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Award Number: DAMD17-02-1-0342

TITLE: Ron in Breast Development and Cancer

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REPORT DATE: October 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual Summary (1 Oct 2003 - 30 Sep 2004)

4. TITLE AND SUBTITLE

Ron in Breast Development and Cancer

5. FUNDING NUMBERS

DAMD17-02-1-0342

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The long-term objective of this project is to define the in vivo role of the receptor tyrosine kinase Ron in mammary gland biology. Virtually nothing is known regarding the function of Ron in the breast. However, two recent studies have shown that Ron is over-expressed and highly phosphorylated in a significant fraction of human and feline breast cancers. To define the in vivo significance of Ron, mice were generated with a targeted ablation of the tyrosine kinase domain of this receptor (TK-/- mice). To determine the impact of Ron in a murine model of breast cancer, the TK-/- mice were crossed to mice expressing the polyoma virus middle T antigen (pMT) under control of the mouse mammary tumor virus promoter. Both TK-/- and control mice expressing pMT develop mammary tumors and lung metastasis. However, a significant decrease in mammary tumor initiation and growth was found in the TK-/- mice compared to controls. This decrease was associated with a significant decrease in microvessel density, decreased cellular proliferation and increased apoptosis. Biochemical analyses showed that the pMT expressing TK-/- tumors had defects in MAPK and AKT activation. Our studies are the first to demonstrate the impact of Ron signaling on tumorigenesis.

14. SUBJECT TERMS

growth factors, receptor tyrosine kinases, cancer

15. NUMBER OF PAGES

18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

In vitro studies have shown that Ron is a potent activator of cellular proliferation, migration, branching morphogenesis and invasion-characteristics that are critical for organogenesis and tumorigenesis. Further, alterations in Ron expression have been documented in a number of human tumors(1-8). However, the strongest link to Ron and human cancers may be in the breast. Recent studies have shown that Ron is expressed in the developing breast and Ron's expression pattern suggests that Ron may be influential in the development of this organ(9). In a study analyzing mammalian breast carcinomas, Ron was found to be expressed at abnormally high levels in a large fraction of primary human and feline tumors analyzed(2, 7, 10). Furthermore, in breast carcinomas grown in vitro, Ron activation resulted in proliferation, migration and invasion, suggesting a role for Ron in breast cancer progression and a metastatic phenotype(7). The purpose of this proposal is to determine the contribution of Ron signaling in normal mammary gland growth and development (Aim 1). This is being accomplished by contrasting breast development in wild-type mice compared to mice with a block in Ron signaling. Secondly, the impact of Ron signaling in the pathogenesis of oncogene-driven mammary gland tumors is being evaluated (Aim 2). This will be accomplished by monitoring tumor kinetics and downstream signaling cascades in a polyoma virus middle T antigen (pMT)-induced model of breast carcinogenesis. During the past funding cycle, studies were focused on Aim 2.

BODY

The work presented in this body is a summary of the findings recently published in Cancer Research, and is attached in the appendix for complete review.

To evaluate the impact of Ron signaling on mammary tumor formation induced by polyoma virus middle T antigen, female mice with and without Ron signaling were crossed to polyoma middle T antigen expressing males. Female TK+/+ and TK-/- mice with and without pMT were used for further analysis. In Figure 1, the kinetics of tumor initiation in pMT-expressing control TK+/+, experimental TK-/-, and heterozygous TK+/- mice were calculated as the percent of tumor-free mice per total number of mice observed over time. No difference bwtween TK+/+ and TK+/- mice was observed, but there was a significant difference ($p<0.05$) between the TK+/+ and TK-/- mice, as determined by logrank analysis. Wild-type control mice with Ron signaling exhibit a significantly increased rate of tumor initiation.

Other parameters of tumor formation were also evaluated, and are summarized in Table 1. There was no difference between the control and experimental groups in the age of the animal at sacrifice. Tumors from TK+/+ mice had increased growth compared to tumors from TK-/- mice. Tumor initiation was significantly reduced in the control animals, forming tumors about a week before a palpable tumor was observed in mice devoid of Ron signaling. The number of mammary glands per mouse with frank palpable tumors was also significantly greater in TK+/+ than in TK-/- mice. The body burden of tumor (tumor mass, g) was about half again as much in the control mice than in the Ron signaling deficient mice over the same period of growth, showing that the growth of mammary tumors induced by polyoma virus middle T is augmented by the presence of Ron signaling.

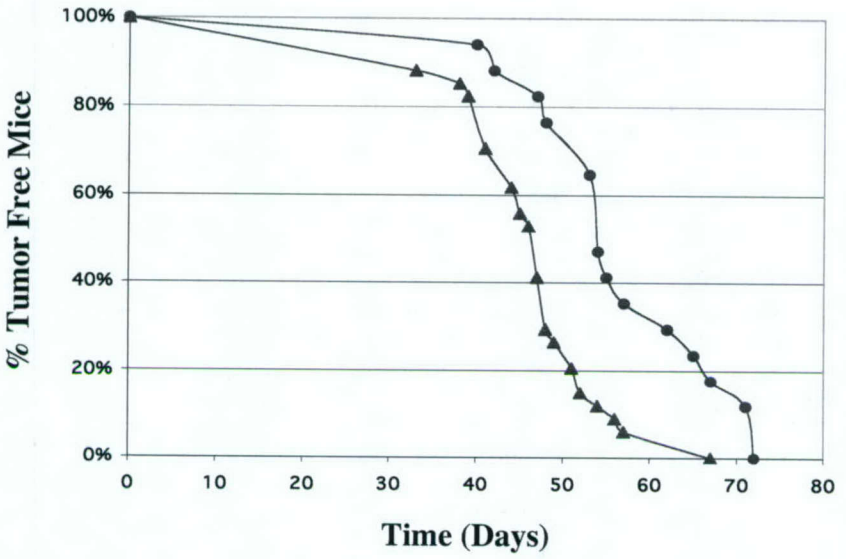

Figure 1: Ron signaling increases tumor initiation. The kinetics of tumor formation in control (pMT+/- TK+/+; n=35) (▲) and experimental (pMT+/- TK-/-; n=17) (●) mice was calculated as the percent of tumor-free mice compared to the total number of mice observed for the initiation of a palpable tumor. The difference between the curves was evaluated by a logrank analysis, and is significantly different ($p<0.05$). Mice lacking Ron signaling capability exhibit a significantly decreased rate of tumor initiation.

Mouse Genotype	Days of Tumor Growth	Days to a Palpable Tumor	Number of Tumors	Body Burden of Tumors, g
pMT+/- TK+/+	82 ± 6 (35)	46 ± 8 (34)	8 ± 0 (35)	4.9 ± 2.7 (35)
pMT+/- TK-/-	80 ± 8 (17)	55 ± 9 (15) *p=0.002*	6 ± 1 (17) *p=0.05*	3.0 ± 1.9 (17) *p=0.012*

Table 1: Growth of mammary tumors induced by polyoma virus middle T is augmented by Ron signaling. The average age of the animal at sacrifice is indicated as days of tumor growth, which did not differ between genotypes. A significant decrease in the days to mammary tumor initiation, and a significant increase in the number of mammary glands with tumors, and in the tumor body burden (tumor mass, g) was seen in pMT+/- TK+/+ mice compared to pMT+/- TK-/- mice. No differences were observed between pMT containing TK+/+ and TK+/- mice. Data shown are mean ± SD (number of mice evaluated).

