DOI:10.1158/0008-5472.CAN-06-2473

Cancer Research

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Cancer Res 2006;66:11967-11974. Published online December 18, 2006.

DOI:10.1158/0008-5472.CAN-06-2473

Research Article

Mammary-Specific Ron Receptor Overexpression Induces Highly Metastatic Mammary Tumors Associated with β-Catenin Activation

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Abstract

Activated growth factor receptor tyrosine kinases (RTK) play pivotal roles in a variety of human cancers, including breast cancer. Ron, a member of the Met RTK proto-oncogene family, is overexpressed or constitutively active in 50% of human breast cancers. To define the significance of Ron overexpression and activation *in vivo*, we generated transgenic mice that overexpress a wild-type or constitutively active Ron receptor in the mammary epithelium. In these animals, Ron expression is significantly elevated in mammary glands and leads to a hyperplastic phenotype by 12 weeks of age. Ron overexpression is sufficient to induce mammary transformation in all transgenic animals and is associated with a high degree of metastasis, with metastatic foci detected in liver and lungs of >86% of all transgenic animals. Furthermore, we show that Ron overexpression leads to receptor phosphorylation and is associated with elevated levels of tyrosine phosphorylated β-catenin and the up-regulation of genes, including *cyclin D1* and *c-myc*, which are associated with poor prognosis in patients with human breast cancers. These studies suggest that Ron overexpression may be a causative factor in breast tumorigenesis and provides a model to dissect the mechanism by which the Ron induces transformation and metastasis. (Cancer Res 2006; 66(24): 11967-74)

Introduction

Overexpression or increased activation of receptor tyrosine kinases (RTK) has been associated with many malignant human cancers, including breast cancer. Breast cancer prognosis has been associated with abnormal RTK expression due to gene amplification, protein overexpression, or abnormal transcriptional regulation (1–3). Moreover, several studies have reported that protein kinase activity is higher in most malignant human cancers compared with normal tissue or in benign tumors (4–7). Recently, the Ron RTK is overexpressed and constitutively active in ~50% of primary breast cancer cases (8) and increased expression of the Ron receptor strongly correlates with the more aggressive phenotype observed in node-negative breast tumors (9). These findings raise the question of whether overexpression or activation of the Ron receptor is a driving force in mammary gland tumorigenesis *in vivo*.

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doi:10.1158/0008-5472.CAN-06-2473

The Ron receptor is a member of a distinct subfamily of RTKs, such as the Met proto-oncogene. In its mature form, Ron exists as a heterodimer composed of a 35-kDa extracellular α chain and a 150-kDa transmembrane-spanning β chain with intrinsic tyrosine kinase activity. On binding its ligand, hepatocyte growth factor-like protein, Ron becomes phosphorylated at key intracellular tyrosine residues that provide docking sites for downstream signaling adapter molecules (10–14). Ligand-stimulated Ron triggers activation of several signaling pathways, including phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase (MAPK), c-Jun NH_2-terminal kinase, and β-catenin, which participate in cell proliferation, differentiation, and migration (11–14). Ron activation induces "invasive growth" of transformed cells, leading to cell-cell dissociation (scattering), cellular proliferation, cell motility, morphologic changes, and increased tumorigenic capacity (13–16).

Several human neoplastic syndromes are associated with activating point mutations in a highly conserved region of the Met tyrosine kinase domain; *in vitro*, these mutations lead to the accumulation and recruitment of the β-catenin signaling pathway (17, 18). The oncogenic potential of Ron receptor gain of function has been assessed *in vitro* by overexpressing either wild-type Ron (WT-Ron) or analogous mutant forms of Ron in transformed cell lines. Our laboratory and others have shown that overexpression of WT-Ron or constitutively active mutant forms of Ron cause increased tumorigenic properties of transformed cell lines, which may be mediated in part by activation of the β-catenin signaling cascade (19, 20).

Whereas these studies suggest that elevated expression of WT-Ron can transform epithelial cells *in vitro*, the potential for Ron overexpression to induce mammary tumorigenesis *in vivo* remains to be established. To investigate the significance of Ron overexpression in the mammary gland, we created transgenic mice overexpressing either WT-Ron or a constitutively active form of Ron (MT-Ron) in the mammary epithelium under the control of the mouse mammary tumor virus (MMTV) promoter. This approach allowed us to directly assess the effect of Ron receptor overexpression on mammary tumorigenesis and metastasis.

These studies show that Ron overexpression or constitutive activation within the mammary gland is sufficient to induce mammary tumors at a high incidence and containing aggressive metastatic potential. Our work suggests that Ron receptor signaling not only plays a vital role in mammary gland tumor formation but also serves as a critical regulator of the complex biological processes used for epithelial cell metastasis. Most importantly, these studies provide an *in vivo* animal model that recapitulates the aggressive phenotype observed in Ron-overexpressing human breast tumors.

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Materials and Methods

Cloning and construction of the Ron transgenes. WT and constitutively active murine *Ron* minigenes were constructed using 5′ genomic DNA and 3′ cDNA fragments of the mouse (m) *Ron* gene (accession nos. U65949 and X74736, respectively; see Fig. 1; refs. 19, 21, 22). A 3.2-kb *Spe*I/*Eco*RI fragment of mRon genomic DNA encompassing exon 1 and half of intron 1 was cloned into pBluescript. Subsequently, a 3.2-kb *Eco*RI m*Ron* genomic DNA fragment encompassing exons 2 to 6 was cloned into *Eco*RI-digested of this plasmid. A 2.8-kb *Age*I/*Xho*I fragment of m*Ron* cDNA encoding exons 4 to 19 was directionally cloned into *Age*I/*Xho*I-digested plasmid, causing a vector harboring a full-length WT mRon minigene. For the constitutively active *Ron* construct, an analogous 2.8-kb *Age*I/*Xho*I fragment of a m*Ron* cDNA harboring a methionine-to-threonine point mutation at amino acid 1,231 of the m*Ron* cDNA sequence (19) was generated.

A 2.3-kb *Bam*HI DNA cassette harboring the MMTV promoter was excised from vector pA9 (23) and cloned into *Bam*HI-digested pIND (Invitrogen, Carlsbad, CA), giving rise to MMTV-pIND. The 8.4-kb *Not*I fragments containing the WT and ΔM1231T m*Ron* minigenes were then cloned into the *Not*I site of MMTV-pIND, causing a vector harboring the MMTV promoter 5′ to the respective m*Ron* minigene constructs. Excision of these plasmids with *Pme*I yielded 10,806-bp MMTV promoter-driven m*Ron* DNA constructs (see Fig. 1).

