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The Ron receptor tyrosine kinase negatively regulates mammary gland branching morphogenesis

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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.

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

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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., 2007; 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 the 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 mRNA 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 Carmine 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 D1× 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, processed, 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 mammary whole mounts, and from images of 6 week-old ovariectomized *RonTK*+/+ and *RonTK*−/− inguinal mammary 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 mm^2 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 involuting glands were isolated 4 and 10 days following pup withdraw. Briefly, frozen glands were homogenized in 1.5× Laemmli Buffer, sonicated, and centrifuged at 12,000 rpm for 15 min. Telomerase immortalized normal human mammary epithelial cells (HMEC) were donated by Dr. Robert Weinberg (MIT, Cambridge, MA) grown in Medium 171 (Cascade Biologics, Portland, OR) plus Mammary Epithelial Growth Supplement (Cascade Biologics, Portland, OR) and antibiotics, then lysed in 1.5× Laemmli Buffer. Human primary adipocytes cells (HPAC) were grown in preadipocyte media (Zen-Bio, Research Triangle Park, NC), and lysed in Laemmli Buffer. Eph4 immortalized normal mouse mammary epithelial cells were grown in DMEM supplemented with 5% FBS and antibiotics, then lysed in Laemmli Buffer. Stromal fat pads were isolated as indicated and lysed in Laemmli Buffer. Protein concentrations were determined using the MicroBCA Kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Rabbit monoclonal anti-phospho-Erk1/2, rabbit polyclonal anti-Erk1,2,3, rabbit polyclonal anti-phospho-Akt, and rabbit monoclonal anti-Akt(pan) were purchased from Cell Signaling Technology (Danvers, MA) and used according to manufacturer's instructions. Anti-Ron β (C-20) rabbit polyclonal antibody was used at a concentration of 0.2 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were detected using peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Antibody detection was performed according to manufacturer's instructions with ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) and developed on film.

Immunohistochemical 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, Burlington, 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 (Epicentre Biotechnologies, Madison, WI) to generate cRNA following the manufacturer's instructions. Biotin-X-X-NHS (Epicentre Biotechnologies, Madison, WI) was used to label the aminoallyl-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 6v.1.1 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 genotype (*RonTK*+/+ and *RonTK*−/−) by a parametric ANOVA assuming equal variance with multiple testing correction Benjamini and Hochberg false discovery rate of $p=0.2$. This resulted in 686 probe sets that differed from the wild-type average raw intensity value, which were then subjected to another parametric ANOVA $p=0.005$ with no multiple testing correction to find differences between genotypes by each age time point (5, 6, and 7 weeks), yielding 114 probes at 5 weeks, 200 probes at 6 weeks, and 106 probes at 7 weeks. A Venn diagram was generated by pooling the genes from individual time points (393 probe sets). Complete raw gene expression data and analyses can be obtained through the Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/geo) accession number GSE16629.

Functional annotation analysis

The 393 gene list generated from the Gene Spring analysis was uploaded onto the DAVID Bioinformatics Resources 2008 National Institute of Allergy and Infections Diseases, NIH website (http://david.abcc.ncifcrf.gov/) and pathway and functional annotation analysis was performed.

