels of RON were found to reduce survival from 59 months to 35 months (Ferrandina *et al.*, 2008).

Targeting RON in the treatment of cancer

Due to the oncogenic effects of RON signaling in epithelial cells, much interest has been piqued to develop chemotherapeutic agents targeting RON in human

malignancies (Wang *et al.*, 2006). There is some molecular evidence that suggests that this approach may be successful, for example, one study demonstrated that reducing RON expression in colon carcinoma cells by shRNA also lowers proliferation and increases apoptotic cell death (Xu *et al.*, 2004). A splice variant of RON known as RONΔ170 (Angeloni *et al.*, 2003), which lacks the C-terminal tyrosine docking sites, acts as a dominant negative in colorectal cancer cells and blocks RON-mediated phosphorylation, activation of downstream effectors, cell proliferation and migration (Wang *et al.*, 2007a). The molecular compound known as curcumin (diferuloylmethane), which is naturally found in certain plants, was found to block the invasive behavior induced by RON signaling in breast carcinoma cells by inhibiting the activation and expression of NFκB (Narasimhan & Ammanamanchi, 2008).

Pharmaceutical companies are currently designing drugs that target RON for human cancer treatment. ImClone Systems, Inc. demonstrated that a humanized monoclonal antibody that targeted the extracellular binding domain of RON reduced RON signaling and lowered the rate of tumor growth in human xenograft models of lung, pancreatic and colon cancers (O'Toole *et al.*, 2006). More recently, Amgen, Inc. has developed a chemical inhibitor of both RON and c-Met that also reduces kinase activity and inhibits tumor growth in colon cancer xenografts (Zhang, Y *et al.*, 2008).

Summary

To summarize, RTKs are strongly implicated in the development and progression of human cancers. The RON RTK exhibits increased expression in several human cancer types, and RON most likely contributes to the severity of the disease. Because of the high expression levels and constitutive activation of RON in breast cancers, my studies examined the potential of RON to transform normal human breast epithelial cells to acquire parameters associated with tumor formation.

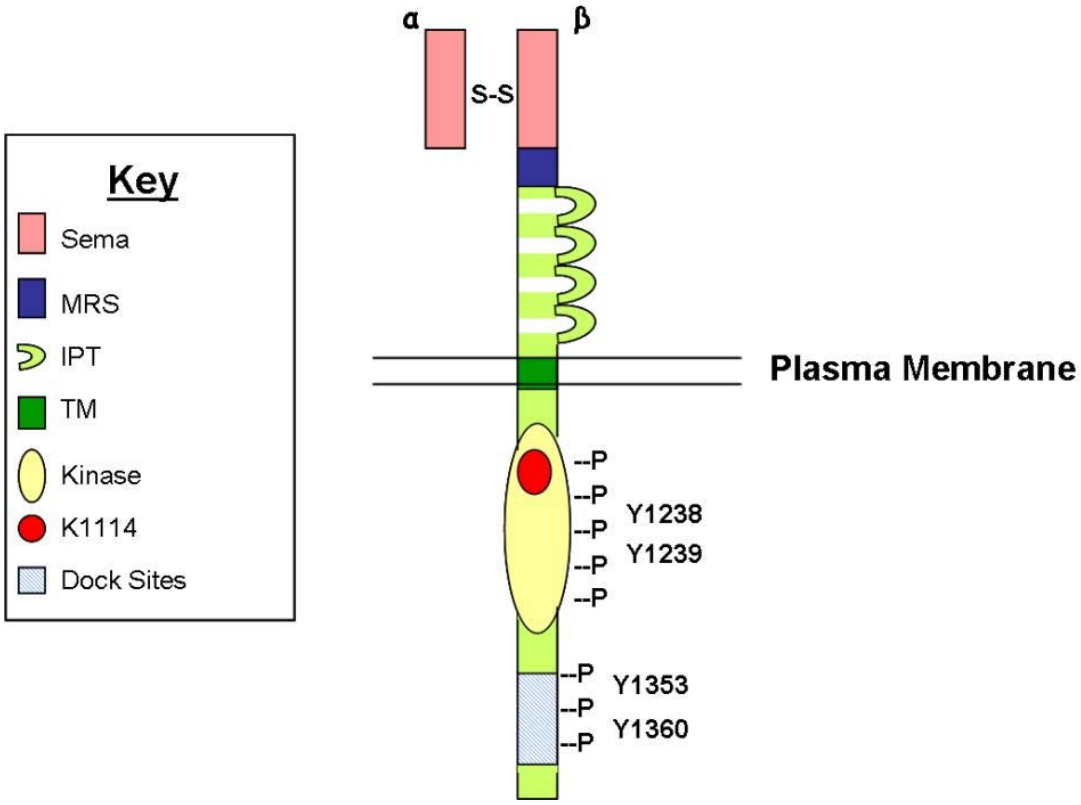

Figure IN.1

Structure of RON RTK. Mature RON is a heterodimer consisting of α and β strands. In the extracellular region, RON contains a Sema domain, which is the ligand-binding site, a Met-related sequence (MRS) domain and an Immunoglobulin-Plexin-Transcription factor domain (IPT). The transmembrane (TM) region spans the plasma membrane. The intracellular domain of RON contains a tyrosine kinase domain with an essential catalytic lysine residue, K1114. Both the kinase domain and the C-terminal region contains several available tyrosines that are phosphorylated after RON activation in order to transduce downstream signals.

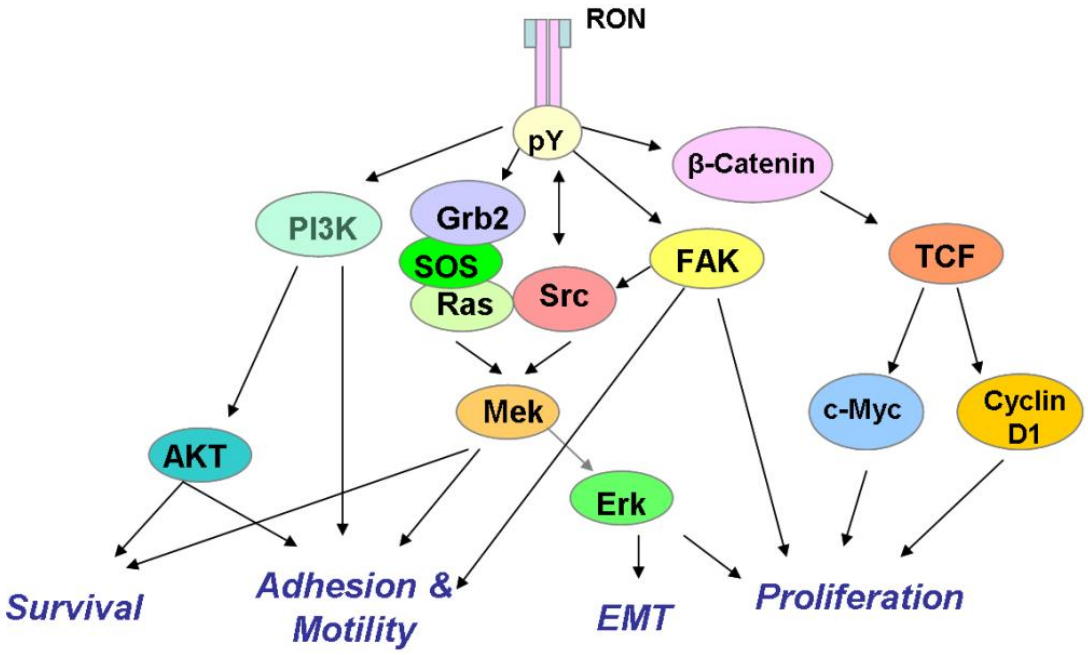

Figure IN.2

Select signaling pathways induced by the RON RTK. RON can activate several downstream pathways in response to MSP binding. Some of these known pathways include the PI3K, MAPK, and β-catenin pathways. These pathways can lead to cell survival, adhesion, migration, proliferation and an epithelial-to-mesenchymal transition (EMT), among other cellular effects.

Chapter 1

Expression of WT-RON in human breast epithelial cells

Introduction

RON appears to play a significant role in breast cancer. Nearly 47% of primary human breast cancers expressed RON, and increased expression of RON was found in several established breast cancer cell lines (Maggiora et al., 1998). Additionally, when mice were engineered to express RON in mammary tissue, 100% of the RON-expressing mice developed tumors, whereas the parental mice did not develop tumors (Zinser *et al.*, 2006). Although increased expression of RON in breast carcinomas is well-documented, less-understood is whether RON can promote cancer progression in the absence of its ligand, MSP. To date, no naturally occurring mutations of RON have been identified in human breast cancers; therefore, it is likely that interactions with other cell receptors or kinases might be responsible for the MSP-independent activation of RON.

In order to determine whether RON contributes to the progression of breast cancers, either in the presence or absence of MSP, we generated a RON-expressing

cell system using the well-characterized MCF-10A human mammary epithelial cell line. The parental cell line, MCF-10, was isolated from normal diploid mammary tissue of a 36-year old woman with fibrocystic disease (Soule *et al.*, 1990). MCF-10A cells are a spontaneously immortalized cell line that are non-transformed, and do not promote tumors in nude mice (Soule et al., 1990). The "A" was designated to mean "attached", as opposed to another isolated cell line called MCF-10F which floated in suspension (Soule et al., 1990). Like normal, non-cancerous cells, MCF-10A cells exhibit a highly adherent phenotype when cultured on plastic tissue culture dishes and retain normal cell-cell junctions, including hemisdesmosomes and desmosomes, and also these cells require exogenous growth factors to be maintained in culture (Russo *et al.*, 1993; Soule et al., 1990). Because MCF-10A cells resemble normal epithelial cells in signaling and morphology, we can introduce stable expression of RON in these cells to determine whether RON-mediated signaling results in aberrant cell behavior.

One unique feature of MCF-10A cells is their ability to grow in three dimensions (3-D), forming acini structures when cultured in Matrigel. Matrigel is a gel-like medium that contains basement membrane (BM) and extracellular matrix (ECM) proteins (Debnath *et al.*, 2003). It has become increasingly clear that culturing cells on plastic tissue culture dishes does not necessarily represent what happens in more biologically-relevant systems in terms of cell morphology or cell signaling (Kass *et al.*, 2007). It is thought that 3-D cultures of MCF-10A cells in ECM may more closely mimic what occurs *in vivo*, as the acini they form structurally

resemble mammary gland acini in the breast (Debnath et al., 2003) compared to the flat, 2-D appearance of MCF-10A cells on plastic. In 3-D, MCF-10A acini initially form as a solid ball of cells. Without contact with the ECM for growth and survival nutrients, over a period of approximately 3 weeks the cells in the interior of the structure die by a process of anoikis. This process eventually produces a polarized, growth-arrested structure that is a hollow sphere surrounded by a single layer of cells (Debnath *et al.*, 2002).

MCF-10A cells are a powerful cell system with which to identify the biological effects of oncogenic signaling by RTKs because they do not already express other oncogenes and they have 3-D growth capacity (Debnath *et al.*, 2003; Muthuswamy *et al.*, 2001). Changes that occur in 3-D MCF-10A acini that would imply oncogenic effects include a cell-filled lumen, a loss of the growth-arrested state, changes in polarity, and the ability to invade the surrounding ECM (Debnath & Brugge, 2005). Much work has been done with MCF-10A cells expressing oncogenes, such as ErbB2 (Muthuswamy et al., 2001; Seton-Rogers *et al.*, 2004), c-Met and CSF-1R (Wrobel *et al.*, 2004), to identify cell transformation events that correlate with changes in 3-D structure. Different RTKs have distinct effects on acini formation, which implies that oncogenic RTKs employ different signaling mechanisms, and may differentially contribute to the cancer phenotype. Activated CSF-1R in MCF-10A cells permits growth of cells in the acinar lumen, promotes increased size of the acini and individual cells were able to migrate out of the structure and invade into the Matrigel (Wrobel et al., 2004). C-Met, on the other

hand, formed branching tubules in 3-D-cultured MCF-10A cells, but otherwise retained the polarized acinar structure (Wrobel et al., 2004). Activation of ErbB2 leads to a larger, multi-acini structure with filled lumens, but does not promote invasive behaviors (Muthuswamy et al., 2001). EGFR activation supports normal development of acini and does not contribute to additional structural changes (Muthuswamy et al., 2001).

This chapter describes the generation and characterization of RON-expressing MCF-10A cells, which were used throughout my studies in order to examine the role of RON in the development and progression of breast cancer.

Results

Characterization of RON expression levels in MCF-10A cells

Endogenous levels of RON were low in the relatively normal epithelial cell line, MCF-10A (For example, RON was undetectable in Figure 1.1a). Consequently, the cells did not respond to MSP in any biological or biochemical assays we have tested to date (For example, see Figures 2.3 & 3.1). Therefore, to examine the contributions of increased RON expression to the progression of breast cancer, I transduced MCF-10A cells with a Phoenix € (Φ€)-generated retrovirus expressing both wild-type human RON and a separate IRES-promoted GFP cassette. Φ€ packaging cells were utilized because the line of MCF-10A cells I transduced

expresses the mouse ecotropic receptor (a kind gift of Dr. J. Brugge, Harvard Medical School), which increases the infection efficiency, and reduces safety concerns found with amphotropic viruses, which can infect humans. Pools of infected cells were selected for high, medium and low GFP expression levels by fluorescence-activated cell sorting (FACS), and, as expected, GFP levels were mimicked by expression levels of RON (Figure 1.1a). I designated these cells as 10A/RON. I also isolated a vector-only negative control cell line, which was designated 10A/Vector. The levels of RON expression in 10A/RON cells were well within the range of expression found in human breast carcinomas, some of which had RON levels that were up to 72-fold higher than the level found in benign tissue (Maggiora et al., 1998). High, medium and low 10A/RON cell lines behaved similarly in all of my experiments; therefore, I utilized the medium-RON expressing MCF-10A cell line throughout this study, unless otherwise noted in the text.

RON is localized at the cell membrane in MCF-10A cells

Generally, RTK's are found at the cell membrane, however, RTKs can also generate signals via intracellular compartments, particularly during the endocytic recycling process (Sorkin & Von Zastrow, 2002). To determine the localization of RON in 10A/RON cells, I used a monoclonal antibody that recognizes the extracellular domain of RON in an immunofluorescent study. No RON expression was exhibited in 10A/Vector cells (Figure 1.1b). As shown in Figure 1.1b, RON is localized to the cell membrane, around the perimeter of the cell. Unfortunately, I

could not determine whether RON was localized to intracellular regions of the cell in the absence of stimulation (Figure 1.1b). The anti-RON antibody I had available for immunofluorescence did not detect RON unless it is added first to live cells. Therefore, the cells were not fixed or permeable, so the antibody could not get inside the cells to determine if RON is signaling within the cells in endocytic vesicles.

Although it has been reported to block RON activation in other systems (R&D Systems, 2009), this anti-RON antibody may stimulate RON activity in MCF-10A cells as judged by receptor tyrosine phosphorylation (Figure 1.2a), and can be added to living cells to look for changes in RON localization after receptor dimerization (Figure 1.2b). For this assay, the anti-RON antibody was combined with the Alexa-Fluor 546 anti-mouse secondary antibody and then added to a live culture of 10A/RON cells combined with uninfected MCF-10A cells growing on coverslips. The combination of 10A/RON with uninfected cells allowed me to conclude that the labeling of RON with this antibody was specific, as RON was not detected in uninfected cells, which do not have GFP expression (Figure 1.2b). After the addition of the antibodies for 1 minute, the cells were then washed with DMEM and imaged every 5 minutes for 1 hour. As is shown in Figure 1.2b, whereas RON was initially located primarily at the cell membrane, it was gradually internalized into the cell after antibody binding, as expected based on previous studies of RON downregulation (Penengo et al., 2003). Unfortunately, this antibody could not be used to visualize the changes in RON distribution after MSP stimulation due to its own effects on RON dimerization and internalization.

10A/RON cells exhibit invasive behaviors when cultured in three-dimensions

To determine whether 10A/RON cells exhibit disruption of normal, polarized acinar structures, I cultured both 10A/RON and 10A/Vector cells using a 3-D overlay method (Debnath et al., 2003). In this assay, a 1:1 mixture of collagen:matrigel was gelled on the bottom of an 8-well chamber slide, and the cells were added on top of the gel in a 2% matrigel-containing media. A solution of collagen and matrigel was used rather than 100% matrigel because previous studies suggested that certain oncogenes promote invasive properties only on matrices containing collagen (Seton-Rogers et al., 2004). Cells were allowed to develop acini for 8 days in regular media, at which point MSP was added to 100ng/ml in the specified cultures and the cells were allowed to grow for a further 13 days. As indicated in Figure 1.3, 10A/Vector cells formed typical-looking acini with a growth-arrested, spherical shape, whereas RON-expressing 10A cells formed long, hollow tubules that extend across the culture dish. Tubulogenesis of 10A/RON cells occured both the presence and absence of MSP stimulation, suggesting that RON promotes MSP-independent structural changes to normal breast epithelial cells. MSP did not appear to promote any additional structural changes in 10A/RON cells cultured in 3-D.

Discussion

I have established an MCF-10A cell-based system with which to study the potential oncogenic effects mediated by the wild-type RON RTK. The signaling mechanisms of some other RTKs have been illuminated in MCF-10A cells, such as CSF-1R and ErbB2 (Muthuswamy et al., 2001; Wrobel et al., 2004). As with other RTKs, RON is located at the cell membrane in unstimulated cells, and is internalized into intracellular vesicles after activation when expressed in MCF-10A cells.

Unlike the 3D structures formed by ErbB2, which are large, multi-acini structures without growth constraints, 10A/RON cell acini form long, connecting tubules. This is in agreement with the initial study that described the ability of RON to induce invasive behavior in mouse liver progenitor cells, in which MSP promoted the formation of tubules in collagen gels (Medico et al., 1996). The formation of tubules is also reminiscent 10A/Met cells when cultured in a collagen/matrigel assay (Wrobel et al., 2004). Further, c-Met expressed in Madin-Darby Canine Kidney Cells (MDCK) cells promotes branching morphogenesis when cultured in a 100% collagen gel (Birchmeier *et al.*, 2003), which implies that the development of tubules in response to c-Met and RON signaling might be a general phenomenon in epithelial cells.

Tubulogenesis is a biological process that is involved in the structural development of the kidney, lung, pancreas and mammary gland among other organs (Rosario & Birchmeier, 2003). This process requires an intricate set of interactions between RTKs, integrins, ECM proteases and several downstream pathways, such as

PI3K and MAPK (Rosario & Birchmeier, 2003). Because cells forming these polarized structures must exhibit active ECM-degrading proteases in order to move into the extracellular space, it is thought that the mechanisms that underlie tubulogenesis in development are potential pathways for tumors to hijack in order invade the basement membrane and eventually form metastatic growths (Rosario & Birchmeier, 2003).

In other 3-D assay studies, the formation of tubules promoted by either Met or RON has been shown to be dependent on their respective ligands (Medico et al., 1996; Wrobel et al., 2004). In 10A/RON cells, however, RON mediated an MSP-independent formation of tubules, which implies that RON may promote MSP-independent signaling in normal breast epithelial cells. The following chapters will describe the work I have completed to examine both MSP-dependent and MSP-independent signaling of RON in the 10A cell system in more detail.

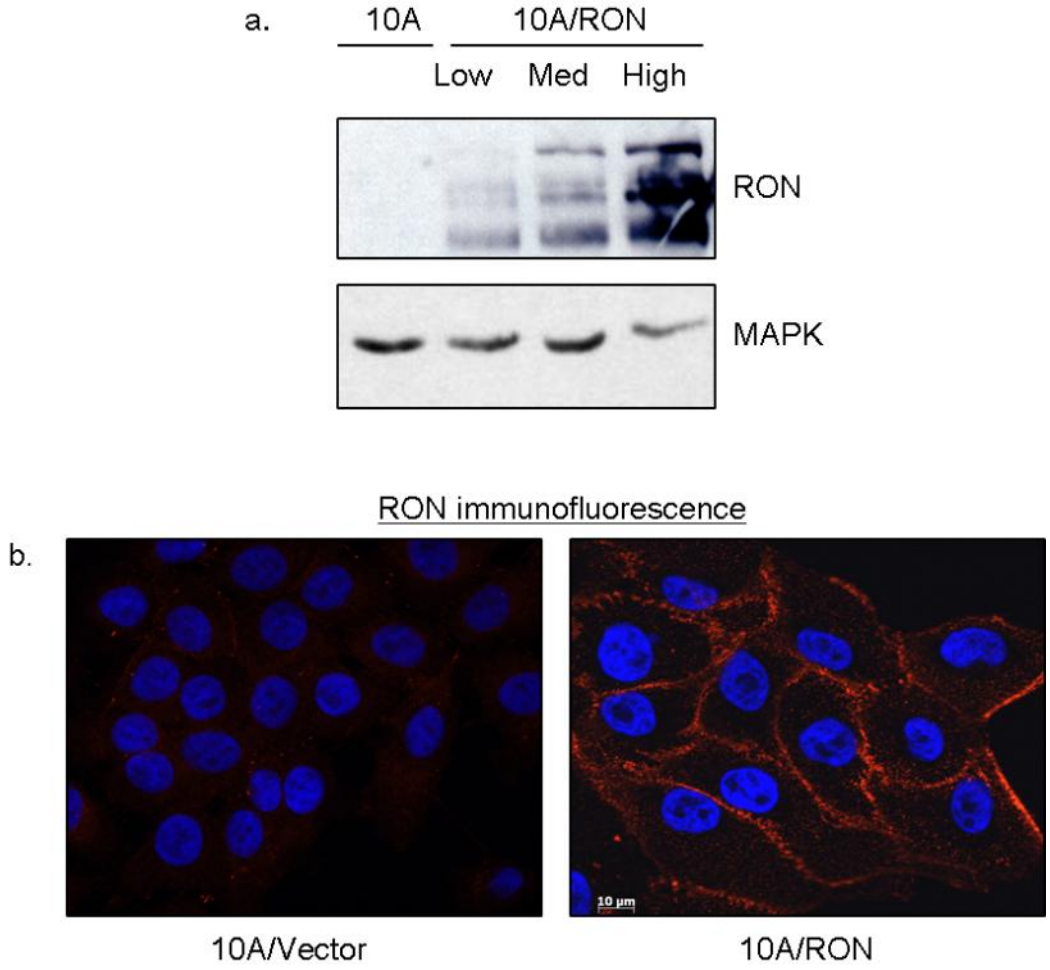

Figure 1.1

Expression of RON in MCF-10A cells. (a) MCF-10A/Vector control and MCF-10A/RON cell lysates were subjected to Western blot for total RON expression. This particular antibody recognizes three different forms of the RON receptor, which is why there are three bands. The upper band is the full-length, unprocessed form of RON. The middle band is a fully-processed form of RON into its αβ heterodimer and it is also glycosylated. The bottom band is a fully-processed, but un-glycosylated form of RON. Presumably it is the middle band that is the active form of the RON receptor. A MAPK blot followed to provide a loading control. (b) 10A/Vector and 10A/RON cells were plated on coverslips for 48 hours before looking for RON expression by immunofluorescence. Blue = DAPI, Red= RON.

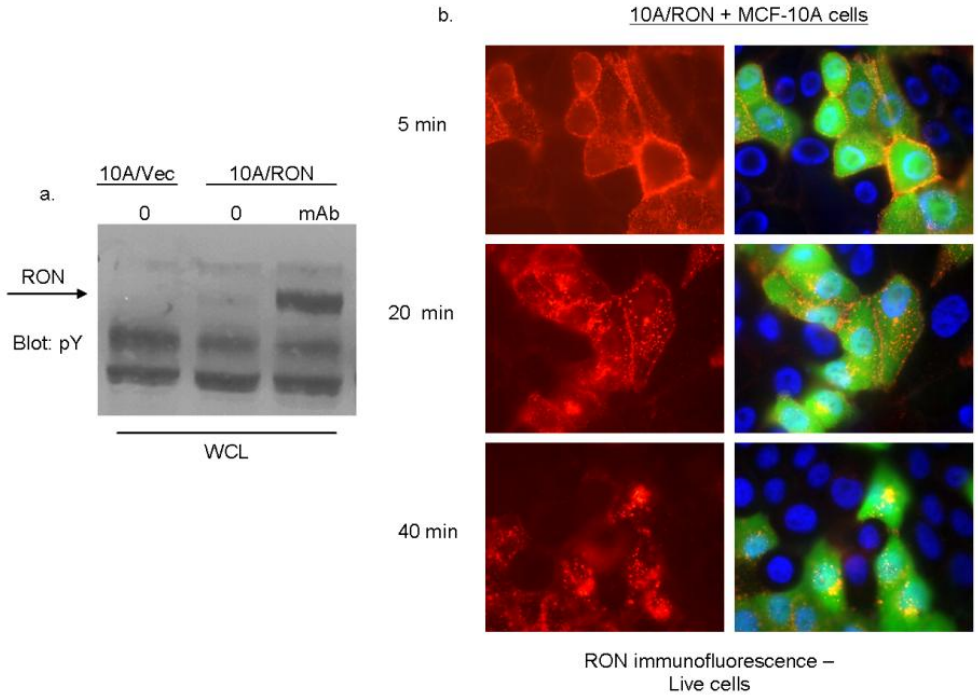

Figure 1.2

RON is internalized after activation in MCF-10A cells. (a) 10A/Vector control and 10A/RON cell lysates were subjected to Western blot for total phospho-tyrosine. Phosphorylated RON is indicated by the arrow. Some background bands are present to indicate loading control. (b) 10A/ecoR (no expression of GFP) and 10A/RON cells were cultured together on the same coverslips. Anti-RON antibodies were mixed with Alexa-Fluor 546 and briefly added to live cells to detect RON expression. Cells were washed with DMEM and images were taken at 5 minute intervals. Blue = DAPI, Green = GFP of infected cells, Red= RON.