Generation and identification of transgenic animals. The Ron minigene constructs were injected into fertilized eggs from FVB/N mice. Positive founders were crossed with WT FVB/N mice (Taconic Laboratory, Germantown, NY) to generate the F_1 offspring. F_1 positive mice were subsequently crossed with WT littermates to generate offspring used in the analyses. Genotype analysis of the transgenic mice was determined by PCR and Southern analyses. For PCR analyses, primers (5′-TGGGTGGTGAGGTCTGCCAACATGAGCTCC-3′) and (5′-CCGTCTTCGGGAGTTAAAGATCAGGGCAAC-3′) were used and produced a 251-bp fragment corresponding to the *Ron* minigene and a 331-bp fragment corresponding to the endogenous *Ron* sequence. Transgenic transmission was further confirmed by Southern analysis using a 708-bp probe. For Southern analysis, DNA was digested with *Bam*HI and the Southern membranes were probed with a PCR-generated fragment (5′-TCCCCAACAACACTCTCTGACATCA-3′ and 5′-ACAAAGGACCTGCAGCCTGAGGTCA-3′). For Southern analyses, a genomic band of 4.7 kb is generated, whereas a band of 3.7 kb is obtained from the transgene insertion. Copy number was determined by comparison to the endogenous allele. All animal procedures were approved by the University of Cincinnati Animal Care and Use Committee.

RNA isolation and Northern analysis. Total RNA was isolated from tissues using Trizol (Invitrogen). RNA-containing membranes were hybridized at 68°C for 24 hours with a mouse *Ron* cDNA. Membranes were exposed to a PhosphorImager (Molecular Dynamics Storm 860 PhosphorImager system, Piscataway, NJ). The membrane was reprobed with a 213-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR fragment (5′-GCTCCTCTCGCCAAGGTTATTC-3′ and 5′-GCTCTGGGATGACTTTGCCTACAG-3′ accession no. MMU09964).

Protein isolation and assays. Tissues were homogenized in protein lysis buffer [50 mmol/L Tris (pH 7.4), 0.5% Triton X-100, 0.5% IGEPAL, 150 mmol/L NaCl, 2 mmol/L EDTA] containing protease inhibitor (Complete Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN) and 1 mmol/L Na_3VO_4. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). After transfer, the membranes were probed with a rabbit polyclonal anti-Ron antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific binding was detected using an anti-rabbit alkaline phosphatase secondary antibody. The membrane was developed using electrochemifluorescence Western detection reagent (Amersham Biosciences, Piscataway, NJ) and images were captured by the AlphaInnotech gel documentation system (San Leandro, CA).

The membrane was stripped and reprobed sequentially with the following antibodies: anti-pRon (Biosource International, Camarillo, CA), anti-β-catenin (Sigma-Aldrich, St. Louis, MO), anti-cyclin D1 (Santa Cruz Biotechnology), anti-MAPK (Upstate Cell Signaling Solutions, Lake Placid, NY), anti-phosphorylated p44/42 MAPK (Thr^{202}/Tyr^{204}; Cell Signaling Technology, Danvers, MA), anti-c-Myc (Neomarkers, Fremont, CA), and anti-actin antibody C4.

For kinase assays, total tissue lysates (1 mg) were incubated with 5 μg of a primary antibody to the extracellular domain of Ron [Ron-α (BD Transduction Laboratories, San Diego, CA) or 41A10 (Imclone Systems, Inc., New York, NY)] followed by incubation with protein G-agarose overnight at 4°C. The immunocomplexes were washed five times with wash buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol] and twice in kinase buffer [20 mmol/L HEPES (pH 7.4), 10% glycerol, 10 mmol/L MgCl2, 10 mmol/L MnCl2, 150 mmol/L NaCl]. For the kinase reactions, immunocomplexes were supplemented with 20 μg myelin basic protein (substrate, Upstate, Billerica, MA) and γ-ATP-^{32}P (10 μCi) and

