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Vitamin D_3 receptor ablation alters mammary gland morphogenesis

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SUMMARY

Postnatal mammary gland morphogenesis is achieved through coordination of signaling networks in both the epithelial and stromal cells of the developing gland. While the major proliferative hormones driving pubertal mammary gland development are estrogen and progesterone, studies in transgenic and knockout mice have successfully identified other steroid and peptide hormones that impact on mammary gland development. The vitamin D_3 receptor (VDR), whose ligand 1,25-dihydroxyvitamin D_3 is the biologically active form of vitamin D_3, has been implicated in control of differentiation, cell cycle and apoptosis of mammary cells in culture, but little is known about the physiological relevance of the vitamin D_3 endocrine system in the developing gland. In these studies, we report the expression of the VDR in epithelial cells of the terminal end bud and subtending ducts, in stromal cells and in a subset of lymphocytes within the lymph node. In the terminal end bud, a distinct gradient of VDR expression is observed, with weak VDR staining in proliferative populations and strong VDR staining in differentiated populations. The role of the VDR in ductal morphogenesis was examined in *Vdr* knockout mice fed high dietary Ca^{2+} which normalizes fertility, serum estrogen and neonatal growth. Our results indicate that mammary glands from virgin *Vdr* knockout mice are heavier and exhibit enhanced growth, as evidenced by higher numbers of terminal end buds, greater ductal outgrowth and enhanced secondary branch points, compared with glands from age- and weight-matched wild-type mice. In addition, glands from *Vdr* knockout mice exhibit enhanced growth in response to exogenous estrogen and progesterone, both in vivo and in organ culture, compared with glands from wild-type mice. Our data provide the first in vivo evidence that 1,25-dihydroxyvitamin D_3 and the VDR impact on ductal elongation and branching morphogenesis during pubertal development of the mammary gland. Collectively, these results suggest that the vitamin D_3 signaling pathway participates in negative growth regulation of the mammary gland.

Key words: Vitamin D receptor, Vitamin D receptor knockout mouse, Mammary gland development, Terminal end bud, 1,25-dihydroxyvitamin D_3

INTRODUCTION

Development of the mammary gland predominantly occurs postnatally, when the gland undergoes extensive ductal elongation and branching during the pubertal period. The process of mammary gland morphogenesis reflects a complex interaction between signaling networks in both the epithelial and stromal compartments of the developing gland. The major proliferative hormones driving pubertal mammary gland development are estrogen, which stimulates ductal elongation, and progesterone, which mediates branching (Silberstein, 2001). Over the last few years, studies in transgenic and knockout mouse models have identified additional steroid hormones, peptide growth factors and receptors that impact on mammary gland development, including transforming growth factor β (TGFβ), parathyroid hormone-related peptide (PTHRP), and the epidermal growth factor (EGF) family of receptors (Hennighausen, 2000; Dunbar and Wysolmerski, 2001).

Another nuclear steroid hormone receptor expressed in mammary gland is the vitamin D_3 receptor (VDR), whose ligand 1,25-dihydroxyvitamin D_3 is the biologically active form of vitamin D_3. 1,25-dihydroxyvitamin D_3 has been shown to modulate differentiation, cell cycle, and apoptosis of stromal and epithelial cells derived from mammary gland and breast cancers (Lefebvre et al., 1995; Simboli-Campbell et al., 1996; Kanazawa et al., 1999; Narvaez and Welsh, 2001). Furthermore, mammary glands from vitamin D_3-deficient mice exhibit impaired Ca^{2+} transport and casein production, supporting a functional role for the vitamin D_3 signaling pathway in lactation (Bhattacharjee et al., 1987; Mezzetti et al., 1988). Using mouse mammary gland organ culture, Mehta et al. (Mehta et al., 1997a) demonstrated that 1,25-dihydroxyvitamin D_3 inhibited the development of DMBA-induced pre-neoplastic lesions. These and other data suggest that VDR signaling may modulate mammary gland development, function or sensitivity to carcinogenesis. To begin to address the role of vitamin D_3 in mammary gland development and carcinogenesis, mammary gland morphogenesis was monitored in *Vdr* knockout mice and their wild-type counterparts using both in vivo and organ culture approaches. Our data indicate that mammary glands that lack

VDR exhibit enhanced ductal extension and branching during puberty in vivo and in response to growth-promoting hormones in vitro, suggesting that the vitamin D_3 signaling pathway participates in negative growth regulation of the mammary gland.

MATERIALS AND METHODS

Animal maintenance

A breeding colony of *Vdr* knockout mice was established from three mice generously provided by Dr Marie Demay (Harvard Medical School, Boston, MA). The phenotype of these mice, generated by targeted ablation of the second zinc finger of the DNA-binding domain of the VDR, closely resembles that of human vitamin D-dependent rickets type II (VDRRII), a genetic disorder characterized by non functional VDR (Li et al., 1997). Mice were genotyped by PCR amplification of DNA isolated from ear punches using primers directed against exon 3, which codes for the second zinc-finger region of the VDR and the neomycin gene, which replaces exon 3 in the *Vdr* knockout animal. To alleviate disturbances in mineral homeostasis and hormonal imbalances (Li et al., 1998), all VDR knockout and wild-type mice were fed a diet containing 2% Ca^{2+}, 1.25% phosphorous and 20% lactose with 2.2 IU vitamin D_3/g (TD96348, Teklad, Madison, WI).

Assessment of mammary gland pubertal development

Eight to 16 weight-matched female mice of each genotype (wild type and knockout) were sacrificed at 4, 5, 6, 7, 8 and 10 weeks of age for analysis of mammary gland development. Blood was removed by cardiac puncture for measurement of serum estradiol using a radioimmunoassay kit from Diasorin (Stillwater, MN). To assess whether the presence of the VDR exerted a gross effect on mammary gland development, wet weights of surgically removed mammary fat pads were obtained at sacrifice from 10-12 animals per genotype between 6 and 10 weeks of age for calculation of organ weight to body weight ratio (expressed as mg/g). One thoracic and one inguinal gland from each mouse were prepared as wholemounts, while the contralateral glands were paraffin embedded. For whole-mount analysis, mammary glands were fixed in Carnoy's fixative and stained overnight in Carmine Alum. Samples were dehydrated, cleared in xylene, mounted and examined on an Olympus SZX12 stereoscope. The extent of ductal outgrowth was measured on inguinal wholemounts as the distance from the center of the lymph node to the leading edge of the ductal mass. The degree of branching was assessed by counting the numbers of primary, secondary and tertiary branch points on inguinal wholemounts. Primary branches were defined as ducts that arose in the nipple region and extended to the leading edge of the gland, secondary branches as ducts that extended from primary ducts and tertiary branches as lateral branches that arose from secondary ducts. Terminal end buds (bulb-like structures with area greater than 0.03 mm^2) were counted manually on inguinal wholemounts.