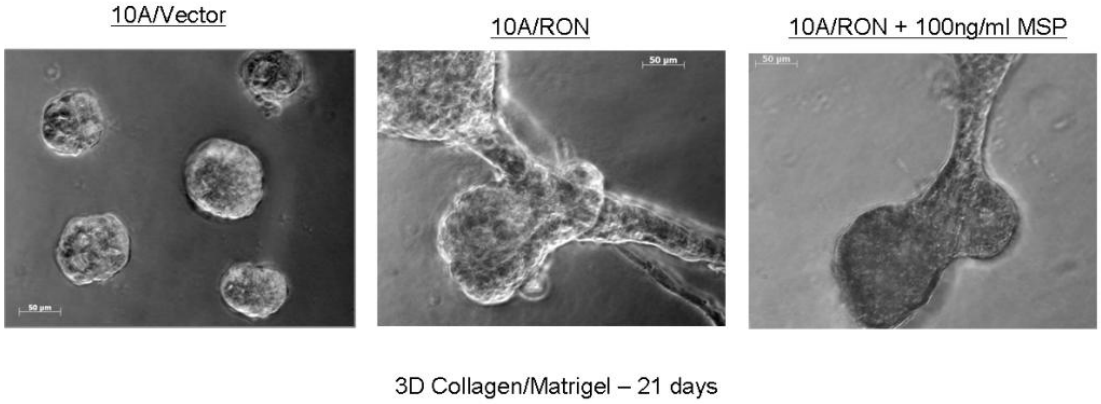

Figure 1.3

RON expression in MCF-10A cells promotes tubule formation in 3-D cultures. 10A/Vector and 10A/RON cells were cultured in a 3-D overlay method with a 1:1 mixture of collagen:matrigel gelled on the bottom of the chamber slide. 15,000 cells/well were added to the gel in 2% matrigel-containing media. Growth media containing 2% matrigel was refreshed every 4 days. MSP (100ng/ml) was added to the specified sample after 8 days in culture. Cultures were imaged using phase-contrast microscopy up until day 21.

Chapter 2

RON exhibits two distinct mechanisms of signaling in MCF-10A cells

Introduction

In cancers, ligand-independent signaling is a common mechanism by which RTKs propagate their downstream biological effects. Although mutations can render RTKs constitutively active, sometimes interactions with other cell signaling moieties can substitute for the requirement of the ligand, thereby promoting oncogenic outcomes.

The intracellular Src kinase frequently augments signaling by RTKs, however, wild-type Src itself is not oncogenic (Biscardi *et al.*, 2000). Structurally, unstimulated Src is found in a conformation in which its carboxy-terminus folds over and blocks the catalytic domain by interaction with the amino-terminal SH3 and SH2 domains (Xu *et al.*, 1999). In order to become fully active, Y418 in the kinase domain of Src must be phosphorylated, and Y529 in the C-terminal region must be dephosphorylated (Brown & Cooper, 1996). This allows the exposure of the kinase domain for activation (Engen *et al.*, 2008). A truncated Src which lacks the C-

terminal autoinhibitory region is constitutively active and is thought to play a role in some colon cancers (Irby *et al.*, 1999).

Src becomes active downstream of RTKs, integrins, FAK, and steroid hormone receptors, among others (Ishizawar & Parsons, 2004). Once activated, Src positively regulates signaling via MAPK, c-JUN kinase (JNK), Rho GTPase and signal transducers and activators of transcription 5b (STAT 5b) (Ishizawar & Parsons, 2004). Src also appears to be involved in the downregulation of cbl, which, in the case of EGFR, leads to the inability of EGFR to be downregulated and degraded, leading to prolonged signaling (Bao *et al.*, 2003; Ishizawar & Parsons, 2004).

In breast carcinomas, the activity of Src promotes tumor progression at least in part by its ability to synergize with the epidermal growth factor receptor (EGFR) (Biscardi *et al.*, 2000; Wilson *et al.*, 1989). Other RTKs also interact with Src kinases to enhance oncogenic signaling in human cancers, including c-Met (Emaduddin *et al.*, 2008) and platelet-derived growth factor receptor (PDGFR) (Ishizawar & Parsons, 2004). Additionally, Src mediated RON activation downstream of β1 integrins in human keratinocytes (Danilkovitch-Miagkova *et al.*, 2000). The fact that two or more kinases cooperate to increase their oncogenic effects may dramatically impact the clinical treatment for those patients whose tumors are co-expressing RTKs with other kinases (Stommel *et al.*, 2007).

Since Src is highly expressed and deregulated in at least 70% of human breast cancers (Ishizawar & Parsons, 2004), it is likely that RON and Src are co-expressed in a number of breast tumors. Furthermore, Src is recognized as an important

contributing factor to breast cancer progression (Ishizawar *et al.*, 2004). In this study, I examined the contributions of RON, and its putative interaction with c-Src, to the progression of breast cancer. RON expression in MCF-10A cells gave rise to evasion of cell death, an increase in spreading and an increased migratory potential. MSP-stimulation of RON was required for RON-mediated cell migration and proliferation, which suggests that MSP-independent functions of RON are not solely a consequence of increased RON expression levels. In addition, Src activity was required for RON-mediated, MSP-independent biological effects. These data imply that RON cooperates with Src in mammary epithelial cells to promote cellular changes that may allow progression toward a fully oncogenic state.

This chapter describes RON-mediated cell signaling in MCF-10A cells. I have determined that the RON receptor exhibits both MSP-dependent and MSP-independent signaling when expressed in MCF-10A cells.

Results

RON exhibits MSP-independent tyrosine phosphorylation in MCF-10A cells

Although MSP is the only known ligand for RON, RON may not require MSP to induce some of its oncogenic effects since RON contributed to cancer progression even in the absence of its MSP and its proteolytic activator, MT-SP1 (Welm *et al.*, 2007). I detected MSP-independent tyrosine phosphorylation of RON with several different phospho-specific antibodies (Figures 2.1a, 2.1b). RON was immune-

precipitated with a RON-specific monoclonal antibody, and total phospho-tyrosine was detected using the anti-phospho-tyrosine antibody, 4G10 (Figure 2.1a). With a similar strategy, RON phosphorylation was also detected by antibodies directed specifically against tyrosine phosphorylated residues in the catalytic site (Y1238/1239) and on C-terminal docking sites (Y1353 and Y1360) of RON (Figure 2.1b). Since phosphorylation of these residues has been shown to lead to signaling by RON, this data suggests that RON may transduce downstream signals in the absence of MSP. RON also exhibited increased phosphorylation on Y1238/1239 and on the Y1360 docking site in the presence of exogenously added MSP (Figure 2.1b). There was no significant increase of phosphorylation on Y1353 with the addition of MSP.

RT-PCR analysis indicated that neither the 10A/Vector nor the 10A/RON cells endogenously express MSP (Figure 2.1c), indicating that the activation of RON is not due to an autocrine signaling loop unique to MCF-10A cells. Similar levels of RON expression in NIH-3T3 cells did not lead to tyrosine phosphorylation of RON in the absence of ligand (Figure 2.2a), indicating that the RON receptor is not generally constitutively active, but rather that the activation seen in MCF-10A cells is a cell-type specific effect. MSP-independent tyrosine phosphorylation of RON is not unique to MCF-10A cells, as I can recapitulate the MSP-independent activation of RON by expressing RON in HeLa cells (Figure 2.2b). Further, I found that a kinase-dead mutant of RON, K1114M (Danilkovitch-Miagkova et al., 2000), did not exhibit any tyrosine phosphorylation in HeLa cells (Figure 2.2b), providing evidence that the kinase activity of RON is necessary for phosphorylation.

MSP-stimulation of RON increases activation of MAPK and AKT but has no effect on the phosphorylation of Src or FAK

I considered possible molecular differences between MSP-dependent and MSP-independent RON signaling in MCF-10A cells by looking at the activation of MAPK (Erk 1 & 2) and AKT, which are both known to be important for MSP-driven activation of RON in other cell systems. In the absence of MSP, RON promoted a low level of MAPK activation and a barely detectable AKT activation; however, MSP significantly increased the activation of both MAPK and AKT (Figure 2.3). Occasionally, activated AKT is detected in 10A/RON cells unstimulated with MSP when the Western blots are overexposed, but it is difficult to detect this with consistency, as the antibody is very weak. With MSP-stimulation, RON interacts with the Src kinase in other cell types (Danilkovitch-Miagkova et al., 2000). Src activation can be monitored by determining if Y418 is phosphorylated. To determine whether RON expression contributed to Src activation in MCF-10A cells, I measured the level of actived Src with pY418-specific antibodies in whole-cell lysates from 10A/Vector and 10A/RON cells. 10A/RON cells exhibited higher constitutive phosphorylation of Src on Y418 compared to 10A/Vector cells, and the addition of MSP did not further increase the activation of Src in either cell type (Figure 2.4a). Since RON has an established role in cell migration, and 10A/RON cells exhibit MSP-independent activation of Src, which has a known role in focal adhesion kinase (FAK) signaling, I considered whether a molecular distinction could be made in FAK phosphorylation between 10A/Vector and 10A/RON cells. FAK was immune-

precipitated from cell lysates with an anti-FAK monoclonal antibody, and tyrosine phosphorylation of FAK was detected using a phospho-tyrosine antibody. These data demonstrated that in 10A/RON cells, FAK, like Src, was constitutively phosphorylated, which was not increased by the addition of MSP. However, 10A/Vector cells also exhibited constitutive activation of FAK in the presence or absence of MSP (Figure 2.4b), implying that Src activation in 10A/RON cells is downstream of a FAK-independent pathway.

Tyrosine-phosphorylation of RON is Src-dependent

Ligand-independent activation of RTKs can occur as a result of interaction with other cell surface receptors or other intracellular kinases. I considered whether the intracellular Src kinase was required for phosphorylation of RON in 10A/RON cells for four reasons: wild-type Src is frequently upregulated in breast cancers (Ottenhoff-Kalff *et al.*, 1992), it is known to enhance RTK signals in cancer (Tice *et al.*, 1999), Src was previously implicated in signaling by RON (Danilkovitch-Miagkova et al., 2000) and 10A/RON cells exhibited MSP-independent Src activation (Figure 2.4a). When Src activity was blocked with the inhibitor PP2, I found that both MSP-dependent and MSP-independent phosphorylation of RON was reduced, although in the presence of MSP, substantially more tyrosine phosphorylation of RON remained after PP2 treatment (Figure 2.5a). These data suggest that MSP-stimulation of RON may promote the activation of additional Src-independent

pathways compared to the pathways activated by MSP-independent signaling of RON.

To extend these observations, I tested the effect of a dominant-negative mutant of Src, (in which the Src active site lysine was mutated to arginine), on the tyrosine phosphorylation of RON in HeLa cells. Both MSP-dependent and MSP-independent phosphorylation of RON were blocked when RON was co-transfected with the dominant-negative Src construct (Figure 2.5b). The discrepancy between the PP2 and DN-Src data regarding the MSP-dependent phosphorylation of RON might be explained by noting that a dose-dependent relationship exists between the concentration of PP2 and the phosphorylation status of RON or that the effect is cell-type specific (data not shown). Taken together, these data imply that Src family kinases play a general role in the tyrosine phosphorylation of RON in epithelial cells, but it is particularly important for signaling in the absence of MSP.

Discussion

My data clearly demonstrate that expression of RON in MCF-10A cells leads to MSP-independent signaling mechanisms to activate both Src and the MAPK cascade. Although I demonstrate that MCF-10A cells do not express MSP (Figure 1.1c), I cannot rule out the possibility that RON can be activated by other, as-yet-undefined ligands. Other studies have demonstrated that ligand-independent signaling by RON leads to activation of the MAPK cascade in Hek 293 human

embryonic kidney cells, which also requires Src kinase activity (Wei et al., 2005). Some evidence suggests that autophosphorylation of RON may provide docking sites for Src (Correll *et al.*, 2006), but my data implies that Src is acting upstream of RON, since blocking Src with PP2 abrogates the MSP-independent RON phosphorylation and reduces MSP-dependent phosphorylation of RON. It will be pertinent to determine what initially contributes to the activation of Src in 10A/RON cells, but not in 10A/Vector cells.

It is possible that RON interacts with other signaling receptors to induce activation of Src. For example, MCF-10A cells most likely exhibit activation of integrins, as these cells have been shown to secrete laminin 5 ((Stahl *et al.*, 1997) and my own observations), which is a component of the ECM that binds to β1 integrins (Stupack, 2007). β1 integrin activation of RON has been shown to contribute to MSP-independent tyrosine phosphorylation of RON (Danilkovitch-Miagkova et al., 2000) and integrin signaling promotes Src activation as well (Playford & Schaller, 2004). In other cell systems, RON can interact with RTKs, such as EGFR and c-Met (Follenzi et al., 2000; Peace et al., 2003), both of which are expressed in MCF-10A cells. However, I found no evidence in my studies that RON can interact with EGFR or c-Met in MCF-10A cells and cross-activation of RON does not occur with the addition of either EGF or HGF (data not shown). However, MCF-10A cells also express other RTKs, such as type I insulin-like growth factor receptor (IGF1R) (Zhang *et al.*, 2007), and in fact, these cells also depend on the addition of insulin for efficient growth in cell culture. Furthermore, RON has been described to interact

with the hyaluronan receptors, RHAMM and CD44 in the presence of MSP (Manzanares et al., 2007; Matzke et al., 2005), therefore, RHAMM and CD44 may also represent potential interacting partners. My data regarding hyaluronan in MCF-10A cells will be discussed in Chapter 4.

Addition of MSP to 10A/RON cells provides increased activation of the MAPK and PI3K pathways above the levels seen in the absence of MSP, and it would be of interest to learn mechanistic differences between signaling that requires MSP and signaling that does not require MSP. It is not known whether the addition of MSP leads to recruitment of a different array of downstream effector molecules than is seen in the absence of MSP. MSP-stimulation of RON induces conformational changes (Yokoyama *et al.*, 2005) that could potentially expose alternative residues to activate distinct pathways, or alter the signal intensity and duration. MSP-activation of RON increased phosphorylation of the docking site Y1360 as well as Y1238/1239 in the catalytic domain (Figure 2.1b), implying that some structural changes do occur in the presence of MSP. In the mouse RON receptor, docking site tyrosines of RON were required for MSP-independent activation of MAPK, but were dispensable for MSP-induced MAPK activation (Wei et al., 2005). An alternative possibility is that MSP-independent signaling only affects a subset of RON receptors within a cell, and that MSP might activate the remaining group of RON receptors, which promotes increased activation of ERK and AKT. This scenario would explain why MSP-dependent and MSP-independent tyrosine phosphorylation of RON appears to occur on the same tyrosine residues (Figure 2.1b), implying that the downstream effectors

binding to the docking site are likely the same in both cases. A future comprehensive analysis of human RON tyrosine phosphorylation sites would appear merited.

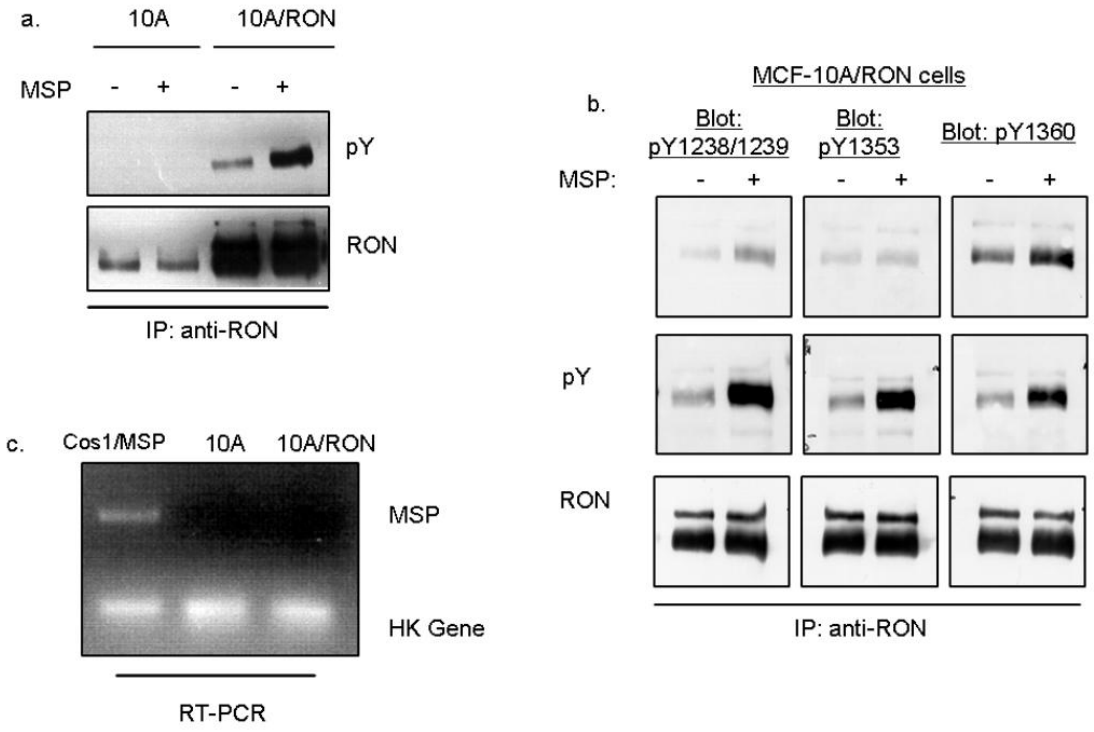

Figure 2.1

RON exhibits MSP-independent tyrosine phosphorylation in MCF-10A cells. (a) 10A/Vector and 10A/RON cells were left untreated or treated with MSP. Cell lysates were immunoprecipitated with an anti-RON antibody and Western blotted for total phospho-tyrosine and total RON expression. (b) 10A/RON cells were processed as in (a), and lysates were Western blotted for specific phospho-tyrosine residues found to be important for RON activation. Y1238/Y1239 are in the catalytic domain and Y1353 and Y1360 are found in the C-terminal docking site region of RON. (c) mRNA was collected and reverse transcribed from Cos1 cells transfected with a MSP-expressing construct, 10A/Vector and 10A/RON cells. MSP was amplified using MSP-specific primers and a general housekeeping gene was amplified to use as a loading control. I am grateful to Irene Ischenko for providing the Western blots for Figure 2.1b.

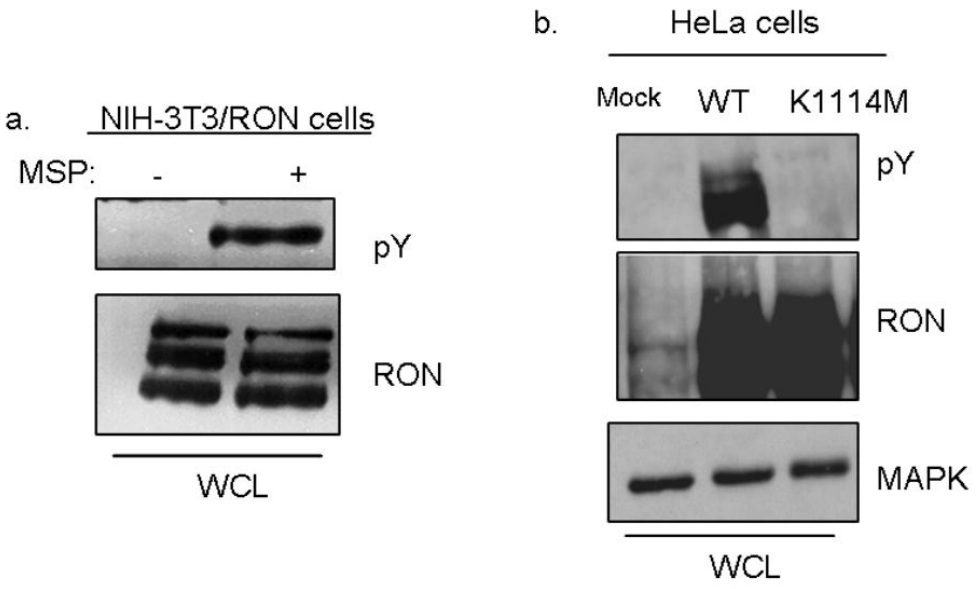

Figure 2.2

MSP-independent activation of RON is cell type specific and requires kinase activity. (a) NIH-3T3 cells were retrovirally infected to express RON, following the same protocol and constructs as used to infect MCF-10A/RON cells. Cells were left untreated or treated with MSP and total cell lysates were subjected to Western blotting for total phospho-tyrosine and total RON expression. (b) HeLa cells were transfected with an empty vector, WT-RON or a kinase-dead RON mutant (K1114M). Cell lysates were subjected to Western blot for total phospho-tyrosine, total RON and MAPK kinase expression as a loading control

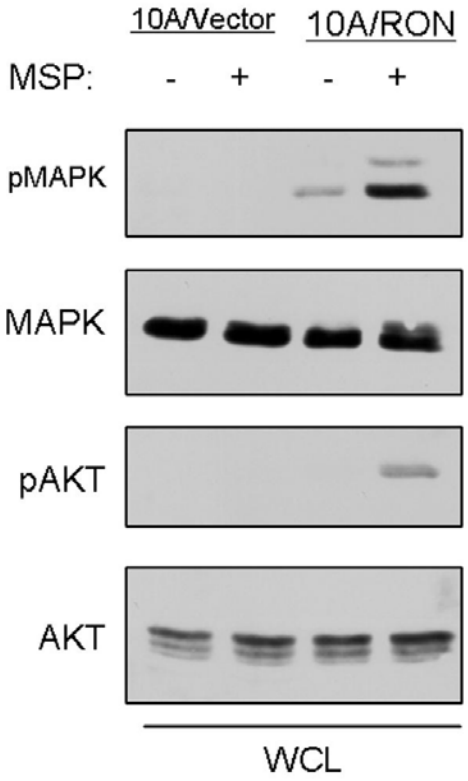

Figure 2.3

RON-mediated signaling in MCF-10A cells. 10A/Vector and 10A/RON cells were serum-starved for 3 hours before being stimulated with 100ng/ml MSP for 30 minutes. Lysates were prepared and subjected to Western blot for phospho-MAPK (Erk1 & 2), MAPK, phospho-AKT (Phospho-serine 473) and total AKT.

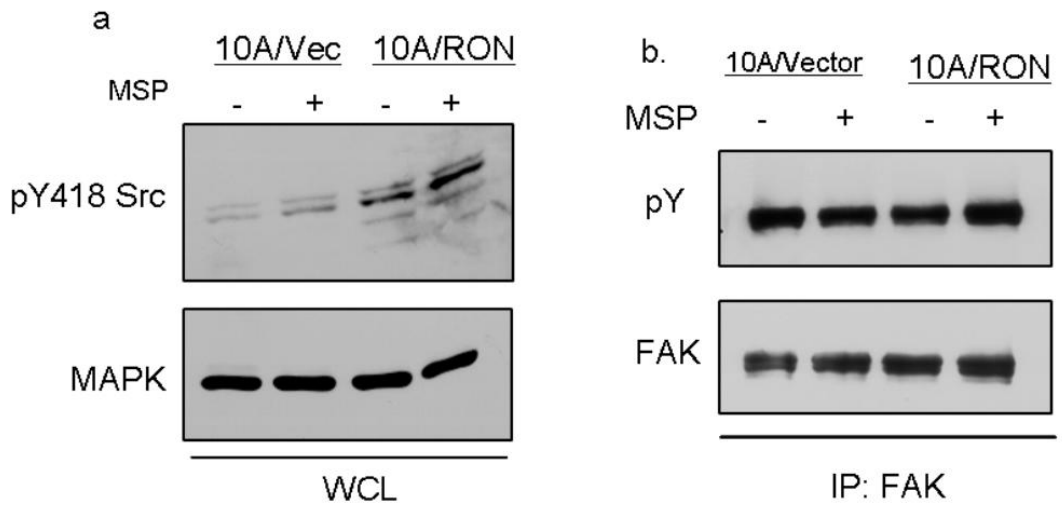

Figure 2.4

Activation of Src Kinase and Focal Adhesion Kinase in 10A cells. (a) Whole cell lysates of 10A/Vector and 10A/RON were Western blotted for Phospho-Src (pY418) and MAPK as a loading control. (b) FAK was immunoprecipitated from 10A/Vector and 10A/RON cell lysates. Total phosphotyrosine and total FAK were detected by Western blot. I am grateful to Irene Ischenko for providing the Wester blots shown in Figure 2.4b.

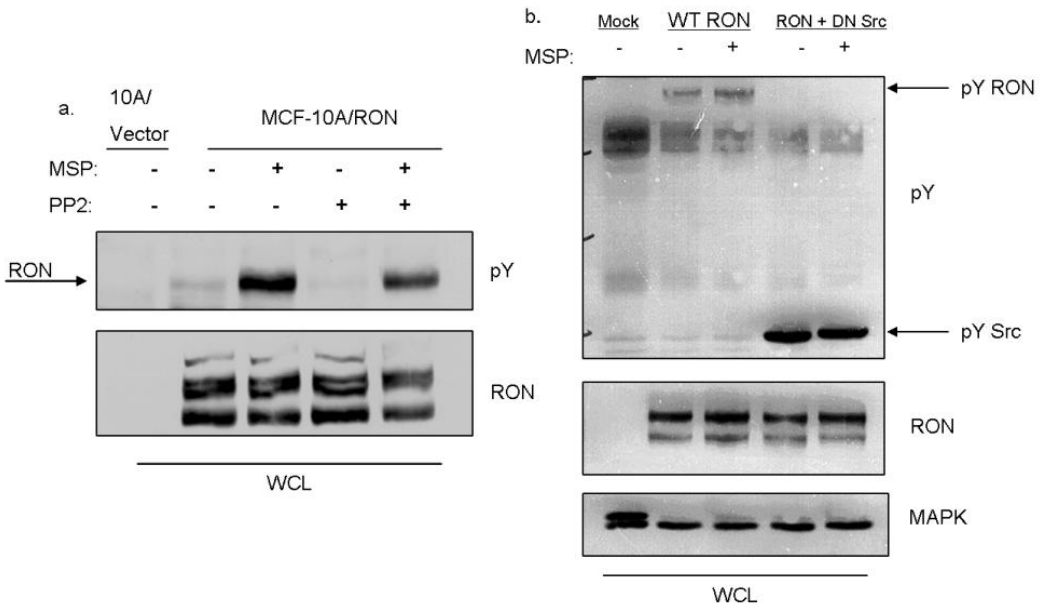

Figure 2.5

Src Kinase activity is required for MSP-independent tyrosine phosphorylation of RON. (a) 10A/RON cells were left untreated, treated with MSP or PP2 or both, as indicated. See Materials and Methods for details. Untreated 10A/Vector cell lysates were used as a negative control for the position of RON. Lysates were Western blotted for total phospho-tyrosine and RON. The arrow indicates the position of RON. (b) HeLa cells were mock transfected or transfected with WT-RON alone or WT-RON with a dominant negative Src construct. Cells were left untreated or treated with MSP. Lysates were Western blotted for total phospho-tyrosine, RON and MAPK. The arrows point out the positions of RON and Src.

