The Ron receptor tyrosine kinase negatively regulates mammary gland branching morphogenesis

Sara E. Meyer a,1, Glendon M. Zinser a,1, William D. Stuart a, Peterson Pathrose a, Susan E. Waltz a,b,⁎

a Department of Cancer and Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, 3125 Eden Ave, Cincinnati, OH 45267-0521, USA
b Department of Research, Shriner’s Hospital for Children, Cincinnati, OH 45267-0521, USA

Abstract

The Ron receptor tyrosine kinase is expressed in normal breast tissue and is overexpressed in approximately 50% of human breast cancers. Despite the recent studies on Ron in breast cancer, nothing is known about the importance of this protein during breast development. To investigate the functional significance of Ron in the normal mammary gland, we compared mammary gland development in wild-type mice to mice containing a targeted ablation of the tyrosine kinase (TK) signaling domain of Ron (TK−/−). Mammary glands from RonTK−/− mice exhibited accelerated pubertal development including significantly increased ductal extension and branching morphogenesis. While circulating levels of estrogen, progesterone, and overall rates of epithelial cell turnover were unchanged, significant increases in phosphorylated MAPK, which predominantly localized to the epithelium, were associated with increased branching morphogenesis. Additionally, purified RonTK−/− epithelial cells cultured ex vivo exhibited enhanced branching morphogenesis, which was reduced upon MAPK inhibition. Microarray analysis of pubertal RonTK−/− glands revealed 393 genes temporally impacted by Ron expression with significant changes observed in signaling networks regulating development, morphogenesis, differentiation, cell motility, and adhesion. In total, these studies represent the first evidence of a role for the Ron receptor tyrosine kinase as a critical negative regulator of mammary development.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Mammary gland development is a highly regulated, intricate, and continuous process throughout the life of an animal beginning in the embryo, and continuing postnatally during puberty, pregnancy, lactation, and involution (Howlin et al., 2006; Watson and Khaled, 2008). Importantly, studies have shown that many of the factors necessary for proper mammary gland development are also deregulated during breast cancer. Therefore, it is imperative to continue to study novel regulators of mammary development in order to gain further insight and understanding of breast tumorigenesis (Dickson et al., 2000; Lanigan et al., 2007).

During normal pubertal mammary gland development in mice (5-10 weeks of age), generation of the mammary ductal tree occurs through two simultaneous morphological processes — ductal elongation and branch formation (Lu et al., 2006; Silberstein, 2001). The mammary epithelium elongates into the fat pad by proliferation of the terminal end buds (TEB) and simultaneous hollowing out of the end buds by apoptosis to form ducts to create the primary ductal network (Silberstein, 2001). There are two types of branching that can occur in a pubertal mouse mammary gland, TEB bifurcation and lateral side branching from existing ducts (Brisken, 2002; Lu et al., 2006). While the exact mechanisms that regulate the length, placement, and number of ducts are not fully understood, proper ductal elongation and branching morphogenesis during puberty are necessary to provide enough surface area for alveoli to form during pregnancy and lactation to supply an adequate amount of milk to nurse young pups.

Branching morphogenesis is subjected to complex positive and negative regulatory signals, generated from the surrounding stroma, serum, and crosstalk between these components and the epithelium that orchestrate the growth of the mammary epithelium during pubertal development (Silberstein, 2001). The ovarian hormones estrogen and progesterone and their receptors induce ductal elongation and side branching, respectively, and are required for proper mammary gland development (Hennighausen and Robinson, 2005). In addition, growing evidence supports that growth factor and receptor tyrosine kinase signaling are essential for proper mammary development and branching morphogenesis (Kumar and Wang, 2002). Receptor tyrosine kinases function to transduce extracellular signals inside the cell, as well as crosstalk with other cell surface molecules (Hendrickson, 2005). It has been shown that receptor tyrosine kinases play a key role in the communication between the mammary epithelium and surrounding mammary gland environment, including stroma and sera, to contribute...
both positive and negative regulatory signals during mammary gland development (Sternlicht, 2006).

Previous studies have shown that the molecules that regulate pubertal mammary gland development are frequently deregulated or overexpressed during mammary tumorigenesis (Kumar and Wang, 2002; Lanigan et al., 2007). Receptor tyrosine kinases are often overexpressed in human breast cancers and are a desirable target for cancer therapeutics (Longati et al., 2001). In addition, mammary-specific overexpression of several receptor tyrosine kinases has been shown to drive mammary tumorigenesis in mice and, in more rare circumstances, metastasis (Cardiff and Kenney, 2007; Robles and Varticovski, 2008).

The membrane spanning receptor tyrosine kinase Ron, a member of the Met family, has recently been shown to be overexpressed in a variety of human cancers including breast cancer (Gaudino et al., 1994; Leonis et al., 1999; Maggiora et al., 1998; Wagh et al., 2008). Importantly, mammary-specific overexpression of Ron in the mouse gives rise to tumors with 100% incidence that progress to mammary carcinomas that metastasize with high frequency (Zinser et al., 2006). The Ron receptor consists of a 35 kDa alpha chain, with ligand binding capacity, joined by disulfide bonds to a 150 kDa beta chain containing the transmembrane and intracellular tyrosine kinase domains (Comoglio and Boccaccio, 1996).

In humans and mice, Ron is expressed in many tissues including mammary gland (Chodosh et al., 2000; Maggiora et al., 1998). Hepatocyte growth factor-like protein (HGFL), also known as macrophage stimulating protein, is the ligand for Ron and is present in the circulation (Gaudino et al., 1994; Wang et al., 1995). Upon binding of HGFL to Ron, receptor dimerization and tyrosine autophosphorylation occurs for activation. Downstream signaling targets of Ron activity include PI3K, Src, FAK, Akt, and MAPK that can lead to proliferation, cell survival, cell motility, cell shape change, and invasion (Danilkovitch and Leonard, 1999; Danilkovitch-Miagkova, 2003).