VDR expression, histology, apoptosis and proliferation

Formalin-fixed mammary glands were embedded in paraffin, sectioned at 5 μM, and stained with Hematoxylin and Eosin Y for routine histological assessment. To detect VDR, formalin-fixed paraffin wax-embedded sections were incubated in 2 N HCl at 37°C for 20 minutes. After rinsing in phosphate-buffered saline (PBS) for 5 minutes, slides were incubated overnight with a rat monoclonal antibody directed against VDR (clone 9A7, Neomarkers, Fremont, CA) at a dilution of 1:60, followed by incubation with anti-rat secondary antibody at a dilution of 1:200. Slides were counterstained with Harris modified Hematoxylin.

Mitotic and apoptotic indices were assessed in the leading edge terminal end buds by quantitative morphometric analysis of bromodeoxyuridine (BrdU) incorporation and in situ terminal transferase mediated dUTP nick end labeling (TUNEL), which are established markers of proliferation and apoptosis. For mitotic index, mice were injected with BrdU 2 hours before sacrifice, and BrdU was localized in sections with a mouse monoclonal biotinylated anti-BrdU (Zymed Laboratories, San Francisco, CA) and the ABC technique followed by diaminobenzidine (Sigma, St Louis, MO). DNA fragmentation was assessed by TUNEL assay using a commercially available kit (Roche Diagnostics, Indianapolis, IN) after optimization of pre-treatment conditions and reagent dilutions to ensure that labeling was restricted to cells with apoptotic morphology. For both assays, tissues were counterstained with Hematoxylin (Harris modified, Fisher Scientific, Pittsburgh, PA).

BrdU incorporation and TUNEL were quantitated in terminal end buds on tissue sections viewed with an Olympus AX70 microscope (40× objective lens) and photographed with the Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI). A minimum of six animals were assessed per genotype, with three to five sections/animal and two to five terminal end buds/section. The photographs were analyzed with image analysis software (Zeiss KS 300) and the percentage of cells that stained positive for BrdU or TUNEL was calculated.

Mammary gland organ culture

To prime glands for adaptation to organ culture, 3-week-old mice were implanted with 21-day release subcutaneous 17 β-estradiol (21.0 μg) and progesterone (21.0 mg) pellets (Innovative Research, Sarasota, FL). Confirming previous reports (Singh et al., 1970), our preliminary studies indicated that a minimum of 15 days was required for optimal adaptation of C57Bl mammary glands to culture. For this reason, wild-type and *Vdr* knockout mice were pretreated for 15-17 days with estrogen and progesterone prior to removal of both inguinal and thoracic glands under sterile conditions. Glands were floated on Dacron rafts in Waymouth's media (Life Technologies, Gaithersburg, MD) and supplemented with 17 β-estradiol (1 ng/ml), progesterone (1 μg/ml), insulin (5 μg/ml), aldosterone (1 μg/ml), hydorcortisone (1 μg/ml) and prolactin (5 μg/ml) as described by Mehta et al. (Mehta et al., 1997b). Glands were cultured in a controlled atmosphere chamber (Bellco Glass, Vineland, NJ) gassed with 95% O_2/5% CO_2 at 37° C for up to 14 days in the presence or absence of 100 nM 1,25-dihydroxyvitamin D_3 (a generous gift from Leo Pharmaceuticals, Ballerup, Denmark). After culture, glands were stained, whole mounted and photographed as described for the pubertal time course studies.

Statistical evaluation

Data are presented as mean±s.e.m., with the number of analyses for each mean indicated. Data were analyzed by Student's *t*-test, and means were considered significantly different if $P<0.05$. All statistical evaluations were performed with Instat software (GraphPad Software, San Diego CA, http://www.graphpad.com).

RESULTS

High Ca^{2+} intake normalizes fertility and growth of Vdr knockout mice

Previous data have indicated that *Vdr* knockout mice fail to thrive because of hypocalcemia and impaired skeletal formation (Li et al., 1997). To determine whether administration of supplemental calcium through dietary means could support normal growth and reproduction in the *Vdr* knockout mice, homozygous wild-type (+/+) and *Vdr* knockout (–/–) mice were weaned onto a high Ca^{2+} diet containing

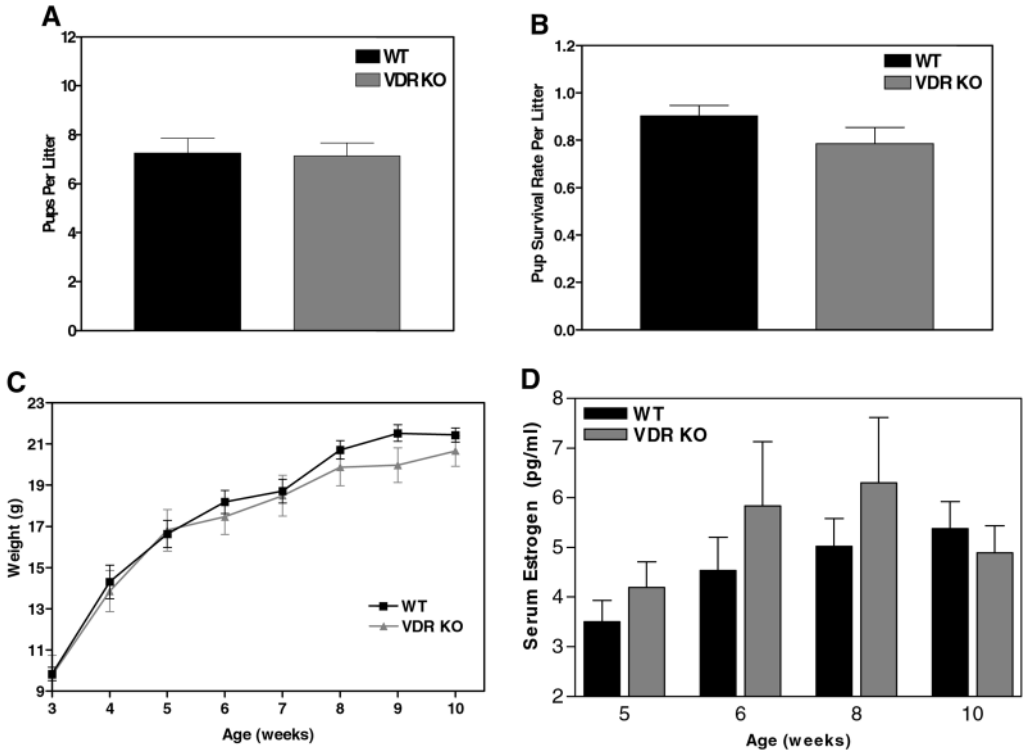

Fig. 1. Fertility and growth of wild-type and *Vdr* knockout mice. Homozygous wild-type and *Vdr* knockout mice maintained on a high Ca^{2+} diet were mated at 8 weeks of age. (A-C) Litter size (A), pup survival to weaning (B) and growth after weaning (C) were monitored for both male and female offspring. (D) Serum estradiol was measured by radioimmunoassay in virgin females. Results are mean±s.e.m. (*n*=7-8); no statistically significant differences were observed between the genotypes.