In an analysis of the possible mechanisms by which Ron signaling may synergize with pMT signaling to influence tumor growth, our studies have identified three main findings. First, overexpression of Ron protein is seen in the mammary tumors induced by pMT. Our previous experiments have demonstrated that Ron overexpression is accompanied by constitutive activation of the receptor, transformation, and increased cell proliferation(11). In the absence of

the tyrosine kinase domain, the overexpression of the truncated receptor will not amplify downstream transformative and proliferative signals.

Second, the lack of Ron signaling has an apparent effect on angiogenesis within the mammary tumors induced by pMT. There has been considerable research conducted on the relationship between angiogenesis and tumor growth. Angiogenesis within human mammary tumors has been correlated with metastatic disease, and poor prognosis(12). Polyoma middle T induced tumors have been shown to be poorly perfused in relationship to their growth(13), and yet tumor growth in this model has been shown to be influenced by the ability of the tumor to recruit microvessels(14). The dramatic reduction in microvessels seen in the pMT+/- TK-/- tumors compared to the pMT+/- TK+/+ tumors, coupled with their reduced growth rate, suggests that Ron signaling may play a role in promoting angiogenesis in this mammary tumor.

Third, the parallel increases in cellular proliferation and cell survival may be mediated by increased activation of MAPK and AKT acting in concert in pMT+/- TK+/+ mammary tumors, compared to tumors in which Ron signaling is absent. Activation of AKT is coupled to activation of phosphatidylinositol 3' kinase (PI3-K). Polyoma middle T antigen transformation is highly dependent on PI3-K. Mice carrying a mutation abolishing the binding site for PI3-K on pMT develop mammary gland hyperplasias that are highly apoptotic, and only develop focal tumors at a late time point(15). A reintroduction of activated AKT into the mouse strain decoupled from pMT-PI3-K interaction restored and accelerated mammary tumorigenesis, with a concomitant reduction in apoptosis in the mammary tumor(15). Ron has previously been shown to mediate both apoptotic and growth signals(16); however, the role of Ron in cell survival may be cell type dependent(16, 17). Adherent epithelial cell survival stimulated by ligand binding of Ron was found to depend on both MAPK and PI3-K/AKT activation, and each pathway independently contributed to overall cell survival(18). These experiments support the argument that the increased activation of both MAPK and AKT in pMT+/- TK+/+ tumors compared to pMT+/- TK-/- tumors, jointly contributes to the increased proliferation, decreased apoptosis, and overall increased tumor growth.

Summary

The mouse model of mammary tumorigenesis induced by MMTV-pMT has been extensively utilized to examine pathways and molecules involved in mammary tumorigenesis and metastasis, including genetic loci(19, 20); putative tumor suppressors(21, 22); and other disease modifying molecules(23-25). Our report is the first to investigate the role of Ron tyrosine kinase signaling in this mammary tumor and metastasis model. We conclude that Ron will play a significant role in breast cancer, and may be an important therapeutic target.

KEY RESEARCH ACCOMPLISHMENTS

- Ron receptor signaling is important for pMT-induced mammary tumor initiation and growth.
- Ron signaling augments microvessel density and mammary cell proliferation and inhibits mammary epithelial apoptosis during pMT-induced mammary tumorigenesis

- Ron signaling synergizes with pMT induced signaling, in part, by impacting proliferative and cell survival pathways, namely MAPK and AKT activation.

REPORTABLE OUTCOMES

One reportable outcome resulting from the research supported by the US Army Medical Research and Material Command is a recent paper published in Cancer Research and included in the appendix. Moreover, the work in this manuscript was presented as a poster the 12th International Conference on Second Messengers and Phosphoproteins on August 3-7, 2004.

In addition to research materials, this Career Development Award had led to an additional grant application from the P.I. The P.I. submitted a revised R01 grant application to the National Institutes of Health/National Cancer Institute in March of 2003. This grant received a 4.8 percentile and was funded as of 6/1/03.

CONCLUSIONS

Our data indicate that Ron receptor signaling may be a new and important therapeutic target in our fight against breast cancer. We have demonstrated that Ron is expressed in the normal mammary epithelium and its expression is increased in a model of oncogene-induced breast cancer. This expression pattern is observed in human and feline cancers. Further, we have shown that Ron signaling participates in normal mouse mammary gland development, regulating branching morphogenesis. This data is the first to directly demonstrate a novel role for this receptor in the mammary gland. As we learn more about Ron regulated signaling events in the breast, we will be in a better position to understand how this organ develops and the processes involved in mammary tumorigenesis and metastasis.

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APPENDICIES

Included in the appendix is a published article entitled "Ron Receptor Signaling Augments Mammary Tumor Formation and Metastasis in a Murine Model of Breast Cancer." The paper was published in Cancer Res 2005; 65: (4). February 15, 2005 and summaries our current accomplishments in this progress report.

Ron Receptor Signaling Augments Mammary Tumor Formation and Metastasis in a Murine Model of Breast Cancer

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Abstract

The tyrosine kinase receptor Ron has been implicated in several types of cancer, including overexpression in human breast cancer. This is the first report describing the effect of Ron signaling on tumorigenesis and metastasis in a mouse model of breast cancer. Mice with a targeted deletion of the Ron tyrosine kinase signaling domain (TK−/−) were crossed to mice expressing the polyoma virus middle T antigen (pMT) under the control of the mouse mammary tumor virus promoter. Both pMT-expressing wild-type control (pMT+/− TK+/+) and pMT+/− TK−/− mice developed mammary tumors and lung metastases. However, a significant decrease in mammary tumor initiation and growth was found in the pMT+/− TK−/− mice compared with controls. An examination of mammary tumors showed that there was a significant decrease in microvessel density, significantly decreased cellular proliferation, and a significant increase in terminal deoxynucleotidyl transferase–mediated nick end labeling–positive staining in mammary tumor cells from the pMT+/− TK−/− mice compared with the pMT+/− TK+/+ mice. Biochemical analyses on mammary tumor lysates showed that whereas both the pMT-expressing TK+/+ and TK−/− tumors have increased Ron expression compared with normal mammary glands, the pMT-expressing TK−/− tumors have deficits in mitogen-activated protein kinase and AKT activation. These results indicate that Ron signaling synergizes with pMT signaling to induce mammary tumor formation, growth, and metastasis. This effect may be mediated in part through the regulation of angiogenesis and through proliferative and cell survival pathways regulated by mitogen-activated protein kinase and AKT. (Cancer Res 2005; 65(4): 1285-93)

Introduction

Ron is one of a unique family of receptor tyrosine kinases, along with the proto-oncogene *Met*, and the avian oncogene *Sea* (1, 2). Ron is the receptor for hepatocyte growth factor–like protein/macrophage stimulating protein (3–6). Binding of hepatocyte growth factor–like protein to Ron induces phosphorylation of key tyrosine residues in the intracellular catalytic domain, followed by phosphorylation of tyrosine residues at the carboxyl terminal that provide docking sites for downstream adapter signal molecules (7–9). Ron activation by ligand binding has been shown to promote responses important for tumorigenesis and metastasis, including cell-cell dissociation (scattering), proliferation, motility, and morphologic changes (10–12).