Ron mRNAs is increasingly expressed throughout pubertal mammary gland development in mice (Chodosh et al., 2000); however, nothing is known about the morphological impact Ron signaling has on pubertal mammary gland development. Based on our previous studies indicating that Ron signaling is sufficient to induce mammary tumorigenesis, we hypothesized that Ron receptor signaling would impact postnatal mouse mammary gland development. To test this hypothesis we compared pubertal mammary gland development (5–10 weeks) in wild-type (RonTK+/+) and Ron tyrosine kinase domain null mice (RonTK−/−). We are the first to report that Ron signaling profoundly impacts pubertal mouse mammary gland development. Surprisingly, we found that in the absence of Ron signaling, RonTK−/− mice exhibited significantly increased ductal extension and branching morphogenesis without significant changes in epithelial cell turnover. Furthermore, using ovariectomized mice we show that mammary glands from RonTK−/− mice also displayed excessive branching morphogenesis, compared to wild-type controls. In conjunction with increased branching, we also observed elevated phosphorylation of Akt and MAPK in RonTK−/− mammary glands compared to controls. Additionally, isolated primary RonTK−/− mammary ductal epithelial fragments (organoids) demonstrated a significant increase in branching morphogenesis, ex vivo, that was blocked by MAPK inhibition. By microarray analysis, deletion of the Ron tyrosine kinase domain significantly altered the genetic profile of pubertal mammary glands in comparison to wild-type control glands with many of the genes grouped into developmental and morphological categories. Taken together, these results demonstrate that the Ron receptor tyrosine kinase is a novel and important regulator of pubertal mouse mammary gland development.

**Materials and methods**

**Animals**

A germline deletion of the tyrosine kinase domain of Ron (RonTK−/−) has been previously described and was backcrossed 8 generations onto the FVB/N background for the studies herein (Peace et al., 2005; Waltz et al., 2001). FVB/N mice containing wild-type Ron (RonTK+/+) were used as controls for all experiments. For ovariectomization experiments, 3 week-old RonTK+/+ and RonTK−/− female mice were ovariectomized and mammary glands were allowed to develop for an additional 3 weeks in the absence of ovarian hormones. At 6 weeks of age, inguinal mammary glands were harvested and prepared for whole mount analyses. All experiments involving animals were performed under protocols approved by the Institutional Animals and Use Committee of the University of Cincinnati.

**Whole mount and histological analyses**

Mammary glands from 5, 6, 7, 8, and 10 week-old virgin female RonTK+/+ and RonTK−/− mice (n = 10 per genotype) were harvested. Thoracic glands were frozen for protein and RNA analysis, one inguinal gland was formalin fixed for histology and immunohistochemistry, while the other was while mounted for morphological assessment. For whole mount preparation, glands were spread on glass slides and fixed overnight in Carnoy’s Fixative, rinsed in 70% ethanol, and transferred into Carnine Alum stain overnight. Glands were rinsed in a graded series of ethanol and cleared in Xylene before mounting with Permount. Images of whole mounts were taken using a Nikon D1x digital camera with a Nikon AF MICRO NIKKOR 105 mm 1:2.8D lens. For histological analysis, glands were fixed overnight in 10% neutral buffered formalin then changed to 70% ethanol, and paraffin embedded. 4 μm-thick sections were stained using Harris Hematoxylin and Eosin. Images of histological sections were taken with a Nikon FX-35DX camera affixed to the Nikon Microphot microscope and Spotcam Advanced software (Nikon).

**Ductal elongation, TEB, and branch point analyses**

Axiovision Release 4.5 software was used to measure ductal elongation, terminal end bud number, and number of secondary and tertiary branch points from images of 5, 6, 7, 8, and 10 week-old RonTK+/+ and RonTK−/− inguinal mammmary whole mounts, and from images of 6 week-old ovariectomized RonTK+/+ and RonTK−/− inguinal mammmary whole mounts. Ductal elongation was measured as the distance from the center of the lymph node to the furthest terminal end bud at the leading edge of the mammary epithelium. All terminal end buds of greater than or equivalent to 0.03 mm² were quantified. The longest primary duct directly above the lymph node was used for branch quantification from one inguinal gland per mouse (n = 10 per genotype). A secondary branch was defined as any branch that bifurcates from the primary duct. A tertiary branch was defined as any branch that bifurcates off a secondary branch.

**BrDU and TUNEL analyses**

Five, 6, and 7 week-old RonTK+/+ and RonTK−/− mice (n = 4 per genotype) were injected intraperitoneally with 10 μl per gram body weight of a 10 mM BrDU solution (Amersham Biosciences, Piscataway, NJ) 2 h prior to sacrifice. The left side thoracic and inguinal glands were snap frozen in liquid nitrogen for use in the microarray analysis procedure. The right side thoracic and inguinal glands were fixed in 10% neutral buffered formalin overnight then changed to 70% ethanol. Mammary glands were processed and embedded in paraffin. 4 μm-thick sections of RonTK+/+ and RonTK−/− inguinal mammary glands were stained using a BrDU Staining Kit (Zymed Laboratories, Inc., San Francisco, CA) and In Situ Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. The percentage of BrDU-positive cells was determined by quantifying the number of BrDU-positive cells out of the total number of cells from 4 end buds and 4 ducts per mouse. The same quantification procedure was also used for TUNEL analysis.
Primary mammary cell purification and stromal fat pad isolation

Mammary epithelial cells were purified from RonTK+/+ and RonTK−/− mice. Two 6–8 week-old female mice per genotype were sacrificed and thoracic and inguinal mammary glands were removed, diced using razor blades, and placed into 25 ml digestion media containing DMEM/F12, 1 mg/ml collagenase ( Worthington Biochemical, Lakewood, NJ), Penicillin/Streptomycin, and 2 mg/ml bovine serum albumin (Sigma, St. Louis, MO) for approximately 2 h at 37 °C with shaking at 200 rpm. Cells were then centrifuged for 5 min at 1000 rpm and supernatant removed. To further dissociate epithelial cells, the pellet was resuspended in DMEM/F12 containing 2 U/ml DNase for 5 min with vigorous shaking. DNase was inactivated with equal volume of DMEM/F12 containing 5% FBS. Organoids were centrifuged for 5 min at 800 rpm, supernatant was removed and pellet was resuspended in 1× PBS plus 5% FBS. To remove fibroblasts, organoids were shaken vigorously then pulse spun for 10 s to a maximum of 1000 rpm, supernatant was removed and repeated 6 additional times. Epithelial organoids were rinsed twice in 1× PBS to remove traces of FBS. For characterization of the mammary fat pad without epithelium, fat pads were dissected, excluding nipple and lymph node, from 3 week-old RonTK+/+ mouse inguinal mammary glands. Fat pads from at least 2 mice were pooled, per sample.