lactose, which facilitates vitamin D_3-independent Ca^{2+} absorption. Breeding pairs of wild-type and *Vdr* knockout mice were continuously maintained on this diet, and litter size and pup survival were monitored. Data from 24 litters indicated that on the high Ca^{2+} diet, both wild-type and *Vdr* knockout females had litters containing approximately seven pups (95% confidence interval, wild type 6.0-8.5; *Vdr* knockout 6.1-8.2), and no significant effect of genotype was detected (Fig. 1A). Pup survival to weaning was close to 90% for both wild-type and *Vdr* knockout mothers, again with no significant differences due to genotype (Fig. 1B). The body weights of mice through puberty were also similar for wild-type and *Vdr* knockout mice fed the high Ca^{2+} diet (Fig. 1C). Although previous studies have suggested that *Vdr* knockout mice exhibit impaired estrogen production and uterine hypoplasia (Kinuta et al., 2000), we found that VDR ablation in the setting of high dietary Ca^{2+} did not significantly alter circulating estradiol during the pubertal period (Fig. 1D). These data indicate that VDR ablation does not adversely affect reproduction or peri-natal survival, as long as sufficient highly absorbable Ca^{2+} is supplied.

Vdr knockout females display enhanced mammary gland ductal morphogenesis

To assess mammary gland ductal development post-weaning, wholemounts of both inguinal and thoracic mammary glands were prepared from virgin wild-type and *Vdr* knockout mice sacrificed weekly from four to 10 weeks of age. Although the gross morphology of glands from prepubertal mice (up to 4 weeks of age) was similar in wild-type and *Vdr* knockout mice (not shown), differences in glandular development were detected in the pubertal period (5-7 weeks of age). Representative wholemounts of inguinal mammary glands

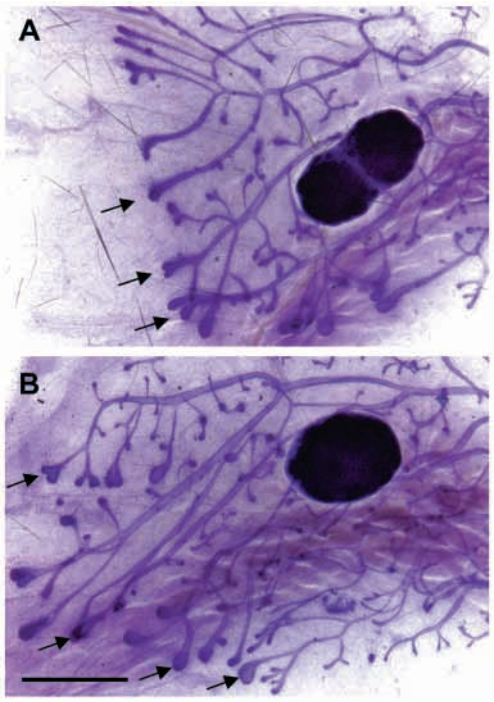

Fig. 2. Representative wholemounts of mammary glands from wild-type and *Vdr* knockout mice. (A,B) Inguinal mammary glands from 6-week-old virgin female wild-type (A) and *Vdr* knockout (B) mice maintained on the high Ca^{2+} diet were fixed in Carnoy's and stained with Carmine Alum to visualize ductal development. Arrows indicate terminal end buds. Note the greater ductal extension and density and more numerous end buds in the gland from the *Vdr* knockout mouse compared with the wild-type mouse. The darkly stained round object within the ducts is the inguinal lymph node. Scale bar: 2 mm.

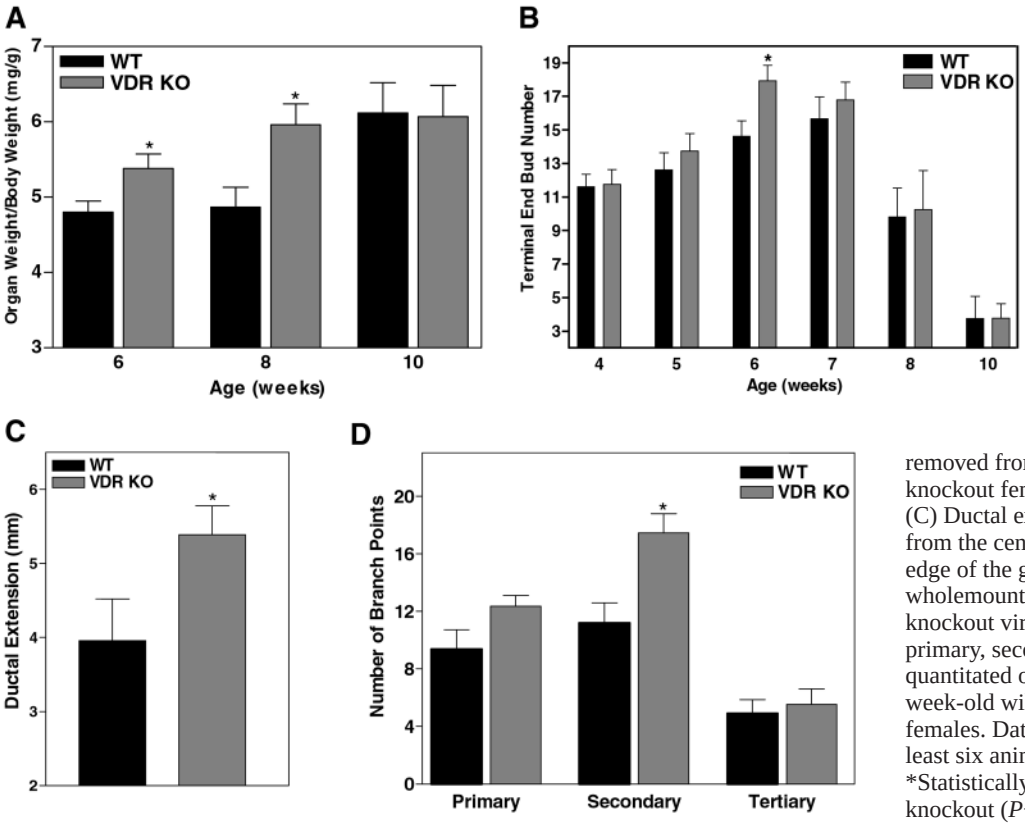

Fig. 3. Quantitative analysis of mammary gland morphogenesis in virgin wild-type and *Vdr* knockout mice. (A) Fresh weights of surgically dissected mammary gland fat pads from virgin wild-type and *Vdr* knockout mice (mean±s.e.m., *n*=10-12 per group), expressed as organ weight to body weight ratio (mg/g). (B) The number of terminal end buds per mammary gland was counted in wholemounts of inguinal glands removed from weight-matched wild-type and *Vdr* knockout females from 4-10 weeks of age. (C) Ductal extension (defined as the distance from the center of the lymph node to the leading edge of the gland) was measured on inguinal wholemounts from 6-week-old wild-type and *Vdr* knockout virgin females. (D) The number of primary, secondary and tertiary branch points was quantitated on inguinal wholemounts from 6-week-old wild-type and *Vdr* knockout virgin females. Data in B-D represent mean±s.e.m. of at least six animals per genotype per time point. *Statistically significant, wild-type versus *Vdr* knockout (P<0.05).