Quantitative real-time PCR

RNA was isolated from whole glands, purified epithelial cells, or from glandular areas devoid of epithelial cells (consisting of adipocytes, fibroblasts and associated stromal cells). The RNA was used to generated cDNA using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. To measure *Ron* transcript expression in wild-type, or *RonTK*+/+ mice, whole gland and purified gland components were analyzed using the following mouse *Ron* primers; Forward: 5′-GTC CCA TTG CAG GTC TGT GTA GA-3′ and Reverse: 5′-CGG AAG CTG TAT CGT TGA TGT C-3′. These primers encompass part of the kinase domain deleted in the *RonTK*−/− mice. An additional set of primers were utilized for Ron that would detect a possible truncated product produced upstream of the TK deletion in the *RonTK*−/− glands. For these experiments, the following primers were utilized: Forward: 5′-TGG AGC CAG TGC TGA CAT C-3′ and Reverse: 5′-GAT AGC GTG AAG TGC CAT G-3′. Human *Ron* expression in immortalized Human Mammary Epithelial Cells (HMEC) and immortalized Human Primary Adipocyte Cells (HPAC) the following human *Ron* primers were used; Forward: 5′-GAC CAG GCC CAG AAT CGA AT-3′, Reverse: 5′-CAG GTC ACC GTG GCA CAT ATA G-3′, and Taqman probe: 5′-TGT GCC ATC AAG TCA CTA AGT CGC ATC A -3′. To confirm microarray results the following genes and corresponding sequences were chosen: *Acpl2* (Forward: 5′-CCT TAA ATT CCC TGC CTC TC-3′; Reverse: 5′-GTT GGG CAG AAG TTT GTG T-3′), *Ceacam10* (Forward: 5′-ACT CCG ATT TCT GTG CGA-3′; Reverse: 5′-AAG AAC GTT TTC CCC TTC G-3′), and *Pcdh17* (Forward: 5′-TCG GAT GTC CAT AAT TCA GAC AGA-3′ and Reverse: 5′-CTG CCT GCT GCC CAT GTA AT-3′). Gene expression values were normalized to 18S (Forward: 5′-AGT CCC TGC CCT TTG TAC ACA-3′; Reverse: 5′-GAT CCG AGG GCC TCA CTA AAC-3′) as internal control. Relative gene expression results are reported. Real-time analyses were repeated twice with similar results using samples from 3 individual mice per genotype.

Results

The Ron receptor is expressed in the mouse mammary gland at specific phases of glandular development

Previous studies on intact mammary glands have shown that *Ron* mRNA expression increases progressively during ductal morphogenesis, is down regulated at the onset of pregnancy, and remains low throughout the remainder of postnatal mammary development (Chodosh et al., 2000). Since the ligand for Ron, HGFL, 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 Eph4, 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, 8, 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, measuring 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 as compared to *RonTK*+/+ controls (Fig. 2B). We also saw a trend