Chapter 3

MSP-dependent and MSP-independent activation of RON give rise to distinct biological consequences some of which require Src kinase activity

Introduction

Several biological events can occur that contribute to the development of cancer, which have been described as the "hallmarks of cancer" (Hanahan & Weinberg, 2000). Some of these events include increased rates of proliferation, survival, adhesion, migration and invasion (Hanahan & Weinberg, 2000). Previous studies have indicated that RON may play a role in all of these processes (see "Introduction" for details). However, it is not clear whether RON can promote these behaviors in the absence of MSP. The evidence that RON can contribute to biological outcomes in the absence of MSP is increasing. For example, in primary erythroid progenitor cells, MSP-independent RON activation promotes an increase in colony growth, and this growth requires the kinase activity of RON (Wei et al., 2005). In addition to RON, the Src kinase also plays a role in signaling that contributes to the hallmarks of cancer due to its involvement in critical downstream pathways, such as

MAPK and FAK. Src enhances the signaling of several kinases which leads to an increase in proliferation, survival, adhesion and migration in a variety of cell systems (Ishizawar & Parsons, 2004).

Since RON is able to promote MSP-independent invasive behavior in 3-D ECM cultures (see Chapter 1) and RON exhibits tyrosine phosphorylation in the absence of MSP (see Chapter 2), I hypothesized that RON may stimulate cancer-promoting behaviors in MCF-10A cells also in the absence of MSP. I further predicted that the Src kinase would be required for all RON-mediated events that occurred in the absence of MSP because of its critical role in MSP-independent tyrosine phosphorylation of RON. This chapter describes the results from these cell-based biological studies, which strongly supports my hypotheses.

Results

RON-mediated proliferation requires MSP

MCF-10A cells require the addition of EGF to proliferate, therefore, by omitting EGF from the regular growth media, I could determine whether 10A/RON cells could proliferate both in the presence and absence of MSP. Approximately 12% of 10A/RON cells were BrdU positive when cultured without the addition of MSP or EGF compared to approximately 7% BrdU incorporation in 10A/Vector cells (Figure 3.1a). In contrast, the addition of MSP to the media of 10A/RON cells led to approximately 30% BrdU incorporation whereas the 10A/Vector cells did not

proliferate in response to MSP (Figure 3.1a). With the addition of EGF, 35% of both 10A/Vector and 10A/RON cells were BrdU positive. These data indicate that MSP-independent activation of RON is not sufficient for cell proliferation however, due to the increased expression level of RON, MSP can act as a mitogen in 10A/RON cells. Furthermore, the MSP-independent activation of RON did not appear to promote additive proliferative effects with EGFR stimulation as these cells proliferate approximately the same as 10A/Vector cells.

RON-mediated migration requires MSP

Although MSP-stimulation of RON can increase epithelial cell migration, it is not clear whether RON-mediated migration can occur in the absence of MSP. Using Transwell filter-based migration assays, I confirmed that MSP induced migration in 10A/RON cells, but in the absence of MSP, 10A/RON cells did not significantly migrate through the filter (Figure 3.1b,c). I concluded that MSP-independent RON activation is not sufficient to promote cell migration in the absence of basally-added growth factors.

Both 10A/Vector and 10A/RON cells migrated in response to EGF; however, at 6 hours after EGF addition, approximately 50% more 10A/RON cells had migrated than 10A/Vector cells (Figure 3.1c). A similar effect was seen 18 hours after adding EGF, when approximately 27% more of 10A/RON cells migrated through the filter than 10A/Vector cells (Figure 3.1b). To determine whether the increased migratory potential of 10A/RON cells was an EGF-specific phenomenon, I repeated the assay

with the addition of the c-Met ligand, hepatocyte growth factor (HGF), since MCF-10A cells express endogenous levels of Met (Montesano *et al.*, 1998). In this case, approximately 30% more 10A/RON cells migrated in response to HGF than 10A/Vector cells after 6 hours and approximately 60% more 10A/RON cells migrated when cells were examined after 18 hours of HGF treatment (Figure 3.1b). These data suggest that although the MSP-independent activation of RON is not sufficient to promote migration on its own, the expression of RON in MCF-10A cells confers an intrinsic migratory advantage of these cells compared to 10A/Vector cells in response to EGF or HGF.

RON-mediated cell spreading is MSP-independent

During the process of cell migration, cells must first adhere to the substratum and then spread before extending lamellipodia to propel them forward (Yamaguchi *et al.*, 2005). I hypothesized that if 10A/RON cells had an ability to adhere and/or spread more efficiently, it would explain the MSP-independent migratory advantage over the 10A/Vector cells. To test this possibility, I allowed the 10A/Vector and 10A/RON cells suspended in DMEM to briefly attach to poly-d-lysine-coated coverslips. After 10 minutes the unattached cells were washed away and the remaining cells were fixed and stained with rhodamine-conjugated phalloidin to visualize the actin filaments. Attached cells exhibited two phenotypes: they were either columnar in shape, which indicated a very early point in the cell attachment process, or the cells were flattened and spread, representing a more advanced stage in the sequence of adhesion events

(Figure 3.2a-c). When measured, the columnar cells were approximately 15-20 microns in diameter or less, whereas spreading cells ranged from 20-40 microns in diameter (data not shown). I quantified the difference between 10A/Vector and 10A/RON cells, and found that approximately 60% of 10A/RON cells exhibited a flattened appearance within 10 minutes, compared with approximately 20% spreading of the 10A/Vector cells (Figure 3.2c). The addition of MSP to this assay did not increase the percentage of spreading 10A/RON cells (Figure 3.2c).

The difference in cell attachment and spreading was also evident between 10A/Vector and 10A/RON cells when newly trypsinized cells were suspended in regular growth media and added to tissue culture plates. In this assay, unbound cells were washed away and the remaining attached cells were imaged at 30 minutes and 150 minutes post-plating (Figure 4.1). These data demonstrate that 10A/RON cells have acquired an ability to attach and spread more efficiently than 10A/Vector cells.

RON-mediated evasion of cell death is MSP-independent

MSP stimulation of RON has been associated with anti-apoptotic activity in epithelial cells (Danilkovitch *et al.*, 2000). To examine whether RON can protect cells from cell death in the absence of MSP, I used a cell death assay in which I incubated cells in DMEM without added growth factors or serum for 72 hours. Floating cells were collected from the media and pooled with the remaining adherent cells after they were removed from the plate by trypsinization. Dead cells were identified by propidium iodide labeling and the percentage of live cellsl was assessed

by FACS analysis. As seen in Figure 3.3a, only 7% of the 10A/Vector cells survived under these conditions in contrast to 31% of the 10A/RON cells. MSP addition minimally increased the survival rate to 34%. These data imply that MSP-independent activation of RON improves 10A/RON cell survival in the absence of growth factors, and that MSP does not significantly contribute to this effect. Very few cells died when cultured in regular media for 3 days (Figure 3.4a). Figure 3.3b presents the phenotypic difference between 10A/Vector and 10A/RON cells when cultured without EGF. After 4 days, most of the 10A/Vector cells were rounded-up, floating in the media, and stained positive for propidium iodide whereas the 10A/RON cells remained strongly adherent and began to proliferate again when growth factors were added to the media (data not shown). The 10A/Vector and 10A/RON cells undergoing cell death in my survival assay appear to be dying by apoptosis, as poly (ADP-ribose) polymerase (PARP) is cleaved in these cells (Figure 3.4b). It was difficult to collect enough 10A/Vector cells for analysis, which is why it is not possible to easily detect the cleaved form of PARP in the 10A/Vector cells. There is a very faint band representing cleaved PARP in the 10A/Vector cells, but it is difficult to see in this figure. It is clear, however, that PARP is cleaved in the dying 10A/RON cells from this data.

Src is required for RON-mediated MSP-independent biological effects

Because tyrosine phosphorylation of RON depends on the activity of Src family kinases (Figure 2.5 a,b), I examined whether Src signaling is required for

RON-mediated biological effects. I found that PP2 nearly abolished the increased survival of 10A/RON cells under serum-starved conditions regardless of whether MSP was added (Figure 3.3a). PP2 treatment had no effect on 10A/Vector and 10A/RON cells cultured in regular growth media for 72 hours (Figure 3.4a). RON-mediated cell spreading was also completely abrogated by PP2 in both the absence and presence of MSP (Figure 3.5a, b). Src activity was not necessary for cell proliferation in response to EGF or MSP, as PP2 treatment did not influence BrdU incorporation (data not shown). However, PP2 abolished cell migration in response to either MSP or EGF (data not shown), which could potentially be due to the effect of PP2 on cell spreading. These data imply the activity of Src family kinases is required for RON-mediated MSP-independent events, but it remains unclear if all MSP-mediated events also depend on Src.

Effects of kinase inhibitors on RON-mediated biological effects

I also determined the influence of PI3K/AKT (LY294002) and MEK/ERK (U0126) inhibitors on RON-mediated biological effects. In contrast to PP2 these inhibitors had no effects on cell spreading either with or without MSP. These two inhibitors did, however, completely abrogate the cell survival properties of RON and reduced MSP-induced Transwell migration by 60-80% (data not shown).

Discussion

My study describes a novel and significant role for MSP-independent RON signaling in cell adhesion, spreading and evasion of cell death. I found that increased expression of RON in breast epithelial cells leads to both MSP-dependent and MSP-independent biological events. In the absence of MSP, RON promoted an increase in cell spreading, cell survival and enhanced growth-factor mediated migration in MCF-10A cells. MSP was required, however, to induce RON-mediated migration and proliferation, implying that RON possesses at least two distinct modes of signaling. Furthermore, I found that Src family kinases contribute to the activity of RON in both the presence and absence of MSP.

Ligand-independent activation of non-mutated RTKs is commonly due to increased expression that allows unregulated receptor dimerization and activation, promoting tumor development (Weiss *et al.*, 1997). In epithelial cells that express high amounts of c-Met, cell attachment mediates ligand-independent tyrosine phosphorylation of c-Met (Wang *et al.*, 2001; Wang, R *et al.*, 1996). In my study, I did not find that MSP-independent tyrosine phosphorylation of RON was a direct result of cell attachment (data not shown). However, I found that 10A/RON cells exhibited a significant increase in cell attachment and spreading compared to 10A/Vector cells, suggesting that cell adhesion may generally regulate the scatter factor receptors.

I hypothesize that the increased adhesive and spreading property of 10A/RON cells explains their enhanced migratory response toward EGF and HGF, as well as

their ability to avoid cell death under serum starved conditions. During migration, 10A/RON cells may attach to the filter and spread out more quickly than 10A/Vector cells, allowing for more efficient movement through the pores. Cell adhesion itself activates survival pathways via integrins or other cell surface receptors (Hofmann *et al.*, 2007; Kang *et al.*, 2007; Muller *et al.*, 2008). The ability of cells to evade intrinsic cell death pathways is a major contributor to tumor development (Mehlen & Puisieux, 2006). Even at the lowest level of RON expression, at least 30% of 10A/RON cells survived serum-free conditions compared to almost no survival of the 10A/Vector cells. It is not known whether the adhesion effects are mediated by interactions of RON with receptors, such as integrins. Although RON is known to interact with β1 integrins (Danilkovitch-Miagkova et al., 2000; Santoro *et al.*, 2003), I could not determine if 10A/RON cells bound more strongly to laminin 5 than did 10A/Vector cells (data not shown).

Src mediates a diverse range of biological processes downstream of RTKs, such as cell survival (Yamamoto *et al.*, 2006), proliferation (Riggins *et al.*, 2006), adhesion and migration (Van Slambrouck *et al.*, 2007). Furthermore, RON signals via Src downstream of integrins (Danilkovitch-Miagkova et al., 2000) and Src was responsible for RON-mediated constitutive activation of MAPK in the absence of MSP (Wei *et al.*, 2005). My data confirms and extends the observations that Src family kinases play a significant role in both MSP-dependent and MSP-independent RON signaling. I found that inhibition of Src family kinases reduced tyrosine phosphorylation of RON and abrogated RON-mediated biological events, with the

exception of cell proliferation, implying that RON utilizes a Src-independent pathway to mediate proliferation in MCF-10A cells. In the case of EGFR, Src mediates EGF-independent transactivation, which promotes differential biological effects than EGF-stimulation of EGFR (Moro *et al.*, 2002; Wu *et al.*, 2002) This is similar to the observations reported here on the MSP-independent activation of RON.

In the presence of MSP, RON activates the MAPK and PI3K pathways ((Danilkovitch-Miagkova, 2003) and Chapter 2, Figure 2.3). I found that MSP-independent signaling by RON also activates MAPK (Chapter 2, Figure 2.3), however, the reduced level of stimulation was not sufficient for RON-mediated proliferation or migration. To examine the role of these pathways further I used the inhibitors LY294002 to block PI3K/AKT or U0126 to block MEK/ERK in biological assays. These pathways were clearly not involved in cell-spreading but did play a role in MSP-dependent and -independent cell survival and also in MSP-induced migration. In addition to MAPK and AKT, there is also the potential that MSP regulates additional or alternative signaling pathways to MSP-independent signaling.

RON activation in cancer is thought to be a late stage event, due to its roles in migration, invasion and development of EMT in tissue culture (Cote *et al.*, 2007; Wang *et al.*, 2004). I propose that in early stage breast cancers, co-expression of Src and RON is sufficient to initiate oncogenic behavior in the absence of MSP. In support of this hypothesis, one recent study concluded that co-expression of RON, MSP and the MSP-activating enzyme (MT-SP1) was a strong prognostic marker for a poor patient survival; however, less than 20% of the samples actually exhibited

expression of all three mRNAs (Welm *et al.*, 2007). Nonetheless, clinical studies have shown that RON expression alone is predictive for a poor patient prognosis in breast cancers (Lee et al., 2005). Taken together, these data offer two major implications. First is that in the majority of RON-expressing breast cancers, RON contributes to oncogenicity in the absence of activated MSP. The second implication is that RON expression and its ligand-independent signaling in breast cancer are early events, and that the later stage disease is characterized by further activation of MSP and MT-SP1.

Nearly all cancer deaths stem from metastatic growths rather than the primary tumors (Mehlen & Puisieux, 2006), and therefore, it is critical to design cancer therapies that target events occurring in early stages of cancer to prevent secondary growths. Several therapeutic strategies have recently been developed to target RTKs in human cancers. These agents are either small molecule inhibitors that block RTK kinase activity or monoclonal antibodies that bind to the RTK and inhibit its signaling (Tagliaferri *et al.*, 2005). Both of these molecular approaches have proven to be clinically successful in many cases, however, strategies targeting the extracellular ligand-binding domain of RTKs may not be successful in treating patients whose tumors exhibit signaling in the absence of ligand. I provide evidence that in the case of RON-expressing mammary tumors, targeting Src in addition to RON could potentially reduce oncogenic behaviors.

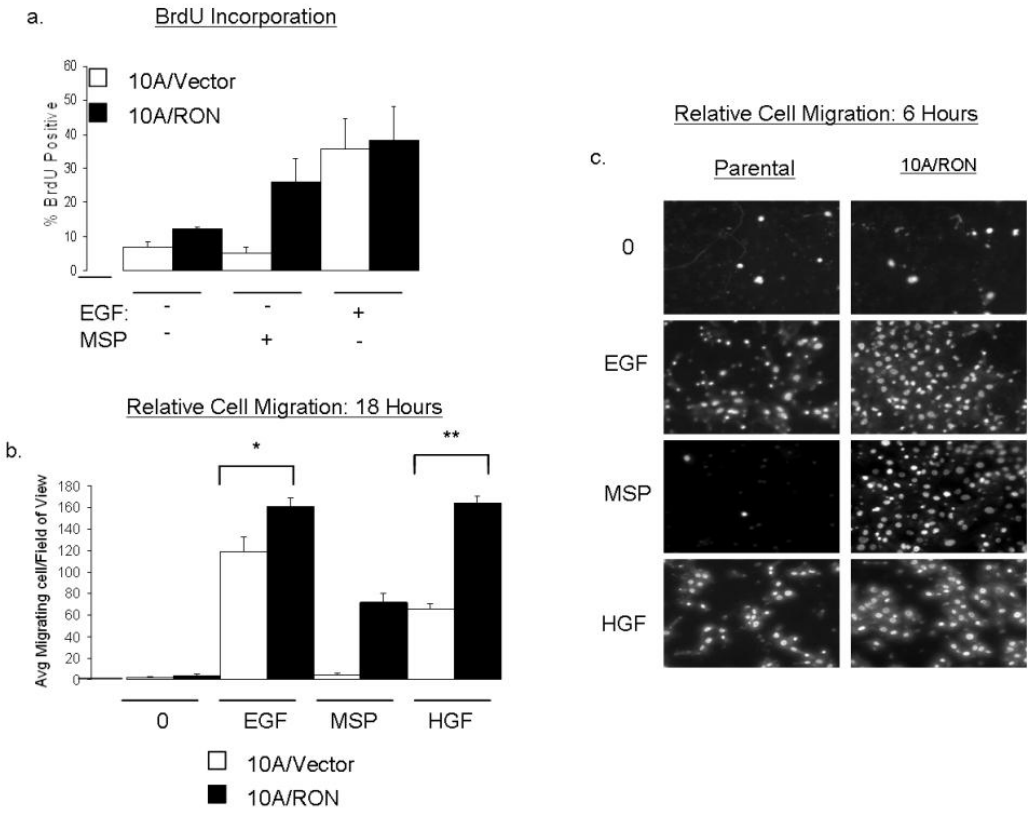

Figure 3.1

RON-mediated cell proliferation and migration require MSP (a) BrdU incorporation into cells that were cultured for 48 hours in regular MCF-10A media that lacked EGF and MSP or contained either EGF or MSP. The percent BrdU-positive cells were determined by counting at least 500 cells/coverslip. (b) Analysis of the number of migrating cells in the presence or absence of various growth factors. * indicates $P<0.02$, $n=5$ ** indicates $P<0.0001$. The error bars for (a) and (b) represent + SEM. (c) Representative images of Transwell migration assay after 6 hours. Each experiment was repeated at least 3 times.

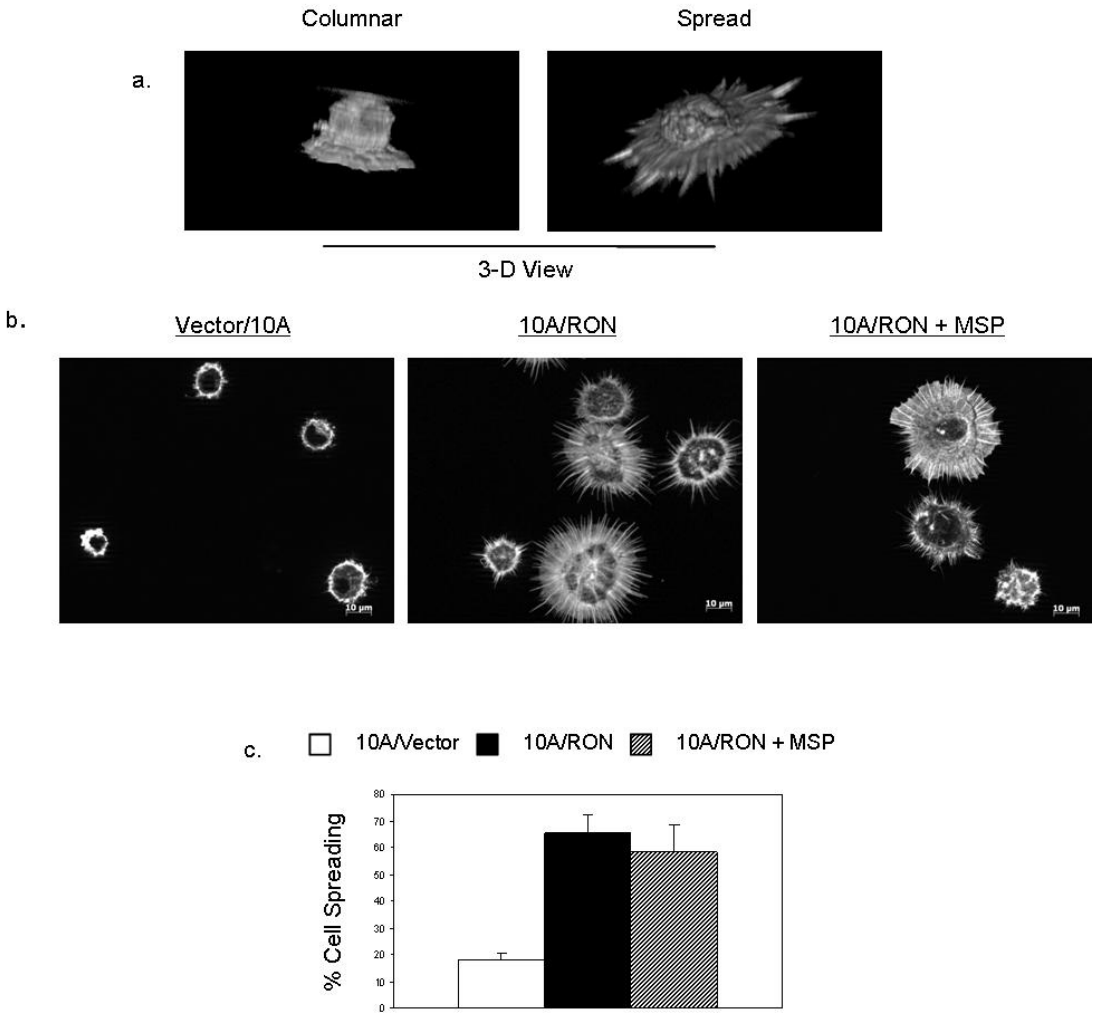

Figure 3.2

RON promotes cell spreading in the absence of MSP. (a) Cells were stained with Rhodamine-Phalloidin in a 10 minute attachment assy. 3-D rendered images from Z-stacks of the 2 basic phenotypes of adherent cells. (b) Representative 2-D images of comparing the phenotype of 10A/Vector and 10A/RON cells in the attachment assay as in (a). (c) Quantitation of the percentage of spread cells. Error bars represent + SEM, n=4, $P<0.001$.

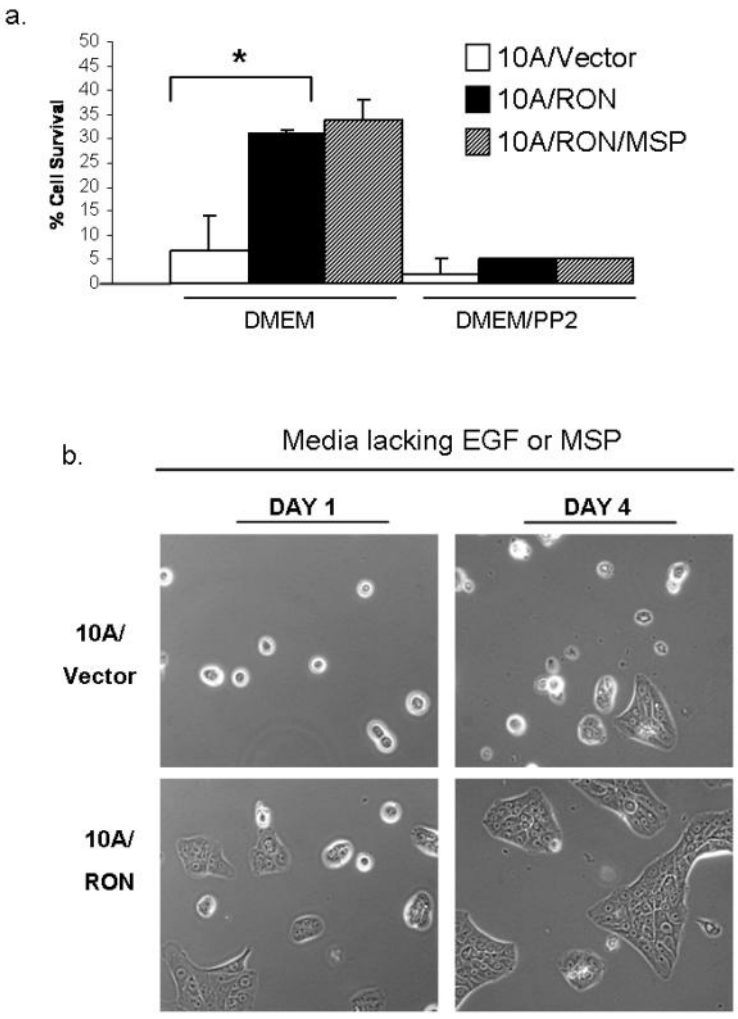

Figure 3.3

RON promotes cell survival in the absence of MSP (a) After 72 hours in serum-free media, floating and attached cells were pooled together, stained for Propidium Iodide (PI) and the percentage of PI-positive cells was determined by FACS. The % of surviving cells is shown (PI-negative cells). * indicates a $P<0.002$. Error bars represent + SEM, n=4. (b) Cells were cultured in regular MCF-10A media that lacked EGF. Phase contrast images were taken on Day 1 and Day 4 to demonstrate cell morphology

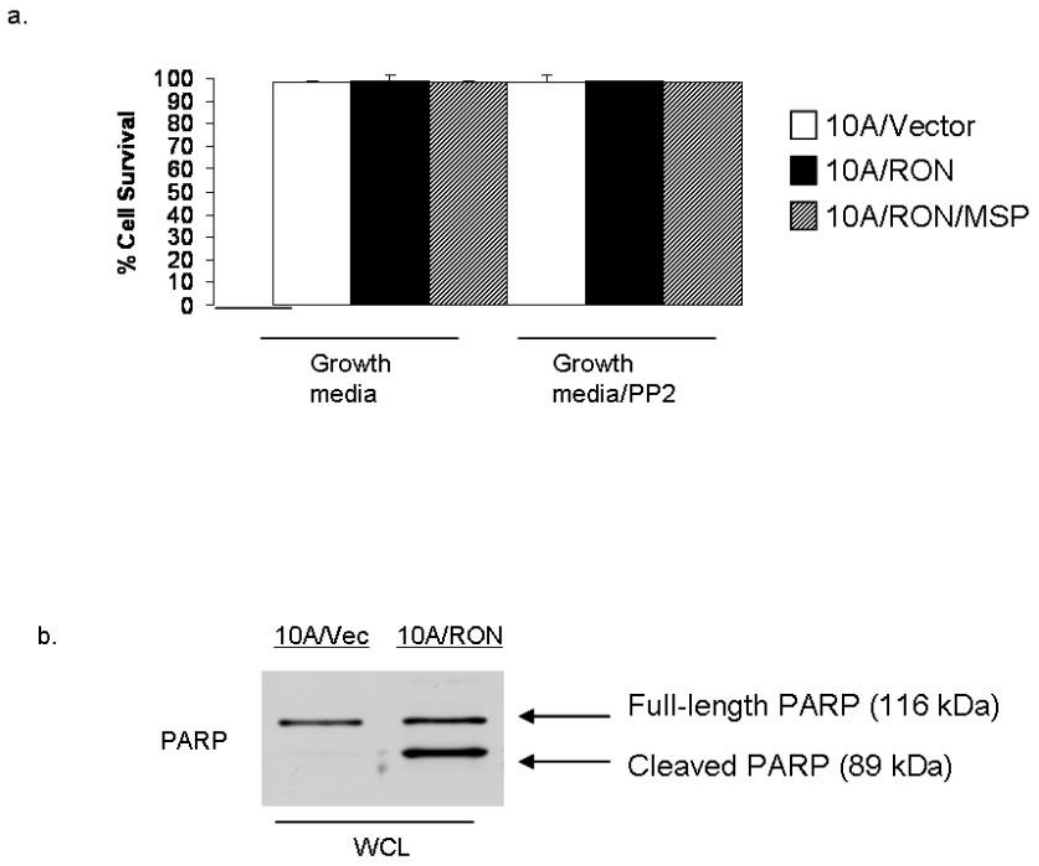

Figure 3.4

Src does not mediate survival in regular growth media and 10A cell death is apoptotic (a) Cells were cultured for 72 hours in regular growth media with and without the addition of PP2, trypsinized, stained for PI. The percentage of PI-positive cells was determined by FACS analysis. The % of surviving cells is shown (PI-negative cells). (b) After 72 hours in DMEM, floating and attached cells were collected and lysed. Shown is a Western blot analysis of PARP using whole cell lysates. The gel does not represent an equal loading of proteins, as there were significantly more 10A/RON cells available for lysis. Although it is difficult to detect, there is, in fact, a faint band seen in the 10A/Vector cells for cleaved PARP.