Primary mammary epithelial organoid cultures

Mammary epithelial organoids purified from RonTK+/+ and RonTK−/− female mice were resuspended in Growth Factor Reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) and plated on Matrigel pre-coated 24-well plates. 171 Medium (Cascade Biologics, Portland, OR) plus Mammary Epithelial Growth Supplement (Cascade Biologics, Portland, OR) was added on top of the matrix/organoid mixture once gelled. Organoids were treated with 2 μM of the MAPK inhibitor PD98059 (Calbiochem, San Diego, CA), or equivalent volume DMSO vehicle control, and cultured for 6 days with media plus inhibitor refreshed every 2 days. On day 6, the number of organoids with buds and/or branches was quantified and the percentage of budding/branching organoids out of total organoids plated was determined. Approximately equal numbers of organoids were plated per genotype and experiments were repeated three times with similar results.

Western analyses

Mammary glands excised from 5, 6, 8, and 10 week-old RonTK+/+ and RonTK−/− mice (n = 8 per genotype) and snap frozen in liquid nitrogen. Glands from pregnant mice were isolated at day 16 following timed matings, lactating glands were isolated at day 10 after birth, and nitrog. Glands from pregnant mice were isolated at day 16 following

Immunochemical analyses

Phosphorylated MAPK (phospho-Erk1/2, Cell Signaling Technology, Danvers, MA) and Ron expression (Santa Cruz Biotechnology, Santa Cruz, CA) were detected on tissue sections from 6 week-old virgin female RonTK+/+ and/or RonTK−/− mice as indicated. Primary antibodies were detected using 0.75 μg/ml goat anti-rabbit biotinylated secondary antibodies. Antibody immunoreactivity was amplified using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA), and visualized using DAB substrate (Vector Laboratories, Burlingame, CA). The sections were counterstained in hematoxylin, dehydrated in a graded series of alcohols ending with Xylene, and mounted. All images were captured using a Nikon FX-35DX camera attached to the Nikon Microphot microscope and Spotcam Advanced software.

Serum steroid hormone assays

Whole blood was collected from 5, 6, and 7 week-old virgin female RonTK+/+ and RonTK−/− mice via cardiac puncture and placed into serum separator tubes. Tubes were spun and serum was placed in a 0.5 ml tube and stored at −80 °C until assays were performed. The serum from these mice was analyzed for circulating estradiol and progesterone levels using 17β-Estradiol ELISA Kit (Cayman Chemical, Ann Arbor, MI) and Progesterone ELISA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Statistical analyses

Statistical significance for all analyses, except for microarray analysis, was determined by a Student’s t-test using Sigma Stat 3.5 software (Cranes Software International, Karnataka, India).

Microarray analysis

RNA was harvested from whole mammary glands of 5, 6, and 7 week-old RonTK+/+ and RonTK−/− virgin female mice using TriZol (Invitrogen, Carlsbad, CA) according to the manufacturers instructions with one modification. To remove fat from the TriZol preparation, centrifugation at 12,000 g for 10 min at 4 °C was performed prior to the addition of chloroform. RNA samples were submitted to the Cincinnati Children’s Hospital Medical Center Affymetrix Core, Cincinnati, Ohio. The Agilent Bioanalyzer 2100 (Hewlett Packard, Palo Alto, CA) using the RNA 6000 Nano Assay was applied to the RNA for quality assessment. Next, 400–500 ng of total RNA per sample was used in the TargetAmp 1-Round Aminoallyl-aRNA Amplification Kit (EpigenInstruments, Madison, WI) to generate cRNA following the manufacture’s instructions. Biotin-X-X-NHS (Epicentre Biotechnologies, Madison, WI) was used to label the aminomethyl-aRNA with biotin. The biotin-labeled cRNA target was then chemically fragmented, and a hybridization cocktail was prepared and hybridized to the Affymetrix Mouse GeneChip 430 2.0 array. The probe arrays were scanned using the Affymetrix GeneChip Scanner 3000 and Genechip Operating Software 1v4 (Affymetrix, Santa Clara, CA).

Gene spring analysis

Changes in gene expression were then analyzed using Gene Spring GX 6v1.1 software. 45,000 probe sets were first filtered on expression
using a minimum raw intensity value greater than or equal to 120. The resulting 32,181 probe sets were then normalized to the median intensity value of all wild-types. Next, all probes were assessed by parametric ANOVA assuming equal variance with multiple testing correction. Jordan and Hochberg false discovery rate of \( p = 0.2 \) was used in the ANOVA analysis.

Real-time analyses were repeated twice with similar results using primers that span the beginning of the tyrosine kinase domain, which is deleted in the "RonTK−/−" mice (Waltz et al., 2001). Correspondingly, Ron mRNA expression was observed during mammary development in wild-type virgin mouse mammary glands 5–8 weeks of age, pregnancy, and lactation (Fig. 1B). Ron protein expression was undetectable in mammary glands harvested on involution day 10 and in mammary fat pads devoid of epithelium (Fig. 1B). Further, Ron protein expression was also observed in the normal mouse mammary epithelial cell line EHP4, and the normal human mammary epithelial cell lines MCF10A and HMEC (Fig. 1B and C–right). Despite the scant Ron mRNA expression in the human primary adipocyte cell line (HPAC) by real-time PCR (Fig. 1C, left), Ron protein was undetectable in these cells by Western analysis (Fig. 1C, right). Immunohistochemical detection of Ron in 6-week-old virgin wild-type mammary glands showed an intense staining pattern of Ron in the mammary epithelium of both ducts and terminal end buds (Fig. 1D). Taken together, these data suggest that Ron is expressed during specific time frames throughout mouse mammary gland development, primarily in the epithelial compartment in both humans and mice with low to undetectable levels of Ron expression in adipose tissue.