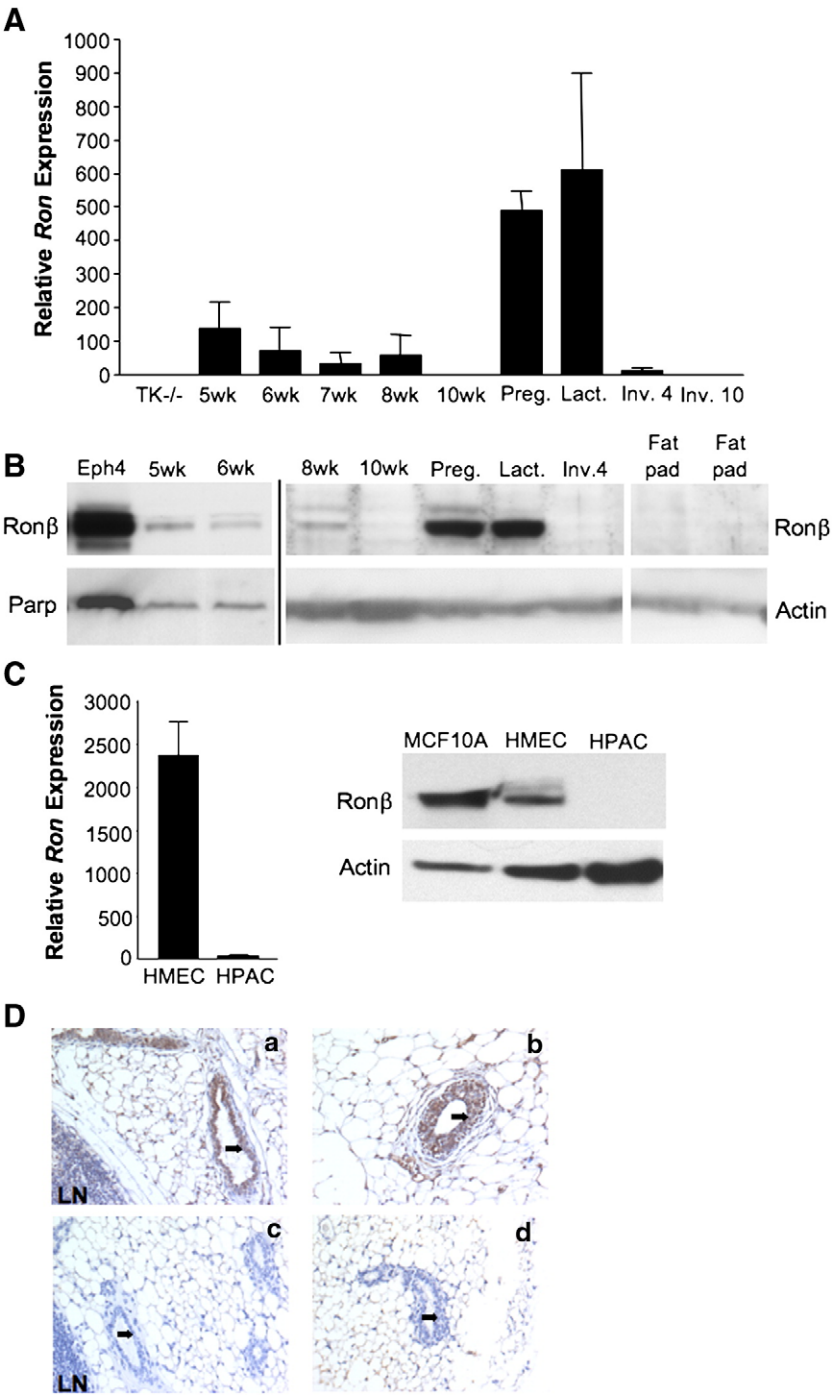

Fig. 1. The Ron receptor is expressed throughout postnatal mouse mammary gland development, predominantly in the epithelium. (A) Real-time PCR analysis for *Ron* mRNA expression using RNA isolated from wild-type 5, 6, 8, and 10 week-old virgin, pregnant (timed mating day 16), lactating (day 10), and 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.