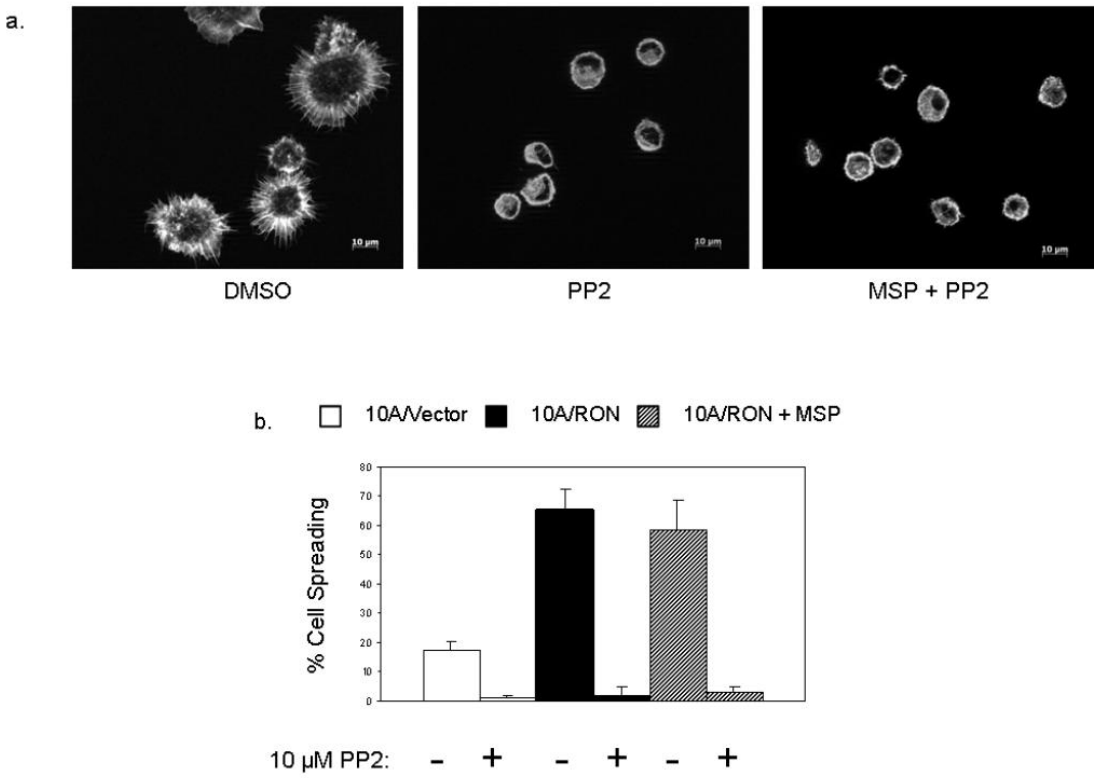

Figure 3.5

Src kinase activity is required for RON-mediated cell spreading (a) 10A/RON cells were stained with Rhodamine-Phalloidin after a 10 minute attachment assay. Cells that were incubated with or without MSP for 10 minutes, then PP2 or DMSO was added for an additional 10 minutes before attachment to the coverslip. (b) Quantitation of the percentage of spread cells in the presence or absence of PP2 treatment. Error bars represent + SEM, n=4, $P<0.001$.

Chapter 4

RON-expressing breast epithelial cells exhibit increased expression of hyaluronan, which is responsible for RON-mediated early adhesion events

Introduction

Hyaluronan (also known as Hyaluronic Acid or HA) is a highly negatively charged glycosaminoglycan consisting of glucoronic acid and N-acetylglucosamine disaccharides and is very large, ranging in size from 10^5-10^7 kDa, and up to 25 μm long (Toole, 2004). Although HA plays a structural role in several normal tissues, it is upregulated in inflammation, wound repair and cancer (Toole & Slomiany, 2008). HA is synthesized on the interior plasma membrane side of the cell surface by hyaluronan synthases (HAS 1, 2, and 3) and, at the same time, is secreted onto the cell surface and into the extracellular matrix (ECM) (Tammi *et al.*, 2002). This is in contrast to most proteoglycans, which are synthesized in the Golgi apparatus and secreted by an exocytic mechanism (Prehm, 1984). In addition to secretion by HAS proteins, new evidence supports a potential role for ABC-family transporters, in particular multi-drug resistance protein 5 (MRP5), in secreting HA into the ECM,

which suggests that HA may be involved in multi-drug resistance that can arise in invasive cancers (Toole & Slomiany, 2008).

HA is upregulated in a number of different cancer types, including breast cancers, and its expression correlated with invasive behaviors (Bertrand *et al.*, 1992; Kimata *et al.*, 1983; Toole *et al.*, 1979). One mechanism for increased HA expression in cancer is an upregulation of HAS genes, and, in fact, increased expression of HAS 2 (Kosaki *et al.*, 1999) and HAS 3 (Liu *et al.*, 2001) correlated with invasive cell behaviors in MDA-231 cells (Udabage *et al.*, 2005b). Further, the cytokine IL-1α promoted upregulation of HA in human inflammatory arthritis models (Deiters & Prehm, 2008) and stromal cells can also release paracrine factors that stimulate the production of HA (Knudson *et al.*, 1984). Similarly, extracellular matrix metalloproteinase inducer (EMMPRIN or CD147) expression in mammary carcinoma cells stimulated HA synthesis, leading to anchorage-independent growth (Marieb *et al.*, 2004).

In human cancers, it is most likely the interaction of HA with its cell surface receptors, namely CD44 and RHAMM (receptor for hyaluronan-mediated motility) that contributes to malignancy, through the increased tethering of HA to the extracellular cell surface as well as increased receptor-mediated cell signaling events (Toole, 2004). CD44 has multiple isoforms that are generated by alternative splicing to include short additional sequences in the membrane-proximal extracellular region. CD44 isoforms may exhibit distinct glycosylation patterns, which can affect signaling by recruiting different binding partners (Hirano *et al.*, 1994; Turley *et al.*, 2002).

However, CD44 standard (CD44s) is the most common variant and the most thoroughly-characterized in regards to HA binding (Aruffo *et al.*, 1990). CD44 interacts with several RTKs, including c-Met (Orian-Rousseau et al., 2002), EGFR (Kim *et al.*, 2008) and ErbB2 (Palyi-Krekk *et al.*, 2008). Through their interactions with RTKs as well as several downstream effectors, such as Src kinase, MAPK (ERK1), and the Gab 1 adaptor proteins, CD44 and RHAMM mediate cell signaling that promotes cell proliferation, survival and migration (Toole, 2004). HA binding to its receptors is associated with activation of the FAK (Fujita *et al.*, 2002) , PI3K (Misra *et al.*, 2008) and NFκB pathways (Bourguignon *et al.*, 2009). Blocking of HA binding, by soluble HA peptides (Hamilton *et al.*, 2007), antisense CD44 cDNA (Harada *et al.*, 2001), soluble CD44 (Peterson *et al.*, 2000) or soluble RHAMM protein (Mohapatra *et al.*, 1996) leads to an attenuation of oncogenic behavior, which suggests that HA might be a potential target in cancer treatment.

The RON RTK has been shown to interact with CD44 (Matzke et al., 2005) as well as with RHAMM (Manzanares *et al.*, 2007). My recent work has shown that ligand-independent signaling of RON in MCF-10A breast epithelial cells resulted in an increase in cell spreading, cell survival and cell migration compared to a parental cell line (Feres *et al.*, 2009). Although I concluded that Src-family kinases were required for these behaviors, it was not clear from my studies what might be acting upstream of Src to confer its activation in MCF-10A cells. One possibility is that RON mediates an upregulation of HA expression, which would promote Src signaling via CD44 or RHAMM.

Based on the studies of HA's effects on ErbB2 signaling, and considering that RON is known to interact with HA receptors, I examined the contribution of HA to the MSP-independent oncogenic behaviors of MCF-10A/RON (10A/RON) cells. I report here that the increased adhesive properties of 10A/RON cells can be explained by an increase in HA on the cell surface compared to MCF-10A/Vector (10A/Vector) cells. Additionally, immediate attachment is disrupted by briefly pre-treating the cells with hyaluronidase.

Results

MCF-10A/RON cells exhibit increased adhesion that is mediated by negatively-charged glycoproteins

My data implies that RON expression in MCF-10A cells promotes an increase in cell attachment. When cell suspensions were added to tissue culture dishes in regular media, significantly more 10A/RON cells were attached to the dish than 10A/Vector cells. This was evident after 30 minutes and continued at least until 150 minutes after the addition of cells (Figure 4.1). My earlier studies also indicated that 10A/RON cells exhibited increased cell adhesion to poly-d-lysine-coated coverslips within 10 minutes and rapid cell spreading ((Feres et al., 2009) and Figure 3.2b). Whereas the spreading was dependent on Src-family kinases, attachment itself did not require Src activity, as the Src inhibitor PP2 did not prevent attachment. This

prompted us to consider alternative, Src-independent mechanisms of MCF-10A cell attachment.

I hypothesized that integrins are most likely not involved in the attachment process since poly-d-lysine is reported not to stimulate integrin signaling. As mentioned earlier, MCF-10A cells have been reported to express Ln-5 (Stahl *et al.*, 1997) which could potentially stimulate β1 integrin signaling. However, my data indicate that both 10A/Vector and 10A/RON express similar amounts of Ln 5 (data not shown), so this would not explain the difference in binding capacities between these two cell types. Furthermore, I tested whether immediate attachment occurred on various ECM proteins, including fibronectin, vitronectin, laminin and collagen, which would be an integrin-dependent attachment event. However, there was no significant difference in attachment to the various substrates, either within 10 minutes or later time-points (data not shown), again suggesting that integrins are not likely to be involved in this particular type of attachment.

Instead, I considered that there could be an attraction between the positively charged lysines on the coverslips and negative charges on the cell surface. It was possible that 10A/RON cells had a greater overall negative charge on their cell surface, which could be the result of an increase in extracellularly deposited glycosaminoglycans (GAGs), such as chondroitin-sulfate proteoglycans (CSPGs) and/or hyaluronan (HA). These molecules are highly negatively charged and both are known to be involved in cell adhesion to ECM (Fuster & Esko, 2005; Toole, 2004). To test for the cell surface expression of these glycoproteins, I stained the cells with

the *Wisteria floribunda* agglutinin (WFA), which is a lectin that binds N-acetylgalactosamine residues on highly negatively charged chondroitin sulfate chains found on some proteoglycans (Young & Williams, 1985). To determine whether there is a change in either expression levels or distribution between 10A/Vector and 10A/RON cells, I allowed the cells to attach to poly-d-lysine-coated coverslips for 10 minutes, washed away unbound cells and labeled the CSPGs on the cell surface with biotinylated WFA which was then fluorescently labeled with Cy3-Streptavidin. As shown in Figure 4.2a, both 10A/Vector and 10A/RON cells appeared to express similar amounts of these CSPGs on the cell surface, however it was not possible to accurately determine whether there was a quantitative difference of cell-surface CSPGs between 10A/RON and 10A/Vector cells with this assay. The CSPGs appeared to be differentially distributed depending on far the cells have spread after attachment. 10A/RON cells exhibited long, CSPG-expressing microspikes compared to 10A/Vector cells. This pattern of localization is similar to that I have shown previously for the distribution of actin after 10 minutes of attachment ((Feres et al., 2009) and Figure 3.2b). Interestingly, despite their strong negative charge, the quantity of CSPGs on the cell surface does not appear to play a direct role in cell spreading, as I noted that cells that exhibit a more dense concentration of CSPGs may not necessarily have a larger cell span (Figure 4.2b).

Hyaluronan (HA) forms the extracellular framework to which other GAGs, including CSPGs, are structurally bound. When HA is cleaved into fragments by hyaluronidase (HAase) (Toole, 2004), this so-called “glue” no longer holds the GAGs

in place (Stern & Jedrzejas, 2006). To determine if the CSPGs on the cells' surface might be responsible for the increased binding of 10A/RON cells, I repeated the attachment assay in the presence of HAase, which would remove the extracellular CSPGs in addition to any HA. Pre-incubation with 300 U/ml of HAase for 10 minutes before allowing the cells to attach to the coverslip for 10 minutes nearly completely abrogated attachment in both 10A/Vector and 10A/RON cells (Figure 4.2c), implying that HA and/or other GAGs in the extracellular matrix are required to mediate the early attachment of 10A cells. Titration of the HAase down to 100 U/ml significantly reduced binding of both 10A/Vector and 10A/RON cells, however, there were significantly more 10A/RON cells attached than 10A/Vector cells (data not shown), further implying that 10A/RON cells exhibit increased adhesion, which might be mediated by an abundance of HA on the cell surface.

10A/RON cells exhibit an increase in HA on the cell surface

Since HAase abrogated attachment of 10A cells, I suspected that in addition to the CSPGs, it was possible that 10A/RON cells produced more HA than 10A/Vector cells. Increased HA would allow for faster anchoring of the cells onto positively charged lysine residues. To compare HA expression between 10A/Vector and 10A/RON cells, I cultured cells in regular MCF-10A media (Debnath *et al.*, 2003) for approximately 48 hours. HA was labeled using biotinylated HA Binding Protein (bHABP) and bHABP was detected with Cy3-Streptavidin. As shown in Figure 4.3, both cell types are positive for HA-staining but 10A/RON cells exhibited an increase

in HA over their cell surface compared to 10A/Vector cells. This can be seen more strongly in Figure 4.3b, which are 2-D images of bHABP staining. In the 10A/Vector cells, the nuclei are highly visible, whereas in the 10A/RON cells, the nuclei are less visible presumably because there is more HA on the cell surface to cover the nuclei. It is possible however, that rather than an increase in total HA protein on the cell surface, the 10A/RON cells may exhibit a change in HA distribution. However, the intracellular stores of HA appeared to be similar in both amount and distribution between the 10A/Vector and 10A/RON cells (Figure 4.3a). Treatment of live cells with 300 U/ml HAase for 1 hour before HABP labeling abrogated nearly all of the extracellular HA, but left behind the intracellular HA, which could potentially be endocytic vesicles containing internalized HA during its receptor recycling process (Toole & Slomiany, 2008). The roles for intracellular stores of HA are unclear, and are currently under investigation (Hascall *et al.*, 2004).

In addition to the increase of HA production of 10A/RON cells, HABP labeling was useful to see a difference in cell morphology on 2-D plastic dishes. When cells were cultured at low densities, and allowed to form islands of cells over the course of 72 hours, the 10A/Vector cells appeared to remain tightly bound with HA expressed around the perimeter of the island of cells, whereas 10A/RON cells did not appear to be tightly bounded by an outline of HA, seemed larger or more spread, often had spaces between each cell and frequently exhibited a display of long extensions between cells (Figure 4.3b and data not shown).

To determine if HA forms microspikes after immediate adhesion in the same pattern as CSPGs and actin, I repeated the 10 minute attachment assay with labeling for bHABP/Cy3. I found that HA was not found in microspike structures, however, it was again clear that the 10A/RON cells were secreting/depositing more HA than 10A/Vector cells onto their cell surfaces (Figure 4.4).

10A/RON cells do not exhibit increased HAS gene expression

Since 10A/RON cells appear to have more HA over their cell surface than 10A/Vector cells, I hypothesized that 10A/RON cells might exhibit an increased level of HAS gene expression to promote larger amounts of HA secretion at the cell membrane. To this end, I used reverse-transcriptase PCR (RT-PCR) to detect HAS 1, HAS 2 and HAS 3 genes in 10A/Vector and 10A/RON cells. For comparison, I looked at the levels of HAS genes in the invasive breast cancer cell line, MDA-231, which is known to exhibit increased expression of HAS 2 (Udabage *et al.*, 2005b) and is thought to play a role in the invasive behaviors of these cells. In Figure 4.5, all three cell lines expressed similarly low levels of HAS 1 expression. As expected, MDA-231 cells expressed high levels of HAS 2 compared to both 10A cell lines, but expressed approximately the same amount of HAS 3 as 10A/Vector and 10A/RON cells. Both 10A/Vector and 10A/RON cells expressed approximately the same levels of HAS 2 and HAS 3, implying that the difference in the amount of HA expression between 10A/Vector and 10A/RON cells is not due to an over-abundance of HA synthases on the cell surface of 10A/RON cells.

MCF-10A cells exhibit expression of both CD44 and RHAMM

In order to mediate hyaluronan-driven cell signaling, cells must express either or both of the HA receptors, CD44 or RHAMM. CD44 and RHAMM also have the potential to tether HA to the cell surface, so that increased expression of these receptors may lead to an increase of HA retention on the cell surface. To examine whether 10A/Vector and 10A/RON cells express these receptors, or if they are expressed in differential amounts, I used RT-PCR to detect expression of RHAMM and the standard form of CD44. Figure 4.5 demonstrates that both 10A/Vector and 10A/RON cells expressed both RHAMM and CD44 at approximately the same level, implying that the increase of HA on 10A/RON cells is unlikely due to a greater number of receptors sequestering HA at the cell surface. However, expression of CD44 and RHAMM also suggests that MCF-10A cells are potentially capable of transducing HA-mediated signals to generate biological responses.

Involvement of Src

Since Src was found to be essential for the cell spreading of MCF-10A cells but not initial cell attachment, I examined whether Src was important for the deposition of HA in either 10A/Vector or 10A/RON cells. When cultured in the Src inhibitor, PP2, MCF-10A cells attach to the tissue culture dish, but do not spread, even after 48 hours in culture (data not shown). If HA mediated the cell spreading effect, I expected cultures containing PP2 would block expression of HA on the cell surface. However, I found that whether the cells were cultured in negative-control

DMSO-containing media or PP2-containing media, both 10A/Vector and 10A/RON cells expressed HA, as noted by staining the cells for HABP, as in the above experiments (Figure 4.6a). Since the DMSO-containing cultures had spread on the plate but the PP2-containing cultures did not, it appears that the HA has a different distribution in the presence of PP2, which is particularly evident in the profile view, in which the PP2-containing cells were much less flat (Figure 4.6b). Furthermore, if 10A/Vector and 10A/RON cells were first treated with HAase to remove cell surface HA, PP2 does not block the new secretion of HA to the cell surface (Figure 4.7). Taken together, these data suggest that Src does not mediate the expression of HA in MCF-10A cells.

Hyaluronidase reduces EGF-driven migration

As shown previously, 10A/RON cells appear to have a migration advantage over 10A/Vector cells (Figure 3.1b, c), which I hypothesized might be due to an increased adhesive property in 10A/RON cells. To determine whether HA was an important mediator of attachment during migration, I incubated 10A/Vector and 10A/RON cells in 200U/ml HAase for 1 hour prior to adding EGF to the bottom well of a Transwell insert. Cells were allowed to migrate for 6 hours, and migrated cells were fixed, stained and counted. Figure 4.8 points out that HAase dramatically reduced the number of cells migrating through the filter within 6 hours in both 10A/Vector and 10A/RON cells, implying that HA is necessary for efficient binding to the filter so that the cells can move through the pores. However, with this assay, I

were not able to determine if HAase reduced cell spreading, which would also likely abrogate cell migration

Discussion

My current study is the first that I know of to describe a correlation between an increase of RON RTK expression and an increase of HA expression in mammary epithelial cells. These results are significant because they point out a potentially novel mechanism by which RON can promote tranformed behavior in normal epithelial cells in the absence of its ligand, MSP. There is a strong correlation between the HA, HAS, and CD44 expression levels and the invasiveness of breast cancer cell lines (Udabage *et al.*, 2005a). Because of the correlation between the expression of RON and HA, it is possible that RON contributes to CD44 and/or RHAMM signaling, the two known HA receptors. The role of RON in RHAMM-mediated signaling is unknown, however, these two receptors were found to associate in primary human lung cells, and blocking the MSP-binding site on RON reduced RHAMM-mediated biological effects (Manzanares et al., 2007). No other reports of RON interaction with RHAMM have yet been reported, however, MCF-10A cells express RHAMM (Figure 4.5), allowing for the possibility of an interaction in this system.

Both RON and its other family member, c-Met, require the CD44 V6 isoform as a co-receptor to transduce their signals in HT-29 and HCT116 colon carcinoma cells (Matzke et al., 2005). In this study, phosphorylation of both c-Met and RON,

downstream activation of ERK and invasion through matrigel was blocked by anti-CD44 V6 antibodies and peptides (Matzke et al., 2005). Although I found that MCF-10A cells express CD44s, I did not gain clear data suggesting that these cells also express the V6 variant (data not shown). It is possible that RON could utilize CD44s or other isoforms as a co-receptors in certain cell types either in the presence or absence of MSP. Additionally, the co-receptor function of CD44 V6 was important in MSP-induced RON signaling, and may not be required for MSP-independent signaling of RON. Studies that block CD44 signaling in 10A/RON cells would be useful to demonstrate whether CD44 was required MSP-independent RON signaling.

I propose that HA is necessary for the early adhesion events in MCF-10A cells, but HA alone is not sufficient to induce cell spreading. Instead, my data suggests that HA mediates the initial binding of MCF-10A cells to poly-d-lysine on glass coverslips, and that because 10A/RON cells have an increased amount of HA on their cell surface, they bind more efficiently than 10A/Vector cells. HA mediates attachment to the ECM in other cell types including prostate and breast cell lines (Draffin *et al.*, 2004). Cell spreading occurs sequentially after adhesion (Zaidel-Bar *et al.*, 2004), so it is likely that the 10A/RON cells exhibit increased cell spreading because of their faster initial binding potential. Other studies have demonstrated that 2-10 minutes after HA-mediated attachment to the substratum, cells form focal adhesions and signaling occurs via integrins, which are activated by ECM proteins (Cohen *et al.*, 2006). My data likely fits this model, with MCF-10A cells potentially activating integrin signaling after HA-mediated attachment to promote cell spreading.

In addition to the baseline level of integrin signaling in 10A/Vector cells that promotes cell-ECM adhesion and spreading, it is possible that RON expression also increases the duration or intensity of integrin signaling due to possible interactions with β1 and β4 integrins in 10A/RON cells based on its known interactions with these integrins in other epithelial cells (Santoro *et al.*, 2003).

Further evidence that the signaling mechanisms differ between the initial adhesion event and subsequent spreading events is implied by the distinct requirements of Src kinase activity. In MCF-10A cells, although Src activation was necessary for cell spreading, it was dispensable for attachment (Feres et al., 2009) and Src activity not involved in the synthesis or retention of HA (Figure 4.6). Studies have demonstrated that Src plays an essential role in the integrin/FAK signaling pathway, which promotes the cell spreading and motility responses (Parsons, 2003). Once activated by integrins, FAK recruits and activates Src at the region of cell-ECM attachment (Siesser & Hanks, 2006) in order to further strengthen cell adhesion (Michael *et al.*, 2009). Src is activated downstream of CD44 (Subramaniam *et al.*, 2007) and also couples CD44 signaling to β1 integrin signaling (Lee, JL *et al.*, 2008). Src may be the link between the switch that occurs between HA/CD44-mediated attachment and integrin/FAK-mediated cell spreading. Blocking Src activation may prevent the switch from occurring, therefore preventing cell spreading and subsequent migration. However, since Src is not important for the synthesis of HA (Figure 4.6), it is also not necessary for the initial attachment event to occur.

The question of how HA is upregulated on the cell surface of 10A/RON cells remains unanswered. Increased amounts of HAS proteins can lead to an increase in HA on the cell surface (Wilkinson *et al.*, 2006). Ligand-activation of other RTKs can also promote HA synthesis, including PDGFR and EGFR (Heldin *et al.*, 1989; Heldin & Pertoft, 1993). PDGFR-stimulation of vascular smooth muscle cells leads to an upregulation of HAS 2 expression, as well as an increase of HA binding proteins that sequester HA around the cell surface and contribute to form a thick pericellular coat (Evanko *et al.*, 2001). Although it has been suggested that increased expression of HA is induced in certain cancers generally through upregulation of the HAS genes (Adamia *et al.*, 2005), I found no evidence that 10A/RON cells produced an abundance of HAS proteins (Figure 4.5). However, increased HAS gene expression may have transiently occurred at a time-point earlier than 48 hours, and I may not have detected this increase in HAS expression in the assay I used. Further, there is some evidence that HAS proteins must be proteolytically modified in order to secrete HA at the cell surface (Hascall et al., 2004), and RON expression might induce a change in the activation status of HAS isoforms rather than affect expression levels itself.