Deletion of the Ron tyrosine kinase domain accelerates pubertal mammary gland development

To examine the functional contribution of Ron during mammary development, a temporal analysis of glandular architecture was undertaken in wild-type mice ("RonTK+/+" and mice with homozygous deletion of the tyrosine kinase domain of Ron ("RonTK−/−")). Developmental analysis of whole mount preparations from 5, 6, 7, and 8 weeks of age and 10-week-old virgin female RonTK−/−/− mouse mammary glands were compared to age and weight-matched RonTK++/+ control glands. Surprisingly, the RonTK−−/− mammary glands displayed accelerated branching morphogenesis as evident by the denser mammary ductal tree compared to RonTK++/+ controls (Fig. 2A). To evaluate the developmental progress of the RonTK++/+ and RonTK−−/− mammary glands, terminal end bud (TEB) number, ductal elongation, and secondary and tertiary branch points were quantified. Ductal outgrowth, measured from the center of the lymph node to the furthest TEB, was significantly increased in RonTK−−/− mammary glands at 6 and 7 weeks of age compared to RonTK++/+ controls (Fig. 2B). We also saw a trend throughout the remainder of postnatal mammary development (Chodosh et al., 2000). Since the ligand for Ron, HGF, is a motility factor that promotes epithelial cell migration (Wang et al., 1996), and since Ron is a member of the c-Met family of receptor tyrosine kinases of which Met and its ligand, hepatocyte growth factor (HGF), have been shown to induce branching morphogenesis (Yang et al., 1995; Yant et al., 1998), we hypothesized that Ron may contribute to the rapid epithelial migration and branching characteristic of mammary ductal morphogenesis. Our first objective was to define the cellular compartment in which Ron is expressed in the mammary gland. Prior studies had only demonstrated Ron mRNA expression from whole gland homogenates. Using primers that span the beginning of the tyrosine kinase domain, which is deleted in the "RonTK−−/−" mice (Waltz et al., 2001), we found that Ron mRNA is expressed in wild-type virgin mouse mammary glands at 5–8 weeks of age, but undetectable at 10 weeks of age (Fig. 1A). Interestingly, Ron expression was also observed in later phases of mammary development including pregnancy (timed mating day 16), lactation, and involution day 4, but was undetectable by involution day 10 (Fig. 1A). We did not detect a PCR product in the RonTK−−/− mammary glands using these primers as predicted (Fig. 1A). However, utilizing a set of primers directed to a region upstream of the TK deletion did amplify a Ron product in the RonTK−−/− glands at levels similar to wild-type glands, suggesting that a truncated Ron transcript is expressed at the mRNA level (data not shown). This is consistent with previously published data characterizing the RonTK−−/− mice (Waltz et al., 2001). Correspondingly, Ron protein expression was observed during mammary development in wild-type virgin mouse mammary glands 5–8 weeks of age, pregnancy, and lactation (Fig. 1B). Ron protein expression was undetectable in mammary glands harvested on involution day 10 and in mammary fat pads devoid of epithelium (Fig. 1B). Further, Ron protein expression was also observed in the normal mouse mammary epithelial cell line EHP4, and the normal human mammary epithelial cell lines MCF10A and HMEC (Fig. 1B and C–right). Despite the scant Ron mRNA expression in the human primary adipocyte cell line (HPAC) by real-time PCR (Fig. 1C, left), Ron protein was undetectable in these cells by Western analysis (Fig. 1C, right). Immunohistochemical detection of Ron in 6-week-old virgin wild-type mammary glands showed an intense staining pattern of Ron in the mammary epithelium of both ducts and terminal end buds (Fig. 1D). Taken together, these data suggest that Ron is expressed during specific time frames throughout mouse mammary gland development, primarily in the epithelial compartment in both humans and mice with low to undetectable levels of Ron expression in adipose tissue.
toward an increase in TEB number at 5–8 weeks in the \RonTK\−/− glands, but these differences did not reach statistical significance in this study (Fig. 2C). At 10 weeks, TEB number significantly decreases in \RonTK\−/− glands compared to \RonTK+/+ controls (Fig. 2C), correlating with the completion of ductal morphogenesis in the \RonTK\−/− glands compared to controls. Most strikingly, secondary and tertiary branch points were significantly increased in mammary glands from 6, 7, and 8 week-old \RonTK\−/− mice as compared to \RonTK+/+ controls.

Fig. 1. The Ron receptor is expressed throughout postnatal mouse mammary gland development, predominantly in the epithelium. (A) Real-time PCR analysis for Ron mRNA expression using RNA isolated from wild-type 5, 6, 8, and 10 week-old virgin, pregnant (timed mating day 16), lactating (day 10), and involuting (days 4 and 10) mouse mammary glands. Involuting mammary glands from \RonTK\−/− mice serve as a negative control with the primer set homologous to sequences spanning the deleted region in the \RonTK\−/− mice. Expression values were normalized to 18S as an internal control and the relative expression level of Ron in each sample is illustrated. (B) Western analysis of Ron expression in wild-type 5, 6, 8, and 10 week-old virgin, pregnant, lactating, and involuting (day 4) mouse mammary glands, 3 week-old wild-type female stromal mammary fat pads devoid of epithelium and lymph node (Fat pad), and normal mouse mammary epithelial cell line Eph4. Parp and Actin serve as loading controls. (C) Real-time PCR analysis of Ron mRNA expression in RNA isolated from normal human mammary epithelial immortalized cell line (HMEC) and normal human primary adipocytes (HPAC) (left). Expression values were normalized to 18S as an internal control and the relative expression level of Ron in each cell line is illustrated. Western analysis of Ron expression in lysates prepared from the normal human mammary epithelial cells MCF10A and HMEC, and normal human primary adipocytes HPAC is depicted on the right. Actin serves as loading control. (D) Mammary gland sections from 6 week-old virgin wild-type mice were subjected to immunohistochemistry with an antibody directed against the Ron receptor tyrosine kinase. Ron positive cells appear brown against the blue hematoxylin counterstain. Ron expression primarily localized to the ductal epithelial cells (a, block arrow) and the terminal end buds (b, block arrow). Staining with an isotype control is shown as a negative control (c and d, block arrows and arrowheads). The lymph node (LN) is indicated for orientation.
In spite of the significant developmental acceleration displayed in the mammary glands from RonTK−/− mice throughout 6–8 weeks of age, ductal extension and branching morphogenesis equalize with the RonTK+/+ mice by 15 weeks of age. Moreover, the RonTK−/− mice are overtly normal with respect to fertility, pregnancy, and lactation.