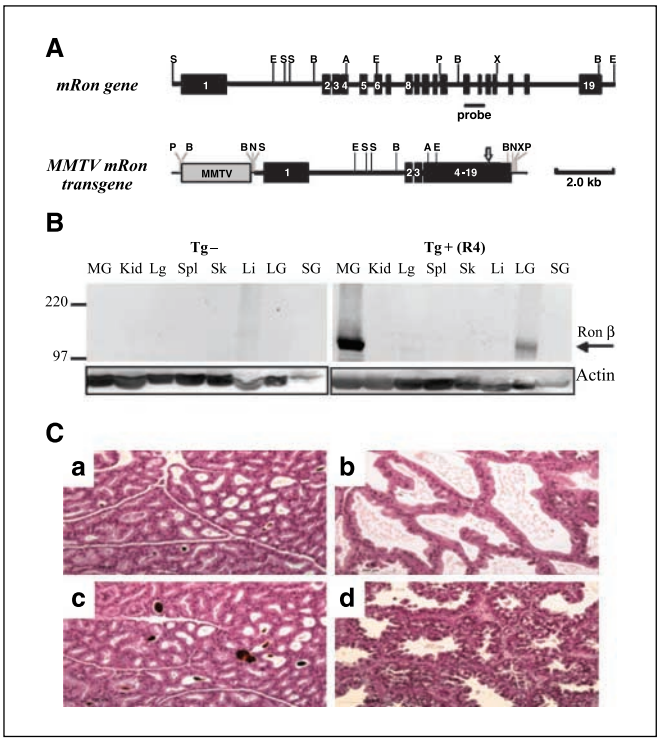


Figure 1. Characterization of the MMTV-Ron transgenic mice. *A,* generation and targeted expression of *Ron* in the mammary gland of transgenic mice. Expression cassette used for the generation of the WT-Ron and constitutively active *Ron* (ΔM1231T, MT-Ron) mice. WT murine *Ron* (m*Ron*) transgene (*bottom*) driven by the MMTV promoter (*gray box*) was constructed as described in Materials and Methods. This MMTV m*Ron* transgene harbors the first three exons (*black boxes*) and introns (*horizontal black line*) of WT m*Ron* genomic DNA followed by, in the correct reading frame, exons 4 to 19 of WT m*Ron* cDNA. The location of a 706-bp genomic DNA probe used in Southern analyses to differentiate transgenic from endogenous WT m*Ron* of transgenic mice is noted below the m*Ron* gene figure (*top*). *Open arrow,* the location of the ΔM1231T point mutation generated from the m*Ron* cDNA sequence, which leads to a constitutively active m*Ron* tyrosine kinase. Restriction enzyme cut sites: *A, Age*I; *B, Bam*HI; *E, Eco*RI; *N, Not*I; *P, Pme*I; *S, Spe*I; *X, Xho*I. *Right,* 2.0-kb ruler. *B,* Ron transgene expression in the mammary gland of transgenic mice. Whole-cell lysates from various tissues taken from WT-Ron transgenic (Tg^+) and nontransgenic (Tg^-) mice were used to assess the level of Ron protein expression. Lysates were subjected to SDS-PAGE and blotted with a polyclonal antibody that recognizes the Ron β chain. *Right,* Ron is highly expressed in the mammary gland of transgenic mice and is also detectable in the lacrimal gland of MMTV-Ron mice; *left,* detection of Ron was not evident in nontransgenic tissues. The membranes were stripped and reprobed with anti-actin antibody to ensure equal protein loading. *MG,* mammary gland; *Kid,* kidney; *Lg,* lung; *Spl,* spleen; *Sk,* skin; *Li,* liver; *LG,* lacrimal gland; *SG,* salivary gland. *C,* histologic differences in lacrimal glands. Enlarged lacrimal glands in MMTV-Ron transgenic animals (*b* and *d*) showed extremely dilated, cystic acini lined by hyperplastic epithelial cells (*d*). This dysplastic architecture is vastly different from that observed in WT animals (*a* and *c*). *Bar*, 200 μm.

DOI:10.1158/0008-5472.CAN-06-2473

incubated for 45 minutes at 37°C. The negative control reactions did not have myelin basic protein. Reactions were terminated by addition of SDS sample buffer and subjected to SDS-PAGE. The gel was fixed in 50% methanol and 5% acetic acid for 10 minutes, dried on a gel dryer, and exposed to a phosphoimager. Band intensities were quantitated using ImageQuant software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

For coimmunoprecipitation experiments, total tissue lysates (1 mg) were incubated with 5 μg of the indicated primary antibodies followed by incubation with protein G-agarose overnight at 4°C. The immunocomplexes were washed three times and bound proteins were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE. Western membranes were then probed with anti-Ron, anti-β-catenin, anti-pRon, and anti-phosphorylated tyrosine (4G10, Upstate Cell Signaling Solutions) primary antibodies as noted above.

Electromobility shift assays were done on whole-cell lysates from nontransgenic and transgenic mammary tissue. Double-stranded oligonucleotides containing the core consensus T-cell factor (TCF)/lymphoid enhancement factor (LEF) binding site, 5′-CTCTGCCGGGCTTTGATCTTTGCTTAACAACA-3′ and 5′-TGTTGTTAAGCAAAGATCAAAGCCCGGCAGAG-3′, were labeled by end labeling reactions using [^{32}P]dATP. Approximately 1.75 pmol of labeled probe were added to 25 μg lysate and incubated for 30 minutes. Protein-DNA complexes were resolved through a nondenaturing 4% polyacrylamide gel and exposed to a phosphoimager.

Tumor development curves. Animals were examined weekly for mammary tumor development by palpation for up to 400 days, and tumor incidence was plotted against time. Data on tumor development was subjected to Kaplan-Meier analysis using GraphPad Prism for Windows (GraphPad Software, San Diego, CA). All mice with excessive tumor burden were euthanized by CO_2 asphyxiation, and mammary glands, tumors, liver, and lungs were removed for histologic examination.

Tissue histology and immunohistochemistry. For whole-mount analysis, mammary glands were fixed in Carnoy's fixative and stained overnight in carmine alum. Samples were dehydrated, cleared in xylene, mounted, and examined on a stereoscope equipped with an Axiovert digital camera.

Tissues were processed as indicated previously (19). Four-micrometer sections of lung and liver tissue were taken at 40 to 200 μm intervals, respectively, along the entire tissue to obtain full coverage of this organ. Sections were stained with H&E for routine histologic examination for metastatic tumor foci. Staining for Ron protein in lung metastasis sections was done on formalin-fixed, paraffin embedded sections. Sections were stained with a rabbit polyclonal antibody specific to Ron (Santa Cruz Biotechnology). The slides were developed using a biotinylated secondary antibody incubated with Vectastain avidin-biotin complex method kit (Vector Laboratories, Burlingame, CA) that was further amplified with a Vector alkaline phosphatase substrate kit (Vector Laboratories).

Results

Generation of transgenic mice expressing WT and constitutively active Ron in the mammary epithelium. To determine whether Ron is sufficient for the oncogenic effects observed in human patients, we generated two new murine models to mimic Ron overexpression and activation observed in human breast cancer specimens. In one model, we generated a minigene construct containing a WT-Ron expression cassette (Fig. 1*A*). In the constitutively active Ron transgene, a point mutation was generated analogous to the mutation observed in human cancers in the RTKs Met, Kit, and Ret (24–28). The ΔM1231T (MT-Ron) point mutation refers to the substitution of a threonine for a methionine at residue 1231 (Fig. 1*A, open arrow*) and has been shown to cause constitutive activation of the receptor, leading to transformation *in vitro* and tumorigenicity *in vivo* (19). Each construct was placed under the transcriptional control of the MMTV promoter (Fig. 1*A*). The expression cassettes were excised from the vector and injected into fertilized eggs from FVB/N mice. Positive founders were crossed with WT FVB/N mice to produce F_1 offspring. Based on Southern analysis of tail biopsies, we identified four transgenic lines for the MMTV-Ron [WT-Ron (R3, R4, R6, and R7)] construct and two MMTV-Ron [ΔM1231T; MT-Ron (M9, M10)] transgenic lines that were studied further. The copy number of each transgenic line was determined by comparing the intensity of the transgenic allele to that of the endogenous allele and varied between one to five copies per line. All founder animals transmitted the transgene to their progeny in Mendelian fashion as determined by PCR with primers designed to detect endogenous Ron and the transgenic copy of the Ron minigene constructs (data not shown).

To test the tissue distribution of the transgene, we isolated mammary gland and other tissues from nontransgenic (Tg^-) and transgenic (Tg^+) mice. A general panel of tissues was selected for analyses, including tissues, in which MMTV promoter expression had been reported previously (29). Protein extracts were generated from each tissue and analyzed for the amount of Ron expression. In all the transgenic mice analyzed, Ron expression was found to be very high in the Tg^+ mammary gland compared with the Tg^- mammary gland. In addition, we also detected transgene expression in the lacrimal gland in both WT-Ron and MT-Ron transgenic lines. In contrast, no transgene expression was found in any of the other tissues that were isolated (Fig. 1*B*). Thus, the transgenes were highly and selectively expressed within mammary tissue and to a limited extent in the lacrimal gland.