from 6-week-old mice are shown in Fig. 2. In comparison with glands from wild-type mice, ductal morphogenesis was accelerated in glands from *Vdr* knockout mice, as evidenced by more extensive ductal elongation and branching in the absence of the VDR. Similar changes were apparent in thoracic glands (not shown). To assess whether alterations in ductal development in *Vdr* knockout mice were sufficient to alter overall size of the mammary gland, wet weights of surgically removed inguinal glands were measured and expressed as a function of body weight. As shown in Fig. 3A, the organ weight:body weight ratio of glands from *Vdr* knockout mice was significantly higher than that of glands from wild-type mice at 6 and 8 weeks of age, but not at 10 weeks of age. The developmental progress of the mammary gland was quantitatively assessed in inguinal gland wholemounts. The number of terminal end buds (defined as bulb-like structures with an area greater than 0.03 m^2) was counted manually for a minimum of eight mice of each genotype over the pubertal developmental time course. As presented in Fig. 3B, the number of terminal end buds increased rapidly between 4 and 6 weeks of age in both wild-type and *Vdr* knockout mice. The peak number of terminal end buds, achieved at 6-7 weeks of age, was significantly higher in glands from *Vdr* knockout (17.9±0.9, *n*=17) mice than in glands from wild-type mice (14.6±0.9, *n*=16) (P<0.05). After 6-7 weeks of age, the number of terminal end buds declined in both genotypes, as expected, as these transient structures undergo differentiation into terminal end ducts by 10 weeks after birth (Silberstein, 2001). The decrease in terminal end bud number with age was not different in wild-type and *Vdr* knockout mice, suggesting that conversion of terminal end buds into terminal end ducts occurs normally in the absence of the VDR.

Further analysis of ductal morphogenesis was conducted on inguinal glands obtained from 6-week-old wild-type and *Vdr* knockout mice, as this was the time point at which the difference in terminal end bud number was noted. As pubertal growth of the mammary gland involves progressive ductal outgrowth towards the periphery of the mammary fat pad (Silberstein, 2001), we measured the length of the ductal network from the center of the lymph node to the leading edge in inguinal glands from 6-week-old wild-type and *Vdr* knockout mice. As shown in Fig. 3C, the ductal outgrowth of glands from *Vdr* knockout mice was significantly higher than that of glands from wild-type mice. To determine whether VDR signaling impacted on ductal branching, we counted the number of primary, secondary and tertiary branch points in wild-type and *Vdr* knockout mice. An increase in the number of secondary branch points was detected in 6-week-old *Vdr* knockout mice, but the number of primary and tertiary branch points was not affected by VDR ablation (Fig. 3D).

Hematoxylin and Eosin staining was used to compare the histomorphology of mammary glands from wild-type and *Vdr* knockout mice. Representative sections from 6-week-old mice showing terminal end buds are presented in Fig. 4. Other than the larger ductal mass and increased branching in glands from *Vdr* knockout mice, no abnormalities in tissue architecture were noted in the absence of the VDR. No obvious differences were detected between wild-type and VDR mammary gland sections stained with Masson's trichrome, which highlights connective tissue (not shown).

VDR is widely expressed in developing mammary gland

Immunohistochemistry with a monoclonal antibody directed against the VDR was used to localize the receptor protein in the mammary gland of wild-type mice. Specific staining for the VDR was found to be temporally and spatially distinct. VDR staining was present in the nuclei of epithelial cells (in both terminal end buds and ducts), of stromal cells surrounding the ducts and of lymphocytes in the lymph node. Consistent with the known nuclear localization of the VDR, no cytoplasmic staining was seen. Representative VDR staining of the inguinal mammary gland from a 6-week-old virgin female wild-type mouse are presented in Fig. 5. In Fig. 5A, VDR staining in a typical terminal end bud is shown. VDR expression was detected in all of the major cell populations of the end bud, including the epithelial cells, cap cells and stromal cells. Although cells positive for VDR were observed throughout the end bud, VDR expression was consistently low in the cells of the proliferative zone at the leading edge (Fig. 5B). Cap cells, which exhibit the highest rate of cell division of any cells in the mammary gland, were infrequently positive for VDR (Fig. 5B, arrowheads). Underneath the cap cell layer, both the intensity and number of VDR-positive epithelial cells (Fig. 5B, arrows) were lower than that detected in the trailing edge of the end bud (Fig. 5C, arrows), where the majority of epithelial cells were strongly positive for VDR. Cells in the dense stromal layer surrounding the end bud (Fig. 5C, arrowheads) were mostly positive for VDR, but the intensity of staining was much lower than that detected in the adjacent epithelial cell layer.

VDR expression in the luminal epithelial cells of mature ducts was examined over the pubertal time course (Fig. 6). The number of positive cells and the intensity of VDR staining was highest in luminal epithelial cells of ducts from young (5- to 7-week-old) mice (Fig. 6A-C). In older (8- to 10-week-old) mice, when the pubertal phase of glandular development was largely completed, VDR expression was downregulated in ductal epithelial cells, and the majority of cells were negative for VDR (Fig. 6D,E). VDR expression was also detected in a subset of cells within the lymph node (Fig. 6F), in stromal cells and in adipocytes, but at low levels that did not change with age.

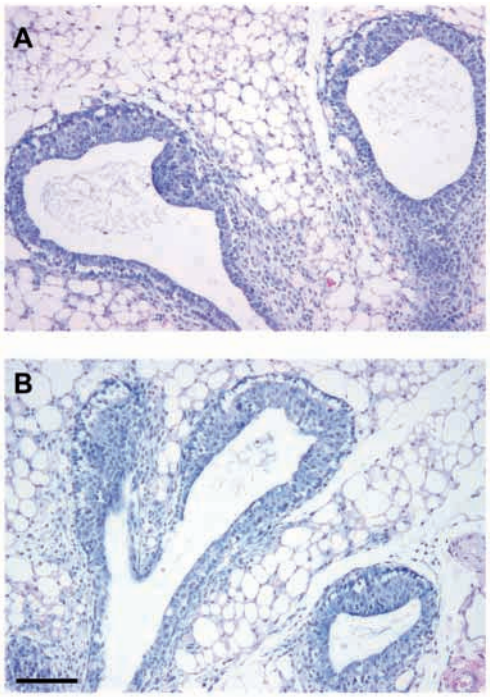

Fig. 4. Morphology of mammary glands from wild-type and *Vdr* knockout mice. Formalin fixed inguinal mammary glands from 6-week-old virgin wild-type (A) and *Vdr* knockout (B) mice were sectioned and stained with Hematoxylin and Eosin. Longitudinal sections through two terminal end buds are shown in each panel. The terminal end bud in B is undergoing bifurcation, which will result in a branch point. Scale bar: 100 μm.