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

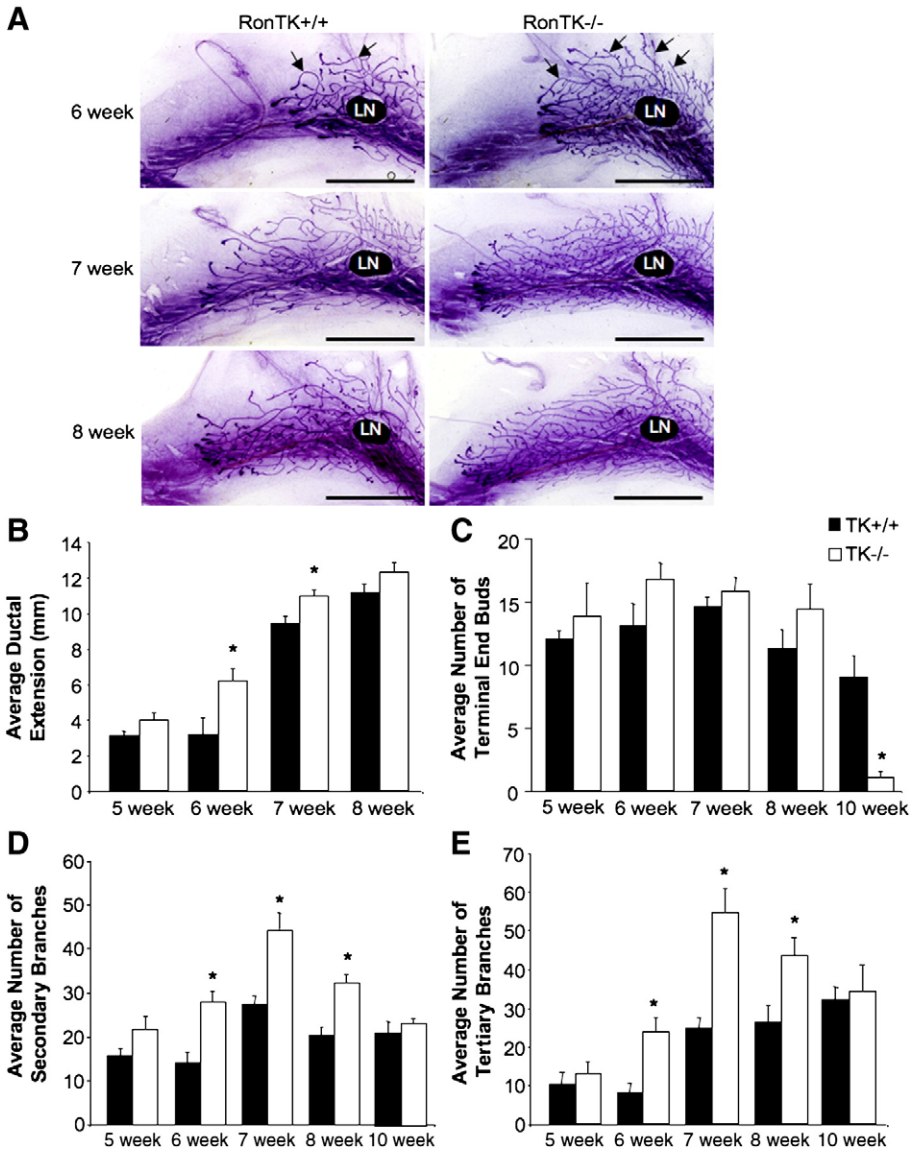

Fig. 2. *RonTK*−/− mice have significantly accelerated pubertal mammary gland development. Mammary glands were isolated from 5, 6, 7, 8, and 10 week-old female *RonTK*+/+ and *RonTK*−/− mice and examined by whole mount analysis. (A) Images of representative *RonTK*+/+ and *RonTK*−/− age and weight-matched 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.

(Figs. 2D and E). 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

Estrogen, a nuclear hormone, responsible for mammary epithelial growth and ductal elongation, and progesterone, also a nuclear hormone, predominately responsible for ductal side branching are both essential for normal pubertal mammary gland development (Feng et al., 2007; Humphreys et al., 1997; Korach et al., 1996). To test if the *RonTK*−/− mice may have accelerated branching morphogenesis due to an alteration in serum hormone levels, we analyzed serum that was isolated from 5, 6, and 7 week-old virgin female *RonTK*+/+ and *RonTK*−/− mice and found no significant differences in circulating estrogen or progesterone levels (Fig. 3A). To further examine the role of Ron in branching morphogenesis in the absence of circulating hormones, *RonTK*+/+ and *RonTK*−/− mice were ovariectomized at 3 weeks of age prior to pubertal onset. Following ovariectomy the mammary glands were allowed to develop for three weeks prior to isolation at 6 weeks of age and analyzed for ductal elongation and secondary and tertiary branch events. Examination of ovariectomized mice at the time of sacrifice confirmed that all ovaries were removed from the mice in this study. Interestingly, mammary glands from ovariectomized *RonTK*−/− mice demonstrated significantly increased secondary and tertiary branching events compared to mammary glands from ovariectomized *RonTK*+/+ controls (Fig. 3B and C). Of note, all end buds in the ovariectomized mice regardless of genotype were considerably smaller than in mice with intact ovaries. Although ovariectomized *RonTK*+/+ mice appear to have similar average numbers of secondary branches as *RonTK*+/+ mice with intact ovaries