The oncogenic potential of overexpressed wild-type Ron or activating point mutations in Ron has been shown *in vitro* by foci formation, increased proliferation, and increased motility. *In vivo*, transformed cells overexpressing Ron produce tumors in nude mice (13). Few studies analyzing the expression of Ron in human tumors have been done to date. However, these studies suggest that Ron overexpression or constitutive activation may be important in several tumor types, including hepatocellular carcinoma, colon and colorectal cancer, and a subset of non–small cell lung cancer (14–17). Importantly, Ron overexpression and activation, indicated by increased phosphorylation, has recently been identified in human breast cancer (18). Overexpression of Ron was found in about 50% of the primary ductal and lobular carcinomas examined (35 of 74 patients), yet was barely detectable in normal breast tissue or in benign breast lesions, including papillomas and fibroadenomas. In support of an important role for Ron in breast cancer, overexpression of Ron has also been found in feline mammary tumors (19). The functions of Ron in cell culture and the presence of activated forms of Ron in tumor tissue lead to the hypothesis that Ron tyrosine kinase function may be important in mammary tumorigenesis.

We previously reported the development of mice containing the targeted deletion of the tyrosine kinase domain of Ron (20). These mice (TK−/−) are phenotypically normal, fertile, and nurse their young. However, several functional defects have been noted in these mice, including the inability to regulate inflammatory responses mediated by ligand binding and activation (20–22). To test the hypothesis that Ron receptor activation and signaling is an important factor in mammary cancer, mice with a defect in Ron signaling (TK−/− mice) were crossed to transgenic mice expressing polyoma virus middle T antigen (pMT) under the control of the mouse mammary tumor virus promoter. Female mice expressing pMT rapidly develop multifocal mammary tumors that metastasize to the lungs (23).

In this report, we show that deletion of Ron signaling produces a significant reduction in mammary tumor development induced by the pMT. Our results suggest that Ron signaling synergizes with pMT signaling to induce mammary tumor formation in this model.

Materials and Methods

Mice. Hemizygous male mice in a FVB/N background expressing pMT under the control of the mouse mammary tumor virus promoter [FVB/N-TgN(MMTVPyVT)634Mul] were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice with a deletion of the Ron tyrosine kinase domain (TK−/−) of Ron have been previously described (20). TK−/− mice were maintained in an FVB/NJ background following seven generations of back crosses. The *MMTV-pMT* transgene was maintained in a hemizygous state by crossing male transgene carriers bred to homozygosity for the deletion

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of the Ron tyrosine kinase domain (pMT+/− TK−/−) to female mice (pMT−/− TK−/−) to produce the experimental group (pMT+/− TK−/−). Male mice (pMT+/− TK+/+) were crossed to wild-type females (pMT−/− TK+/+) to produce the control group (pMT+/− TK+/+). Male mice (pMT+/− TK+/+) were crossed to females homozygous for the deletion of the Ron tyrosine kinase domain (pMT−/− TK−/−) to produce *MMTV-pMT* transgenic positive, Ron kinase domain deletion heterozygous animals (pMT+/− TK+/−). Transmission of the *MMTV-pMT* transgene and the Ron genomic locus was determined by PCR using primers that have been described previously (20, 24). Only female mice were examined for tumor formation. The mice were maintained and sacrificed under approved animal care protocols in an Association for Assessment of Laboratory Animal Care–accredited facility.

Tumor Parameters. The time to the formation of a palpable tumor was determined by manual palpation. The number of glands containing tumors was determined by examination at sacrifice. The tumor burden was calculated by determining the total tumor mass at the time of sacrifice.

Mammary Tumor Histology and Immunohistochemistry. Mammary tumors were formalin fixed, processed, and paraffin embedded. Parallel 4 μm sections were used for different analyses. Sections were stained with H&E for histologic examination. Images were obtained using a Zeiss (Thornwood, NY) microscope equipped with an Axiovert digital camera. The left abdominal mammary gland was taken from mice at 49 days to evaluate proliferation in discrete tumor nodules. Proliferation of mammary cells was examined following i.p. injection of 0.1 mL of 5-bromo-2′-deoxyuridine (BrdUrd) solution 2 hours before sacrifice using a cell proliferation kit (Amersham Pharmacia Biotech, Piscataway, NJ). Paraffin-embedded mammary tissue sections were stained following the manufacturer's guidelines. Positively staining cells were visualized using a horseradish peroxidase–conjugated secondary antibody, stained with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO), then counterstained with hematoxylin and permanently coverslipped. Measurement of the size of a tumor nodule or tumor area examined and counting the number of positively staining cells in the tumor nodule was done on a Macintosh computer using the public domain NIH Image software program (developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/). Five areas per tumor from four mice per group were evaluated. The proliferation index was calculated as the number of cells incorporating BrdUrd per area of tumor nodule in square inches.

Apoptosis was evaluated by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining using an *in situ* cell death detection kit (Roche, Indianapolis, IN) following the manufacturer's guidelines. The sections were treated with an alkaline phosphatase conjugated secondary antibody, and positively staining cells were visualized with Fast Red (Sigma-Aldrich). The slides were then mounted with Immu-Mount (Shanndon, Pittsburg, PA) for evaluation. The apoptotic index was calculated as the number of cells positive for TUNEL staining per area of tumor nodule in square inches as described for proliferation above. Three to five areas per tumor from four to six control and experimental mice were evaluated.

Staining for Ron protein in mammary tumor sections was done on formalin-fixed, paraffin-embedded sections of mammary tumors at 49 days. Antigen retrieval was done by heating the slides in a citric acid-sodium citrate buffer. Sections were stained with a mouse antibody specific to the extracellular α-chain of Ron (Ron α, BD Transduction Laboratories, San Diego, CA). The slides were developed using a horseradish peroxidase–conjugated secondary antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), stained with DAB, and counterstained with hematoxylin. As a negative control, a serial section was treated with normal mouse IgG in place of the primary antibody.

Microvessel staining in mammary tumor sections was done on formalin-fixed, paraffin-embedded sections of mammary tumors at 52 ± 3 days. Five to seven areas per tumor from four mice per group were evaluated. Antigen retrieval was done by heating the slides in a citric acid-sodium citrate buffer. Microvessels were visualized by staining with rabbit anti-human von Willebrand factor (DakoCytomation, Carpinteria, CA). The slides were developed using a horseradish peroxidase conjugated secondary antibody (Vectastain ABC kit, Vector Laboratories), stained with DAB, and counterstained with hematoxylin. As a negative control, a serial section was treated with normal mouse IgG in place of the primary antibody.

Lung Histology. Lung tissue was prepared by perfusion with PBS, followed by perfusion with formalin, formalin fixed, processed, and paraffin embedded. The left lung was routinely sectioned. Four-micrometer sections were taken at 200-μm intervals along the entire lobe to obtain full coverage of this organ. Sections were stained with H&E for routine histologic examination. The number of metastases per section was counted, and the maximum number of metastases was used for statistical analysis. The lung section containing the maximum number of metastases was digitized and area measurements of the lung section and of the metastatic foci were done on a Macintosh computer using the public domain NIH Image program.

Northern Analysis. Total RNA was isolated from tumor tissue of experimental and control animals using Trizol (Invitrogen, Carlsbad, CA). RNA (30 μg) was separated on a 1.2% agarose gel, transferred to a nylon membrane, and stained with methylene blue to detect 18 and 28 S rRNA. The membrane was hybridized with a polyoma specific sequence generated by PCR using oligonucleotides that have been previously described (25).

Western Analysis. Western analysis of protein lysates from mammary tumor tissue of experimental and control mice at 55 ± 4 days was done essentially as described (13). Western membranes were probed with antibodies to either the extracellular α-chain of Ron (Ron α, BD Transduction Laboratories), phospho-mitogen-activated protein kinase (MAPK, Biosource, Camarillo, CA), MAPK (Biosource), phospho-AKT (Upstate Biotechnology, Lake Placid, NY), or AKT (Upstate Biotechnology). The anti-C4 actin antibody was a gift of Dr. James Lessard (Cincinnati Children's Hospital Medical Center, Cincinnati, OH).