During propagation of the mice, lacrimal gland swelling was apparent in several transgene-positive mice from both WT-Ron and MT-Ron lines. To analyze the effect of Ron overexpression within this tissue, we isolated a subset of lacrimal glands from Tg^- and Tg^+ mice and processed them for histologic analysis. The expression of Ron in the lacrimal gland produced epithelial hyperplasia that caused dilated and cystic acini (Fig. 1*C*). However, no further progression of this phenotype was observed.

Effect of Ron overexpression on mammary gland development and transformation. To assess whether mammary-specific Ron overexpression would alter normal mammary development, we isolated mammary glands from Tg^- and Tg^+ 12-week-old virgin mice for whole-mount and histologic analysis. Normal mammary gland development is seen by whole-mount analysis in glands from the Tg^- mice at 12 weeks of age, displaying an abundance of secondary and tertiary branch points and thin mammary ducts that fill the mammary fat pad and show little evidence of alveolar development (Fig. 2*A*). In contrast, mammary glands from Tg^+ mice, WT-Ron and MT-Ron, display pronounced ductal ectasia with dramatic ductal thickening and regression of tertiary branches, resulting in stubby, dilated ducts and acinar hyperplasia (Fig. 2*B*).

Histologic examination of mammary glands from 12-week-old mice show the dilated ducts and multilayered epithelium found in the glands of Ron transgenic mice (Fig. 2*D*) compared with the glands of Tg^- mice that have a well-organized columnar epithelium causing an organized ductal morphology (Fig. 2*C, arrowhead*). In addition, secretory vacuolization was apparent in Tg^+ mammary glands (Fig. 2*D, arrow*), resembling a mammary morphology from a pregnant female. The mammary gland from Tg^+ mice also contained small clusters of epithelial hyperplasia (Fig. 2*D, block arrow*). By 4 months of age, Tg^+ glands have a large number of well-developed hyperplastic alveolar nodules (data not shown).

DOI:10.1158/0008-5472.CAN-06-2473

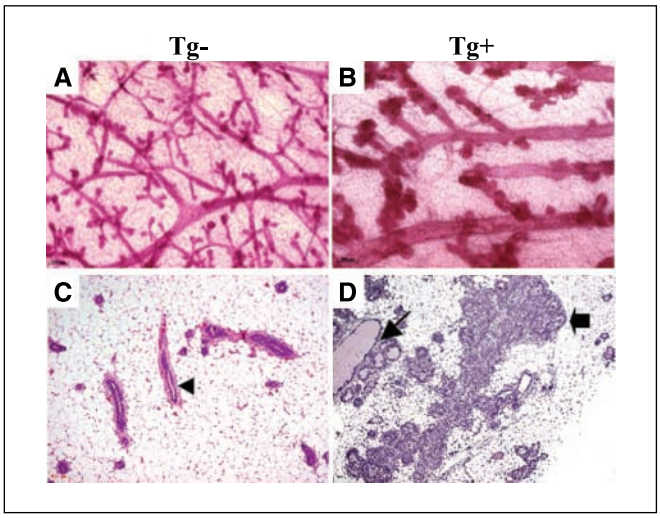


Figure 2. Mammary-specific expression of the MMTV-Ron transgene induces mammary hyperplasia and alveolar development. *A* and *B,* whole mounts from 12-week-old nulliparous nontransgenic (*A*) and MMTV-Ron (WT-Ron; *B*) mice. The mammary-specific expression of MMTV-Ron produced acinar hyperplasia and distended ductal morphology (*B*) compared with nontransgenic tissue, which shows normal glandular morphology with thin ducts and abundant secondary and tertiary branches (*A*). *C* and *D,* H&E-stained sections of mammary gland from WT mice showing normal glandular morphology with thin ducts (*arrowhead*; *C*) and from transgenic glands showing epithelial hyperplasia *(block arrow*) and secretory vacuolization with distended ducts (*arrow*; *D*). *Bar*, 200 μm (*A* and *B*) or 100 μm (*C* and *D*).

This assessment of transgenic mammary gland morphology is consistent with prior observations that this receptor family regulates events controlling branching morphogenesis (30, 31). Moreover, these data suggest that Ron overexpression is sufficient to induce ductal hyperplasia and ultimately breast tumorigenesis. To follow tumor development in these animals, weekly palpation of multiparous WT-Ron and MT-Ron Tg^+ mice was done. Tumor kinetics were plotted by monitoring tumor development as the percentage of tumor-free mice versus time (Fig. 3*A*). The nontransgenic mice did not develop mammary tumors. Given that all of the four WT-Ron transgenic lines and both of the MT transgenic lines produced similar results regardless of the integration site and copy number, the data from the WT lines were pooled and likewise, the data from the MT lines were pooled. By 189 days of age, 50% of the constitutively active MT-Ron mice had palpable mammary tumors compared with the WT-Ron transgenic mice that reached 50% tumor incidence at day 202. Although tumor latency was significantly different between transgenic lines as determined by Kaplan-Meier analysis, the overall tumor incidence was similar in both transgenic lines, producing 100% tumor incidence.

The tumor morphology that developed as a result of Ron overexpression was diverse, ranging from the most prevalent form being adenocarcinoma (Fig. 3*B, a*) with a varying degree of desmoplastic epithelial malignancy (Fig. 3*B, b*) to a more papillary carcinoma (Fig. 3*B, c*). The tumors that developed contained several mitotic figures, an increased nuclear to cytoplasmic ratio, and a high degree of pleomorphism that resembles what is observed in aggressive human disease. Tumors from both transgenic lines were highly invasive, as shown by the local invasion of the underlying connective tissue stroma and musculature (Fig. 3*B, d, arrow*).

Mammary-specific Ron overexpression leads to highly metastatic mammary tumors. The tumors that arose in WT-Ron and MT-Ron transgenic mice displayed a very aggressive phenotype. On microscopic evaluation, the Ron-overexpressing tumor cells were locally invasive as shown by the penetration of tumor cells into the underlying connective stroma and muscle tissue in Fig. 3*B* (*d*). Furthermore, distant metastatic foci were found in the lung and liver both grossly at dissection and on histologic analyses (Fig. 4*A*). Overexpression of Ron induced metastases in a high percentage of mice with a majority of the Tg^+ animals having foci in the lungs and livers (Fig. 4*B*). Although studies were not focused on the initiation of metastatic events in this model, visible metastatic lung foci were observed as early at 110 days of age in mice overexpressing WT-Ron. The dramatic percentage of animals with metastases suggests that Ron overexpression causes an aggressive mammary tumor phenotype and is similar to the aggressive nature of human breast cancers observed overexpressing Ron (9).