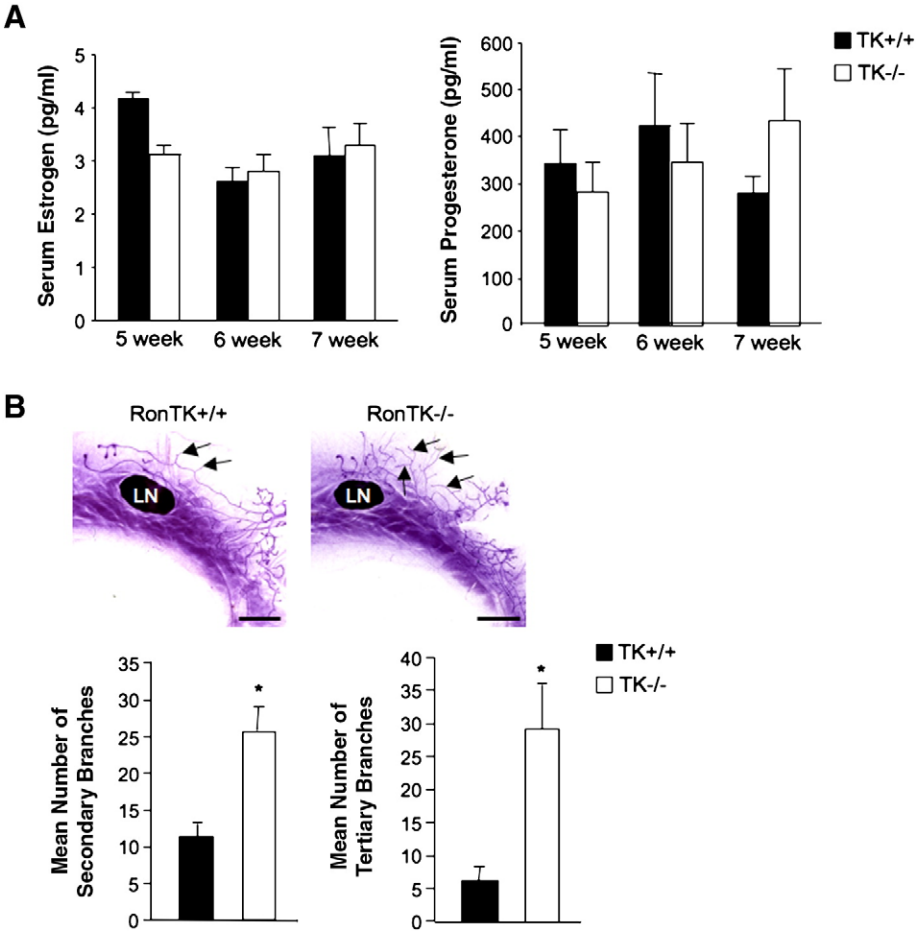

Fig. 3. *RonTK*−/− mammary glands display significantly increased branching after ovariectomization. Average circulating estrogen and progesterone levels were measured in serum isolated from 5, 6, and 7 week-old *RonTK*+/+ and *RonTK*−/− mice (A). (B) *RonTK*+/+ and *RonTK*−/− mice were ovariectomized at 3 weeks of age and sacrificed at 6 weeks of age, at which point whole mounts were generated from their mammary glands and representative images are shown (top). The mammary lymph node (LN) is indicated for orientation. Arrows point to representative branches observed. Scale bar; 2 mm. The average number of secondary and tertiary branch points per genotype was quantified and graphed (C). *$p<0.05$ 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