Statistical Analysis. ANOVA statistical analysis was done using the StatView program (SAS Institute, Cary, NC). Growth curve differences between the two genotypes were evaluated by log rank analysis using the same statistical software package.

Results

To evaluate the impact of Ron signaling on mammary tumor formation induced by pMT, female mice with and without Ron signaling were crossed to pMT-expressing males. Female TK+/+ and TK−/− mice with and without pMT were used for further analysis.

In Fig. 1*A*, the kinetics of tumor initiation in pMT-expressing control TK+/+, experimental TK−/−, and heterozygous TK+/− mice were calculated as the percent of tumor-free mice per total number of mice observed over time. No difference between TK+/+ and TK+/− mice was observed, but there was a significant difference ($P < 0.05$) between the TK+/+ and TK−/− mice, as determined by log rank analysis. Wild-type control mice with Ron signaling exhibit a significantly increased rate of tumor initiation.

The expression of pMT in TK+/+ and TK−/− tumors was also evaluated (Fig. 1*B*). Figure 1*B* (*top*) shows a Northern analysis of total RNA from several independent pMT+/− TK+/+ and pMT+/− TK−/− tumors, hybridized with a sequence specific for pMT. Figure 1*B* (*bottom*), shows the 18S and 28S RNA from this Northern analysis to show approximately equal RNA loading. No difference in the expression of pMT between TK+/+ and TK−/− tumors was observed.

Other parameters of tumor formation were also evaluated and are summarized in Table 1. There was no difference between the control and experimental groups in the age of the animal at sacrifice. Tumors from TK+/+ mice had increased growth compared with tumors from TK−/− mice. Tumor initiation was

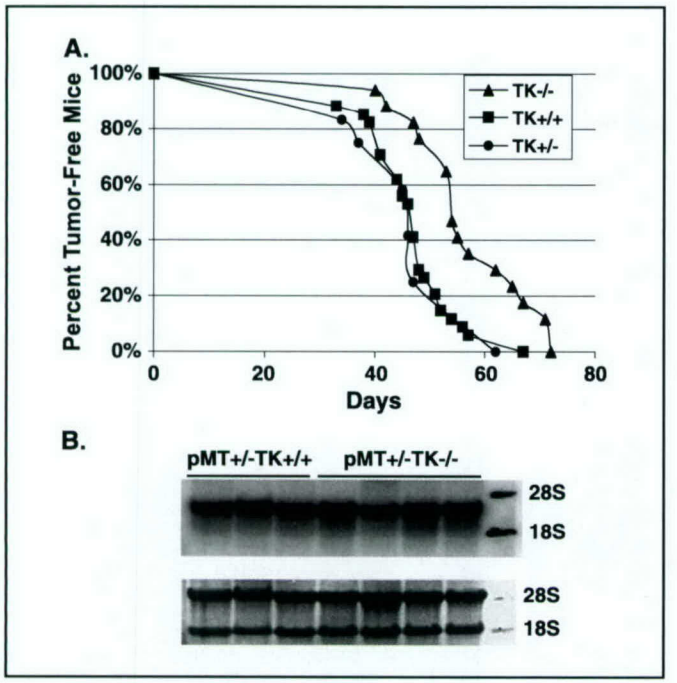

Figure 1. Ron signaling increases tumor initiation. *A*, The kinetics of tumor formation in control (pMT+/− TK+/+, $n = 35$) (■), heterozygous (pMT+/− TK+/−; $n = 12$) (●), and experimental (pMT+/− TK−/−; $n = 17$) (▲) mice was calculated as the percent of tumor-free mice compared with the total number of mice observed for the initiation of a palpable tumor. The difference between the curves was evaluated by a log rank analysis. No difference in tumor initiation was found between mice homozygous or heterozygous for the Ron tyrosine kinase domain. A significant different was found between the control and experimental groups ($P < 0.05$). Mice lacking Ron signaling capability exhibit a significantly decreased rate of tumor initiation (*B*). Total RNA from three pMT+/− TK+/+ and four pMT+/− TK−/− mice was subjected to Northern analysis and hybridized to a probe specific for pMT (*top*) The ethidium bromide–stained 18S and 28S is shown to evaluate loading (*bottom*).

significantly reduced in the control animals, forming tumors about a week before a palpable tumor was observed in mice devoid of Ron signaling. The number of mammary glands per mouse with frank palpable tumors was also significantly greater in TK+/+ than in TK−/− mice. The body burden of tumor (tumor mass, grams) was about half again as much in the control mice than in the Ron signaling–deficient mice over the same period of growth, showing that the growth of mammary tumors induced by pMT is augmented by the presence of Ron signaling. In a comparison of F1 hybrid animals, we found that there was no significant difference in tumor latency or tumor mass between wild-type and heterozygous Ron TK–deficient mice (Table 1). These results suggest that the phenotypic changes observed in the TK−/− mice are a result of the Ron allele and not due to modification by other genetic loci.

Tumors were examined by light microscopy for morphologic comparison. Figure 2*A* and *B* shows representative tumors from control and experimental mice taken at 51 ± 2 days of age. The early-stage tumors from both genotypes presented as well-differentiated adenocarcinomas with minimal necrosis. Late-stage tumors (89 ± 5 days of age) from TK+/+, TK−/−, and TK+/− mice (Fig. 2*C-E*) were moderately differentiated adenocarcinomas with areas demonstrating moderate to severe cystic patterns, many with luminal secretions. Necrosis varied from minimal to extensive. In architecture and cytology, the mammary tumors from control and experimental animals were similar. However, the increased cell mass of tumor in the control animals was apparent at early stages of tumor growth (Fig. 2*A* and *B*), and remained striking even at late stages (Fig. 2*C-E*). The extensive tumor mass in heterozygous TK+/− animals resembled that seen in the control TK+/+ group.

The mammary tumors induced by pMT express Ron protein, as shown in Fig. 2*F-G*. Serial sections from a pMT +/− TK+/+ tumor are shown. A control section using normal mouse IgG in place of the primary antibody against Ron is shown in Fig. 2*F*. The lack of staining in the control section establishes the specificity of the Ron antibody. Our previous work also showed the specificity of this antibody by blocking Ron immunoreactivity with exogenously added Ron recombinant protein (26). Specific immunohistochemical staining for Ron protein in the mammary tumor section in Fig. 2*G*, indicates that Ron is expressed in the mammary epithelium during pMT-induced transformation.

Tumors arising from pMT metastasize to the lungs (23). To facilitate comparison, the entire left lung was sectioned at intervals, and each section examined for metastatic foci. Figure 3 shows a representative section of lung from control (*A* and *C*) and experimental (*B* and *D*) mice, examined at low magnification (*A* and *B*) and at high magnification (*C* and *D*). The distinctive appearance of the metastatic foci in the lung tissue is apparent. There were no morphologic differences between metastatic foci in the lungs of pMT +/− TK+/+, pMT +/− TK+/−, or pMT +/− TK−/− animals. Metastatic foci were quantitated in two ways, and the data are summarized graphically in Fig. 2*E* and *F*. The section of the left lung containing the greatest number of metastatic foci was examined, and the maximum number of foci counted, shown in Fig. 2*E*. The area covered by the metastatic foci, compared with the total area of the lung section in which they were counted, is presented in Fig. 2*F*. Although the difference is not statistically significant, there is a trend to a greater number of metastatic foci, and a larger area covered by

Table 1. Growth of mammary tumors induced by pMT is augmented by Ron signaling

Mouse genotype	Days of tumor growth	Days to a palpable tumor	No. of tumors	Body burden of tumors (g)
pMT+/− TK+/+	82 ± 6 (35)	46 ± 8 (34)	8 ± 0 (35)	4.9 ± 2.7 (35)
pMT+/− TK+/−	83 ± 6 (12)	47 ± 10 (12), $P = 0.40$	7 ± 2 (12), $P = 0.39$	4.3 ± 2.5 (12), $P = 0.31$
pMT+/− TK−/−	80 ± 8 (17)	55 ± 9 (15), $P = 0.002$	6 ± 1 (17), $P = 0.05$	3.0 ± 1.9 (17), $P = 0.012$

NOTE: The average age of the animal at sacrifice is indicated as days of tumor growth, which did not differ between genotypes. A significant decrease in the days to mammary tumor initiation and a significant increase in the number of mammary glands with tumors and in the tumor body burden (tumor mass, g) was seen in pMT+/− TK+/+ mice compared with pMT+/− TK−/− mice. No differences were observed between pMT containing TK+/+ and TK+/− mice. Data shown are mean ± SD (number of mice evaluated).