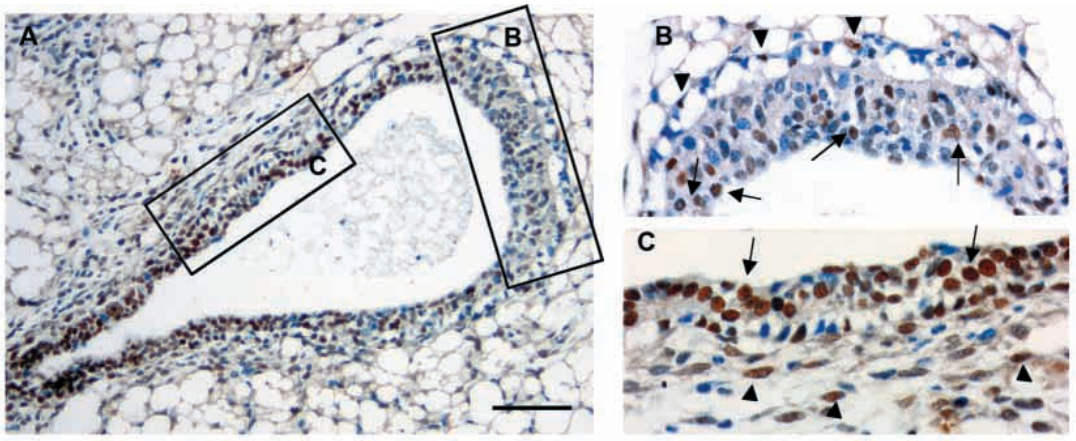

Fig. 5. Expression of the VDR in mammary gland terminal end bud of wild-type mice. Formalin fixed sections of mammary gland from a 6-week-old virgin wild-type mouse were subjected to immunohistochemistry with a monoclonal antibody directed against VDR. VDR positive cells appear brown against the blue Hematoxylin counterstain. (A) VDR was localized to the nuclei of both stromal and epithelial cells in the terminal end bud. VDR expression was low in the leading edge (box B) compared to the trailing edge (box C). (B) Higher magnification of boxed area B (leading edge of terminal end bud) from A, showing weak VDR staining in highly proliferative cap cells (arrowheads) and epithelial body cells (arrows). (C) Higher magnification of boxed area C (trailing edge of terminal end bud) from A, showing strong nuclear VDR positivity in the luminal and myo-epithelial cells (arrows) and weaker but detectable staining in stromal cells (arrowheads). Scale bar, 50 μm.

Enhanced morphogenesis in *Vdr* knockout mice is not associated with deregulated cell proliferation or apoptosis

As 1,25-dihydroxyvitamin D_3 has been implicated in the control of cell cycle and apoptosis, we quantitated mitotic and apoptotic indices, using BrdU incorporation and TUNEL, in terminal end buds of inguinal mammary glands from 6-week-old wild-type and *Vdr* knockout mice. In glands from both wild-type and *Vdr* knockout animals, DNA synthesis (BrdU incorporation) was prominent in outer body cells and cap cells of the terminal end bud (Fig. 7A,B), and no quantitative differences in mitotic index (% of BrdU labeled cells) were detected between the two genotypes (wild-type: 25.7±2.0, n=8; *Vdr* knockout: 25.5±2.0, n=6). These values are consistent with those reported by Humphreys et al. (Humphreys et al., 1996) for BrdU labeling in murine terminal end buds. Apoptotic cells, as identified by both morphology and TUNEL positivity, were frequently detected in the body cells of the terminal end buds of both wild-type and *Vdr* knockout mice (Fig. 7C,D), and no quantitative differences were detected when multiple sections were assessed. Collectively, these data indicate that VDR ablation does not result in deregulation of either cell proliferation or cell death in the terminal end bud.