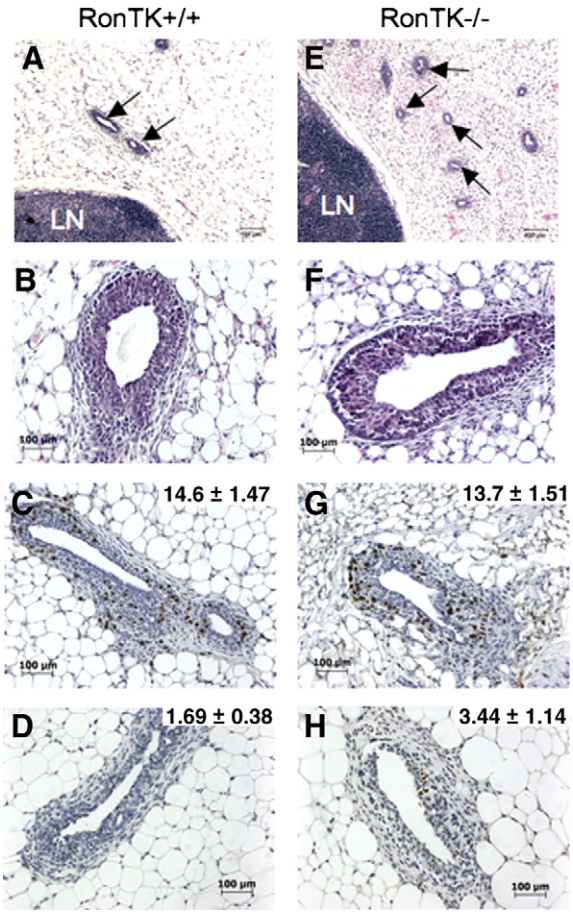

Fig. 4. Histological appearances and rates of proliferation and cell death of *RonTK*+/+ and *RonTK*−/− mammary epithelium. 6 week-old *RonTK*+/+ (A–D) and *RonTK*−/− (E–H) mammary glands were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Representative sections are shown demonstrating 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.

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 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

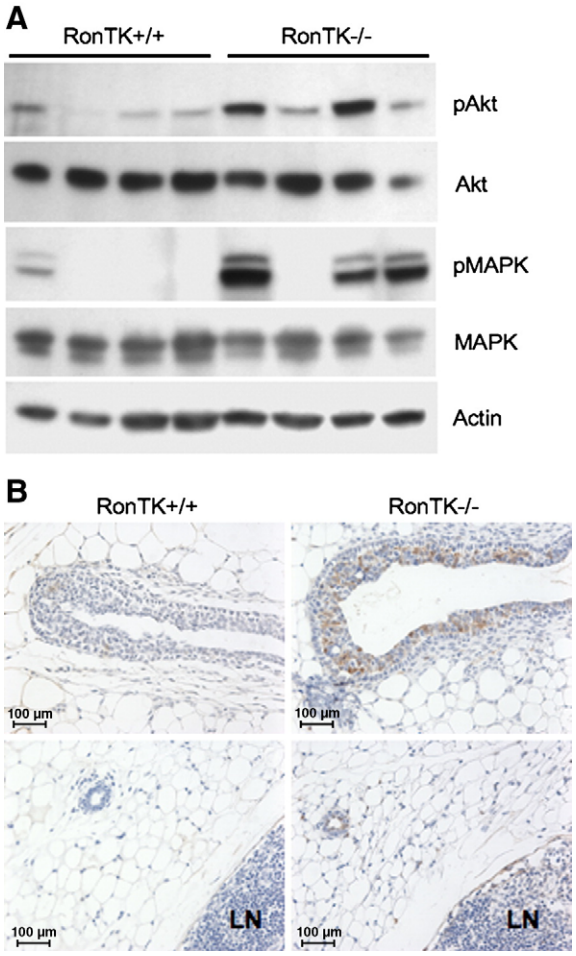

Fig. 5. *RonTK*−/− mouse mammary glands contain significantly increased phosphorylated Akt and MAPK. (A) Western analyses of proteins isolated from mammary glands using anti-phospho Akt (pAkt), anti-Akt(pan), anti-phospho-MAPK (pMAPK), and anti-total MAPK. Actin served as loading control. Each lane represents 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.