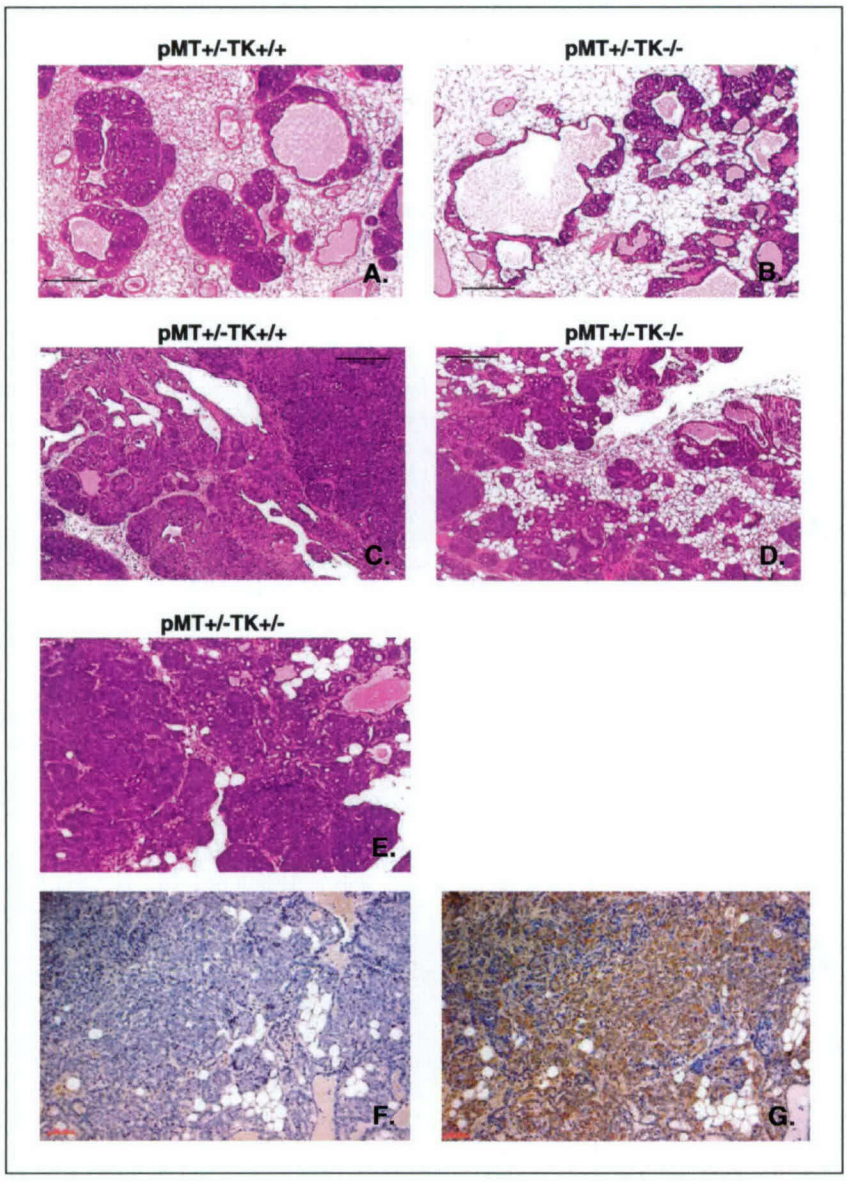

Figure 2. Mammary tumors of pMT+/− TK+/+ and pMT+/− TK−/− mice are morphologically similar but exhibit different growth. Representative sections from mammary tumors are shown at different stages of tumor growth. Tumor cells are visible in both pMT+/− TK+/+ (*A*) and pMT+/− TK−/− (*B*) mammary glands taken at 51 ± 2 days. By 89 ± 5 days of growth, the tumors show more extensive growth in pMT+/− TK+/+ (*C*) and pMT+/− TK+/− (*E*) than pMT+/− TK−/− (*D*) mammary glands. *F-G*, expression of Ron protein in pMT-induced mammary tumors. *F*, control section without primary antibody. *G*, specific staining for Ron protein in pMT+/− TK+/+ section from a tumor at 52 days of growth. *Black bars*, 200 μm; *red bar*, 50 μm.

the foci, in the lungs of the pMT +/− TK+/+ mice compared with the pMT +/− TK−/− mice. In these experiments, there was no correlation between either the tumor burden or the total number of days of tumor growth and either the number of metastatic foci or the area of the lung occupied by the metastatic foci, underscoring the complex nature of the metastatic process and suggesting that the decrease in metastatic load is not simply due to a delay in lung colonization.

The growth of the pMT +/− TK+/+ tumors compared with pMT +/− TK−/− tumors suggested that several mechanisms might be operating, either singly or collectively, to repress the growth of polyoma induced tumors in the absence of signaling from Ron. Therefore, mammary tumor sections were examined by immunohistochemistry for angiogenesis (microvessel formation), cellular proliferation, and apoptosis.

Cellular proliferation was evaluated by immunohistochemical staining of mammary tumor sections after *in vivo* incorporation of BrdUrd. Mammary tumors were collected from mice of both genotypes between 49 and 55 days of age. The tumor collection at this early time point allowed the examination of cells in discrete tumor foci. Representative tumor sections with this proliferation marker are illustrated in Fig. 4*A* and *B*. An increased number of proliferating cells are observed in the pMT +/− TK+/+ mammary tumor Fig. 4*A*, compared with the pMT +/− TK−/− tumor Fig. 4*B*. TUNEL staining was used to analyze apoptotic cells within the mammary tumors. Representative tumor sections collected at 49 to 55 days of age containing TUNEL-positive cells are presented in Fig. 4*C* and *D*, and tumor sections collected at 89 ± 6 days are shown in Fig. 4*E-G*. A decreased number of TUNEL-positive are seen in the mammary tumor from the pMT +/− TK+/+ mouse (Fig. 4*C* and *E*), compared with the pMT +/− TK−/− mouse (Fig. 4*D* 4*D* and *G*). Equivalent numbers of TUNEL-positive cells were seen in heterozygous pMT +/− TK+/− and wild-type pMT +/− TK+/+ tumors (Fig. 4*F* and *E*).

The number of proliferating and TUNEL-positive cells were counted and normalized to the area of the tumor foci. The

summary of these measurements is presented in Table 2. A significantly greater number of proliferating cells are present in pMT-induced mammary tumors from pMT +/− TK+/+ mice compared with pMT +/− TK−/− mice ($P < 0.02$). Moreover, significantly fewer TUNEL-positive cells are present in the mammary tumors from pMT +/− TK+/+ mice than pMT +/− TK−/− mice ($P < 0.02$) at both early and late time points. No difference was seen between pMT +/− TK+/+ and pMT +/− TK+/− tumors in TUNEL-positive staining cells.