As indicated, many transgenic mice displayed evidence of gross metastasis as depicted in a representative lung sample (Fig. 4*A, a*). Interestingly, many metastatic foci were observed growing within the vasculature of the lungs and liver (Fig. 4*A, b, asterisk*; data not

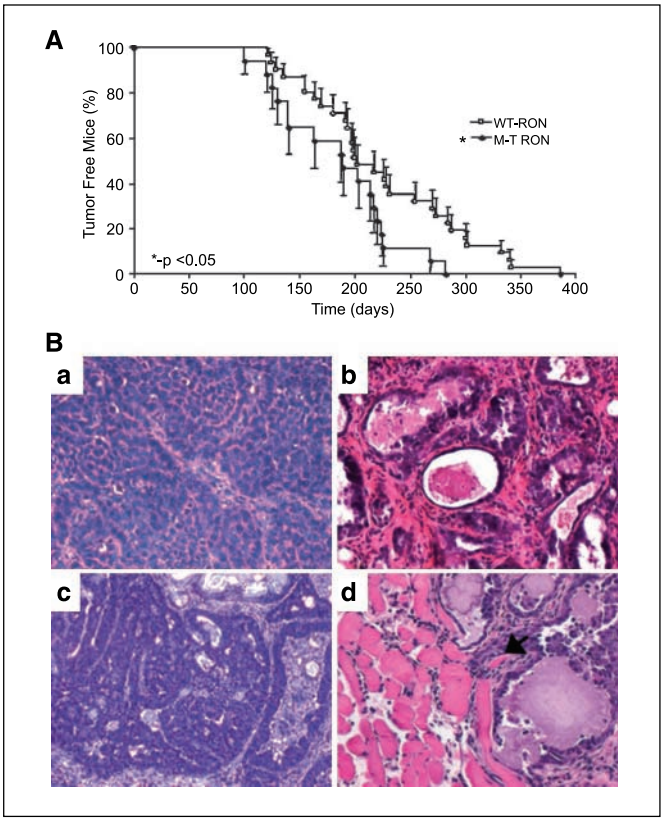
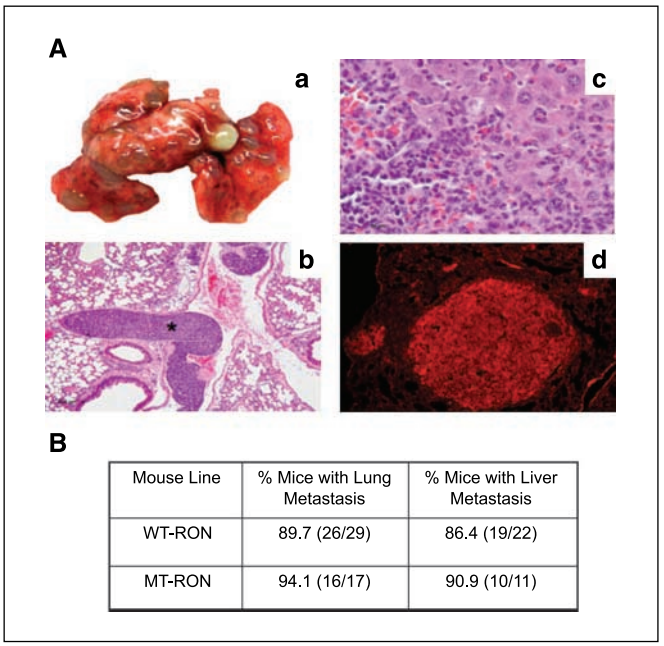


Figure 3. Expression of WT-Ron and MT-Ron in mammary gland induces tumor formation. *A,* kinetics of mammary tumor development in WT-Ron and MT-Ron mice. Transgenic lines had a similar tumor incidence but had a tumor latency that was significantly shorter in MT-Ron mice with median tumor formation being 189 days compared with WT-Ron mice that had median tumor development time of 202 days. $P < 0.05$. *B,* expression of WT-Ron and MT-Ron in mammary gland induces a variety of mammary tumor phenotypes in transgenic mice. Representative mammary tumors from transgenic mice display phenotypes consistent with adenocarcinomas (*a*), characterized by large cells and regions of desmoplasia (*b*). Other tumor phenotypes resembled papillary myoepithelial carcinomas that display signs of localized invasion (*c*) as tumor cells migrate through the underlying muscle tissue (*arrow*; *d*).

DOI:10.1158/0008-5472.CAN-06-2473



Mouse Line	% Mice with Lung Metastasis	% Mice with Liver Metastasis
WT-RON	89.7 (26/29)	86.4 (19/22)
MT-RON	94.1 (16/17)	90.9 (10/11)

Figure 4. Presence of metastatic foci at distant sites in MMTV-Ron transgenic mice. *A,* representative illustrations of metastatic foci within the MMTV-Ron tissues. *a,* a gross representation of metastatic foci found in lung tissue derived from primary mammary tumors induced by Ron overexpression. *b,* section of the lung displaying metastatic foci from the primary mammary tumor that developed as a result of Ron overexpression in the mammary gland. The metastatic foci were found growing in the vessels of the lung (*asterisk*) as well as foci that have traversed the vessel wall to form a colony within the lung parenchyma (data not shown). *c,* representative section of the liver also revealed metastatic foci. The foci found in the liver displayed similar growth characteristics to those found in the lung with metastatic cells associated with vessels and metastatic loci that were more invasive, migrating into the liver parenchyma with associated liver damage (hemorrhagic necrosis due to tumor-associated vessel blockage). *d,* immunohistochemistry was used to determine Ron expression status within the metastatic lung foci. Lung tissue was stained with an anti-Ron antibody, which shows positive staining (*red*) in the foci. *B,* summary of metastatic foci as described in Materials and Methods.

shown). These data indicate that Ron-overexpressing mammary cells are hematogenously disseminated, become lodged in select organs, and are capable of growing within the vessels of select target organs. In addition, metastatic cells were also seen efficiently invading into normal lung and liver parenchyma with metastatic mammary tumor cells evident among the hepatocytes of the liver (Fig. 4*A, c*). The metastatic foci found in the lung and liver distinctly resembled the primary mammary tumor cells and displayed mitotic figures that were not observed in sections of lung or liver from control animals. To determine if these distal foci retained Ron expression, metastatic tumor sections were examined by immunohistochemistry. As shown in Fig. 4*A* (*d*), intense Ron expression was observed in the metastatic foci.