Rather than an increase in HA synthesis or modifications, there are several alternative mechanisms by which RON expression could lead to retention of HA on the cell surface, including the induction of a variety of HA binding proteins, which help to stabilize HA in the pericellular region. HA can also be anchored to the cell surface by either of its receptors (Toole, 2004). If RON exhibits an association with

either CD44 or RHAMM, the expression of RON in MCF-10A cells might induce increased retention of the HA at the cell surface by tethering it to its receptors. Furthermore, RON expression may reduce hyaluronidase (HYAL) activity, which would reduce HA turnover and maintain its expression for a longer period of time. Interestingly, HYAL 2 binds to RON in human bronchial epithelial cells and HYAL 2 may also be negatively regulated in jaagsiekte sheep retrovirus (JSRV)-transformed lung tumors that exhibit RON expression (Danilkovitch-Miagkova *et al.*, 2003). This data suggests a correlation might exist between RON and hyaluronidases.

The introduction of RON into MCF-10A cells leads to a change in cell morphology when cultured in 2-D that is not easily seen with phase contrast microscopy, however, it can be visualized when the cell surface is first labeled with HABP/Cy3-Streptavidin (Figure 4.3b). Compared to 10A/Vector cells, it is evident that the 10A/RON cells are significantly flatter and the cell-cell junctions so not appear to be as tight (Figure 4.3b). A similar situation occurs when 10A/RON cells are cultured in media lacking EGF in that cells remain much flatter and better attached to both the tissue culture plate and other cells compared to10A/Vector cells, which barely attach to the tissue culture plate at all (Figure 3.3b). I hypothesized that the change in morphology was due to an increase in adhesive properties of the cells, mediated by increased HA binding. Morphologically, my results are in conflict with a previous study that suggested that *inhibition* of HA binding in vascular smooth muscle cells with HA oligosaccarides lead to a more flattened morphology, although this could be explained by the cell type difference (Evanko *et al.*, 1999). However,

this study also showed that blocking HA with oligosaccharides in smooth muscle cells reduced cell migration in response to PDGF (Evanko *et al.*, 1999; Evanko *et al.*, 2007), which is similar to my studies in which hyaluronidase reduced MSP and EGF-mediated cell migration through Transwell inserts.

An increase in HA expression in the pericellular region can lead to biological effects that are independent of signaling events generated by its interactions with CD44 or RHAMM. Fragmentation of a variety of ECM proteins produce inflammation (Adair-Kirk & Senior, 2008) and HA degradation products can also promote a pro-inflammatory response (Termeer *et al.*, 2000; Weigel *et al.*, 1986). In the absence of microbial stimuli, inflammation is often triggered by HA (Yamasaki *et al.*, 2009). Through its binding to Toll-like receptors, HA fragments upregulate NF-κB, promote leukocyte adhesion (Oertli *et al.*, 1998) and promote migration of several classes of immune regulator cells, such as dendritic cells and macrophages (Termeer *et al.*, 2002). This data implies that HA has the potential to activate the innate immune system, providing an additional means of signal propagation in cancer cells. Inflammation is commonly seen in human cancers and is a often a poor prognostic marker (Ono, 2008; Ruegg, 2006). For example, inflammatory breast carcinoma is the most aggressive type of breast cancer and has a high rate of mortality (Walshe & Swain, 2005). In addition to the contribution of HA to the inflammatory process, some studies also suggest that MSP-stimulation of RON leads to an upregulation of pro-inflammatory cytokines and reactive oxygen species (Suzuki *et al.*, 2008). It would be useful to examine an *in vivo* model of inflammation

in which RON expression can be correlated with HA expression, as my cell-based system is not sufficient for these types of studies. An *in vivo* model would also provide the framework for studying the recruitment of immune cells and other changes in the microenvironment in response to induction of RON expression with or without activation by MSP.

In summary, when examining a role for RTK signaling in tumor progression, it is important to look beyond canonical ligand-receptor interactions that induce cell signaling and consider the broad spectrum of potential receptor interactions within the cell. The role of the ECM in controlling cell behavior is now well-established; and the extent of its influence on RTK signaling is still being intensely investigated. RTKs do not act in isolation within the context of a cancer cell, and studies must continue to delineate these interactions for cancer therapies to be successful.

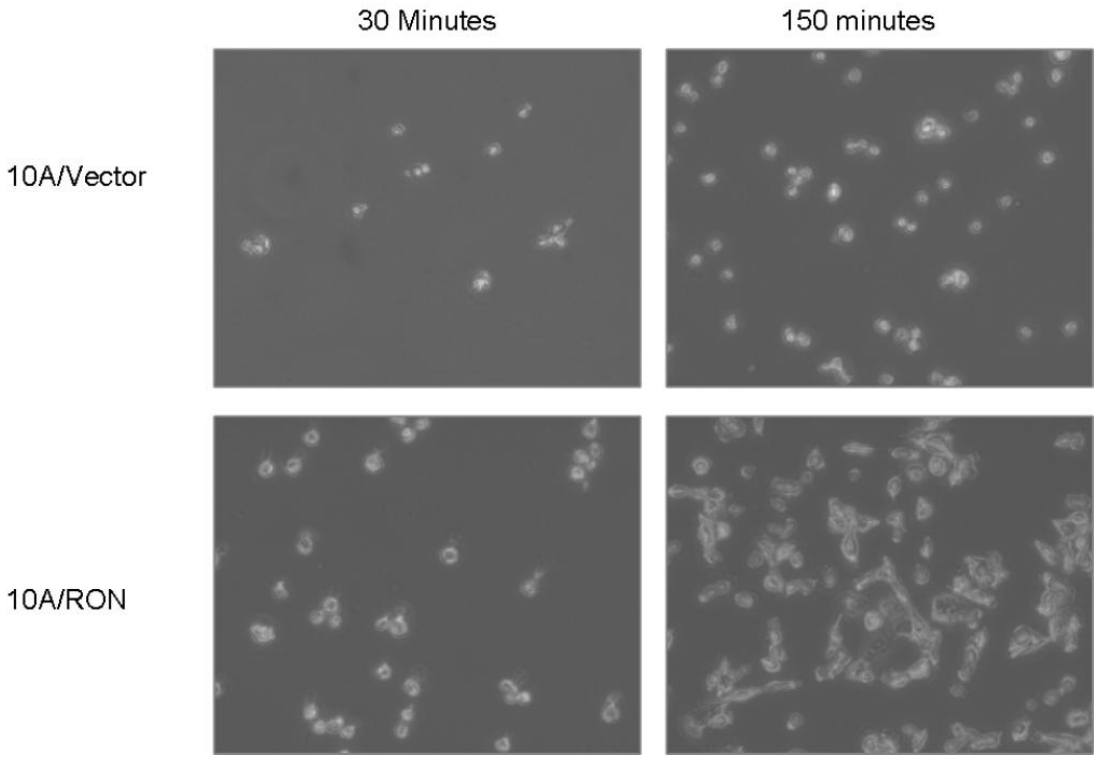

Figure 4.1

10A/RON cells exhibit increased attachment to tissue culture plates. 10A/Vector and 10A/RON cells were trypsinized and added to tissue culture plates. After attaching for either 30 or 150 minutes, the unbound cells were washed away with DMEM, and cell images were collected via phase contrast microscopy.

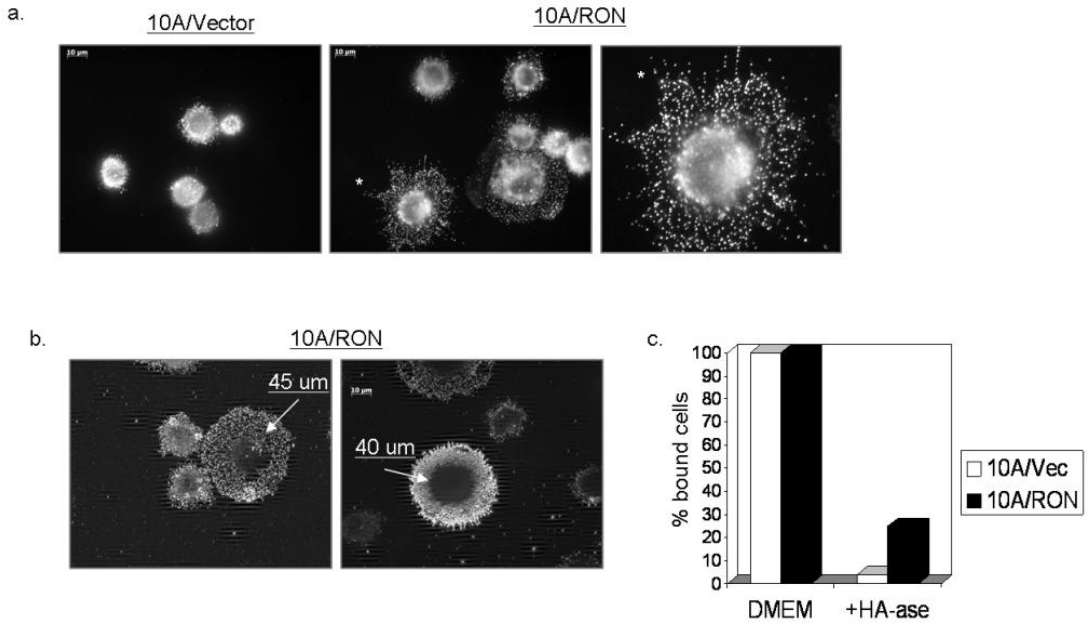

Figure 4.2

Chondroitin Sulfate Proteoglycans are found in microspikes of spreading cells. (a & b) 10A/Vector and 10A/RON cells were allowed to attach to poly-d-lysine coated coverslips for 10 minutes. Bound cells were labeled with biotinylated-WFA and detected by Cy3-streptavidin. The asterisk denotes a cell with microspikes and is the magnified image in the 3rd panel. The span of the cells in panel b was measured to show that a larger cell span does not necessarily correlate with the amount of CSPGs on the cell surface. (c) The relative number of 10A/Vector and 10A/RON cells was determined after 300 U/ml HAase was added to the cells 10 minutes prior to the 10 minute attachment assay.

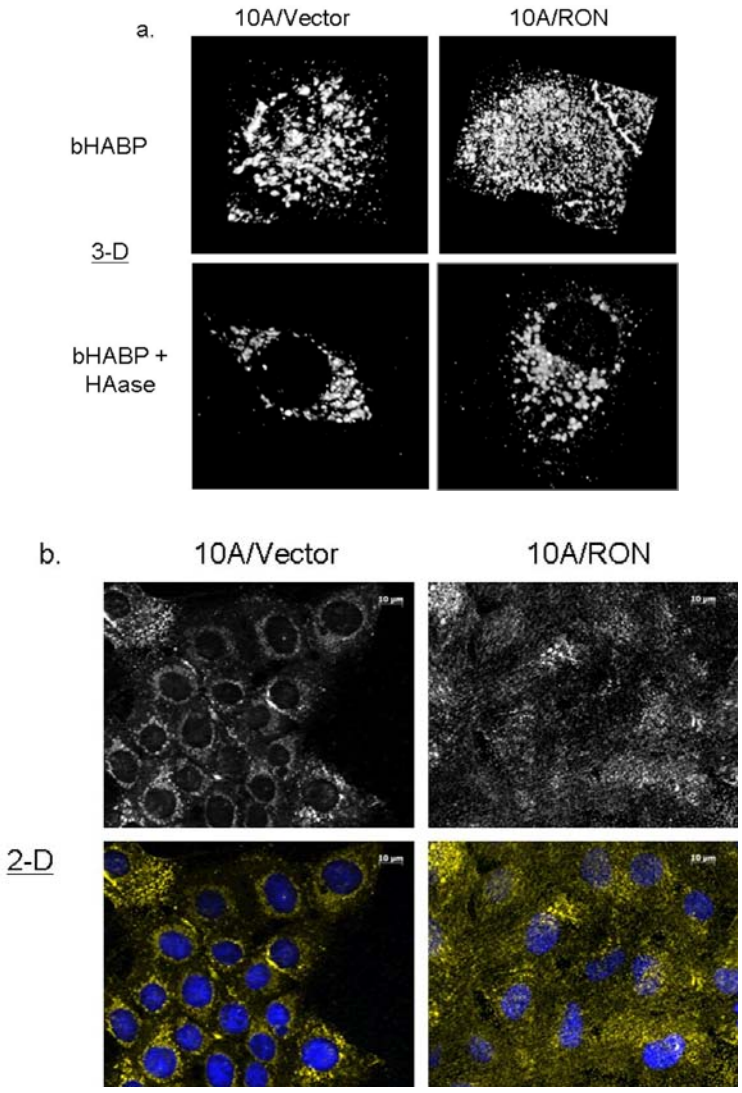

Figure 4.3

10A/RON cells exhibit increased cell surface Hyaluronan expression. (a) 10A/Vector and 10A/RON cells were cultured for 48 hours in regular media. Cells were incubated with biotinylated-Hyaluronan binding protein (bHABP). BHABP was detected with Cy3-Streptavidin. Images are 3-D rendered Z-stacks taken with Apotome processing of individual cells. The top panel shows cells untreated with HAase, and the bottom panel shows cells that were treated with 300U/ml HAase for 1 hour prior to adding bHABP. (b) Cells were labeled as in (a). Images are of 2-D Apotome-processed representative 10A/Vector and 10A/RON cells. The yellow is a pseudo-color in order to make the HABP staining more visible.

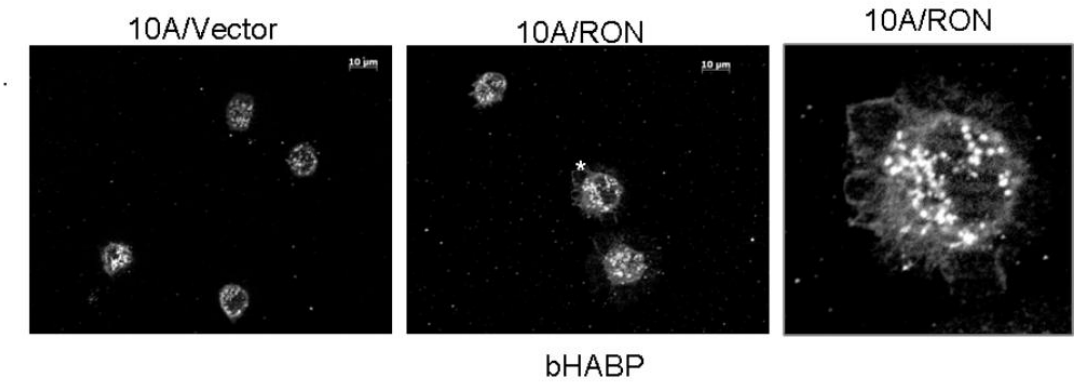

Figure 4.4

HA does not localize to actin microspikes. 10A/RON cells were allowed to attach to poly-d-lysine coated coverslips for 15 minutes before removing unbound cells. Remaining cells were stained with biotinylated-HABP/Cy3-Streptavidin. The asterisk denotes the cell that is magnified in the end panel to show that HA is present on these cells.

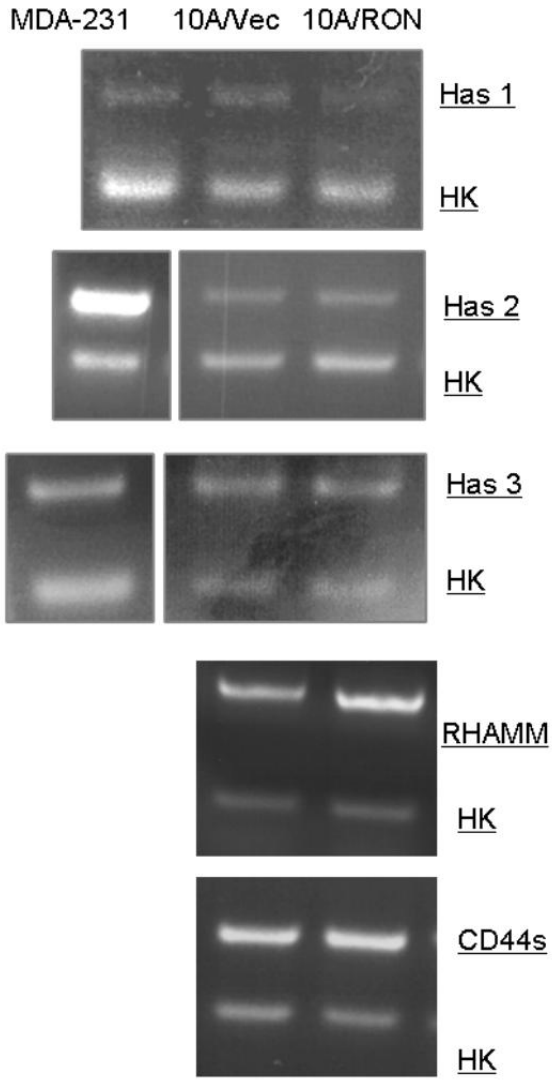

Figure 4.5

10A/RON cells do not exhibit increased expression of HA Synthases (HAS), CD44s or RHAMM mRNA. 10A/RON cells were cultured for 48 hours before collecting mRNA and using RT-PCR methods to detect HAS 1, HAS 2, HAS 3, RHAMM and CD44s, as well as a generic housekeeping gene (60S subunit of human RPLP1) for cell loading. MDA-231 is an invasive breast cancer cell line that was specifically used as a positive control for HAS 2, but was included in all of the HAS studies for comparison to 10A cells.

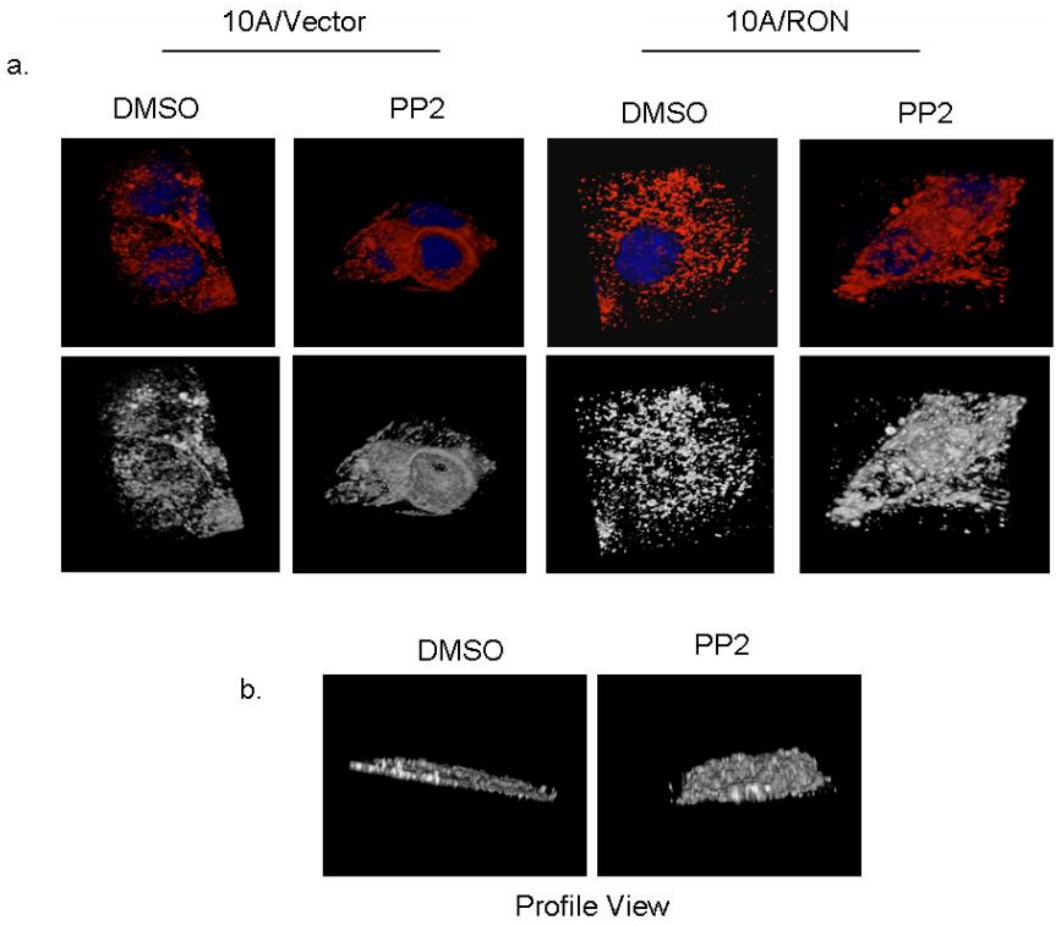

Figure 4.6

HA synthesis does not require Src kinase activity. 10A/Vector and 10A/RON cells were cultured on coverslips for 48 hours in the presence of DMSO or PP2. Cells were then labeled with biotinylated HABP/Cy3-Streptavidin. Images shown are 3-D rendered Z-stacks. (a) Front view. (b) Profile view of 10A/RON cells treated with DMSO or PP2.

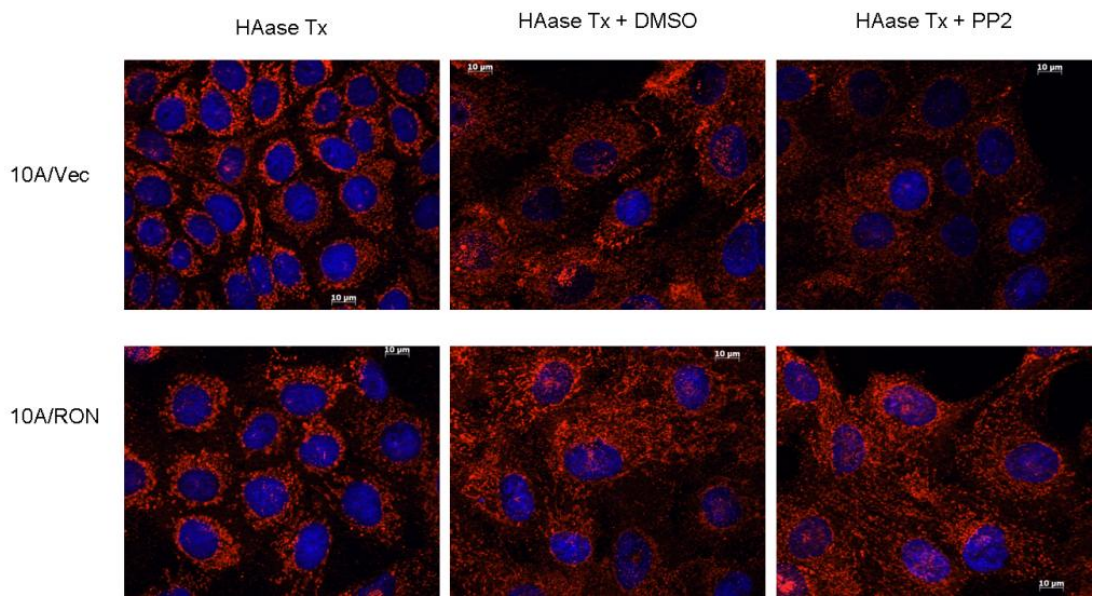

Figure 4.7

Src is not required for the secretion of HA in 10A cells. 10A/Vector and 10A/RON cells were cultured on coverslips for 48 hours and treated with 200U/ml HAase for 1 hour. Regular growth media was replaced with either DMSO (negative control) or 10μM PP2 and the cells were cultured for 20 hours at 37 °C, at which point the cells were fixed and labeled with bHABP/Cy3 to visualize HA.

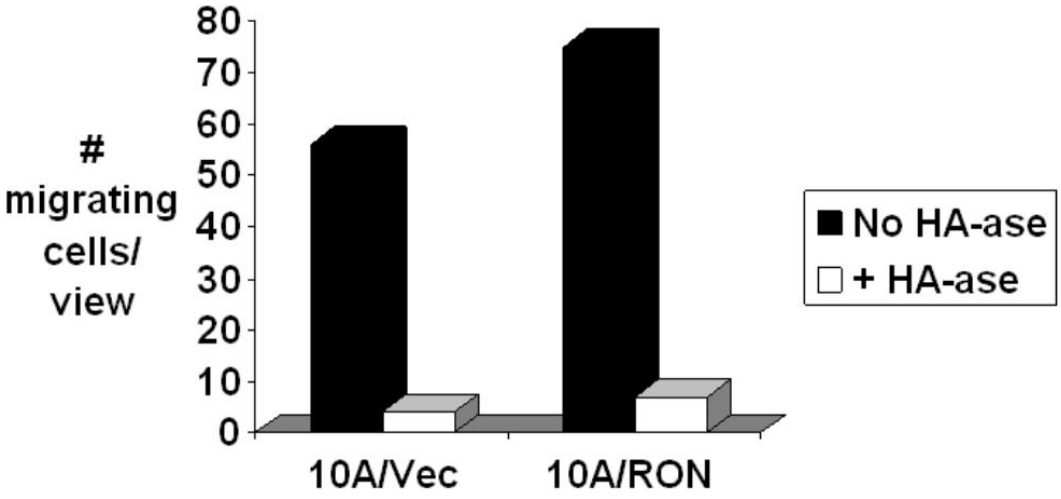

Figure 4.8

Hyaluronidase reduces cell migration. 10A/Vector and 10A/RON cells were allowed to migrate through Transwell filters for 6 hours. Cells were either left untreated or treated with 300 U/ml HAase for 1 hour prior to adding growth factors in the bottom chamber. Shown here is representative data from one experiment, however, this experiment was repeated at least 3 times with similar results.

Chapter 5

Concluding Remarks

Summary of data

The work presented herein describes the contributions of the RON RTK to the development and progression of human breast cancers. My studies have concluded that RON elicited both MSP-dependent and MSP-independent signaling when expressed in MCF-10A mammary epithelial cells. The intracellular Src kinase mediated MSP-independent tyrosine phosphorylation of RON, and may also be involved in RON signaling after MSP-stimulation. Biologically, whereas RON-mediated cell proliferation and migration required the addition of MSP, 10A/RON cells exhibited increased cell survival, adhesion, spreading and invasion through ECM in the absence of MSP. Furthermore, MSP-independent cell survival and cell spreading required the kinase activity of Src, as did growth factor-driven migration (see Table 5.1 for a summary of data). I have shown that Src plays an essential role in MSP-independent signaling by RON, but the role of Src after MSP-stimulation of RON is less clear. Src activity was not needed for MSP-dependent cell proliferation,

and the Src inhibitor, PP2, only partially reduced RON phosphorylation after MSP stimulation.