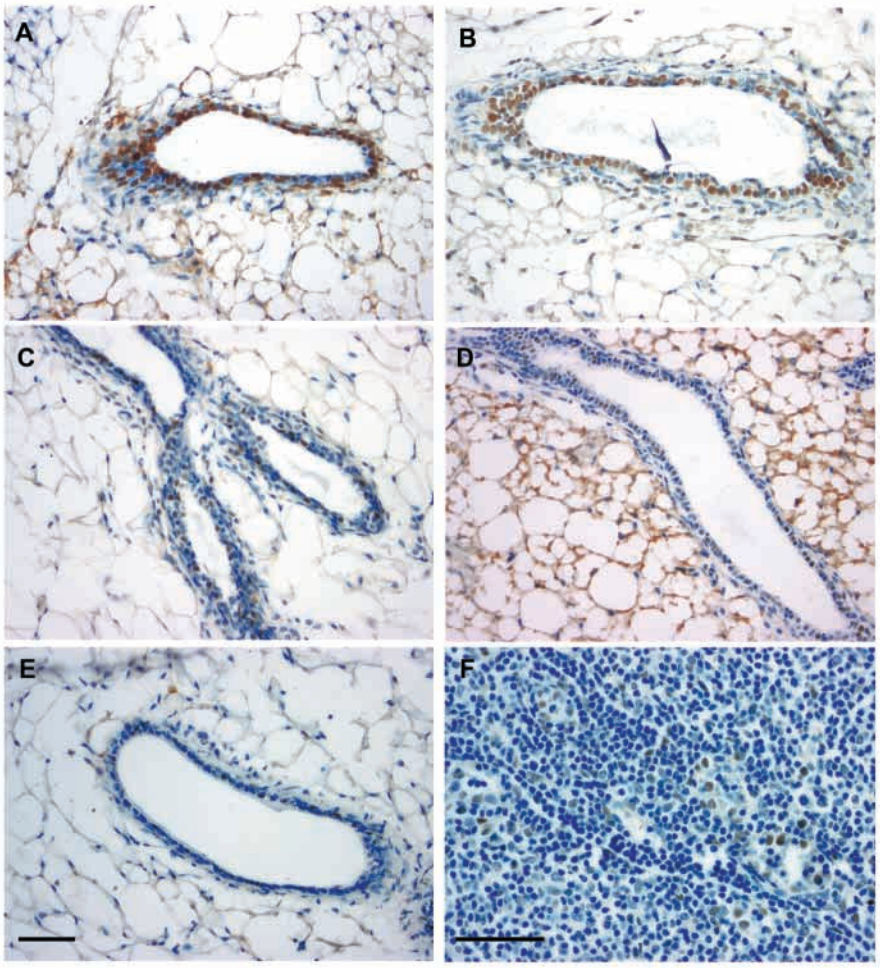

Fig. 6. Expression of the VDR in mature ducts and lymph node of wild-type mice. Formalin fixed sections of mammary glands (A-E) and lymph node (F) from virgin wild-type mice were subjected to immunohistochemistry with a monoclonal antibody directed against VDR. VDR-positive cells appear brown against the blue Hematoxylin counterstain. The number of cells positive for nuclear VDR staining was highest in ducts of mice examined at 5 (A) and 6 (B) weeks of age. Both the number of VDR-positive cells and the intensity of VDR staining declined in mice at 7 (C), 8 (D) and 10 (E) weeks of age. VDR expression was also detected in a subset of lymphocytes within the central lymph node (F), but this did not change with age. Scale bars: 50 μm.

VDR ablation enhances mammary gland responsiveness to exogenous hormones

As 1,25-dihydroxyvitamin D_3 has been shown to down regulate estrogen and progesterone receptors in vitro (Stoica et al., 1999; Swami et al., 2000), we hypothesized that a potential role of the VDR in mammary gland is to oppose estrogen and progesterone signaling. If so, then VDR ablation would be expected to enhance the sensitivity of the gland to estrogen and progesterone, hormones that drive ductal outgrowth and branching (Atwood et al., 2000). To test this hypothesis, we examined mammary glands from wild-type and *Vdr* knockout mice before and after exposure to estrogen and progesterone both in vivo and in organ culture. As demonstrated in Fig. 8A,B, wholemounts of glands from unsupplemented 5-week-old mice were immature, with little evidence of side branching and no consistent differences caused by genotype. After 15 days of estrogen and progesterone supplementation (administered via subcutaneously implanted pellets), 5-week-old thoracic and inguinal gland pairs were removed, and one gland was examined by wholemount analysis while the contralateral gland was placed in serum free organ culture and exposed to lactogenic hormones for an additional 10 days. Immediately upon removal, glands from hormone-primed *Vdr* knockout mice consistently exhibited more extensive branching than glands from wild-type mice (Fig. 8C,D). When placed in organ culture, glands from hormone-primed wild-type and *Vdr* knockout mice experienced further growth and development of alveolar buds (Fig. 8E,F, compare with the contralateral glands not placed in culture shown in Fig. 8C,D). After 10 days in culture, glands from *Vdr* knockout mice consistently displayed enhanced ductal morphogenesis and lobuloalveolar development relative to glands from wild-type mice.

Finally, we tested whether mammary glands from estrogen- and progesterone-primed wild-type and *Vdr* knockout mice differentially responded to 1,25-dihydroxyvitamin D_3, the ligand for the VDR, in vitro. After 15 days of estrogen and progesterone supplementation, thoracic and inguinal gland pairs from wild-type and *Vdr* knockout mice were placed in serum-free organ culture with lactogenic hormones. For each genotype, one gland of each pair was exposed to ethanol

vehicle, while the contralateral gland was exposed to 100 nM 1,25-dihydroxyvitamin D_3. After 14 days of culture in the absence of 1,25-dihydroxyvitamin D_3, glands from wild-type (Fig. 9A) and *Vdr* knockout (Fig. 9B) mice displayed even more extensive lateral branching and alveolar bud development than that observed after 10 days in culture (Fig. 8E,F), and differences between genotypes were minimized. However, in the contralateral glands from wild-type mice exposed to 1,25-dihydroxyvitamin D_3, side branching and alveolar bud development was markedly suppressed (Fig.9C, arrows), supporting the concept that VDR activation suppresses hormone-induced signaling pathways. Exposure of glands from *Vdr* knockout mice to 1,25-dihydroxyvitamin D_3 did not alter morphology or branching (Fig. 9D), ruling out the possibility that the observed effects of 1,25-dihydroxyvitamin D_3 on mammary gland morphogenesis in wild-type mice are mediated through alternative receptors or non-genomic mechanisms. Collectively, these in vitro data provide evidence that 1,25-dihydroxyvitamin D_3, through the VDR, directly impacts on mammary gland development.

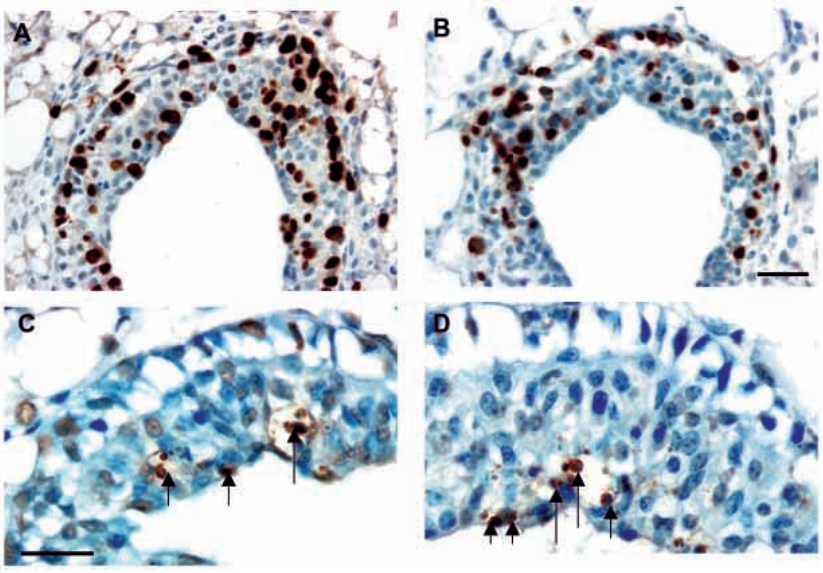

Fig. 7. DNA synthesis and apoptosis in terminal end buds of wild-type and *Vdr* knockout mice. Immunohistochemistry was performed on mammary gland sections from 6-week-old wild-type (A,C) and *Vdr* knockout (B,D) mice to detect BrdU incorporation (A,B) and TUNEL (C,D). For both BrdU and TUNEL, positive cells appear brown against the blue Hematoxylin counterstain. Arrows in C,D indicate TUNEL-positive cells with characteristic apoptotic morphology. No differences in either proliferation or apoptosis were noted to be due to genotype. Scale bars: 50 μm.