Ron overexpression activates signaling cascades involved in cell proliferation/survival and associates with β-catenin. To ensure that the observed mammary tumors retained Ron expression, we isolated RNA and protein from Tg^- and Tg^+ mammary tissue to compare transgene expression within the mammary gland and in the resulting mammary tumors. In contrast to the Tg^- mammary tissue, in which *Ron* expression was undetectable, *Ron* mRNA expression was observed in the hyperplastic mammary ducts in all Tg^+ lines and noticeably higher expression in palpable mammary tumor tissue (Fig. 5*A*). Protein isolated from the palpable tumor samples showed a similar pattern of Ron expression by Western analyses, with a dramatic increase in Ron expression in all tumor samples compared with the Tg^- tissue (Fig. 5*B*). To determine if Ron overexpression in this transgenic model results in constitutive receptor activation, an antibody that recognizes the phosphorylated active form of Ron was used. As shown by Western analysis of mammary tissue lysates, a significant amount of phosphorylated Ron protein is present in mammary extracts from all the WT-Ron and MT-Ron transgenic lines (Fig. 5*B*; data not shown). This increased Ron phosphorylation is similar to the overexpression and increased receptor phosphorylation observed in human breast cancers and suggests that Ron activation may be driving cell proliferation and survival signals.

In addition to increased Ron phosphorylation, we used mammary gland extracts from Tg^- and several different Tg^+ mice and did *in vitro* kinase assays. Using immunoprecipitation of these lysates with two different antibodies that recognize the extracellular domain of Ron, we were able to show a significant increase in kinase activity from the transgenic lysates compared with control tissue (Fig. 5*C*). These results suggest that the increased phosphorylation of Ron observed in Fig. 5*B* correlates with increases in kinase activity.

To examine the downstream signaling pathways regulated in response to Ron activation, we analyzed the protein expression patterns of signaling molecules involved in cell cycle progression and survival. Normal mammary tissue from Tg^- mice was compared with mammary tumor tissue taken from various Tg^+ lines by Western analysis. Figure 5*D* shows the consistent increases observed in key signaling molecules (i.e., increases in β-catenin, cyclin D1, and c-Myc) found in the Ron-mediated breast tumors. In addition, increased amounts of phosphorylated MAPK were observed.

The morphology of the Ron-induced mammary tumors have striking similarities to tumors that develop in β-catenin-overexpressing animal models (32, 33). These data suggest that Ron and β-catenin signaling may cooperate to produce very aggressive mammary tumors. To elucidate the interaction of Ron and β-catenin, we did immunoprecipitations of lysates from Tg^- and Tg^+ mammary tissue using antibodies directed against β-catenin, Ron, and IgG. Western membranes of the immune complexes were blotted with antibodies for Ron and phosphorylated Ron to assess the association of active (phosphorylated) Ron with β-catenin. As shown in Fig. 6*A*, β-catenin associates with Ron and phosphorylated Ron as evident by the high protein expression in the Tg^+ mammary tissue. To determine if this association is present for Ron as well, we conversely immunoprecipitated with Ron and blotted for Ron, β-catenin, and phosphorylated tyrosine. Western analyses indicate that β-catenin and Ron coprecipitate from transgenic mammary cell lysates and that β-catenin seems to be tyrosine phosphorylated in this complex (Fig. 6*B*). Moreover, a high level of tyrosine phosphorylation was observed in the mammary tissue lysates. To determine if the increased amount of β-catenin observed in the mammary cell lysate was associated with an increase in the activation of β-catenin target genes, electromobility shift assays were done using a consensus β-catenin/TCF/LEF binding sequence. For these analyses, mammary tumor (Tg^+) and nontransgenic mammary tissue (Tg^-) lysates were incubated with a probe specific for the β-catenin/TCF binding site (Fig. 6*C*). A protein:DNA complex is present when mammary tumor extract alone or in combination with nonspecific inhibitor is incubated in the reaction. To test for binding specificity, a 100-fold excess of unlabeled specific or nonspecific competitor was added to the

DOI:10.1158/0008-5472.CAN-06-2473

reaction. As is apparent in Fig. 6*C*, the specific competitor efficiently competes the binding complex, whereas the nonspecific competitor has no effect. To determine if β-catenin was present in the protein:DNA complex from the transgenic mammary tumor lysates, the addition of anti-β-catenin antibody was added to the reaction. With this addition, we saw a band shift as a result of the β-catenin/anti-β-catenin complex migrating toward the top of the gel (Fig. 6*C*; data not shown). Relatively no active β-catenin complexes were seen in the Tg^- mammary gland lysates, further supporting the up-regulation and transcriptional activation of β-catenin in the mammary extracts from the Ron-overexpressing glands (Fig. 6*C*).

Discussion

Overexpression of Ron is associated with human breast cancer and is found in a significant percentage of infiltrating carcinomas (8). In this study, we examined the effect of overexpressing the Ron receptor in the mammary epithelium. Our studies provide the first *in vivo* evidence that Ron overexpression in the mammary gland is sufficient to induce the onset of mammary tumors with a short latency and a highly metastatic phenotype. These data suggest that Ron participates in mammary cell transformation, cell dissociation, and cell migration leading to a high degree of metastasis to distant secondary tissues. Tumorigenesis in these transgenic mouse lines was correlated with elevated expression of Ron and an increase in Ron-associated tyrosine kinase activity within the mammary gland. Ron overexpression produced mammary tumors in 100% of the transgenic mice and distant metastatic foci in ~90% of the animals assessed. Moreover, mammary tumor lysates isolated from transgenic mice display increased β-catenin expression and up-regulated β-catenin target genes cyclin D1 and c-myc. Our experimental observations support the clinical observations that Ron overexpression is associated with poor clinical outcome (8, 9) and a more aggressive phenotype as shown in a variety of *in vitro* assays (13, 19).