Microvessel formation in the mammary tumors was evaluated by immunohistochemical staining with endothelial cell marker, von Willebrand factor. Figure 5 shows representative tumor sections from pMT+/− TK+/+ (*A* and *B*) and pMT+/− TK−/− (*C* and *D*) mice. Serial sections treated with normal mouse IgG in place of the primary antibody (*A* and *C*) do not show vessel staining, demonstrating the specificity of this analysis. An increased number of microvessels is seen in the pMT +/− TK+/+ tumor section (Fig. 5*B*), compared with the pMT +/− TK−/− tumor (Fig. 5*D*). The number of microvessels was counted and normalized to the area of the tumor foci, to determine microvessel density. These measurements are also presented in Table 2. A significantly greater number of microvessels are present in pMT-induced mammary tumors from pMT +/− TK+/+ mice compared with pMT +/− TK−/− mice ($P < 0.02$).

To elucidate the molecular mechanism underlying this striking difference in the growth of pMT-induced mammary tumors mediated by Ron signaling, Western analyses were done (Fig. 6). Ron expression and the activation of proliferation- and apoptosis-associated molecules were evaluated.

In Fig. 6*A*, a representative Western analysis of Ron protein expression in mammary tumor and normal mammary gland lysates is shown. These analyses used an antibody that recognizes the extracellular domain of Ron, which is intact in both the TK+/+ and TK−/− mice. The same membrane was stripped and reprobed with an antibody that recognizes actin to show equivalent protein loading in each lane. Equivalent levels of Ron protein are expressed in normal mammary glands of TK+/+ (lanes 1 and 2) and TK−/− (Fig. 6, *lanes 5* and *6*)) mice. Compared to the level of Ron expression in normal mammary glands, the pMT-expressing tumors from pMT+/− TK+/+ (Fig. 6, *lanes 3* and *4*)) and pMT+/− TK−/− (Fig. 6, *lanes 7* and *8*) mice express 5-fold and 4-fold higher levels of Ron protein. The ligand for Ron is expressed in the liver, excreted into the bloodstream, and circulates in an inactive form until it is activated at the cell surface. Normal mammary tissue and mammary tumor tissue was analyzed for the presence of hepatocyte growth factor–like protein, the ligand for Ron, by Western and Northern analysis. Hepatocyte growth factor–like protein was not detected in

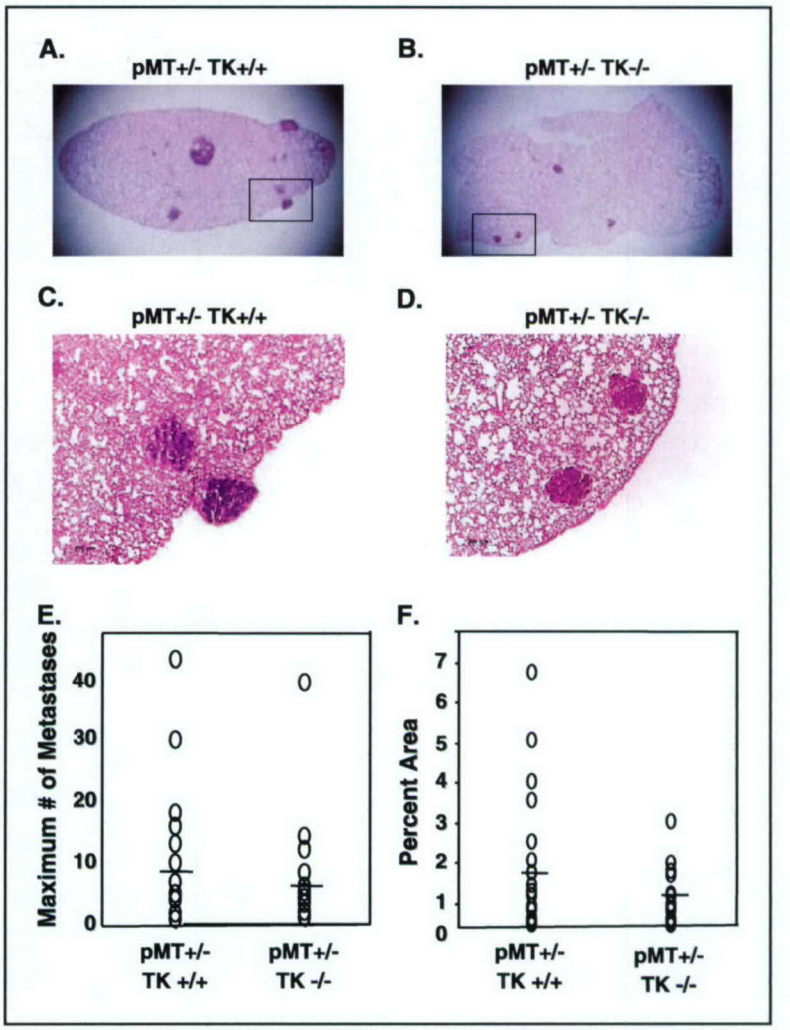

Figure 3. Lung metastasis of pMT–induced tumors is reduced in experimental animals lacking Ron signaling. *A* and *B*, representative whole lung sections, showing the typical appearance of metastases within the lung, are shown for pMT+/− TK+/+ (*A*) and pMT+/− TK−/− (*B*) lungs taken at 90 days of growth. Higher magnification images of representative metastases, corresponding to the outlined areas in (*A*) and (*B*) are also shown for pMT+/− TK+/+ (*C*) and pMT+/− TK−/− (*D*) lungs. No morphologic difference between lung metastases from control or experimental mice was observed (*C-D*). The extent of lung metastasis between the experimental and control animal groups was evaluated (pMT+/− TK+/+, $n = 21$; pMT+/− TK−/−, $n = 20$). The number of metastatic foci in a lung section was counted, and the maximum number of metastases observed in a section of the lung is shown (*E*). The area covered by metastatic cells compared with the total area of the lung section was also measured and these values shown in (*F*).