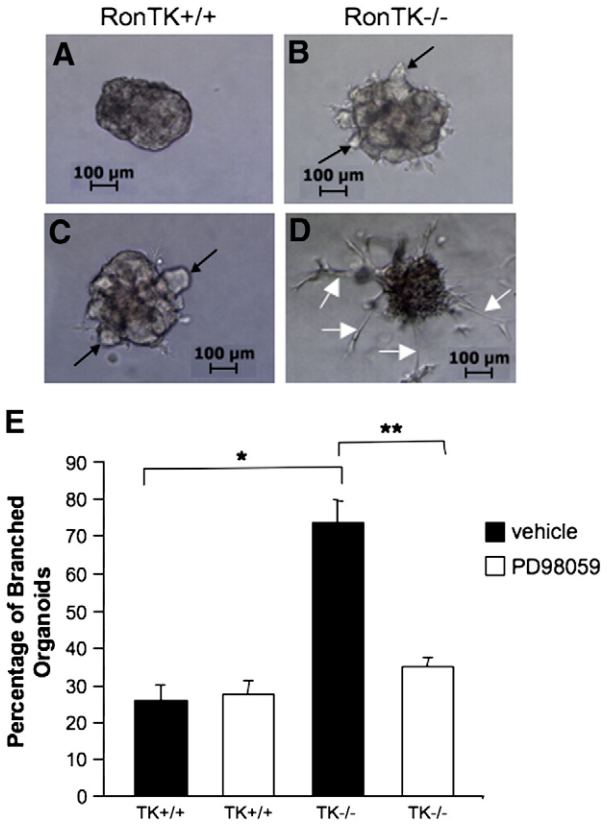

Fig. 6. *RonTK*−/− primary mammary epithelial organoids display significantly advanced branching in vitro that is reduced upon MAPK inhibition. Primary mammary epithelial cells were purified from female *RonTK*+/+ (A and C) and *RonTK*−/− (B and D) mice and cultured in Matrigel for 6 days. (A–D) Representative images of organoids cultured in growth medium described in Materials and methods with no protrusions, buds (black arrows), or branches (white arrows). Images represent the extent of branching exhibited in each genotype. Scale bar; 100 μm. (E) *RonTK*+/+ and *RonTK*−/− organoids were cultured in Matrigel for 6 days treated with 2 μM PD98059 or equivalent volume vehicle control. The number of organoids with branches were quantified in multiple independent experiments, averaged, and depicted as percentage of branched organoids. At least 260 organoids were quantified per group.

independent *RonTK*+/+ mice per time point were averaged as baseline controls for comparison to *RonTK*−/− experimental animals. ANOVA analyses identified 393 genes that were significantly differentially expressed in *RonTK*−/− mammary glands compared to *RonTK*+/+ control mice over 5, 6, and 7 weeks of age are illustrated in a heat map (Fig. 7A), and are depicted temporally in a Venn diagram in Fig. 7B. A complete list of the genes in the heat map and Venn diagram can be found in the Supplementary Tables S1 and S2–S4, respectively. These analyses have provided several types of information. First, a number of genes that change during each developmental window based on Ron expression were identified. Interestingly, the majority of genes (188 genes) are differentially expressed solely at the 6-week time point (Supplemental Table S3). Second, genes that overlap in any two of the three time points were found (Supplemental Table S5). Only 7 genes fell into this category including *Mrpl3*, *Mid1*, *Cdkal1*, *Pcdh17*, and three other unstudied sequences. Finally, 10 genes were differentially expressed following Ron ablation at all time points including *Tmem30a*, *Mapk6*, *Nck1*, *Acpl2*, *Srprb*, and other unstudied sequences (Supplemental Table S5).

Using DAVID functional annotation analyses on the 686 genotypically differentially expressed genes, a number of biological processes were significantly altered including morphogenesis, cell motility, adhesion, and development (Fig. 7C, Supplemental Tables S6–S12). These results demonstrate that Ron tyrosine kinase receptor signaling significantly affects that genetic profile of the developing mammary gland.

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 TGFβ limits branching morphogenesis during pubertal mouse mammary development, and overexpression of TGFβ 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