Additionally, RON expression in MCF-10A cells induced the secretion of HA onto the cell surface by an unknown mechanism. HA appears to be necessary for the more efficient adhesion and subsequent cell spreading noted in the 10A/RON cells compared to the 10A/Vector cells since removal of HA from the cell surface abrogates cell attachment and spreading. The increased adhesive quality of 10A/RON cells may explain why more of these cells migrated in response to other growth factors, such as EGF or HGF, as compared to 10A/Vector cells. Finally, Src kinase activity was dispensable for the increased production of HA.

Model for MSP-independent signaling of RON

With this data, I have generated a model to describe the phenomenon of MSP-independent signaling of RON in MCF-10A cells, as shown in Figure 5.1. In my model, the expression of RON induces increased secretion or retention of HA onto the cell surface, although, at this point, it is not clear how HA is upregulated in 10A/RON cells. Both 10A/Vector and 10A/RON cells express and may activate CD44 and RHAMM by HA binding. However, RON's association with CD44 and RHAMM may produce a more robust signaling response to the additional amount of HA, promoting an increase in Src kinase activity above the baseline level found in 10A/Vector cells. Once Src is activated, Src can phosphorylate and activate the RON receptor, which in turn, phosphorylates Src, in a positive feedback loop. Finally, the

additional levels of activated Src in 10A/RON cells ultimately lead to activation of the MAPK pathway, paving the way for changes in cell behavior that are not produced in 10A/Vector cells. In my model, the addition of MSP directly activates the RON receptor, which promotes signaling of both the MAPK and AKT pathways, as noted in Figure 2.3. Src activity is not required for the tyrosine phosphorylation of RON when MSP is present. HA-induced signaling via CD44 or RHAMM would not have an effect on MSP-mediated RON activation in my model.

Overall impact of the study

Recently, it has become clear that cancer cells do not promote disease in isolation, and that the surrounding microenvironment of the cell plays an essential role in determining cancer cell behavior. In order to become invasive, cancer cells must develop mechanisms to hijack the normal control exerted by cross-talk between cells and their environment. There are several possible ways in which the cells can influence their microenvironment, including, as appears to be the case in 10A/RON cells, upregulation of HA to drive additional receptor-mediated cell signaling events. The upregulation of GAGs and/or other matrix components can also increase the tensional force of the ECM, which in turn, elicits a mechanosensory response in cells, ultimately leading to alterations in cell behavior toward a more invasive phenotype (Butcher *et al.*, 2009).

Considering the high levels of RON in breast and other types of human cancers and the poor clinical outcome of patients with RON-expressing tumors, it is

essential to understand the underlying mechanisms by which RON generates its signaling and consequent cellular behavior. In addition to MSP-independent activation of intracellular signaling cascades, my study provides evidence that RON expression could potentially affect the cell microenvironment of mammary epithelial cells. This suggests that the RON RTK is capable of providing the necessary cues to cancer cells for promoting more aggressive and invasive behaviors.

Although the majority of RON studies have been in the context of MSP-dependent signaling, my study clearly indicates that RON can generate MSP-independent cell signaling to promote changes in cell behavior. As mentioned previously, monoclonal antibodies have been designed to target the MSP-binding domain of RON for therapeutic use in human cancers (O'Toole *et al.*, 2006). However, if MSP is not required for RON to exert its oncogenic effects, targeted therapies using such antibodies will not have an impact on reducing the disease state. Instead, targeting RON along with its signaling mediators, such as Src, or potential binding partners, such as CD44 and/or RHAMM may prove a more successful strategy. In addition, a more current strategy to alleviate cancer progression may also include inhibiting components of the microenvironment, including the stromal and immune cells as well as ECM proteins (Ronnov-Jessen & Bissell, 2009).

Future projects

My model paves the way for several future studies in which to delineate mechanisms of RON signaling in normal mammary epithelial cells. Specifically of

value would be to explore the necessity of CD44 and/or RHAMM to act as co-receptors in MSP-independent signaling of RON. Some studies have shown success in blocking signaling by specific CD44 isoforms with peptides (Matzke et al., 2005) as well as with a human monoclonal CD44 antibody (Hermes-1), which blocks HA binding (Li *et al.*, 2006). Furthermore, shRNA could be designed to target either CD44 or RHAMM. Studies to determine the mechanisms by which RON expression leads to an increase of cell surface HA might provide useful information. For example, it could be determined whether an increased association between CD44/RHAMM and RON mediates the tethering of HA to the cell surface. One could also examine another possible mechanism of HA accumulation on the cell surface such as whether any of the HAS isoforms are inappropriately down-regulated after secreting HA.

Finally, there is mounting evidence that the process of inflammation dramatically contributes to the development of cancers (Lu *et al.*, 2006). Interestingly, HA is a key player in inflammatory diseases, including human cancers (Heldin *et al.*, 2008). Although there is some evidence that RON might be involved in the regulation of pro-inflammatory cytokines when expressed in macrophages (Wilson et al., 2008), the role of RON in the context of inflammation in cancer is poorly-defined. Studies to examine whether RON-induced HA expression in the microenvironment of mammary cells contributes to the development of inflammation and the progression of cancer would be interesting and worthwhile. Currently, a mammary-specific RON-expressing mouse model exists that might be a good system

with which to study RON and its contributions to inflammation, as these mice form MSP-independent breast tumors and metastatic growths in the lungs (Peace et al., 2005).

	MSP Required	Src Required	MAPK Required	PI3K Required
Proliferation	Yes	No	NT	NT
Adhesion	No	No	No	No
Spreading	No	Yes	No	No
Migration	Yes	Yes	Part.	Part.
Survival	No	Yes	Yes	Yes

Table 5.1

Summary of Results

(**No**: Not needed, **Yes**: required, **NT**: Not Tested, **Part.**: Partially required)

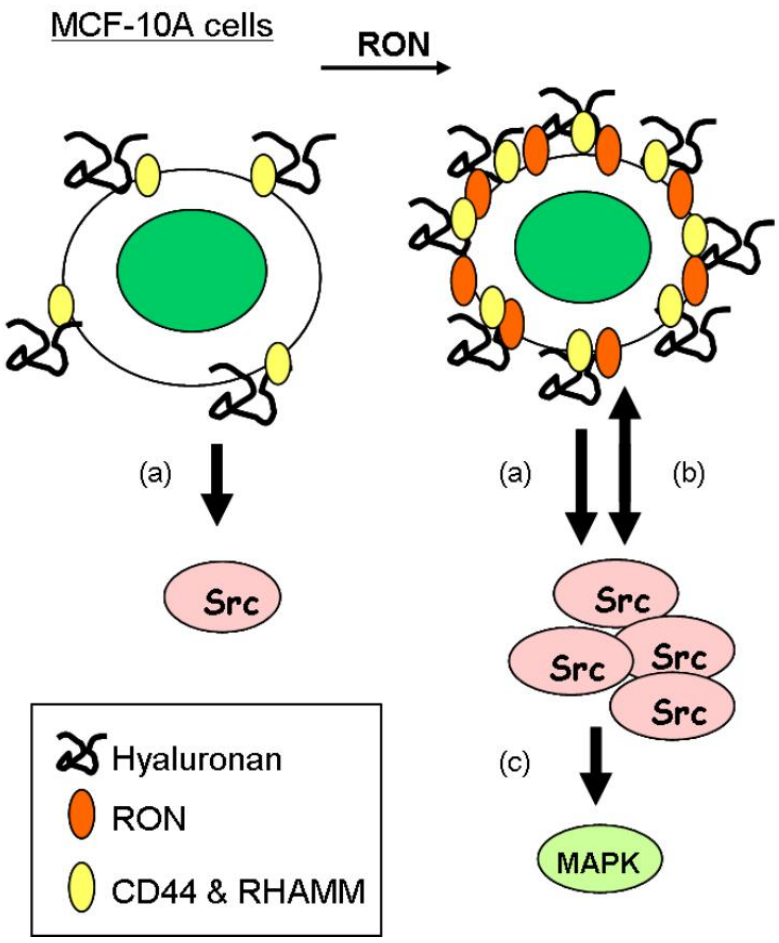

Figure 5.1

Proposed model for MSP-independent RON signaling. 10A/RON cells exhibit an increase of HA expression on their cell surface, which has the potential to drive excessive signaling via CD44 and RHAMM. I do not know how RON expression leads to an increase of HA expression. It is also not yet clear whether an association of RON with CD44 and/or RHAMM is required for this increase in cell signaling. HA-mediated signaling promotes Src kinase activation (a), which in turn, tyrosine phosphorylates RON (b). Once RON is active, it can also phosphorylate Src (b), maintaining a positive feedback loop. When Src activity increases, I can detect an increase in MAPK activity (c), even in the absence of MSP

Materials and Methods

Materials and antibodies

Recombinant human MSP and the anti-MSP Receptor antibody used for immunofluorescence and immunoprecipitation were from R&D Systems (Minneapolis, MN, USA). EGF was from Peprotech (Rocky Hill, NJ, USA). HGF was a gift from Amgen (Thousand Oaks, CA, USA). The biotinylated *Wisteria Floribunda* Agglutinin (bWFA) was kind gift of Dr Joel Levine (Stony Brook University). Poly-d-Lysine (P6407), BrdU, bovine testes hyaluronidase, propidium iodide (P4864) and anti-BrdU antibody (Clone BU-33) were from Sigma-Aldrich (St. Louis, MO, USA). AlexFluor 546 (A11030), rhodamine-phalloidin (R415) and Hoechst 33342 were from Molecular Probes/Invitrogen (Eugene, OR, USA). Cy3/Streptavidin was from Jackson Immunolabs (West Grove, PA). Biotinylated HABP was from Northstar Bioproducts (East Falmouth, MA, USA). Antibodies for Western blots and immunoprecipitations were: 4G10 and anti-MAPK from Upstate Biotechnology, Inc (Lake Placid, NY, USA), anti-phospho-MAPK and anti-FAK from BD Transduction Laboratories (San Diego, CA, USA), anti-FAK (sc-1688), anti-RON (sc-22) and all anti-phospho-RON rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PARP antibodies and rabbit anti-phospho-AKT (Ser 473) was from Cell Signaling (Danvers, MA, USA).

Anti-Src pY418 rabbit antibodies were from BioSource (Camarillo, CA, USA). PP2, U0126 and LY294002 were from BioMol (Plymouth Meeting, PA, USA). Growth factor reduced-matrigel was purchased from BD Biosciences. Vitrogen (collagen) was purchased from Angiotech BioMaterials, (Palo Alto, CA). ECM cell Adhesion Array kit was purchased from Chemicon/Millipore (Billerica, MA, USA).

Cell culture

Cell culture materials were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). NIH-3T3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and Pen/Strep (5 ml of a 100X solution). MCF-10A/ecoR cells were a kind gift from Dr. Joan Brugge (Harvard Medical School), and MCF-10A cells were from the ATTC. 10A/Vector and 10A/RON cells were cultured in the tissue culture media as described (Debnath et al., 2003). pUSE-DN-Src, was provided by Dr. WT Miller (SUNY Stony Brook). HeLa cells were transfected with Fugene (Roche Applied Science, Indianapolis, IN, USA). The K1114M kinase-dead RON mutant was generated using a GeneEditor mutagenesis kit (Promega, Madison, WI, USA) with the previously reported oligonucleotide (Danilkovitch-Miagkova et al., 2000).

Generation of MCF-10A/RON cell lines

Retroviruses expressing wild-type human RON were generated by transfection of Phoenix E cells with REBNA-RON-IRES-GFP retroviral vector

(Petrenko *et al.*, 1999). Virus-containing supernatant from the Phoenix E cells was added to MCF-10A/ecoR cells for 6 hours, and then replaced with regular growth media. The same protocol was followed to generate a negative control, vector only cell line. In both RON-infected and Vector-infected cells, GFP positive cells were selected by FACS. See the Nolan Laboratory website for further details on generating retroviruses with the Phoenix E cell system (http://www.stanford.edu/group/nolan/index.html).

Proliferation Assays

Cells were cultured on coverslips for 48 hours in specified media, pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 2 hours, washed with phosphate-buffered saline (PBS), fixed with methanol, and treated with 4N HCl for 30 minutes. BrdU was labeled with an anti-BrdU antibody and detected using AlexaFluor 546 secondary antibody. The percent of BrdU-positive cells was determined by counting at least 500 cells per coverslip.

Transwell migration assay

Cells were starved overnight in DMEM/F-12 media containing 1% horse serum. 100,000 cells were added to the top well of a 24-well, 0.8 micron pore Transwell filter insert (Costar, Corning, NY, USA). Media added to the bottom well contained no growth factors, 10ng/ml EGF, 100 ng/ml MSP or 2 ng/ml HGF. After 6 or 18 hours, cells that remained in the top chamber were removed with a cotton swab,

the migrated cells were fixed in 2% paraformaldehyde, and DNA was labeled with Hoechst. The number of migrating cells per field of view was counted on a fluorescent microscope under a 20X magnification.

Immunoblotting

All immunoprecipitations and immunoblots were performed as described (Yokayama et al 2005) using a 9% polyacrylamide gel.

Apoptosis assay

Attached cells and floating cells were pooled together, and were stained with Propidium Iodide. The percentage of PI- positive cells was determined using a BD FACSCalibur™ system (BD, Franklin Lakes, NJ, USA). PP2 (10 μM) was added to the cultures after the cells were allowed to settle on the dish overnight.

Adhesion and attachment assays

Cells were incubated on poly-d-lysine coated coverslips in DMEM for 10 minutes. Unbound cells were washed away with PBS. Attached cells were fixed with 3.7% paraformaldehyde for 15 minutes at 4°C, incubated with 0.1% Triton-X 100 for 2 minutes and rhodamine-conjugated phalloidin was added as per commercial protocol. Coverslips were mounted with Prolong Gold (Molecular Probes). Cells were visualized with an Axiovert 200M (Zeiss, Thornwood, NY, USA) using a 63X oil DIC lens and the images were analyzed using the Axiovision software (Zeiss).

Reverse-Transcriptase PCR

Cellular RNA was isolated with RNeasy kit (Qiagen, Valencia, CA, USA), and reverse transcribed to cDNA using the Superscript II reverse transcriptase system (Invitrogen). MSP was detected using primers previously described (Zalcenstein *et al.*, 2006). For MSP detection, the parameters were: 30 seconds at 94°C, 45 seconds at 57°C and 45 seconds at 72°C for 36 cycles. HAS 1, 2, and 3 were detected using primers previously described (Stuhlmeier & Pollaschek, 2004). For HAS 1 detection, the parameters were: 30 seconds at 94°C, 45 seconds at 57°C and 1 minute at 72°C for 36 cycles. For HAS 2 detection, the parameters were: 30 seconds at 94°C, 40 seconds at 56°C and 40 seconds at 72°C for 36 cycles. For HAS 3 detection, the parameters were: 30 seconds at 94°C, 40 seconds at 58°C and 40 seconds at 72°C for 35 cycles. CD44s was detected with previously described primers (Hudson *et al.*, 1995). For CD44s detection, the PCR parameters were 1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C. RHAMM was detected from primers published in (Assmann *et al.*, 1998). For RHAMM detection, the PCR parameters were 1 minute at 95°C, 1 minute at 63°C and 1 minutes at 72°C. The house-keeping gene (60S region of the human RPLP1 gene) that was used as a loading control was a kind gift from Dr. Edward Chan (Stony Brook University).

Immunofluorescence

RON localization was detected by incubating the primary anti-RON antibody at a concentration of 1:250 (R&D Systems) with the secondary Alexa Fluor 546 (1:500) for 5 minutes at room temperature, then adding the mixture to living cells grown on coverslips for approximately 1 minute. Cells were washed with DMEM to remove the antibodies and fresh DMEM was used for the assay. Live cells were visualized with an Axiovert 200M (Zeiss, Thornwood, NY, USA) using a 63X oil DIC lens and the images were analyzed using the Axiovision software (Zeiss). To label cells with biotinylated *Wisteria floribunda* agglutinin (bWFA), cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes, washed with PBS, 1:100 bWFA in 1% BSA added for 90 minutes at room temperature, washed, 1:5000 Cy3-Streptavidin for 40 minutes at room temperature, washed, stained with Hoechst (1:1000) to label the DNA and mounted on coverslips with Prolong Gold. To label cells with biotinylated HA-binding protein (bHABP), cells were grown on coverslips for 48 hours, washed with PBS, fixed with 100% Methanol at -20°C for 20 minutes and blocked in 3% BSA for 1-2 hours. Biotinylated HABP (1:200) was added to the cell overnight. After washing with PBS, the bHABP was detected by a 1:5000 solution of Cy3-Streptavidin for 1 hour. Cells were washed, the DNA was labeled with Hoechst and the coverslips were mounted with Prolong Gold (Molecular Probes). The images were taken as described above for RON localization.

3-D Assays

The 3-D assays were completed essentially as described in (Debnath et al., 2003), except that instead of 100% Matrigel bottom layer, I used a 1:1 mixture of Matrigel:Vitrogen (Collagen). I allowed the gelling to occur in a humid chamber for approximately 12 hours, and rehydrated the gel with DMEM for 1 hour before adding the cell suspension. The top layer of media included 2% Matrigel. Fresh media with 2% matrigel was added every 4 days. The cells were imaged using a 10X phase contrast lens with Axiovision Software (Zeiss).

References

Abd El-Rehim, DM, Pinder, SE, Paish, CE, Bell, JA, Rampaul, RS, Blamey, RW, et al. (2004). Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma *Br J Cancer*, **91,** 1532-42.

ACS. (2008). *Cancer Facts & Figures 2008*. American Cancer Society: Atlanta.

Adair-Kirk, TL and Senior, RM. (2008). Fragments of extracellular matrix as mediators of inflammation *Int J Biochem Cell Biol*, **40,** 1101-10.

Adamia, S, Maxwell, CA and Pilarski, LM. (2005). Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer *Curr Drug Targets Cardiovasc Haematol Disord*, **5,** 3-14.

Angeloni, D, Danilkovitch-Miagkova, A, Ivanov, SV, Breathnach, R, Johnson, BE, Leonard, EJ, et al. (2000). Gene structure of the human receptor tyrosine kinase RON and mutation analysis in lung cancer samples *Genes Chromosomes Cancer*, **29,** 147-56.

Angeloni, D, Danilkovitch-Miagkova, A, Ivanova, T, Braga, E, Zabarovsky, E and Lerman, MI. (2007). Hypermethylation of Ron proximal promoter associates with lack of full-length Ron and transcription of oncogenic short-Ron from an internal promoter *Oncogene*, **26,** 4499-512.

Angeloni, D, Danilkovitch-Miagkova, A, Miagkov, A, Leonard, EJ and Lerman, MI. (2004). The soluble sema domain of the RON receptor inhibits macrophage-stimulating protein-induced receptor activation *J Biol Chem*, **279,** 3726-32.

Angeloni, D, Duh, FM, Moody, M, Dean, M, Zabarovsky, ER, Sentchenko, V, et al. (2003). C to A single nucleotide polymorphism in intron 18 of the human MST1R (RON) gene that maps at 3p21.3 *Mol Cell Probes*, **17,** 55-7.

Artigiani, S, Comoglio, PM and Tamagnone, L. (1999). Plexins, semaphorins, and scatter factor receptors: a common root for cell guidance signals? *IUBMB Life*, **48,** 477-82.

Aruffo, A, Stamenkovic, I, Melnick, M, Underhill, CB and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate *Cell*, **61,** 1303-13.

Assmann, V, Marshall, JF, Fieber, C, Hofmann, M and Hart, IR. (1998). The human hyaluronan receptor RHAMM is expressed as an intracellular protein in breast cancer cells *J Cell Sci*, **111 (Pt 12),** 1685-94.

Bache, KG, Slagsvold, T and Stenmark, H. (2004). Defective downregulation of receptor tyrosine kinases in cancer *Embo J*, **23,** 2707-12.

Bao, J, Gur, G and Yarden, Y. (2003). Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors *Proc Natl Acad Sci U S A*, **100,** 2438-43.

Baselga, J. (2006). Targeting tyrosine kinases in cancer: the second wave *Science*, **312,** 1175-8.

Bertrand, P, Girard, N, Delpech, B, Duval, C, d'Anjou, J and Dauce, JP. (1992). Hyaluronan (hyaluronic acid) and hyaluronectin in the extracellular matrix of human breast carcinomas: comparison between invasive and non-invasive areas *Int J Cancer*, **52,** 1-6.

Bezerra, JA, Carrick, TL, Degen, JL, Witte, D and Degen, SJ. (1998). Biological effects of targeted inactivation of hepatocyte growth factor-like protein in mice *J Clin Invest*, **101,** 1175-83.

Bhatt, AS, Welm, A, Farady, CJ, Vasquez, M, Wilson, K and Craik, CS. (2007). Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway *Proc Natl Acad Sci U S A*, **104,** 5771-6.

Birchmeier, C, Birchmeier, W, Gherardi, E and Vande Woude, GF. (2003). Met, metastasis, motility and more *Nat Rev Mol Cell Biol*, **4,** 915-25.

Biscardi, JS, Ishizawar, RC, Silva, CM and Parsons, SJ. (2000). Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer *Breast Cancer Res*, **2,** 203-10.

Blanco-Aparicio, C, Renner, O, Leal, JF and Carnero, A. (2007). PTEN, more than the AKT pathway *Carcinogenesis*, **28,** 1379-86.

Borges, E, Jan, Y and Ruoslahti, E. (2000). Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain *J Biol Chem*, **275,** 39867-73.

Bork, P, Doerks, T, Springer, TA and Snel, B. (1999). Domains in plexins: links to integrins and transcription factors *Trends Biochem Sci*, **24,** 261-3.

Bourguignon, LY, Xia, W and Wong, G. (2009). Hyaluronan-mediated CD44 interaction with p300 and SIRT1 regulates beta-catenin signaling and NFkappaB-specific transcription activity leading to MDR1 and Bcl-xL gene expression and chemoresistance in breast tumor cells *J Biol Chem*, **284,** 2657-71.

Brown, MT and Cooper, JA. (1996). Regulation, substrates and functions of src *Biochim Biophys Acta*, **1287,** 121-49.

Burridge, K, Fath, K, Kelly, T, Nuckolls, G and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton *Annu Rev Cell Biol*, **4,** 487-525.

Butcher, DT, Alliston, T and Weaver, VM. (2009). A tense situation: forcing tumour progression *Nat Rev Cancer*, **9,** 108-22.

Cabodi, S, Morello, V, Masi, A, Cicchi, R, Broggio, C, Distefano, P, et al. (2009). Convergence of integrins and EGF receptor signaling via PI3K/Akt/FoxO pathway in early gene Egr-1 expression *J Cell Physiol*, **218,** 294-303.

Cantaluppi, V, Biancone, L, Romanazzi, GM, Figliolini, F, Beltramo, S, Galimi, F, et al. (2008). Macrophage stimulating protein may promote tubular regeneration after acute injury *J Am Soc Nephrol*, **19,** 1904-18.

Carafoli, F, Chirgadze, DY, Blundell, TL and Gherardi, E. (2005). Crystal structure of the beta-chain of human hepatocyte growth factor-like/macrophage stimulating protein *Febs J*, **272,** 5799-807.

Chen, BH, Tzen, JT, Bresnick, AR and Chen, HC. (2002). Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells *J Biol Chem*, **277,** 33857-63.

Chen, HC, Appeddu, PA, Isoda, H and Guan, JL. (1996). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase *J Biol Chem*, **271,** 26329-34.

Chen, HC and Guan, JL. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase *Proc Natl Acad Sci U S A*, **91,** 10148-52.

Chen, YQ, Zhou, YQ, Angeloni, D, Kurtz, AL, Qiang, XZ and Wang, MH. (2000). Overexpression and activation of the RON receptor tyrosine kinase in a panel of human colorectal carcinoma cell lines *Exp Cell Res*, **261,** 229-38.

Chen, YQ, Zhou, YQ, Fisher, JH and Wang, MH. (2002a). Targeted expression of the receptor tyrosine kinase RON in distal lung epithelial cells results in multiple tumor formation: oncogenic potential of RON in vivo *Oncogene*, **21,** 6382-6.

Chen, YQ, Zhou, YQ, Fu, LH, Wang, D and Wang, MH. (2002b). Multiple pulmonary adenomas in the lung of transgenic mice overexpressing the RON receptor tyrosine kinase. Recepteur d'origine nantais *Carcinogenesis*, **23,** 1811-9.

Cheng, HL, Liu, HS, Lin, YJ, Chen, HH, Hsu, PY, Chang, TY, et al. (2005). Co-expression of RON and MET is a prognostic indicator for patients with transitional-cell carcinoma of the bladder *Br J Cancer*, **92,** 1906-14.

Chiang, AC and Massague, J. (2008). Molecular basis of metastasis *N Engl J Med*, **359,** 2814-23.

Chou, TY, Chiu, CH, Li, LH, Hsiao, CY, Tzen, CY, Chang, KT, et al. (2005). Mutation in the tyrosine kinase domain of epidermal growth factor receptor is a predictive and prognostic factor for gefitinib treatment in patients with non-small cell lung cancer *Clin Cancer Res*, **11,** 3750-7.

Cohen, M, Kam, Z, Addadi, L and Geiger, B. (2006). Dynamic study of the transition from hyaluronan- to integrin-mediated adhesion in chondrocytes *Embo J*, **25,** 302-11.

Collesi, C, Santoro, MM, Gaudino, G and Comoglio, PM. (1996). A splicing variant of the RON transcript induces constitutive tyrosine kinase activity and an invasive phenotype *Mol Cell Biol*, **16,** 5518-26.

Comoglio, PM, Tamagnone, L and Boccaccio, C. (1999). Plasminogen-related growth factor and semaphorin receptors: a gene superfamily controlling invasive growth *Exp Cell Res*, **253,** 88-99.

Comoglio, PM and Trusolino, L. (2002). Invasive growth: from development to metastasis *J Clin Invest*, **109,** 857-62.

Conrotto, P, Corso, S, Gamberini, S, Comoglio, PM and Giordano, S. (2004). Interplay between scatter factor receptors and B plexins controls invasive growth *Oncogene*, **23,** 5131-7.

Correll, PH, Iwama, A, Tondat, S, Mayrhofer, G, Suda, T and Bernstein, A. (1997). Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase *Genes Funct*, **1,** 69-83.

Correll, PH, Paulson, RF and Wei, X. (2006). Molecular regulation of receptor tyrosine kinases in hematopoietic malignancies *Gene*, **374,** 26-38.