DISCUSSION

In this study, we have used mice with a targeted ablation of the VDR to provide evidence of a role for this nuclear receptor and its ligand, 1,25-dihydroxyvitamin D_3, in mammary gland morphogenesis. Our major finding is that mammary glands from virgin *Vdr* knockout females display enhanced ductal morphogenesis

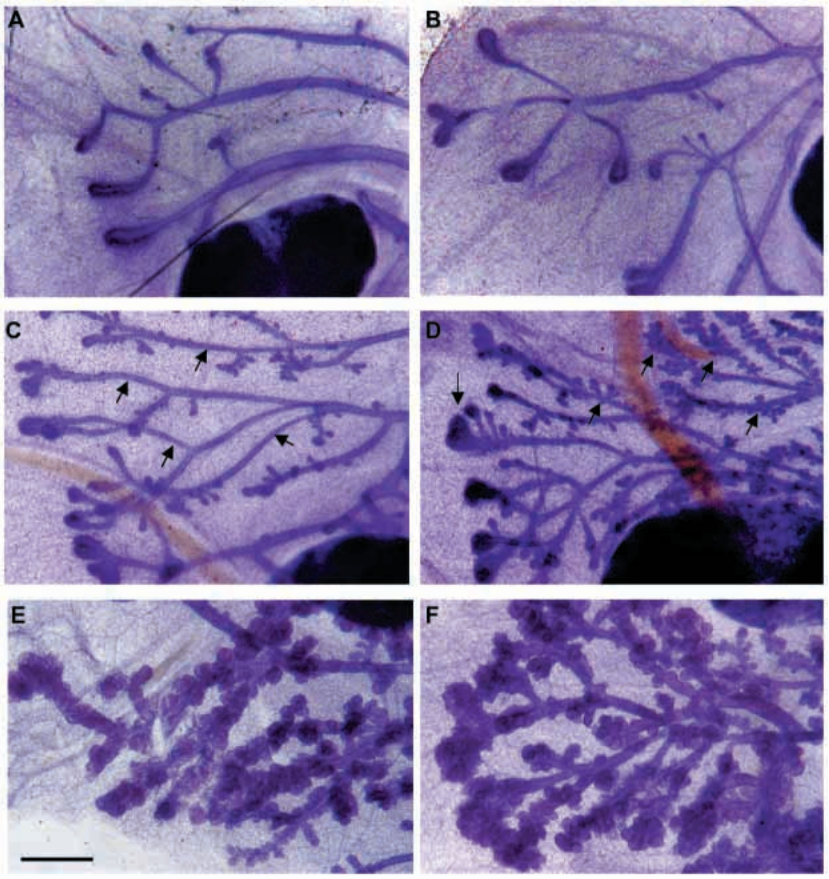

Fig. 8. Response of mammary glands from wild-type and *Vdr* knockout to exogenous hormones. Representative wholemounts of mammary glands removed from wild-type (A,C,E) and *Vdr* knockout (B,D,F) mice before and after estrogen plus progesterone supplementation. Wholemounts shown in A (wild-type) and B (*Vdr* knockout) are representative of glands removed from 5-week-old animals that were not supplemented with estrogen and progesterone. Wholemounts shown in C (wild-type) and D (*Vdr* knockout) represent glands removed at 5 weeks of age after 15 days in vivo supplementation with estrogen and progesterone via subcutaneous pellets. Glands shown in E (wild-type) and F (*Vdr* knockout) are the contralateral glands to those shown in C (wild-type) and D (*Vdr* knockout), which were removed after 15 days in vivo supplementation with estrogen and progesterone and placed in organ culture in the presence of estrogen, progesterone and lactogenic hormones for 10 additional days. Arrows indicate alveolar growth and side branching in D and undeveloped ducts in C. Images are representative of 10 glands per genotype examined. Scale bar: 500 μm.

compared with age- and weight-matched wild-type mice, suggesting that the VDR participates in a growth inhibitory signaling network during mammary gland pubertal development. Specifically, glands from *Vdr* knockout mice are significantly heavier and display increased branching, greater ductal extension and higher numbers of undifferentiated terminal end bud structures compared with glands from wild-type animals. It is well accepted that the terminal end buds are the targets for chemical carcinogens in the mammary gland, and that the number of highly proliferative undifferentiated cells is positively correlated with sensitivity to transformation (Russo and Russo, 1998). Thus, the increased terminal end bud number in the glands of *Vdr* knockout mice may translate to enhanced susceptibility to chemically induced mammary tumorigenesis. This concept is consistent with previous reports that 1,25-dihydroxyvitamin D_3 inhibits DMBA induced pre-neoplastic lesions in normal murine mammary glands (Mehta et al., 1997a), and that vitamin D_3 analogs can prevent carcinogen induced breast cancer in rats (Anzano et al., 1994).

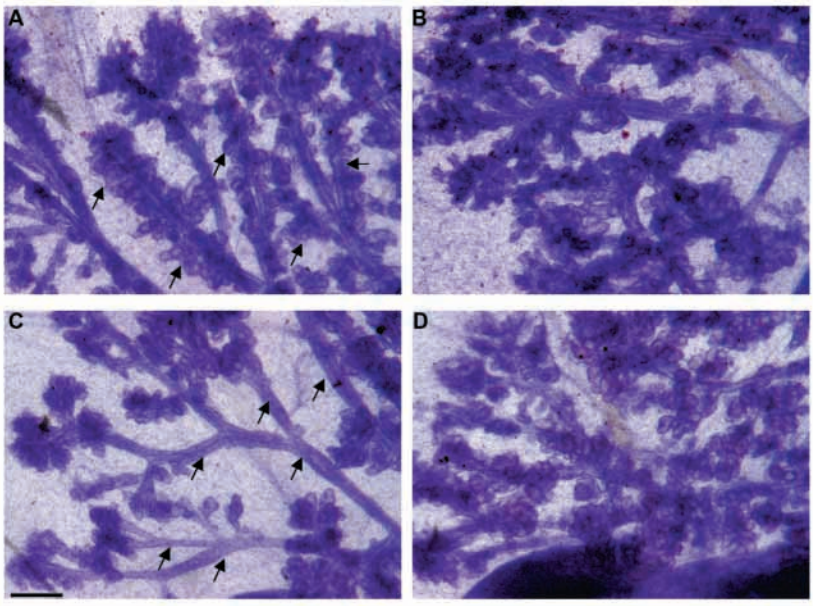

Fig. 9. Effect of 1,25-dihydroxyvitamin D_3 on in vitro growth of mammary glands from wild-type and *Vdr* knockout mice. Inguinal mammary gland pairs were removed from wild-type (A,C) and *Vdr* knockout (B,D) mice pre-treated with estrogen and progesterone for 15 days and placed in organ culture with estrogen, progesterone and lactogenic hormones for 14 days. One gland from each pair was exposed to ethanol vehicle (A,B), while the contralateral gland was exposed to 100 nM 1,25-dihydroxyvitamin D_3 (C,D). Glands were removed from culture, stained and wholemounted after 14 days in vitro growth. Arrows indicate alveolar growth and side branching in A and undeveloped ducts in C. Images are representative of 10 gland pairs per genotype examined. Scale bar: 300 μm.