The Ron receptor regulates mammary branching morphogenesis in ovariectomized mice

Fig. 2. RonTK−/− mice have significantly accelerated pubertal mammary gland development. Mammary glands were isolated from 5, 6, 7, 8, and 10 week-old female RonTK+/+ and RonTK−/− mice and examined by whole mount analysis. (A) Images of representative RonTK−/− and RonTK−/− age and weight-matched mammary glands illustrate the denser ductal network (arrows indicate representative ductal/branch structures) and accelerated epithelial fat pad penetration in RonTK−/− mice observed throughout the developmental time course. The mammary lymph node (LN) is indicated for orientation. Scale bar; 5 mm. The developmental parameters average ductal elongation (B), terminal end bud number (C), and number of secondary (D) and tertiary branch points (E) were quantified as described in the materials and methods and graphed as averages per group. *p<0.05 compared to the corresponding control group.
(compare Figs. 2D and 3B), the ovariectomized mice had significantly fewer secondary branches overall (and correspondingly less ductal morphogenesis) compared to their wild-type counterparts, and the lengths of these secondary branches were significantly blunted. In total, these data suggest that Ron signaling may regulate mammary branching morphogenesis, at least in part, independent of ovarian hormone stimulation.

**RonTK−/− mammary cell turnover is similar to RonTK+/+ controls**

We hypothesized that a potential mechanism by which Ron might regulate mammary gland development is by modulation of mammary epithelial cell homeostasis (i.e. through changes in cell proliferation and/or cell death). While the histological appearance of RonTK−/− (Figs. 4E–F) ductal structures and terminal end buds were similar to those of the RonTK+/+ control glands (Figs. 4A–B), an overall increase in the number of ductal structures in RonTK−/− glands was dramatically apparent (Fig. 4E vs. A). To examine mammary epithelial cell turnover rates, mammary glands from 5, 6, and 7 week-old RonTK+/+ and RonTK−/− mice were analyzed for proliferation by bromodeoxyuridine (BrdU) incorporation and for apoptosis by terminal nick end labeling (TUNEL). Similar percentages of BrdU (Figs. 4C and G) and TUNEL staining (Figs. 4D and H) epithelial cells were detected in the end buds, as well as ducts (data not shown), at all time points analyzed suggesting that Ron ablation does not alter mammary epithelial cell turnover.

Ron signaling in the mammary gland negatively regulates the activation of signaling pathways critical for enhanced branching morphogenesis

Previous evidence has shown that Akt and MAPK are important for mammary branching morphogenesis (Fata et al., 2007; Kumar and Wang, 2002). Given that these are hallmark signaling pathways downstream of the Ron receptor tyrosine kinase, we tested whether the impact of Ron signaling on mammary branching morphogenesis may modulate the regulation of the Akt and/or MAPK signaling. Western analysis of proteins isolated from whole mammary glands from 6 week-old RonTK+/+ and RonTK−/− mice revealed significantly increased phosphorylation of Akt and MAPK proteins in RonTK−/− mammary glands (Fig. 5A). Moreover, immunohistochemical analyses of mammary glands show elevated phosphorylation of MAPK (Fig. 5B) localized predominantly in the mammary epithelium of RonTK−/− mice, as compared with RonTK+/+ controls. Alternatively, immunohistochemical analysis of Akt phosphorylation showed a more diffuse staining pattern in the mammary stromal fat pad and epithelium, with more staining observed overall in RonTK−/− glands as compared to RonTK+/+ controls (data not shown).

Ron tyrosine kinase and MAPK signaling regulate mammary epithelial branching morphogenesis in ex vivo cultures

To test for the importance of Ron signaling emanating from isolated mammary epithelial cells, branching morphogenesis was
inhibition resulted in nearly complete inhibition of branching morphogenesis of RonTK−/− mammary epithelial organoids compared to vehicle treated controls (Fig. 6E). At a dose of 5 μM, PD98059 completely blocked branching in both RonTK+/+ and RonTK−/− organoids, however sphere formation was compromised suggesting toxicity associated at this dose. In a similar set of studies, 5 μM of the Akt inhibitor LY294002 resulted in a modest 10% decrease in the number of RonTK+/+ mammary epithelial organoids with branches and a 15% decrease in RonTK−/− organoids with branches compared to vehicle treated controls. These differences, however, were not statistically significant. Higher concentrations of LY294002 also compromised sphere formation, again suggesting toxicity effects. Taken together, these data suggest that MAPK activity in the RonTK−/− mammary epithelium is critical for branching morphogenesis ex vivo.  