Constitutively active splice variants of Ron have also been identified in human colon cancer cell lines and tissues (34–36), making Ron an obvious target for further understanding aggressive disease. Therefore, we produced and characterized various transgenic lines that overexpress either the WT form of Ron or a constitutively active form of Ron within the mammary gland to assess and better understand the biology behind Ron overexpression. The *Ron* transgene constructs were driven by the well-characterized MMTV promoter, which produced high expression of Ron within the mammary gland and displayed no detectable expression in most other tissues. However, we detected minimal expression in the lacrimal gland, which is consistent with the tissue-specific pattern of transgene expression in other MMTV-driven systems (37). The effect of Ron overexpression within the mammary gland is evident by 12 weeks of age, inducing a mammary phenotype consisting of thick ductal branches, decreased side branching, and the development of hyperplastic alveolar nodules. The hyperplastic nodules quickly form palpable mammary tumors in a majority of the transgenic mice by 8 months of age. These results are similar to what has been reported for the well-documented MMTV-*neu* animal model (29). However, the presence of metastatic foci in the

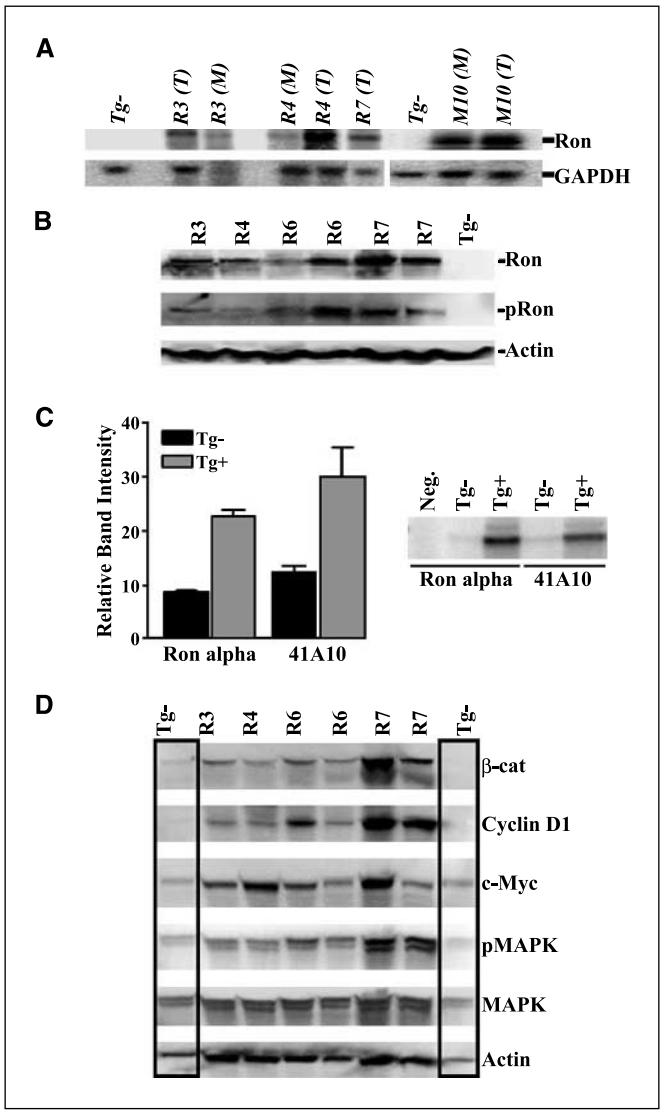


Figure 5. Expression analysis of the mammary epithelium of MMTV-Ron transgenic mice. *A,* Northern hybridization analysis of RNA isolated from nontransgenic and transgenic mammary tissue analyzed for *Ron* expression. The level of *Ron* expression increases in palpable mammary tumor tissue (*T*) compared with mammary gland tissue without palpable mammary tumors (*M*) from transgenic animals. No expression is detected in nontransgenic mammary tissue (Tg^-). Membranes were stripped and reprobed for GAPDH to ensure equal loading. *B,* Western analysis of protein isolated from tumor tissue of transgenic and mammary tissue of nontransgenic mice. Tissue extracts were assayed for total Ron expression. Membranes were then probed with a phosphorylated Ron (*pRon*) antibody to assess the amount of activated Ron expression in the tumor tissue compared with the normal mammary gland. Membranes were stripped and reprobed with an antibody to actin C4 to control for protein loading. *C, in vitro* kinase activity associated with Ron from Tg^- and Tg^+ (WT-Ron) mammary tissue using myelin basic protein as the substrate. *Left,* histogram of the average band intensity of phosphorylated substrate derived from three separate kinase reactions using different mammary lysates and Ron pull-down antibodies (Ron-α and 41A10). *Columns,* relative and intensity; *bars,* SE. *, $P < 0.05$ compared with the Tg^- lysate. *Right,* a representative *in vitro* kinase assay is shown from nontransgenic (Tg^-) and WT-Ron (Tg^+) mammary tissue using two different antibodies directed against the extracellular domain of Ron. The negative control (*Neg.*) depicts reaction without substrate. *D,* Ron overexpression elevates the level of β-catenin in tumor tissue and corresponding β-catenin target genes, including *cyclin D1* and *c-myc*. Increased expression of activated phosphorylated MAPK (*pMAPK*) was observed compared with total MAPK expression, which shows no change in the total MAPK expression between transgenic tumor tissue and nontransgenic mammary tissue. A significant increase of these cycling-associated genes is only detected in transgenic tissue compared with nontransgenic tissue.