Cote, M, Miller, AD and Liu, SL. (2007). Human RON receptor tyrosine kinase induces complete epithelial-to-mesenchymal transition but causes cellular senescence *Biochem Biophys Res Commun*, **360,** 219-25.

Cowin, AJ, Kallincos, N, Hatzirodos, N, Robertson, JG, Pickering, KJ, Couper, J, et al. (2001). Hepatocyte growth factor and macrophage-stimulating protein are upregulated during excisional wound repair in rats *Cell Tissue Res*, **306,** 239-50.

Danilkovitch-Miagkova, A. (2003). Oncogenic signaling pathways activated by RON receptor tyrosine kinase *Curr Cancer Drug Targets*, **3,** 31-40.

Danilkovitch-Miagkova, A, Angeloni, D, Skeel, A, Donley, S, Lerman, M and Leonard, EJ. (2000). Integrin-mediated RON growth factor receptor phosphorylation requires tyrosine kinase activity of both the receptor and c-Src *J Biol Chem*, **275,** 14783-6.

Danilkovitch-Miagkova, A, Duh, FM, Kuzmin, I, Angeloni, D, Liu, SL, Miller, AD, et al. (2003). Hyaluronidase 2 negatively regulates RON receptor tyrosine kinase and mediates transformation of epithelial cells by jaagsiekte sheep retrovirus *Proc Natl Acad Sci U S A*, **100,** 4580-5.

Danilkovitch-Miagkova, A, Miagkov, A, Skeel, A, Nakaigawa, N, Zbar, B and Leonard, EJ. (2001). Oncogenic mutants of RON and MET receptor tyrosine kinases cause activation of the beta-catenin pathway *Mol Cell Biol*, **21,** 5857-68.

Danilkovitch, A, Donley, S, Skeel, A and Leonard, EJ. (2000). Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells *Mol Cell Biol*, **20,** 2218-27.

Danilkovitch, A, Skeel, A and Leonard, EJ. (1999). Macrophage stimulating protein-induced epithelial cell adhesion is mediated by a PI3-K-dependent, but FAK-independent mechanism *Exp Cell Res*, **248,** 575-82.

De Maria, R, Maggiora, P, Biolatti, B, Prat, M, Comoglio, PM, Castagnaro, M, et al. (2002). Feline STK gene expression in mammary carcinomas *Oncogene.*, **21(11),** 1785-90.

Debnath, J and Brugge, JS. (2005). Modelling glandular epithelial cancers in three-dimensional cultures *Nat Rev Cancer*, **5,** 675-88.

Debnath, J, Mills, KR, Collins, NL, Reginato, MJ, Muthuswamy, SK and Brugge, JS. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini *Cell*, **111,** 29-40.

Debnath, J, Muthuswamy, SK and Brugge, JS. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures *Methods*, **30,** 256-68.

Degen, SJ, Stuart, LA, Han, S and Jamison, CS. (1991). Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: expression during development *Biochemistry*, **30,** 9781-91.
Deiters, B and Prehm, P. (2008). Inhibition of hyaluronan export reduces collagen degradation in interleukin-1 treated cartilage *Arthritis Res Ther*, **10,** R8.
DiGiovanna, MP, Stern, DF, Edgerton, SM, Whalen, SG, Moore, D, 2nd and Thor, AD. (2005). Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients *J Clin Oncol*, **23,** 1152-60.
Donate, LE, Gherardi, E, Srinivasan, N, Sowdhamini, R, Aparicio, S and Blundell, TL. (1994). Molecular evolution and domain structure of plasminogen-related growth factors (HGF/SF and HGF1/MSP) *Protein Sci*, **3,** 2378-94.
Draffin, JE, McFarlane, S, Hill, A, Johnston, PG and Waugh, DJ. (2004). CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells *Cancer Res*, **64,** 5702-11.
Eder, JP, Vande Woude, GF, Boerner, SA and Lorusso, PM. (2009). Novel Therapeutic Inhibitors of the c-Met Signaling Pathway in Cancer *Clin Cancer Res*, **15,** 2207-14.
Eisinger, DA and Ammer, H. (2008). Delta-opioid receptors activate ERK/MAP kinase via integrin-stimulated receptor tyrosine kinases *Cell Signal*, **20,** 2324-31.
Emaduddin, M, Bicknell, DC, Bodmer, WF and Feller, SM. (2008). Cell growth, global phosphotyrosine elevation, and c-Met phosphorylation through Src family kinases in colorectal cancer cells *Proc Natl Acad Sci U S A*, **105,** 2358-62.
Engen, JR, Wales, TE, Hochrein, JM, Meyn, MA, 3rd, Banu Ozkan, S, Bahar, I, et al. (2008). Structure and dynamic regulation of Src-family kinases *Cell Mol Life Sci*, **65,** 3058-73.
Evanko, SP, Angello, JC and Wight, TN. (1999). Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells *Arterioscler Thromb Vasc Biol*, **19,** 1004-13.
Evanko, SP, Johnson, PY, Braun, KR, Underhill, CB, Dudhia, J and Wight, TN. (2001). Platelet-derived growth factor stimulates the formation of versican-hyaluronan aggregates and pericellular matrix expansion in arterial smooth muscle cells *Arch Biochem Biophys*, **394,** 29-38.
Evanko, SP, Tammi, MI, Tammi, RH and Wight, TN. (2007). Hyaluronan-dependent pericellular matrix *Adv Drug Deliv Rev*, **59,** 1351-65.
Feldner, JC and Brandt, BH. (2002). Cancer cell motility--on the road from c-erbB-2 receptor steered signaling to actin reorganization *Exp Cell Res*, **272,** 93-108.
Feres, KJ, Ischenko, I and Hayman, MJ. (2009). The RON receptor tyrosine kinase promotes MSP-independent cell spreading and survival in breast epithelial cells *Oncogene*, **28,** 279-88.

Ferrandina, G, Martinelli, E, Petrillo, M, Prisco, MG, Zucconi, A, Santaguida, S, et al. (2008). Prognostic role of the recepteur d'origine nantais (RON) expression in ovarian cancer patients *Gynecol Oncol*, **111,** 237-43.

Fidler, IJ. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited *Nat Rev Cancer*, **3,** 453-458.

Follenzi, A, Bakovic, S, Gual, P, Stella, MC, Longati, P and Comoglio, PM. (2000). Cross-talk between the proto-oncogenes Met and Ron *Oncogene*, **19,** 3041-9.

Franke, TF. (2008). PI3K/Akt: getting it right matters *Oncogene*, **27,** 6473-88.

Fujita, Y, Kitagawa, M, Nakamura, S, Azuma, K, Ishii, G, Higashi, M, et al. (2002). CD44 signaling through focal adhesion kinase and its anti-apoptotic effect *FEBS Lett*, **528,** 101-8.

Funakoshi, H and Nakamura, T. (2001). Identification of HGF-like protein as a novel neurotrophic factor for avian dorsal root ganglion sensory neurons *Biochem Biophys Res Commun*, **283,** 606-12.

Fuster, MM and Esko, JD. (2005). The sweet and sour of cancer: glycans as novel therapeutic targets *Nat Rev Cancer*, **5,** 526-42.

Gaudino, G, Avantaggiato, V, Follenzi, A, Acampora, D, Simeone, A and Comoglio, PM. (1995). The proto-oncogene RON is involved in development of epithelial, bone and neuro-endocrine tissues *Oncogene*, **11,** 2627-37.

Gaudino, G, Follenzi, A, Naldini, L, Collesi, C, Santoro, M, Gallo, KA, et al. (1994). RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP *Embo J*, **13,** 3524-32.

Germano, S, Barberis, D, Santoro, MM, Penengo, L, Citri, A, Yarden, Y, et al. (2006). Geldanamycins trigger a novel Ron degradative pathway, hampering oncogenic signaling *J Biol Chem*, **281,** 21710-9.

Giordano, S, Corso, S, Conrotto, P, Artigiani, S, Gilestro, G, Barberis, D, et al. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met *Nat Cell Biol*, **4,** 720-4.

Giordano, S, Maffe, A, Williams, TA, Artigiani, S, Gual, P, Bardelli, A, et al. (2000). Different point mutations in the met oncogene elicit distinct biological properties *FASEB J.*, **14,** 399-406.

Guo, A, Villen, J, Kornhauser, J, Lee, KA, Stokes, MP, Rikova, K, et al. (2008). Signaling networks assembled by oncogenic EGFR and c-Met *Proc Natl Acad Sci U S A*, **105,** 692-7.

Haglund, K, Sigismund, S, Polo, S, Szymkiewicz, I, Di Fiore, PP and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation *Nat Cell Biol*, **5,** 461-6.

Hamilton, SR, Fard, SF, Paiwand, FF, Tolg, C, Veiseh, M, Wang, C, et al. (2007). The hyaluronan receptors CD44 and Rhamm (CD168) form complexes with ERK1,2 that sustain high basal motility in breast cancer cells *J Biol Chem*, **282,** 16667-80.

Hanafusa, H, Torii, S, Yasunaga, T and Nishida, E. (2002). Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway *Nat Cell Biol*, **4,** 850-8.

Hanahan, D and Weinberg, RA. (2000). The hallmarks of cancer *Cell*, **100,** 57-70.
Harada, N, Mizoi, T, Kinouchi, M, Hoshi, K, Ishii, S, Shiiba, K, et al. (2001). Introduction of antisense CD44S CDNA down-regulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells *Int J Cancer*, **91,** 67-75.
Hascall, VC, Majors, AK, De La Motte, CA, Evanko, SP, Wang, A, Drazba, JA, et al. (2004). Intracellular hyaluronan: a new frontier for inflammation? *Biochim Biophys Acta*, **1673,** 3-12.
Heldin, P, Karousou, E, Bernert, B, Porsch, H, Nishitsuka, K and Skandalis, SS. (2008). Importance of hyaluronan-CD44 interactions in inflammation and tumorigenesis *Connect Tissue Res*, **49,** 215-8.
Heldin, P, Laurent, TC and Heldin, CH. (1989). Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts *Biochem J*, **258,** 919-22.
Heldin, P and Pertoft, H. (1993). Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells *Exp Cell Res*, **208,** 422-9.
Helfrich, BA, Raben, D, Varella-Garcia, M, Gustafson, D, Chan, DC, Bemis, L, et al. (2006). Antitumor activity of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (ZD1839, Iressa) in non-small cell lung cancer cell lines correlates with gene copy number and EGFR mutations but not EGFR protein levels *Clin Cancer Res*, **12,** 7117-25.
Hildebrand, JD, Schaller, MD and Parsons, JT. (1995). Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase *Mol Biol Cell*, **6,** 637-47.
Hirano, H, Screaton, GR, Bell, MV, Jackson, DG, Bell, JI and Hodes, RJ. (1994). CD44 isoform expression mediated by alternative splicing: tissue-specific regulation in mice *Int Immunol*, **6,** 49-59.
Hofmann, C, Obermeier, F, Artinger, M, Hausmann, M, Falk, W, Schoelmerich, J, et al. (2007). Cell-cell contacts prevent anoikis in primary human colonic epithelial cells *Gastroenterology*, **132,** 587-600.
Hsu, PY, Liu, HS, Cheng, HL, Tzai, TS, Guo, HR, Ho, CL, et al. (2006). Collaboration of RON and epidermal growth factor receptor in human bladder carcinogenesis *J Urol*, **176,** 2262-7.
Hudson, DL, Sleeman, J and Watt, FM. (1995). CD44 is the major peanut lectin-binding glycoprotein of human epidermal keratinocytes and plays a role in intercellular adhesion *J Cell Sci*, **108 (Pt 5),** 1959-70.
Huff, JL, Jelinek, MA, Borgman, CA, Lansing, TJ and Parsons, JT. (1993). The protooncogene c-sea encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor *Proc Natl Acad Sci U S A*, **90,** 6140-4.
Hunter, T. (1997). Oncoprotein networks *Cell*, **88,** 333-46.
Hunter, T. (2000). Signaling--2000 and beyond *Cell*, **100,** 113-27.
Hunter, T and Eckhart, W. (2004). The discovery of tyrosine phosphorylation: it's all in the buffer! *Cell*, **116,** S35-9, 1 p following S48.

Irby, RB, Mao, W, Coppola, D, Kang, J, Loubeau, JM, Trudeau, W, et al. (1999). Activating SRC mutation in a subset of advanced human colon cancers *Nat Genet*, **21,** 187-90.

Ischenko, I, Petrenko, O, Gu, H and Hayman, MJ. (2003). Scaffolding protein Gab2 mediates fibroblast transformation by the SEA tyrosine kinase *Oncogene*, **22,** 6311-8.

Ishizawar, R and Parsons, SJ. (2004). c-Src and cooperating partners in human cancer *Cancer Cell*, **6,** 209-14.

Ishizawar, RC, Tice, DA, Karaoli, T and Parsons, SJ. (2004). The C terminus of c-Src inhibits breast tumor cell growth by a kinase-independent mechanism *J Biol Chem*, **279,** 23773-81.

Iwama, A, Wang, MH, Yamaguchi, N, Ohno, N, Okano, K, Sudo, T, et al. (1995). Terminal differentiation of murine resident peritoneal macrophages is characterized by expression of the STK protein tyrosine kinase, a receptor for macrophage-stimulating protein *Blood*, **86,** 3394-403.

Jeffers, M, Fiscella, M, Webb, CP, Anver, M, Koochekpour, S and Vande Woude, GF. (1998). The mutationally activated Met receptor mediates motility and metastasis *Proc Natl Acad Sci U S A*, **95,** 14417-22.

Johnson, GL and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases *Science*, **298,** 1911-2.

Kang, HG, Jenabi, JM, Zhang, J, Keshelava, N, Shimada, H, May, WA, et al. (2007). E-cadherin cell-cell adhesion in ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase *Cancer Res*, **67,** 3094-105.

Kass, L, Erler, JT, Dembo, M and Weaver, VM. (2007). Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis *Int J Biochem Cell Biol*, **39,** 1987-94.

Kim, HJ and Bar-Sagi, D. (2004). Modulation of signalling by Sprouty: a developing story *Nat Rev Mol Cell Biol*, **5,** 441-50.

Kim, Y, Lee, YS, Choe, J, Lee, H, Kim, YM and Jeoung, D. (2008). CD44-epidermal growth factor receptor interaction mediates hyaluronic acid-promoted cell motility by activating protein kinase C signaling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP-2 *J Biol Chem*, **283,** 22513-28.

Kimata, K, Honma, Y, Okayama, M, Oguri, K, Hozumi, M and Suzuki, S. (1983). Increased synthesis of hyaluronic acid by mouse mammary carcinoma cell variants with high metastatic potential *Cancer Res*, **43,** 1347-54.

Klaus, A and Birchmeier, W. (2008). Wnt signalling and its impact on development and cancer *Nat Rev Cancer*, **8,** 387-98.

Knudson, W, Biswas, C and Toole, BP. (1984). Interactions between human tumor cells and fibroblasts stimulate hyaluronate synthesis *Proc Natl Acad Sci U S A*, **81,** 6767-71.

Kosaki, R, Watanabe, K and Yamaguchi, Y. (1999). Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity *Cancer Res*, **59,** 1141-5.
Larsen, J, Meyer, S, Steinlein, P, Beug, H and Hayman, MJ. (1993). Transformation of chicken bone marrow cells by the v-ski oncogene *Oncogene*, **8,** 3221-8.
Lee, CT, Chow, NH, Su, PF, Lin, SC, Lin, PC and Lee, JC. (2008). The prognostic significance of RON and MET receptor coexpression in patients with colorectal cancer *Dis Colon Rectum*, **51,** 1268-74.
Lee, JL, Wang, MJ, Sudhir, PR and Chen, JY. (2008). CD44 engagement promotes matrix-derived survival through the CD44-SRC-integrin axis in lipid rafts *Mol Cell Biol*, **28,** 5710-23.
Lee, WY, Chen, HH, Chow, NH, Su, WC, Lin, PW and Guo, HR. (2005). Prognostic significance of co-expression of RON and MET receptors in node-negative breast cancer patients *Clin Cancer Res*, **11,** 2222-8.
Lengyel, E, Prechtel, D, Resau, JH, Gauger, K, Welk, A, Lindemann, K, et al. (2005). C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu *Int J Cancer*, **113,** 678-82.
Leonard, EJ and Skeel, AH. (1978). Isolation of macrophage stimulating protein (MSP) from human serum *Exp Cell Res*, **114,** 117-26.
Leonard, EJ and Skeel, AH. (1979). Enhancement of spreading, phagocytosis and chemotaxis by macrophage stimulating protein (MSP) *Adv Exp Med Biol*, **121B,** 181-94.
Leonis, MA, Toney-Earley, K, Degen, SJ and Waltz, SE. (2002). Deletion of the Ron receptor tyrosine kinase domain in mice provides protection from endotoxin-induced acute liver failure *Hepatology*, **36,** 1053-60.
Levitzki, A and Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development *Science*, **267,** 1782-8.
Li, L, Heldin, CH and Heldin, P. (2006). Inhibition of platelet-derived growth factor-BB-induced receptor activation and fibroblast migration by hyaluronan activation of CD44 *J Biol Chem*, **281,** 26512-9.
Liang, TJ, Reid, AE, Xavier, R, Cardiff, RD and Wang, TC. (1996). Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors *J Clin Invest*, **97,** 2872-7.
Lilien, J and Balsamo, J. (2005). The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin *Curr Opin Cell Biol*, **17,** 459-65.
Liu, C, Li, Y, Semenov, M, Han, C, Baeg, GH, Tan, Y, et al. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism *Cell*, **108,** 837-47.
Liu, N, Gao, F, Han, Z, Xu, X, Underhill, CB and Zhang, L. (2001). Hyaluronan synthase 3 overexpression promotes the growth of TSU prostate cancer cells *Cancer Res*, **61,** 5207-14.
Lu, H, Ouyang, W and Huang, C. (2006). Inflammation, a key event in cancer development *Mol Cancer Res*, **4,** 221-33.

Lu, Y, Yao, HP and Wang, MH. (2008). Significance of the entire C-terminus in biological activities mediated by the RON receptor tyrosine kinase and its oncogenic variant RON160 *J Exp Clin Cancer Res*, **27,** 55.

Lutz, MA and Correll, PH. (2003). Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C zeta *J Leukoc Biol*, **73,** 802-14.

Ma, PC, Jagadeeswaran, R, Jagadeesh, S, Tretiakova, MS, Nallasura, V, Fox, EA, et al. (2005). Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer *Cancer Res*, **65,** 1479-88.

Ma, PC, Kijima, T, Maulik, G, Fox, EA, Sattler, M, Griffin, JD, et al. (2003). c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions *Cancer Res*, **63,** 6272-81.

Maggiora, P, Lorenzato, A, Fracchioli, S, Costa, B, Castagnaro, M, Arisio, R, et al. (2003). The RON and MET oncogenes are co-expressed in human ovarian carcinomas and cooperate in activating invasiveness *Exp Cell Res*, **288,** 382-9.

Maggiora, P, Marchio, S, Stella, MC, Giai, M, Belfiore, A, De Bortoli, M, et al. (1998). Overexpression of the RON gene in human breast carcinoma *Oncogene.*, **16(22),** 2927-33.

Manzanares, D, Monzon, ME, Savani, RC and Salathe, M. (2007). Apical oxidative hyaluronan degradation stimulates airway ciliary beating via RHAMM and RON *Am J Respir Cell Mol Biol*, **37,** 160-8.

Marieb, EA, Zoltan-Jones, A, Li, R, Misra, S, Ghatak, S, Cao, J, et al. (2004). Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production *Cancer Res*, **64,** 1229-32.

Marmor, MD and Yarden, Y. (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases *Oncogene*, **23,** 2057-70.

Matzke, A, Herrlich, P, Ponta, H and Orian-Rousseau, V. (2005). A five-amino-acid peptide blocks Met- and Ron-dependent cell migration *Cancer Res*, **65,** 6105-10.

McKay, MM and Morrison, DK. (2007). Integrating signals from RTKs to ERK/MAPK *Oncogene*, **26,** 3113-21.

Medico, E, Mongiovi, AM, Huff, J, Jelinek, MA, Follenzi, A, Gaudino, G, et al. (1996). The tyrosine kinase receptors Ron and Sea control "scattering" and morphogenesis of liver progenitor cells in vitro *Mol Biol Cell*, **7,** 495-504.

Mehlen, P and Puisieux, A. (2006). Metastasis: a question of life or death *Nat Rev Cancer*, **6,** 449-58.

Meloche, S and Pouyssegur, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition *Oncogene*, **26,** 3227-39.

Michael, KE, Dumbauld, DW, Burns, KL, Hanks, SK and Garcia, AJ. (2009). FAK Modulates Cell Adhesion Strengthening via Integrin Activation *Mol Biol Cell*.

Miranda, M and Sorkin, A. (2007). Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms *Mol Interv*, **7,** 157-67.

Misra, S, Obeid, LM, Hannun, YA, Minamisawa, S, Berger, FG, Markwald, RR, et al. (2008). Hyaluronan constitutively regulates activation of COX-2-mediated cell survival activity in intestinal epithelial and colon carcinoma cells *J Biol Chem*, **283,** 14335-44.

Mitra, SK and Schlaepfer, DD. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells *Curr Opin Cell Biol*, **18,** 516-23.

Mohapatra, S, Yang, X, Wright, JA, Turley, EA and Greenberg, AH. (1996). Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression *J Exp Med*, **183,** 1663-8.

Monga, SP, Mars, WM, Pediaditakis, P, Bell, A, Mule, K, Bowen, WC, et al. (2002). Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes *Cancer Res*, **62,** 2064-71.

Montesano, R, Soriano, JV, Malinda, KM, Ponce, ML, Bafico, A, Kleinman, HK, et al. (1998). Differential effects of hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis *Cell Growth Differ*, **9,** 355-65.

Morimoto, AM and Hayman, MJ. (1994). Role of oligomerization of the S13 Env-Sea oncoprotein in cell transformation *J Virol*, **68,** 1837-42.

Morin, PJ. (1999). beta-catenin signaling and cancer *Bioessays*, **21,** 1021-30.

Moro, L, Dolce, L, Cabodi, S, Bergatto, E, Boeri Erba, E, Smeriglio, M, et al. (2002). Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines *J Biol Chem*, **277,** 9405-14.

Muller, EJ, Williamson, L, Kolly, C and Suter, MM. (2008). Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *J Invest Dermatol*, **128,** 501-16.

Muraoka, RS, Sun, WY, Colbert, MC, Waltz, SE, Witte, DP, Degen, JL, et al. (1999). The Ron/STK receptor tyrosine kinase is essential for peri-implantation development in the mouse *J Clin Invest*, **103,** 1277-85.

Muthuswamy, SK, Li, D, Lelievre, S, Bissell, MJ and Brugge, JS. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini *Nat Cell Biol*, **3,** 785-92.

Nakamura, T, Nishizawa, T, Hagiya, M, Seki, T, Shimonishi, M, Sugimura, A, et al. (1989). Molecular cloning and expression of human hepatocyte growth factor *Nature*, **342,** 440-3.

Narasimhan, M and Ammanamanchi, S. (2008). Curcumin blocks RON tyrosine kinase-mediated invasion of breast carcinoma cells *Cancer Res*, **68,** 5185-92.

Nishigaki, K, Thompson, D, Hanson, C, Yugawa, T and Ruscetti, S. (2001). The envelope glycoprotein of friend spleen focus-forming virus covalently interacts with and constitutively activates a truncated form of the receptor tyrosine kinase Stk *J Virol*, **75,** 7893-903.

O'Toole, JM, Rabenau, KE, Burns, K, Lu, D, Mangalampalli, V, Balderes, P, et al. (2006). Therapeutic implications of a human neutralizing antibody to the macrophage-stimulating protein receptor tyrosine kinase (RON), a c-MET family member *Cancer Res*, **66,** 9162-70.

Oertli, B, Beck-Schimmer, B, Fan, X and Wuthrich, RP. (1998). Mechanisms of hyaluronan-induced up-regulation of ICAM-1 and VCAM-1 expression by murine kidney tubular epithelial cells: hyaluronan triggers cell adhesion molecule expression through a mechanism involving activation of nuclear factor-kappa B and activating protein-1 *J Immunol*, **161,** 3431-7.

Okigaki, M, Komada, M, Uehara, Y, Miyazawa, K and Kitamura, N. (1992). Functional characterization of human hepatocyte growth factor mutants obtained by deletion of structural domains *Biochemistry*, **31,** 9555-61.

Okino, T, Egami, H, Ohmachi, H, Takai, E, Tamori, Y, Nakagawa, K, et al. (1999). Presence of RON receptor tyrosine kinase and its splicing variant in malignant and non-malignant human colonic mucosa *Int J Oncol*, **15,** 709-14.

Ono, M. (2008). Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy *Cancer Sci*, **99,** 1501-6.

Orian-Rousseau, V, Chen, L, Sleeman, JP, Herrlich, P and Ponta, H. (2002). CD44 is required for two consecutive steps in HGF/c-Met signaling *Genes Dev*, **16,** 3074-86.

Ottenhoff-Kalff, AE, Rijksen, G, van Beurden, EA, Hennipman, A, Michels, AA and Staal, GE. (1992). Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product *Cancer Res*, **52,** 4773-8.

Palyi-Krekk, Z, Barok, M, Kovacs, T, Saya, H, Nagano, O, Szollosi, J, et al. (2008). EGFR and ErbB2 are functionally coupled to CD44 and regulate shedding, internalization and motogenic effect of CD44 *Cancer Lett*, **263,** 231-42.

Park, CH, Lee, MH, Chang, ES, Song, BJ and Jung, SS. (2005). Expression of c-met gene in invasive ductal carcinomas *Breast Cancer Research*, **7,** P12.

Park, M, Dean, M, Kaul, K, Braun, MJ, Gonda, MA and Vande Woude, G. (1987). Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors *Proc Natl Acad Sci U S A*, **84,** 6379-83.

Parsons, JT. (2003). Focal adhesion kinase: the first ten years *J Cell Sci*, **116,** 1409-16.

Patton, KT, Tretiakova, MS, Yao, JL, Papavero, V, Huo, L, Adley, BP, et al. (2004). Expression of RON Proto-oncogene in Renal Oncocytoma and Chromophobe Renal Cell Carcinoma *Am J Surg Pathol*, **28,** 1045-50.