Deletion of the Ron tyrosine kinase domain yields profound effects on gene expression during mammary gland development

To examine the role of Ron during mammary development in more detail, we compared the gene expression profiles of mammary gland mRNA harvested from 5, 6, and 7 week-old RonTK+/+ and RonTK−/− female mice. The mRNA was subjected to microarray using the murine 430 chip from Affymetrix. The hybridization profiles from three

analyzed in primary mammary epithelial organoid cultures purified from virgin female RonTK+/+ and RonTK−/− mice. The isolated organoids were embedded in growth factor reduced Matrigel, cultured for 6 days, and analyzed for branching morphogenesis. No branching morphogenesis was evident on the day of organoid embedding. Fig. 6A shows representative images of the predominant phenotypes of RonTK+/+ and RonTK−/− organoid structures observed which included spheres (Fig. 6A), buds (black arrows), and branches (white arrows). The number of organoids displaying buds and branches out of the total number of organoids present was quantified. As shown graphically in Fig. 6E, significantly more RonTK−/− (Figs. 6B and D) mammary epithelial organoids developed buds and branches (71%) compared to the RonTK+/+ (Figs. 6A and C) organoids (32%), of which the predominant phenotype was sphere formation (Fig. 6A). Given that phosphorylated MAPK was primarily localized to the mammary epithelium of the RonTK−/− mice (Fig. 5B), we hypothesized that this activity was responsible for the exaggerated branching in the mutant animals. To test whether MAPK activity played a role in branching morphogenesis in the RonTK−/− mammary epithelium, RonTK−/− and RonTK+/+ organoids embedded in Matrigel were treated with or without the MAPK inhibitor PD98059 at a final concentration of 2 μM for six days. MAPK

Fig. 4. Histological appearances and rates of proliferation and cell death of RonTK+/+ and RonTK−/− mammary epithelium. 6 week-old RonTK+/+ (A–D) and RonTK−/− (E–H) mammary glands were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Representative sections are shown demonstrating the histological appearance of the mammary ducts (A and E), terminal end buds (B and F). Arrows depict the increased number of ductal structures observed in the RonTK−/− glands (E) compared to controls (A). Cellular proliferation and death were analyzed by immunohistochemical detection of BrdU incorporation in end buds (C and G) and TUNEL staining of the end buds (D and H). The percentage ± S.E.M of BrdU and TUNEL-positive end bud staining (n = 4) is indicated in the upper right hand image corner (C, D, G, and H). Images are all from representative 6 week-old mouse mammary glands. Scale bar; 100 μm.

Fig. 6A shows representative images of the predominant phenotypes of RonTK+/+ and RonTK−/− organoid structures observed which included spheres (Fig. 6A), buds (black arrows), and branches (white arrows). The number of organoids displaying buds and branches out of the total number of organoids present was quantified. As shown graphically in Fig. 6E, significantly more RonTK−/− (Figs. 6B and D) mammary epithelial organoids developed buds and branches (71%) compared to the RonTK+/+ (Figs. 6A and C) organoids (32%), of which the predominant phenotype was sphere formation (Fig. 6A). Given that phosphorylated MAPK was primarily localized to the mammary epithelium of the RonTK−/− mice (Fig. 5B), we hypothesized that this activity was responsible for the exaggerated branching in the mutant animals. To test whether MAPK activity played a role in branching morphogenesis in the RonTK−/− mammary epithelium, RonTK−/− and RonTK+/+ organoids embedded in Matrigel were treated with or without the MAPK inhibitor PD98059 at a final concentration of 2 μM for six days. MAPK

Fig. 5. RonTK−/− mouse mammary glands contain significantly increased phosphorylated Akt and MAPK. (A) Western analyses of proteins isolated from mammary glands using anti-phospho Akt (pAkt), anti-Akt(pan), anti-phospho-MAPK (pMAPK), and anti-total MAPK. Actin served as loading control. Each lane represents an individual mouse. Immunohistochemical analysis of phosphorylated MAPK localization using an anti-phospho-MAPK antibody (B) as illustrated by brown staining in the end buds (top panels) and ducts (bottom panels) of RonTK+/+ and RonTK−/− mammary glands. Scale bar; 100 μm.
To validate the gene expression changes observed in our microarray analyses, real-time quantitative PCR was used. Gene expression changes of Ceacam10, Acpl2, and Pcdh17 in RonTK−/− mammary glands were examined and values were normalized to 18S as loading control and graphed as fold change relative to RonTK+/+ controls (Table 1). Microarray results for these genes suggested an increasing trend in Ceacam10 over time, a 2-fold increase in Pcdh17, and a 60% reduction in Acpl2 transcripts in RonTK−/− mammary glands compared to RonTK+/+. Importantly, our real-time PCR recapitulated the trends observed in gene expression found by microarray analysis for transcripts tested.

Discussion

Pubertal mammary gland development is a complex and tightly controlled process. Many positive regulators of mammary development are known, however, very few negative regulators have been identified (Sternlicht, 2006). We report for the first time evidence supporting an important role for the receptor tyrosine kinase Ron in mammary gland development. Importantly, we showed that Ron is expressed during pubertal mammary development (5–8 weeks). These data correspond with Ron expression analyses published previously (Chodosh et al., 2000). Interestingly, we observed Ron expression in mammary glands during later stages of mammary gland development including pregnancy and lactation, which was not detected previously by Chodosh et al., 2000. This discrepancy may be due to strain-specific differences between the mice, differences in the times at which the tissues were taken, or differences in methods of detection. Immunohistochemistry on mouse mammary glands and Western analysis of human and mouse mammary epithelial cells showed that Ron is expressed in the mammary epithelium. Ron expression was undetectable in mammary fat pads by Western analysis of 3 week-old mouse mammary fat pads devoid of epithelium and in human primary adipocytes. While this data suggests the Ron is expressed predominantly in the mammary epithelium, we cannot preclude, however, that Ron may be expressed in other cell types within the mammary gland during pubertal development.

To test the role of Ron during mammary gland development, RonTK−/− mice with a targeted deletion of the tyrosine kinase domain of Ron were analyzed for mammary growth and morphogenesis compared to wild-type RonTK+/+ mice. We found significantly accelerated ductal elongation and significantly increased branching morphogenesis during pubertal mammary development in RonTK−/− mice compared to age and weight-matched controls. This finding was unexpected given that the majority of receptor tyrosine kinases previously examined in the mammary gland play a positive role in both promoting morphogenesis and breast tumorigenesis, and that overexpression of the Ron receptor also promotes breast tumorigenesis (Lanigan et al., 2007; Niemann et al., 1998; Soriano et al., 1995; Sternlicht, 2006; Wiesen et al., 1999; Xie et al., 1997; Yant et al., 1998; Zinser et al., 2006). However, there is precedent for negative regulation of mammary gland development by a receptor tyrosine kinase, which is also overexpressed in breast cancer (Sternlicht, 2006). The well-studied receptor tyrosine kinase ligand TGFβ3 limits branching morphogenesis during pubertal mouse mammary development, and overexpression of TGFβ3 is one mechanism leading to breast tumor progression (Joseph et al., 1999; Nam et al., 2008; Pollard, 2001).