In conjunction with assessment of mammary gland development, our studies provide the first report of VDR expression and localization during pubertal mammary gland development. In wild-type mice, the VDR protein is localized to the terminal end bud, the subtending ducts and the stromal compartment. Specific cell types positive for VDR included the body and cap cells of the terminal end bud, and the luminal and myoepithelial cells of the ducts. Of potential significance with respect to the observed effects of VDR ablation, VDR expression is not temporally or spatially uniform in the mammary gland. In ductal epithelial cells, VDR expression is highest in the early pubertal period and decreases as the gland matures. In the terminal end bud, the highly proliferative cells at the leading edge express low levels of VDR, whereas the more differentiated epithelial cells in the trailing edge are strongly positive for VDR. The lower level of VDR expression in epithelial cells at the leading edge of the terminal end bud may indicate that 1,25-dihydroxyvitamin D_3 is not a crucial regulator of cell turnover in this region, which is consistent with the lack of effect of VDR ablation on proliferation and apoptosis in the terminal end bud. The strong expression of the VDR at the trailing edge of the bud, where cells differentiate into ductal and myoepithelial cells (Humphreys et al., 1996; Richert et al., 2000) suggests that VDR is upregulated during mammary epithelial cell differentiation, a finding that is consistent with recent studies of VDR expression in relation to differentiation in skin (Zineb et al., 1998) and intestine (Kallay et al., 2001). Localization of the VDR in the trailing edge is also compatible with a role for 1,25-dihydroxyvitamin D_3 in regulation of side branching, which is driven by estrogen and progesterone. Progesterone receptor in particular, which is downregulated by 1,25-dihydroxyvitamin D_3 in breast cancer cells, has been localized to early branch points of developing ducts (Atwood et al., 2000).

Although systemic effects of VDR ablation may have impacted mammary gland morphogenesis, the presence of the VDR in mammary cells from wild-type animals suggests that the enhanced morphogenesis of the mammary gland in *Vdr* knockout mouse is intrinsic to the gland. This suggestion is supported by our organ culture studies which demonstrated that 1,25-dihydroxyvitamin D_3 suppresses side branching and alveolar outgrowth of glands from wild-type mice exposed to lactogenic hormones but has no effect on glands from *Vdr* knockout mice. Our data are consistent with reports that 1,25-dihydroxyvitamin D_3 directly inhibits DMBA induced pre-neoplastic lesions in mouse mammary gland organ culture (Mehta et al., 1997a). Further studies will be necessary to determine if 1,25-dihydroxyvitamin D_3 mediates its effects predominantly through VDR present in the epithelial or stromal cells.

Despite our finding of altered mammary gland morphogenesis during puberty and in response to lactogenic hormones, circulating estradiol is not altered in Ca^{2+}-supplemented *Vdr* knockout mice during the pubertal growth period. Furthermore, *Vdr* knockout female mice are fertile and are capable of supporting litters of the same size as wild-type mice, as long as Ca^{2+} homeostasis is maintained by the high Ca^{2+} diet. These findings contrast with earlier reports of reduced estrogen production, uterine hypoplasia and impaired reproduction in a distinct *Vdr* knockout mouse model created

by targeted disruption of exon 2, rather than exon 3, of the *Vdr* gene (Kinuta et al., 2000). However, our data are consistent with a more recent study that demonstrated normal fertility of this distinct *Vdr* knockout mouse strain when maintained on a high Ca^{2+} diet (Johnson and DeLuca, 2001). Thus, although differences in the severity of the phenotype may exist between these two *Vdr* knockout mouse models, our data confirm that reproductive capacity is maintained in the absence of the VDR. Preliminary examination of mammary gland whole mounts from pregnant and lactating animals indicated no impairment in lobuloalveolar development of the *Vdr* knockout mice used in this study. Specifically, none of the mammary gland defects known to be associated with estrogen or progesterone deficiency (Silberstein, 2001; Dunbar and Wysolmerski, 2001) were noted in the *Vdr* knockout mice at any time, including during pregnancy and lactation. However, based on earlier reports of reduced synthesis of casein and α-lactalbumin in vitamin D_3-deficient rodents (Bhattacharjee et al., 1987; Mezzetti et al., 1988), it remains possible that there are subtle differences in the expression of genes for milk proteins between wild-type and *Vdr* knockout mice.

Since the VDR functions as a ligand-dependent transcription factor, an important future goal will be to identify the specific downstream targets that are altered in the mammary gland of VDR knockout mice. 1,25-dihydroxyvitamin D_3 is known to exert anti-proliferative and pro-apoptotic effects via inhibition of signaling by mitogenic factors (such as 17β-estradiol, IGF1 and EGF) and via induction of negative growth regulators such as TGFβ (Yang et al., 2001). Interactions between the vitamin D_3 and estrogen signaling pathways have been well studied in breast cancer cells, where 1,25-dihydroxyvitamin D_3 transcriptionally downregulates human estrogen receptor α expression through a negative vitamin D_3 response element in the promoter region (Stoica et al., 1999), leading to reduction in downstream gene expression, including that of the progesterone receptor (Swami et al., 2000). These findings suggest the intriguing possibility that the estrogen and/or progesterone signaling pathways may be upregulated in the mammary gland of the VDR knockout mouse, a suggestion that is consistent with our observation that hormone-stimulated growth is enhanced in the absence of the VDR. Upregulation of progesterone signaling is also consistent with our observation of enhanced branching in the *Vdr* knockout mouse, as the progesterone receptor has been preferentially implicated in promotion of cellular proliferation leading to side-branching (Shyamala et al., 1998; Atwood et al., 2000). An alternative, and not mutually exclusive, possibility is downregulation of TGFβ signaling in the *Vdr* knockout mice, as 1,25-dihydroxyvitamin D_3 up regulates TGFβ1 in mouse mammary organ cultures (Mehta et al., 1997a) and a vitamin D_3 response element has been identified in the human TGFβ2 promoter (Wu et al., 1999). Most notably, TGFβ is known to inhibit ductal growth (Dunbar and Wysolmerski, 2001), and mammary glands from mice harboring dominant-negative TGFβ RII exhibit increased epithelial branching (Joseph et al., 1999). The *Vdr* knockout mouse represents a valuable tool to examine these and other potential downstream effectors of vitamin D_3 signaling in the mammary gland.

In summary, these studies have defined the cellular localization of VDR in mouse mammary gland and have used the *Vdr* knockout mouse to demonstrate a role for the VDR in suppressing ductal elongation and branching morphogenesis during pubertal development. Our data provide the first in vivo evidence that 1,25-dihydroxyvitamin D_3 and the VDR impact on mammary gland development.

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