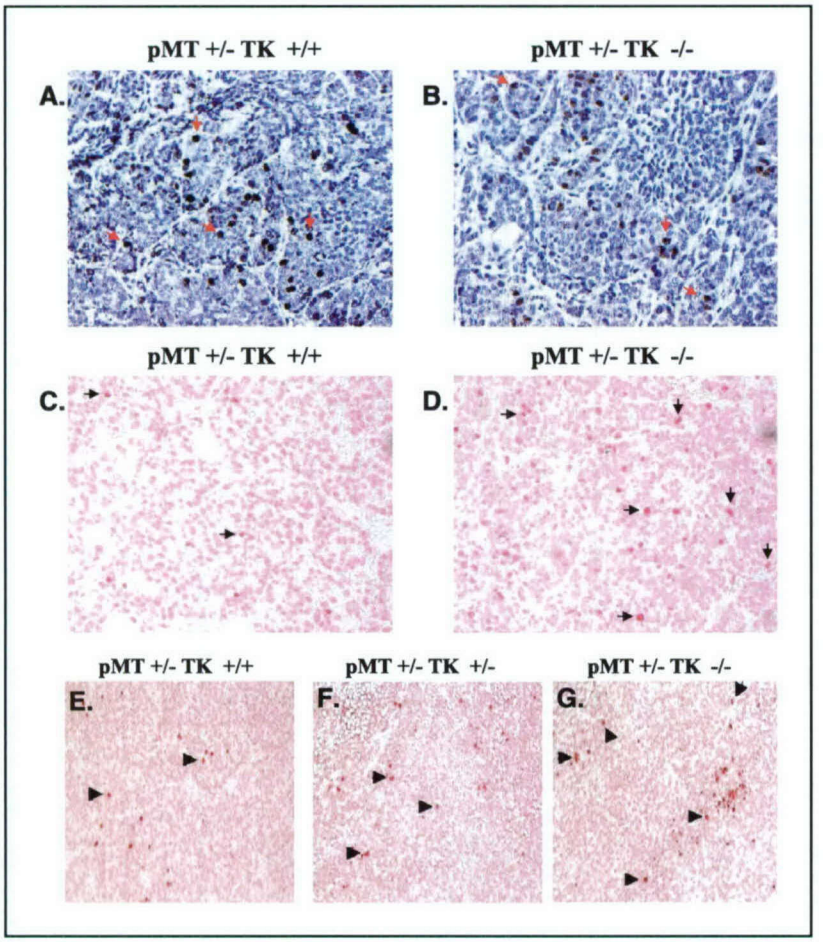

Figure 4. Increased proliferation and reduced TUNEL-positive staining of cells in mammary tumors from pMT+/− TK+/+ compared with pMT+/− TK−/− mice. Proliferative cells in tumor tissue were detected by immunohistochemical staining for BrdUrd incorporation. Representative sections from pMT+/− TK+/+ (*A*), and pMT+/− TK−/− (*B*) tumors taken from mice at 49 days of age. *Red arrows*, representative BrdUrd staining cells. Apoptotic cells in tumor tissue were detected by the immunohistochemical staining for TUNEL-positive cells. Representative sections from pMT+/− TK+/+ (*C*), and pMT+/− TK−/− (*D*), tumors at 51± 2 days, and from pMT+/− TK+/+ (*E*), pMT+/− TK+/− (*F*), and pMT+/− TK−/− (*G*), at 89 ± 5 days are shown. *Black arrows*, representative TUNEL-positive cells. Original magnification ×100.

mammary tissue (data not shown). This suggests that overexpression of Ron in mammary tumors leads to ligand-independent activation of this receptor and is consistent with previous findings on Ron overexpression (13).

Activation of Ron has been shown to activate MAPK, a well-known regulator of cell proliferation (27, 28). In Fig. 6*B*, a representative Western analysis of MAPK level and activation in tumor lysates from pMT+/− TK+/+ (Fig. 6, *lanes 1-3*) and pMT+/−

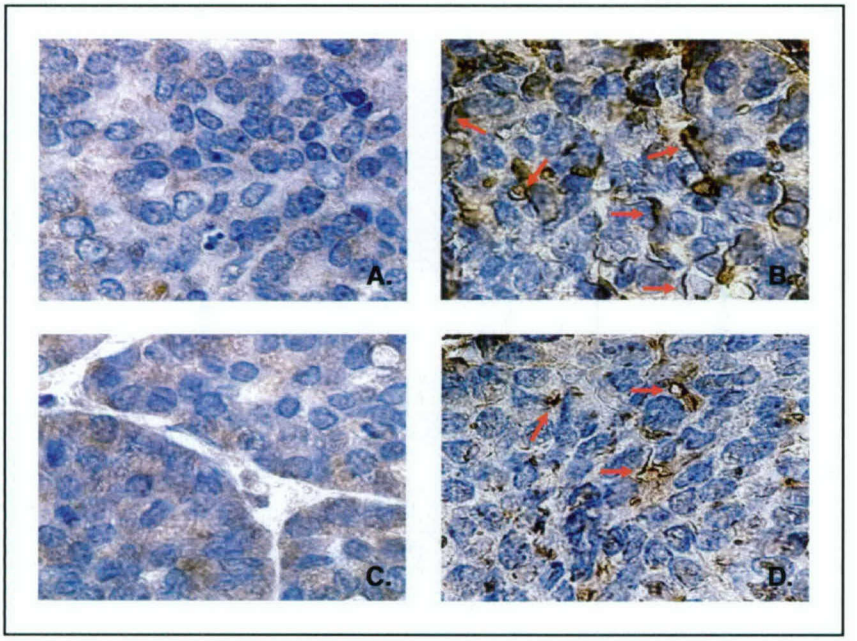

Figure 5. Increased microvessel density in mammary tumors from pMT+/− TK+/+ compared with pMT+/− TK−/− mice. Microvessels in tumor tissue were detected by immunohistochemical staining (Red arrows). Representative sections from pMT+/− TK+/+ (*A* and *B*), and pMT+/− TK−/− (*C* and *D*) tumors taken from mice at 52 ± 3 days of age are shown. *A* and *C*, control serial sections treated with normal mouse IgG in place of the primary antibody for pMT+/− TK+/+ (*A*) and pMT+/− TK−/− (*C*) tumors. Sections of pMT+/− TK+/+ (*B*) and pMT+/− TK−/− (*D*) tumors stained with an anti-von Willebrand factor antibody. Magnification, ×100.

Table 2. Ron signaling regulates cell growth, TUNEL staining, and microvessel density in mammary tumors induced by pMT

Mouse genotype	Proliferative cells (in^2)	TUNEL-positive cells (in^2)	Microvessel density microvessels (in^2)
pMT+/− TK+/+	10.2 ± 4.7	2.1 ± 0.8, 1.5 ± 0.8[a]	4.1 ± 0.39
pMT+/− TK+/−	ND	1.1 ± 0.7,[a] $P > 0.05$	ND
pMT+/− TK−/−	4.7 ± 2.7, $P < 0.02$	3.7 ± 1.5, $P < 0.02$; 2.3 ± 0.7,[a] $P < 0.002$	2.6 ± 0.16, $P < 0.02$

NOTE: A significant increase in proliferation, a significant decrease in TUNEL positive cells, and a significant increase in microvessel density was seen in mammary tumors in the pMT +/− TK+/+ mice compared with pMT +/− TK−/− mice. No differences were observed between pMT-containing TK+/+ and TK+/− mice. Tumors were collected at 51 ± 2 days or at 89 ± 5 days ([a]). Data shown are mean ± SD. Abbreviation: ND, not determined.

TK−/− (Fig. 6, *lanes 4-6*) mice is shown. Phosphorylated MAPK (p44 and p42) are indicated. The same blot was stripped and reprobed with an antibody that recognizes nonphosphorylated p44 and p42 MAPK to determine the total amount of MAPK in each sample. Equivalent amounts of MAPK are found in pMT+/− TK+/+ and pMT+/− TK−/− tumors. However, the phosphorylated form of MAPK is 1.5-fold greater in pMT+/− TK+/+ tumors than in pMT+/− TK−/− tumors.

Phosphatidylinositol-3-kinase (PI3K) has also been shown to interact directly with the Ron receptor (7, 29) resulting in the downstream activation of AKT and increased cell proliferation (30, 31). In Fig. 6*C*, a representative Western analysis of AKT level and activation in tumor lysates from pMT+/− TK+/+ (Fig. 6, *lanes 1-3*) and pMT+/− TK−/− (Fig. 6, *lanes 4-6*) mice is shown. Phosphorylated AKT (p60) is indicated. The same blot was stripped and reprobed with an antibody that recognizes nonphosphorylated AKT to determine the total amount of AKT in each sample. Equivalent amounts of AKT are found in pMT+/− TK+/+ and pMT+/− TK−/− tumors. However, the phosphorylated form of AKT is 1.5-fold greater in pMT+/− TK+/+ tumors than in pMT+/− TK−/− tumors.