The classical initiator and positive regulators of mammary gland development are estrogen and progesterone. During the estrus cycle the rise in estrogen stimulates the mammary epithelium to proliferate, and progesterone can stimulate branching. The loss of either signaling pathway results in a severely stunted mammary rudiment (Sternlicht et al., 2006). Based on our discovery that Ron negatively regulates mammary gland development, we hypothesized that RonTK−/− mice may have altered estrogen or progesterone signaling that leads to this phenotype. However, our analysis of circulating serum levels of
estrogen and progesterone showed the contrary and found no differences between RonTK−/− and RonTK+/+ mice. Additionally, mammary glands from RonTK−/− mice ovariectomized at 3 weeks of age displayed significantly increased branching by 6 weeks of age compared to ovariectomized RonTK+/+ controls, which is consistent with the phenotype observed in animals with intact ovaries. Therefore, our data suggests that Ron signaling may regulate mammary branching morphogenesis in the absence of ovarian hormone stimulation. It is possible that the RonTK−/− mammary glands are more sensitive to other factors, such as growth factors, that also contribute to mammary branching morphogenesis, and are therefore less affected by ovariectomy. It is also important to note that while removal of the ovaries severely compromises estrogen and progesterone levels, low amounts of these hormones are produced by other organs and may account for the ductal morphogenesis observed.

An alternative mechanism by which Ron could negatively regulate mammary development is by limiting cell turnover rates of the mammary epithelium. In tumor cells, Ron overexpression has been reported to modulate cell proliferation and apoptosis, however, nothing is known about the influence of Ron on cell turnover in the normal mammary epithelium.

Fig. 7. RonTK−/− mammary glands have significantly altered gene expression patterns compared to RonTK+/+ controls during development. RNA isolated from the mammary glands of 5, 6, and 7 week-old RonTK+/+ and RonTK−/− female mice was run on a Affymetrix Genechip Array. Probe sets were first filtered based on a minimum raw expression value of 120, then RonTK+/+ probes were averaged as a baseline control for comparison. An ANOVA was performed comparing genes altered in RonTK−/− mammary glands to the baseline control. (A) Gene tree order represents 393 genes altered in RonTK−/− mammary glands according to age, where each gene is represented as a single colored line that corresponds to an expression value. Each vertical column represents a single mouse, and each age group contains three individual mice. (B) A Venn diagram was constructed from the gene list used in A to demonstrate genes altered in RonTK−/− mammary glands compared to controls that are unique to 5, 6, or 7 weeks of age, or are shared in common over two or more age groups. Some of the genes shared in common between 5 and 7 weeks of age are Mrpl3, Mid1, and Cdh1; 6 and 7 weeks is Pcdh17; 5, 6, and 7 weeks are Mapk6, Nck1, Acpl1, SrpB, and Synaptotagmin binding. (C) DAVID functional annotation software was used to group genes into biological processes that were significantly altered in RonTK−/− mammary glands compared to RonTK+/+ baseline controls.
We continued our investigation of Ron signaling by assessing Akt and MAPK, two known downstream targets of Ron receptor signaling. Activation of Akt and MAPK has been shown to be essential for branching morphogenesis in numerous organs (Davies, 2002). Phosphorylation of Akt and MAPK were examined in RonTK+/+ and RonTK−/− mammary glands by Western and immunohistochemical analyses. Strikingly, RonTK−/− mammary glands demonstrated increased phosphorylation of both Akt and MAPK, however, while Akt phosphorylation appeared more diffuse throughout the mammary glands, MAPK phosphorylation primarily localized to the epithelium. To examine whether Ron regulation of MAPK activity was required for modulation of branching morphogenesis, RonTK+/− mammary organoids embedded in Matrigel were treated with the MAPK inhibitor PD98059, which yielded a dramatic decrease in the number of organoids with buds and branches. Treatment of RonTK+/+ organoids with the MAPK inhibitor at the same concentration had no significant impact on branching in Matrigel. Although a dose of PD98059 that blocked branching in both RonTK+/+ and RonTK−/− organoids was achieved, there were signs of toxicity associated with lack of proper sphere formation at this concentration. Conversely, treatment of RonTK+/+ and RonTK−/− organoids with the Akt inhibitor LY294002 resulted in a modest reduction in the percent of organoids with branches, but was not statistically significant or dependent on genotype. While Akt signaling does not appear to be an important factor in the differential branching of mammary organoids under our culture conditions, these data do not exclude the potential importance of Akt activity in branching morphogenesis in RonTK+/+ or RonTK−/− mice in vivo, in the context of the other cellular compartments where we also observed Akt phosphorylation by immunohistochemistry. Given that the Ron receptor is well established as an activator of Akt and MAPK activity (Camp et al., 2005; Danilkovitch and Leonard, 1999), we were surprised to observe increased Akt and MAPK phosphorylation in RonTK−/− mammary glands. This result is seemingly contradictory to a previous study wherein loss of Ron tyrosine kinase in the MMTV-polyoma-middle T antigen (PyMT) breast cancer model resulted in reduced Akt and MAPK phosphorylation (Peace et al., 2005) in lysates from Ron-deficient mammary tumors compared to Ron expressing tumors. Our current study, however, differs significantly compared to the tumor biology studies previously reported. First, the studies herein are examining the normal physiologic levels of Ron during mammary gland development versus examining the loss of Ron function during tumorigenesis whereby Ron expression levels are dramatically upregulated in the mammary tumors. Second, the PyMT model itself is dependent on robust Akt activation in mammary tumors directly coupled to polyoma-middle T antigen overexpression and the mechanisms by which Ron may modulate PyMT signaling and de novo mammary gland development (which is not dependent on this viral oncogene) may be different. Third, it is also not clear as to whether increased phosphorylation of Akt and MAPK in RonTK−/− mouse mammary glands is a direct or indirect consequence of the loss of Ron. Interestingly, abrogation of the transmembrane receptor tyrosine kinase IGF-IR in mouse prostate, also increased phosphorylation of Akt and MAPK (Sutherland et al., 2008). Together, these data suggest that receptor tyrosine kinases are able to differentially regulate Akt and MAPK activation in a context specific manner.