Pawson, T. (1995). Protein modules and signalling networks *Nature*, **373,** 573-80.

Peace, BE, Hill, KJ, Degen, SJ and Waltz, SE. (2003). Cross-talk between the receptor tyrosine kinases Ron and epidermal growth factor receptor *Exp Cell Res*, **289,** 317-25.

Peace, BE, Toney-Earley, K, Collins, MH and Waltz, SE. (2005). Ron receptor signaling augments mammary tumor formation and metastasis in a murine model of breast cancer *Cancer Res*, **65,** 1285-93.

Penengo, L, Rubin, C, Yarden, Y and Gaudino, G. (2003). c-Cbl is a critical modulator of the Ron tyrosine kinase receptor *Oncogene*, **22,** 3669-79.

Persons, DA, Paulson, RF, Loyd, MR, Herley, MT, Bodner, SM, Bernstein, A, et al. (1999). Fv2 encodes a truncated form of the Stk receptor tyrosine kinase *Nat Genet*, **23,** 159-65.

Peschard, P and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases *Cancer Cell*, **3,** 519-23.

Peters, JM, McKay, RM, McKay, JP and Graff, JM. (1999). Casein kinase I transduces Wnt signals *Nature*, **401,** 345-50.

Peterson, RM, Yu, Q, Stamenkovic, I and Toole, BP. (2000). Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites *Am J Pathol*, **156,** 2159-67.

Petrenko, O, Beavis, A, Klaine, M, Kittappa, R, Godin, I and Lemischka, IR. (1999). The molecular characterization of the fetal stem cell marker AA4 *Immunity*, **10,** 691-700.

Playford, MP and Schaller, MD. (2004). The interplay between Src and integrins in normal and tumor biology *Oncogene*, **23,** 7928-46.

Ponzetto, C, Bardelli, A, Zhen, Z, Maina, F, dalla Zonca, P, Giordano, S, et al. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family *Cell*, **77,** 261-71.

Prehm, P. (1984). Hyaluronate is synthesized at plasma membranes *Biochem J*, **220,** 597-600.

Puri, N, Ahmed, S, Janamanchi, V, Tretiakova, M, Zumba, O, Krausz, T, et al. (2007). c-Met is a potentially new therapeutic target for treatment of human melanoma *Clin Cancer Res*, **13,** 2246-53.

Puri, N and Salgia, R. (2008). Synergism of EGFR and c-Met pathways, cross-talk and inhibition, in non-small cell lung cancer *J Carcinog*, **7,** 9.

R&D Systems. (2009).

Rai, SK, Duh, FM, Vigdorovich, V, Danilkovitch-Miagkova, A, Lerman, MI and Miller, AD. (2001). Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation *Proc Natl Acad Sci U S A*, **98,** 4443-8.

Ren, H, Yang, BF and Rainov, NG. (2007). Receptor tyrosine kinases as therapeutic targets in malignant glioma *Rev Recent Clin Trials*, **2,** 87-101.

Rena, G, Guo, S, Cichy, SC, Unterman, TG and Cohen, P. (1999). Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B *J Biol Chem*, **274,** 17179-83.

Renshaw, MW, Price, LS and Schwartz, MA. (1999). Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase *J Cell Biol*, **147,** 611-8.

Riggins, RB, Thomas, KS, Ta, HQ, Wen, J, Davis, RJ, Schuh, NR, et al. (2006). Physical and functional interactions between Cas and c-Src induce tamoxifen resistance of breast cancer cells through pathways involving epidermal growth factor receptor and signal transducer and activator of transcription 5b *Cancer Res*, **66,** 7007-15.

Roepstorff, K, Grovdal, L, Grandal, M, Lerdrup, M and van Deurs, B. (2008). Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer *Histochem Cell Biol*, **129,** 563-78.

Romond, EH, Perez, EA, Bryant, J, Suman, VJ, Geyer, CE, Jr., Davidson, NE, et al. (2005). Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer *N Engl J Med*, **353,** 1673-84.

Ronnov-Jessen, L and Bissell, MJ. (2009). Breast cancer by proxy: can the microenvironment be both the cause and consequence? *Trends Mol Med*, **15,** 5-13.

Ronsin, C, Muscatelli, F, Mattei, MG and Breathnach, R. (1993). A novel putative receptor protein tyrosine kinase of the met family *Oncogene*, **8,** 1195-202.

Rosario, M and Birchmeier, W. (2003). How to make tubes: signaling by the Met receptor tyrosine kinase *Trends Cell Biol*, **13,** 328-35.

Rubin, JS, Bottaro, DP and Aaronson, SA. (1993). Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product *Biochim Biophys Acta*, **1155,** 357-71.

Ruegg, C. (2006). Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions *J Leukoc Biol*, **80,** 682-4.

Ruest, PJ, Shin, NY, Polte, TR, Zhang, X and Hanks, SK. (2001). Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src *Mol Cell Biol*, **21,** 7641-52.

Ruscetti, SK. (1999). Deregulation of erythropoiesis by the Friend spleen focus-forming virus *Int J Biochem Cell Biol*, **31,** 1089-109.

Russo, J, Calaf, G and Russo, IH. (1993). A critical approach to the malignant transformation of human breast epithelial cells with chemical carcinogens *Crit Rev Oncog*, **4,** 403-17.

Santoro, MM, Gaudino, G and Marchisio, PC. (2003). The MSP receptor regulates alpha6beta4 and alpha3beta1 integrins via 14-3-3 proteins in keratinocyte migration *Dev Cell*, **5,** 257-71.

Santoro, MM, Penengo, L, Minetto, M, Orecchia, S, Cilli, M and Gaudino, G. (1998). Point mutations in the tyrosine kinase domain release the oncogenic and metastatic potential of the Ron receptor *Oncogene*, **17,** 741-9.

Schaller, MD. (2001). Paxillin: a focal adhesion-associated adaptor protein *Oncogene*, **20,** 6459-72.

Schaller, MD, Hildebrand, JD, Shannon, JD, Fox, JW, Vines, RR and Parsons, JT. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src *Mol Cell Biol*, **14,** 1680-8.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases *Cell*, **103,** 211-25.

Senchenko, VN, Liu, J, Loginov, W, Bazov, I, Angeloni, D, Seryogin, Y, et al. (2004). Discovery of frequent homozygous deletions in chromosome 3p21.3 LUCA and AP20 regions in renal, lung and breast carcinomas *Oncogene*, **23,** 5719-28.

Seton-Rogers, SE, Lu, Y, Hines, LM, Koundinya, M, LaBaer, J, Muthuswamy, SK, et al. (2004). Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells *Proc Natl Acad Sci U S A*, **101,** 1257-62.

Siesser, PM and Hanks, SK. (2006). The signaling and biological implications of FAK overexpression in cancer *Clin Cancer Res*, **12,** 3233-7.

Slamon, DJ, Clark, GM, Wong, SG, Levin, WJ, Ullrich, A and McGuire, WL. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene *Science*, **235,** 177-82.

Smith, DR, Vogt, PK and Hayman, MJ. (1989). The v-sea oncogene of avian erythroblastosis retrovirus S13: another member of the protein-tyrosine kinase gene family *Proc Natl Acad Sci U S A*, **86,** 5291-5.

Soman, NR, Correa, P, Ruiz, BA and Wogan, GN. (1991). The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions *Proc Natl Acad Sci U S A*, **88,** 4892-6.

Sorkin, A and Von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds *Nat Rev Mol Cell Biol*, **3,** 600-14.

Soule, HD, Maloney, TM, Wolman, SR, Peterson, WD, Jr., Brenz, R, McGrath, CM, et al. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10 *Cancer Res*, **50,** 6075-86.

Stahl, S, Weitzman, S and Jones, JC. (1997). The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis *J Cell Sci*, **110 (Pt 1),** 55-63.

Stern, R and Jedrzejas, MJ. (2006). Hyaluronidases: their genomics, structures, and mechanisms of action *Chem Rev*, **106,** 818-39.

Stommel, JM, Kimmelman, AC, Ying, H, Nabioullin, R, Ponugoti, AH, Wiedemeyer, R, et al. (2007). Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies *Science*, **318,** 287-90.

Stuhlmeier, KM and Pollaschek, C. (2004). Glucocorticoids inhibit induced and non-induced mRNA accumulation of genes encoding hyaluronan synthases (HAS): hydrocortisone inhibits HAS1 activation by blocking the p38 mitogen-activated protein kinase signalling pathway *Rheumatology (Oxford)*, **43,** 164-9.

Stupack, DG. (2007). The biology of integrins *Oncology (Williston Park)*, **21,** 6-12.

Subramaniam, V, Vincent, IR, Gardner, H, Chan, E, Dhamko, H and Jothy, S. (2007). CD44 regulates cell migration in human colon cancer cells via Lyn kinase and AKT phosphorylation *Exp Mol Pathol*, **83,** 207-15.

Sunpaweravong, P, Sunpaweravong, S, Puttawibul, P, Mitarnun, W, Zeng, C, Baron, AE, et al. (2005). Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma *J Cancer Res Clin Oncol*, **131,** 111-9.

Suzuki, Y, Funakoshi, H, Machide, M, Matsumoto, K and Nakamura, T. (2008). Regulation of cell migration and cytokine production by HGF-like protein (HLP) / macrophage stimulating protein (MSP) in primary microglia *Biomed Res*, **29,** 77-84.

Tagliaferri, P, Tassone, P, Blotta, S, Viscomi, C, Grillone, F, Budillon, A, et al. (2005). Antitumor therapeutic strategies based on the targeting of epidermal growth factor-induced survival pathways *Curr Drug Targets*, **6,** 289-300.

Tammi, MI, Day, AJ and Turley, EA. (2002). Hyaluronan and homeostasis: a balancing act *J Biol Chem*, **277,** 4581-4.

Tang, CK, Gong, XQ, Moscatello, DK, Wong, AJ and Lippman, ME. (2000). Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer *Cancer Res*, **60,** 3081-7.

Teal, HE, Ni, S, Xu, J, Finkelstein, LD, Cheng, AM, Paulson, RF, et al. (2006). GRB2-mediated recruitment of GAB2, but not GAB1, to SF-STK supports the expansion of Friend virus-infected erythroid progenitor cells *Oncogene*, **25,** 2433-43.

Termeer, C, Benedix, F, Sleeman, J, Fieber, C, Voith, U, Ahrens, T, et al. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4 *J Exp Med*, **195,** 99-111.

Termeer, CC, Hennies, J, Voith, U, Ahrens, T, Weiss, JM, Prehm, P, et al. (2000). Oligosaccharides of hyaluronan are potent activators of dendritic cells *J Immunol*, **165,** 1863-70.

Thangasamy, A, Rogge, J and Ammanamanchi, S. (2008). Regulation of RON tyrosine kinase-mediated invasion of breast cancer cells *J Biol Chem*, **283,** 5335-43.

Thiery, JP. (2002). Epithelial-mesenchymal transitions in tumour progression *Nat Rev Cancer*, **2,** 442-54.

Thomas, RM, Toney, K, Fenoglio-Preiser, C, Revelo-Penafiel, MP, Hingorani, SR, Tuveson, DA, et al. (2007). The RON receptor tyrosine kinase mediates oncogenic phenotypes in pancreatic cancer cells and is increasingly expressed during pancreatic cancer progression *Cancer Res*, **67,** 6075-82.

Tice, DA, Biscardi, JS, Nickles, AL and Parsons, SJ. (1999). Mechanism of biological synergy between cellular Src and epidermal growth factor receptor *Proc Natl Acad Sci U S A*, **96,** 1415-20.

Tilghman, RW and Parsons, JT. (2008). Focal adhesion kinase as a regulator of cell tension in the progression of cancer *Semin Cancer Biol*, **18,** 45-52.

Toole, BP. (2004). Hyaluronan: from extracellular glue to pericellular cue *Nat Rev Cancer*, **4,** 528-39.
Toole, BP, Biswas, C and Gross, J. (1979). Hyaluronate and invasiveness of the rabbit V2 carcinoma *Proc Natl Acad Sci U S A*, **76,** 6299-303.
Toole, BP and Slomiany, MG. (2008). Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells *Semin Cancer Biol*, **18,** 244-50.
Trusolino, L, Bertotti, A and Comoglio, PM. (2001). A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth *Cell*, **107,** 643-54.
Turley, EA, Noble, PW and Bourguignon, LY. (2002). Signaling properties of hyaluronan receptors *J Biol Chem*, **277,** 4589-92.
Udabage, L, Brownlee, GR, Nilsson, SK and Brown, TJ. (2005a). The over-expression of HAS2, Hyal-2 and CD44 is implicated in the invasiveness of breast cancer *Exp Cell Res*, **310,** 205-17.
Udabage, L, Brownlee, GR, Waltham, M, Blick, T, Walker, EC, Heldin, P, et al. (2005b). Antisense-mediated suppression of hyaluronan synthase 2 inhibits the tumorigenesis and progression of breast cancer *Cancer Res*, **65,** 6139-50.
Ullrich, A and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity *Cell*, **61,** 203-12.
van den Akker, E, van Dijk, T, Parren-van Amelsvoort, M, Grossmann, KS, Schaeper, U, Toney-Earley, K, et al. (2004). Tyrosine kinase receptor RON functions downstream of the erythropoietin receptor to induce expansion of erythroid progenitors *Blood*, **103,** 4457-65.
Van Slambrouck, S, Grijelmo, C, De Wever, O, Bruyneel, E, Emami, S, Gespach, C, et al. (2007). Activation of the FAK-src molecular scaffolds and p130Cas-JNK signaling cascades by alpha1-integrins during colon cancer cell invasion *Int J Oncol*, **31,** 1501-8.
Walshe, JM and Swain, SM. (2005). Clinical aspects of inflammatory breast cancer *Breast Dis*, **22,** 35-44.
Waltz, SE, Eaton, L, Toney-Earley, K, Hess, KA, Peace, BE, Ihlendorf, JR, et al. (2001). Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses *J Clin Invest*, **108,** 567-76.
Waltz, SE, Toms, CL, McDowell, SA, Clay, LA, Muraoka, RS, Air, EL, et al. (1998). Characterization of the mouse Ron/Stk receptor tyrosine kinase gene *Oncogene*, **16,** 27-42.
Wang, D, Shen, Q, Chen, YQ and Wang, MH. (2004). Collaborative activities of macrophage-stimulating protein and transforming growth factor-beta1 in induction of epithelial to mesenchymal transition: roles of the RON receptor tyrosine kinase *Oncogene*, **23,** 1668-80.
Wang, D, Shen, Q, Xu, XM, Chen, YQ and Wang, MH. (2005). Activation of the RON receptor tyrosine kinase attenuates transforming growth factor-beta1-induced apoptotic death and promotes phenotypic changes in mouse intestinal epithelial cells *Carcinogenesis*, **26,** 27-36.

Wang, MH, Cox, GW, Yoshimura, T, Sheffler, LA, Skeel, A and Leonard, EJ. (1994a). Macrophage-stimulating protein inhibits induction of nitric oxide production by endotoxin- or cytokine-stimulated mouse macrophages *J Biol Chem*, **269,** 14027-31.

Wang, MH, Dlugosz, AA, Sun, Y, Suda, T, Skeel, A and Leonard, EJ. (1996a). Macrophage-stimulating protein induces proliferation and migration of murine keratinocytes *Exp Cell Res*, **226,** 39-46.

Wang, MH, Iwama, A, Skeel, A, Suda, T and Leonard, EJ. (1995). The murine stk gene product, a transmembrane protein tyrosine kinase, is a receptor for macrophage-stimulating protein *Proc Natl Acad Sci U S A*, **92,** 3933-7.

Wang, MH, Julian, FM, Breathnach, R, Godowski, PJ, Takehara, T, Yoshikawa, W, et al. (1997). Macrophage stimulating protein (MSP) binds to its receptor via the MSP beta chain *J Biol Chem*, **272,** 16999-7004.

Wang, MH, Kurtz, AL and Chen, Y. (2000). Identification of a novel splicing product of the RON receptor tyrosine kinase in human colorectal carcinoma cells *Carcinogenesis*, **21,** 1507-12.

Wang, MH, Lao, WF, Wang, D, Luo, YL and Yao, HP. (2007a). Blocking Tumorigenic Activities of Colorectal Cancer Cells by a Splicing RON Receptor Variant Defective in the Tyrosine Kinase Domain *Cancer Biol Ther*, **6**.

Wang, MH, Lee, W, Luo, YL, Weis, MT and Yao, HP. (2007b). Altered expression of the RON receptor tyrosine kinase in various epithelial cancers and its contribution to tumourigenic phenotypes in thyroid cancer cells *J Pathol*, **213,** 402-11.

Wang, MH, Ronsin, C, Gesnel, MC, Coupey, L, Skeel, A, Leonard, EJ, et al. (1994b). Identification of the ron gene product as the receptor for the human macrophage stimulating protein *Science*, **266,** 117-9.

Wang, MH, Skeel, A and Leonard, EJ. (1996b). Proteolytic cleavage and activation of pro-macrophage-stimulating protein by resident peritoneal macrophage membrane proteases *J Clin Invest*, **97,** 720-7.

Wang, MH, Wang, D and Chen, YQ. (2003). Oncogenic and invasive potentials of human macrophage-stimulating protein receptor, the RON receptor tyrosine kinase *Carcinogenesis*, **24,** 1291-300.

Wang, MH, Yao, HP and Zhou, YQ. (2006). Oncogenesis of RON receptor tyrosine kinase: a molecular target for malignant epithelial cancers *Acta Pharmacol Sin*, **27,** 641-50.

Wang, R, Ferrell, LD, Faouzi, S, Maher, JJ and Bishop, JM. (2001). Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice *J Cell Biol*, **153,** 1023-34.

Wang, R, Kobayashi, R and Bishop, JM. (1996). Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor *Proc Natl Acad Sci U S A*, **93,** 8425-30.

Wei, X, Ni, S and Correll, PH. (2005). Uncoupling ligand-dependent and -independent mechanisms for mitogen-activated protein kinase activation by the murine Ron receptor tyrosine kinase *J Biol Chem*, **280,** 35098-107.

Weidner, KM, Sachs, M and Birchmeier, W. (1993). The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells *J Cell Biol*, **121,** 145-54.

Weigel, PH, Fuller, GM and LeBoeuf, RD. (1986). A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing *J Theor Biol*, **119,** 219-34.

Weiss, FU, Daub, H and Ullrich, A. (1997). Novel mechanisms of RTK signal generation *Curr Opin Genet Dev*, **7,** 80-6.

Welm, AL, Sneddon, JB, Taylor, C, Nuyten, DS, van de Vijver, MJ, Hasegawa, BH, et al. (2007). The macrophage-stimulating protein pathway promotes metastasis in a mouse model for breast cancer and predicts poor prognosis in humans *Proc Natl Acad Sci U S A*, **104,** 7570-5.

Wickramasinghe, D and Kong-Beltran, M. (2005). Met activation and receptor dimerization in cancer: a role for the Sema domain *Cell Cycle*, **4,** 683-5.

Wilkinson, TS, Bressler, SL, Evanko, SP, Braun, KR and Wight, TN. (2006). Overexpression of hyaluronan synthases alters vascular smooth muscle cell phenotype and promotes monocyte adhesion *J Cell Physiol*, **206,** 378-85.

Willett, CG, Wang, MH, Emanuel, RL, Graham, SA, Smith, DI, Shridhar, V, et al. (1998). Macrophage-stimulating protein and its receptor in non-small-cell lung tumors: induction of receptor tyrosine phosphorylation and cell migration *Am J Respir Cell Mol Biol*, **18,** 489-96.

Wilson, CB, Ray, M, Lutz, M, Sharda, D, Xu, J and Hankey, PA. (2008). The RON receptor tyrosine kinase regulates IFN-gamma production and responses in innate immunity *J Immunol*, **181,** 2303-10.

Wilson, LK, Luttrell, DK, Parsons, JT and Parsons, SJ. (1989). pp60c-src tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src *Mol Cell Biol*, **9,** 1536-44.

Wrobel, CN, Debnath, J, Lin, E, Beausoleil, S, Roussel, MF and Brugge, JS. (2004). Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture *J Cell Biol*, **165,** 263-73.

Wu, W, Graves, LM, Gill, GN, Parsons, SJ and Samet, JM. (2002). Src-dependent phosphorylation of the epidermal growth factor receptor on tyrosine 845 is required for zinc-induced Ras activation *J Biol Chem*, **277,** 24252-7.

Xiao, ZQ, Chen, YQ and Wang, MH. (2000). Requirement of both tyrosine residues 1330 and 1337 in the C-terminal tail of the RON receptor tyrosine kinase for epithelial cell scattering and migration *Biochem Biophys Res Commun*, **267,** 669-75.

Xu, W, Doshi, A, Lei, M, Eck, MJ and Harrison, SC. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism *Mol Cell*, **3,** 629-38.

Xu, XM, Wang, D, Shen, Q, Chen, YQ and Wang, MH. (2004). RNA-mediated gene silencing of the RON receptor tyrosine kinase alters oncogenic phenotypes of human colorectal carcinoma cells *Oncogene*, **23,** 8464-74.

Xu, XM, Zhou, YQ and Wang, MH. (2005). Mechanisms of cytoplasmic {beta}-catenin accumulation and its involvement in tumorigenic activities mediated by oncogenic splicing variant of the receptor originated from Nantes tyrosine kinase *J Biol Chem*, **280,** 25087-94.

Yamaguchi, H, Wyckoff, J and Condeelis, J. (2005). Cell migration in tumors *Curr Opin Cell Biol*, **17,** 559-64.

Yamamoto, N, Mammadova, G, Song, RX, Fukami, Y and Sato, K. (2006). Tyrosine phosphorylation of p145met mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells *J Cell Sci*, **119,** 4623-33.

Yamasaki, K, Muto, J, Taylor, KR, Cogen, AL, Audish, D, Bertin, J, et al. (2009). NLRP3/cryopyrin is necessary for IL-1beta release in response to hyaluronan, an endogenous trigger of inflammation in response to injury *J Biol Chem*.

Yamazaki, H, Fukui, Y, Ueyama, Y, Tamaoki, N, Kawamoto, T, Taniguchi, S, et al. (1988). Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-erbB) in human brain tumors *Mol Cell Biol*, **8,** 1816-20.

Yarden, Y and Sliwkowski, MX. (2001). Untangling the ErbB signalling network *Nat Rev Mol Cell Biol*, **2,** 127-37.

Yokoyama, N, Ischenko, I, Hayman, MJ and Miller, WT. (2005). The C terminus of RON tyrosine kinase plays an autoinhibitory role *J Biol Chem*, **280,** 8893-900.

Yoon, S and Seger, R. (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions *Growth Factors*, **24,** 21-44.

Yoshimura, T, Yuhki, N, Wang, MH, Skeel, A and Leonard, EJ. (1993). Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on chromosome 3 *J Biol Chem*, **268,** 15461-8.

Young, NM and Williams, RE. (1985). Assignment of lectins specific for D-galactose or N-acetyl-D-galactosamine to two groups, based on their circular dichroism *Can J Biochem Cell Biol*, **63,** 268-71.

Yu, H, Gong, X, Luo, X, Han, W, Hong, G, Singh, B, et al. (2008). Co-expression of EGFRvIII with ErbB-2 enhances tumorigenesis: EGFRvIII mediated constitutively activated and sustained signaling pathways, whereas EGF-induced a transient effect on EGFR-mediated signaling pathways *Cancer Biol Ther*, **7**.

Yujiri, T, Sather, S, Fanger, GR and Johnson, GL. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption *Science*, **282,** 1911-4.

Zabarovsky, ER, Lerman, MI and Minna, JD. (2002). Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers *Oncogene*, **21,** 6915-35.

Zaidel-Bar, R, Cohen, M, Addadi, L and Geiger, B. (2004). Hierarchical assembly of cell-matrix adhesion complexes *Biochem Soc Trans*, **32,** 416-20.

Zalcenstein, A, Weisz, L, Stambolsky, P, Bar, J, Rotter, V and Oren, M. (2006). Repression of the MSP/MST-1 gene contributes to the antiapoptotic gain of function of mutant p53 *Oncogene*, **25,** 359-69.

Zhang, H, Pelzer, AM, Kiang, DT and Yee, D. (2007). Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin *Cancer Res*, **67,** 391-7.

Zhang, K, Yao, HP and Wang, MH. (2008). Activation of RON differentially regulates claudin expression and localization: role of claudin-1 in RON-mediated epithelial cell motility *Carcinogenesis*, **29,** 552-9.

Zhang, Y, Kaplan-Lefko, PJ, Rex, K, Yang, Y, Moriguchi, J, Osgood, T, et al. (2008). Identification of a novel recepteur d'origine nantais/c-met small-molecule kinase inhibitor with antitumor activity in vivo *Cancer Res*, **68,** 6680-7.

Zhao, L and Vogt, PK. (2008). Class I PI3K in oncogenic cellular transformation *Oncogene*, **27,** 5486-96.

Zhao, S, Ammanamanchi, S, Brattain, M, Cao, L, Thangasamy, A, Wang, J, et al. (2008). Smad4-dependent TGF-beta signaling suppresses RON receptor tyrosine kinase-dependent motility and invasion of pancreatic cancer cells *J Biol Chem*, **283,** 11293-301.

Zhou, DH, Pan, G, Zheng, C, Zheng, JJ, Yian, LP and Teng, XD. (2008). Expression of the RON receptor tyrosine kinase and its association with gastric carcinoma versus normal gastric tissues *BMC Cancer*, **8,** 353.

Zhou, GL, Zhuo, Y, King, CC, Fryer, BH, Bokoch, GM and Field, J. (2003). Akt phosphorylation of serine 21 on Pak1 modulates Nck binding and cell migration *Mol Cell Biol*, **23,** 8058-69.

Zhou, YQ, He, C, Chen, YQ, Wang, D and Wang, MH. (2003). Altered expression of the RON receptor tyrosine kinase in primary human colorectal adenocarcinomas: generation of different splicing RON variants and their oncogenic potential *Oncogene*, **22,** 186-97.

Zinser, GM, Leonis, MA, Toney, K, Pathrose, P, Thobe, M, Kader, SA, et al. (2006). Mammary-specific Ron receptor overexpression induces highly metastatic mammary tumors associated with beta-catenin activation *Cancer Res*, **66,** 11967-74.

Zwick, E, Bange, J and Ullrich, A. (2001). Receptor tyrosine kinase signalling as a target for cancer intervention strategies *Endocr Relat Cancer*, **8,** 161-73.