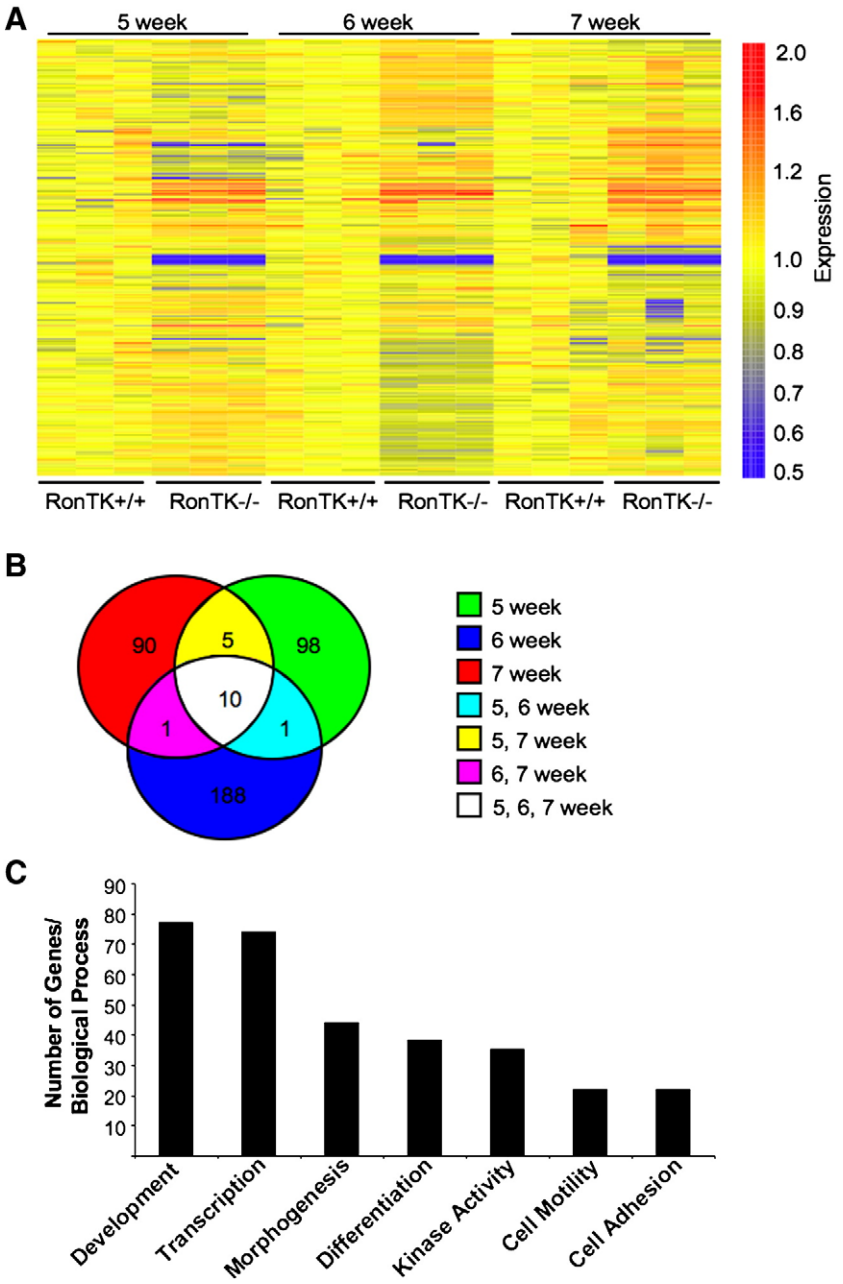

Fig. 7. *RonTK*−/− mammary glands have significantly altered gene expression patterns compared to *RonTK*+/+ controls during development. RNA isolated from the mammary glands of 5, 6, and 7 week-old *RonTK*+/+ and *RonTK*−/− female mice was run on a Affymetrix Genechip Array. Probe sets were first filtered 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 *Cdkal1*; 6 and 7 weeks is *Pcdh17*; 5, 6, and 7 weeks are *Mapk6*, *Nck1*, *Acpl1*, *Srprb*, 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.

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, such that deletion of the tyrosine kinase domain of Ron would increase these rates and accelerate glandular development. 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

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

Genes	Fold change	
	Microarray	Real-time PCR
Ceacam10		
5 weeks	1.44	1.08
6 weeks	1.94	2.08
7 weeks	4.76	2.49
Acpl2		
5 weeks	0.31	0.26
6 weeks	0.29	0.33
7 weeks	0.36	0.30
Pcdh17		
5 weeks	2.62	2.32
6 weeks	3.12	2.72
7 weeks	1.82	1.71

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 overtime 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).

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,

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 (Kouros-Mehr and Werb, 2006). Together, these microarray data support the conclusion that Ron likely impacts mammary development through a process that is multifactoral.

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 pubertal mammary gland development.

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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.

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