Microarray and functional annotation analyses of genes altered in pubertal mammary glands from RonTK−/− mice compared to RonTK+/+ controls revealed that several key cellular processes were significantly altered by deletion of the Ron tyrosine kinase domain including development, transcription, morphogenesis, differentiation, kinase activity, and cell adhesion. It is evident that all these processes are essential during pubertal mammary gland development (Hinck and Silberstein, 2005; Sternlicht, 2006; Sternlicht et al., 2006). Gene families differentially regulated in RonTK−/− mammary glands within these functional categories include Wnt, sprouty, laminin,

Table 1
Real-time PCR validation of mammary gland microarray analysis.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change</th>
<th>Microarray</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccnd10</td>
<td>5 weeks</td>
<td>1.44</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>1.94</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>7 weeks</td>
<td>4.76</td>
<td>2.49</td>
</tr>
<tr>
<td>Aplp2</td>
<td>5 weeks</td>
<td>0.31</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>7 weeks</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>Pcdh17</td>
<td>5 weeks</td>
<td>2.62</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>3.12</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>7 weeks</td>
<td>1.82</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Based on the microarray analyses, RonTK−/− gene raw expression values are shown as fold change relative to the average RonTK+/+ value. For real-time PCR, RonTK−/− relative gene expression values were first normalized to 18S as a loading control, and then expressed as fold change of RonTK−/− expression relative to RonTK+/+ control.

(Wagh et al., 2008). To test this, RonTK+/+ and RonTK−/− mice injected with BrdU were analyzed for BrdU incorporation as a measure of proliferation, and TUNEL staining, as a measure of potential cell death, in mammary gland end buds and ducts. Although mammary glands from RonTK−/− mice harbor significantly more ductal structures than RonTK+/+ controls, there were no significant differences in the percentage of proliferating or apoptotic cells in the ductal and end bud epithelium between RonTK+/+ and RonTK−/− glands. In both genotypes, the end buds displayed the greater amounts of proliferation (approximately 17%) and apoptotic staining (approximately 3%) when compared to ducts as expected (Hinck and Silberstein, 2005). Our finding is consistent with others that have shown that while some cell proliferation is essential in budding epithelium to extend the bud, it may not be necessary for bud formation or branching morphogenesis (Nogawa et al., 1998; Spooner et al., 1989).

To determine whether modification of Ron receptor function in the mammary epithelium is responsible for the observed modulation of branching morphogenesis in vivo, ductal epithelial fragments (organoids) from RonTK+/+ and RonTK−/− mice were purified, and embedded in a three-dimensional Matrigel matrix. After 6 days in culture, the majority, 71%, of RonTK−/− organoids, and only 32% of RonTK+/+ organoids formed buds and branches. This significant increase in RonTK−/− mammary organoid branching suggests that the loss of Ron receptor expression in the mammary epithelium is sufficient to increase branching morphogenesis ex vivo. Our results, however, do not exclude the possibility that loss of Ron in other cellular compartments may contribute to the branching morphogenic phenotype in vivo. In contrast to the in vivo setting where differences in branching morphogenesis early in pubertal development resolve over time between the RonTK+/+ and RonTK−/− glands, the differences between the RonTK+/+ and RonTK−/− organoids cultured in Matrigel do not normalize over extended periods of time (up to three weeks). This finding supports the prospect that other factors may be playing a role to orchestrate branching morphogenesis in vivo. We also investigated the possibility that treatment of RonTK+/+ organoids ex vivo with the Ron ligand, HGFL, would inhibit branching morphogenesis. Interestingly, however, we found that addition of HGFL had no additional effect on branching in culture. This finding could be due to several factors. First, it is not known whether the Matrigel or the mammary epithelial growth supplement (which is derived from pituitary extracts) in which the organoids were grown contain HGFL. In addition, it is not known whether a component of the organoid cultures produces HGFL. There is also a possibility that crosstalk between Ron and other signaling pathways may regulate branching morphogenesis independent of HGFL. Cross-talk with Ron and other receptor tyrosine kinases has been reported by a number of independent groups (Danilkovitch-Miagkova and Leonard, 2001).
protocadherin, and ephrin. We have validated several targets identified by the microarray, which have known functions with broad implications and may potentially play a role in our model, but have not yet been studied with respect to mammary development including the glycoprotein Ceacam10 and acid phosphatase Acpl2. Two genes found by DAVID analysis in the cell adhesion and motility cellular processes important for branching morphogenesis are the tyrosine kinase adapter Nck1 (data not shown) and protocadherin Pcdh17. The overall changes observed in our studies are consistent with other microarray analyses implementing these gene families in mammary gland development (Kouroso-Mehr and Werk, 2006). Together, these microarray data support the conclusion that Ron likely impacts mammary development through a process that is multifactorial.

In summary, we have shown that ablation of Ron receptor tyrosine kinase accelerates pubertal mammary gland development. Markedly, the loss of Ron impacts mammary gland branching morphogenesis independently of ovarian estrogen and progesterone stimulation. Moreover, based on three-dimensional ex vivo analyses, the absence of Ron in the epithelium is sufficient to produce branching. Ron receptor ablation alters Akt and MAPK phosphorylation, which is known to be essential for proper mammary epithelial branching morphogenesis. Finally, deletion of Ron tyrosine kinase profoundly alters the genetic program in the pubertal mammary gland. Taken together, our data demonstrate that Ron is an important regulator of events important to produce branching. Ron receptor tyrosine kinase accelerates pubertal mammary gland development.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.06.028.

References