DOI:10.1158/0008-5472.CAN-06-2473

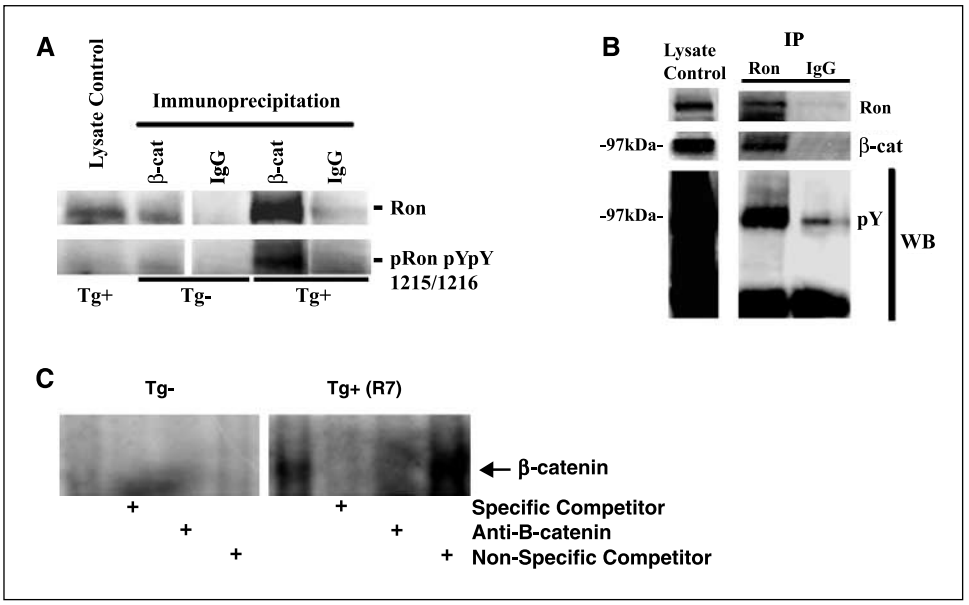


Figure 6. Immunoprecipitation of mammary tissue lysates showing an association between Ron and β-catenin. *A,* whole-cell lysates from nontransgenic (Tg^-) and transgenic (Tg^+) mammary tissue were immunoprecipitated with either an anti-β-catenin or anti-mouse IgG. The precipitated immunocomplexes were then analyzed by Western analyses with anti-Ron and pRon antibodies. Activated Ron protein immunoprecipitates with β-catenin. *B,* Ron- and IgG-precipitated immunocomplexes were further analyzed by Western analyses with anti-Ron, β-catenin (*β-cat*), and phosphorylated tyrosine (*pY*) antibodies. A whole-cell lysate control was provided to show total levels of each protein. *C,* electromobility shift assays were done using a consensus β-catenin/TCF/LEF binding sequence. Mammary tissues extracts were loaded in the presence (+) or absence (−) of specific competitor, an antibody to β-catenin, or a nonspecific competitor (nonspecific probe). A strong protein: DNA complex was observed in mammary tumor tissue from the transgenic mice. This complex was specific for our target sequence and contained β-catenin. Minimal complex formation was observed in the nontransgenic mammary tissue.

lungs and liver of such a high percentage of transgenic mice is unique to this model of Ron overexpression.

As reported previously, Ron expression is barely detectable in normal mammary epithelial cells and benign breast lesions but is overexpressed or constitutively activated in 50% of primary breast cancers (8). We have shown similar results in this transgenic model of breast cancer, in which Ron expression was not detectable in nontransgenic mammary tissue and increased significantly in lysates from palpable mammary tumors. It has been suggested that Ron promotes invasive growth but does not initiate or participate in the early stages of transformation (9, 13). Our experimental data would suggest otherwise, showing the oncogenic potential of Ron when overexpressed, inducing mammary gland transformation and promoting invasive growth characteristics *in vivo*. We suggest that Ron participates in the early stages of mammary gland transformation as well as assists in the progression of mammary tumor growth and a highly metastatic phenotype.

The mammary tumors that developed as a result of Ron overexpression had an appearance that was variable, consisting of adenocarcinomas with varying degrees of desmoplastic epithelial malignancy. The tumors that developed as a result of Ron overexpression had a high degree of pleomorphism and an increase in the nuclear to cytoplasmic ratio, which closely mimics what is observed in more aggressive human breast cancers. Furthermore, these tumors resembled the complex carcinomas that arise due to mutations in the Wnt signaling pathway, including mice with Wnt-1, Wnt-10b, β-catenin transgenes, and adenomatous polyposis coli mutations (32, 33). Characteristics of these Wnt pathway–induced tumors are well-developed stroma, myoepithelial and acinar glandular differentiation, and squamous metaplasia. The histologic phenotype of our tumors suggests altered Wnt signaling, which is further supported by our molecular studies showing an up-regulation of β-catenin and various target genes of the Wnt signaling pathway.

Regardless of the variable morphology, the tumors were uniformly aggressive and metastatic. The unexpected finding that a majority of the mice contained distant metastases may have important clinical implications. Many metastatic lesions were found in the vessels of the lungs and liver and some were also shown invading normal lung and liver tissue, suggesting that Ron overexpression increases cell motility/invasiveness *in vivo*, which has been suggested previously by *in vitro* invasion assays (36, 38, 39).

To support the cooperative involvement of Ron and β-catenin, we showed evidence that active Ron associates with β-catenin and elevates expression of β-catenin target genes. Previous reports have shown that oncogenic mutants of the Met family have the ability to activate the β-catenin pathway when expressed in NIH3T3 or Madin-Darby canine kidney cells (17). In support of our work, which shows an up-regulation of β-catenin as a result of Ron overexpression, Bullions and Levine (12) showed *in vitro* that activated Ron causes a tyrosine phosphorylation of β-catenin that correlates with an increase in cytoplasmic β-catenin and consequent transcriptional activation via TCF-4 transcription factor leading to the up-regulation of c-myc and cyclin D1 gene expression. We also showed that β-catenin was present and capable of binding the TCF/LEF transcription site found upstream of various target genes that were up-regulated due to Ron overexpression. Furthermore, silencing of Ron gene expression in colon cancer cell lines leads to diminished β-catenin expression (40). These studies and ours suggest that the β-catenin pathway may be involved in Ron-mediated tumorigenesis in the mammary gland.

Additionally, Ron-overexpressing mammary tumors also showed an activated MAPK pathway that has been shown previously to be

DOI:10.1158/0008-5472.CAN-06-2473

essential for cell transformation by Met (41–43), suggesting that Ron overexpression may induce or associate with various pathways required for mammary tumor initiation and progression. The association of Ron with various signaling cascades involving cell proliferation and survival suggests that mammary cells overexpressing Ron may have a survival advantage in the environment associated with invasion and distant metastasis. Further understanding the interaction of Ron, β-catenin, and other critical signaling modalities within mammary epithelial cells will offer a better understanding into how to treat Ron-overexpressing tumors that have a highly metastatic phenotype.

Acknowledgments

Received 7/6/2006; revised 9/6/2006; accepted 10/10/2006.

Grant support: Public Health Service grants CA100002 (S.E. Waltz) and DK073552 (S.E. Waltz), the Digestive Diseases Research Development Center grant DK064403 (S.E. Waltz and M.A. Leonis), NIH National Cancer Institute Core Phenotyping Award (R24 CA95784), Department of Defense Career Development Award DAMD17-02-1-0342 (S.E. Waltz), and a Children's Digestive Health and Nutrition Foundation Young Investigator Development Award (M.A. Leonis).

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We thank investigators from the University of Cincinnati including Jerilyn Girardi for her contribution to the mouse colony, Dr. James Lessard for the gift of the actin antibody, and Dr. El Mustapha Bahassi for assistance with the kinase assays.

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