Discussion

The experiments detailed in this report are the first to directly examine the impact of Ron receptor tyrosine kinase signaling on mammary tumor initiation and growth, by using an experimental mouse model of breast cancer induced by mammary-specific expression of pMT. In this model, lack of Ron signaling significantly increases tumor latency, significantly reduces tumor growth, and may reduce tumor metastasis. Significantly increased cellular proliferation, reduced TUNEL-positive staining, and increased microvessel formation is found in wild-type pMT-expressing tumors compared with pMT-induced tumors lacking Ron signaling. The tumors produced by pMT overexpress Ron protein. Increased activation of MAPK and AKT is seen in wild-type pMT expressing tumors compared with pMT-induced tumors lacking Ron signaling. Although the formal possibility exists that a recessive linked locus may be responsible for the effect attributed to the deletion of the Ron tyrosine kinase domain, the lack of any difference in tumor characteristics between the wild-type and heterozygous Ron TK–deficient mice strongly argues that the phenotypic and biochemical changes observed in the TK−/− mice are a result of the Ron allele and not due to modification by other genetic loci.

Multiple mechanisms by which Ron signaling may synergize with pMT signaling to influence tumor growth have been revealed by our immunohistochemical and biochemical analysis of pMT-expressing tumors produced in the presence and absence of an intact tyrosine kinase domain of Ron. First, overexpression of Ron protein is seen in the mammary tumors induced by pMT. Interestingly, a recent series of experiments in our laboratory has determined that activation of Ras in a mouse model of skin carcinogenesis also up-regulates Ron (32). Our previous experiments have shown that Ron overexpression is accompanied by constitutive activation of the receptor, transformation, and increased cell proliferation (13). In the absence of the tyrosine kinase domain, the overexpression of the truncated receptor will not amplify downstream transformative and proliferative signals.

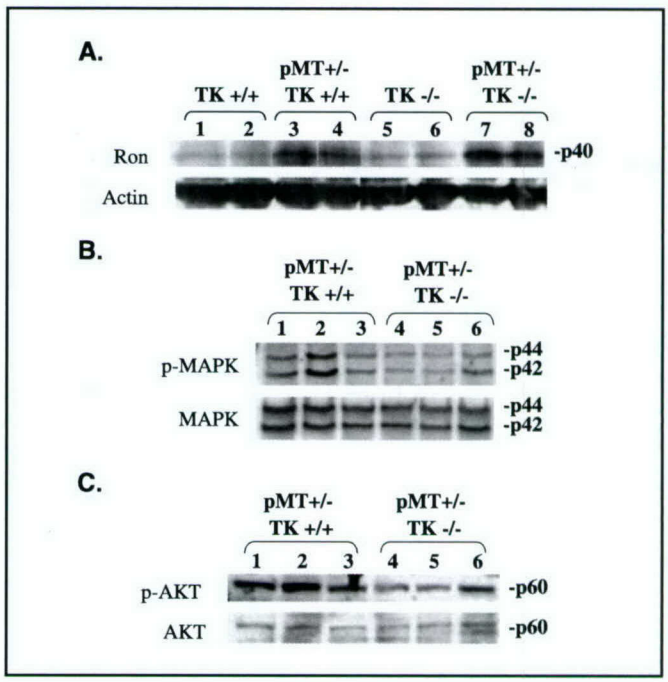

Figure 6. Western analysis of mammary tissue lysates. *A*, representative Western analysis of Ron protein expression in tumor lysates from pMT+/− TK+/+ (*lanes 3 and 4*) and pMT+/− TK−/− mice (*lanes 7 and 8*), compared with non-tumor-bearing TK+/+ (*lanes 1 and 2*) and TK−/− (*lanes 5 and 6*) mice is shown. The same membrane was stripped and reprobed with an antibody that recognizes actin to show equivalent protein loading in each lane. *B*, representative Western analysis of MAPK level and activation in tumor lysates from pMT+/− TK+/+ (*lanes 1-3*) and pMT+/− TK−/− (*lanes 4-6*) mice is shown. Phosphorylated MAPK (*p-MAPK*, p44 and p42) are indicated. The same membrane was stripped and reprobed with an antibody that recognizes nonphosphorylated p44 and p42 MAPK to determine the total amount of MAPK in each sample. *C*, representative Western analysis of AKT level and activation in tumor lysates from pMT+/− TK+/+ (*lanes 1-3*) and pMT+/− TK−/− (*lanes 4-6*) mice is shown. Phosphorylated AKT (*p-AKT*, p60) is indicated. The same blot was stripped and reprobed with an antibody that recognizes nonphosphorylated AKT to determine the total amount of AKT in each sample.

Second, the lack of Ron signaling has an apparent effect on angiogenesis within the mammary tumors induced by pMT. There has been considerable research conducted on the relationship between angiogenesis and tumor growth. Angiogenesis within human mammary tumors has been correlated with metastatic disease, and poor prognosis (33). Polyoma middle T–induced tumors have been shown to be poorly perfused in relationship to their growth (34), and yet tumor growth in this model has been shown to be influenced by the ability of the tumor to recruit microvessels (35). The dramatic reduction in microvessels seen in the pMT+/− TK−/− tumors compared with the pMT+/− TK+/+ tumors, coupled with their reduced growth rate, suggests that Ron signaling may play a role in promoting angiogenesis in this mammary tumor.

Third, the parallel increases in cellular proliferation and cell survival may be mediated by increased activation of MAPK and AKT acting in concert in pMT+/− TK+/+ mammary tumors, compared with tumors in which Ron signaling is absent. Activation of AKT is coupled to activation of PI3K. Polyoma middle T antigen transformation is highly dependent on PI3K. Mice carrying a mutation abolishing the binding site for PI3K on pMT develop mammary gland hyperplasias that are highly apoptotic, and only develop focal tumors at a late time point (36). A reintroduction of activated AKT into the mouse strain decoupled from pMT-PI3K interaction restored and accelerated mammary tumorigenesis, with a concomitant reduction in apoptosis in the mammary tumor (36). Ron has previously been shown to mediate both apoptotic and growth signals (7); however, the role of Ron in cell survival may be cell type dependent (7, 22). Adherent epithelial cell survival stimulated by ligand binding of Ron was found to depend on both MAPK and PI3K/AKT activation, and each pathway independently contributed to overall cell survival (37). These experiments support the argument that the increased activation of both MAPK and AKT in pMT+/− TK+/+ tumors, compared with pMT+/− TK−/− tumors, jointly contributes to the increased proliferation, decreased apoptosis, and overall increased tumor growth.

The mouse model of mammary tumorigenesis induced by *MMTV-pMT* has been extensively used to examine pathways and molecules involved in mammary tumorigenesis and metastasis, including genetic loci (38, 39), putative tumor suppressors (40, 41), and other disease-modifying molecules (24, 42, 43). Our report is the first to investigate the role of Ron tyrosine kinase signaling in this mammary tumor and metastasis model. We conclude that Ron will play a significant role in breast cancer and may be an important therapeutic target.

Acknowledgments

Received 11/14/2003; revised 11/17/2004; accepted 12/8/2004.

Grant support: NIH training grant T-32-HL07752 (B.E. Peace) and Public Health Service grants HD36888 and CA100002, Department of Defense Career Development Award DAMD17-02-1-0342, and University of Cincinnati (Cincinnati, Ohio) grant from the Women's Health Program and the Breast Cancer Angels, Inc. (S.E. Waltz).

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