Loss of vitamin D receptor signaling from the mammary epithelium or adipose tissue alters pubertal glandular development

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Johnson AL, Zinser GM, Waltz SE. Loss of vitamin D receptor signaling from the mammary epithelium or adipose tissue alters pubertal glandular development. Am J Physiol Endocrinol Metab 307: E674–E685, 2014. First published August 19, 2014; doi:10.1152/ajpendo.00200.2014.—Vitamin D3 receptor (VDR) signaling within the mammary gland regulates various postnatal stages of glandular development, including puberty, pregnancy, involution, and tumorigenesis. Previous studies have shown that vitamin D3 treatment induces cell-autonomous growth inhibition and differentiation of mammary epithelial cells in culture. Furthermore, mammary adipose tissue serves as a depot for vitamin D3 storage, and both epithelial cells and adipocytes are capable of bioactivating vitamin D3. Despite the pervasiveness of VDR in mammary tissue, individual contributions of epithelial cells and adipocytes, as well as the VDR-regulated cross-talk between these two cell types during pubertal mammary development, have yet to be investigated. To assess the cell-type specific effect of VDR signaling during pubertal mammary development, novel mouse models with mammary epithelial- or adipocyte-specific loss of VDR were generated. Interestingly, loss of VDR in either cellular compartment accelerated ductal morphogenesis with increased epithelial cell proliferation and decreased apoptosis within terminal end buds. Conversely, VDR signaling specifically in the mammary epithelium modulated hormone-induced alveolar growth, as ablation of VDR in this cell type resulted in precocious alveolar development. In examining cellular cross-talk ex vivo, we show that ligand-dependent VDR signaling in adipocytes significantly inhibits mammary epithelial cell growth in part through the vitamin D3-dependent production of the cytokine IL-6. Collectively, these studies delineate independent roles for vitamin D3-dependent VDR signaling in mammary adipocytes and epithelial cells in controlling pubertal mammary gland development.

vitamin D receptor; mammary gland development; mammary adipocyte; mammary epithelium; cell cross-talk; paracrine signaling

A NETWORK OF INTEGRATED SIGNALING MECHANISMS involving numerous cell types, including those within the mammary stroma and epithelium, govern postnatal mammary gland development. In rodents, the mammary stroma is composed largely of adipocytes and preadipocytes as well as fibroblasts, endothelial cells, and immune cells (33). In addition to direct stimulation of epithelial cell growth, hormone- and growth factor-induced signaling mechanisms in stromal cells support the formation and function of surrounding epithelial ducts, which invade and eventually fill the mammary fat pad through abundant forward-driving proliferation and extensive branching morphogenesis (25, 38, 39, 45). A large body of previous work has shed light upon the endocrine-stimulated expansion of the epithelium during pubertal mammary gland morphogenesis. This body of work includes the growth-promoting actions of estrogen and progesterone (43), growth hormone (29, 48), epidermal growth factor (9), insulin-like growth factor I (IGF-I) (23, 26), hepatocyte growth factor, and neuregulin (51). Negative regulators of mammary development, including transforming growth factor-β1 (TGF-β1), are also crucial to glandular formation at puberty (13, 44, 51). Many of these proteins mediate mammary morphogenesis through stromal-epithelial cross-talk, targeting receptors on a subset of cell types that initiate autocrine and/or paracrine signaling that modulates epithelial cell growth.

The vitamin D receptor (VDR) is a nuclear receptor whose activation is largely ligand dependent and is involved in several regulatory processes of mammary gland development, including ductal outgrowth, branching morphogenesis, and response to ovarian hormones (55). Vitamin D3 as the active ligand 1,25-dihydroxyvitamin D3 (1,25D3) has been shown to regulate various cellular functions, including proliferation, apoptosis, and differentiation (50). Bioactivation of 25-hydroxyvitamin D3 (25OHD3), an inactive form that is the most prevalent circulating form of vitamin D3, to 1,25D3 requires hydroxylase activation by the mitochondrial enzyme CYP27B1, which is highly expressed in the kidneys, where it regulates systemic concentrations of 1,25D3. Because of the broad expression of CYP27B1 in various extrarenal tissues (19), it is widely accepted that extrarenal bioactivation of vitamin D3 can occur locally within tissues, including the mammary gland (42, 47). The VDR is expressed in numerous cell types within the mammary gland, including epithelial cells and stromal cells such as adipocytes, preadipocytes, fibroblasts, and lymphocytes (8, 55). VDR plays an important role in regulating pubertal mammary gland development, as VDR-knockout mice exhibit an increase in the number of terminal end buds (TEB) and accelerated ductal extension and have a greater number of secondary branch points compared with age-matched wild-type (WT) controls (55). However, studies using the global VDR-knockout (KO) mouse model lack the ability to identify the cell type-specific contribution to VDR-mediated growth modulation during mammary gland development. Of importance is that adipose tissue has the unique ability to store lipophilic 25OHD3, a precursor to the active form of vitamin D3, and therefore, it may serve as an important paracrine source of vitamin D3 for VDR signaling in surrounding cells (5, 37). Both mammary adipocytes and epithelial cells have been shown to exhibit active VDR signaling and express CYP27B1 for synthesis of local 1,25D3 (8). Moreover, recent in vitro coculture experiments have shown that VDR signaling in WT adipocytes is capable of inhibiting the growth of WT and VDR-deficient mammary epithelial organoids, suggesting...
that VDR expression in adipocytes may play an important role in negatively regulating epithelial cell growth (8). However, the specific role of mammary adipocyte VDR signaling during mammary gland development remains speculative.

In this study, we show that VDR signaling in both mammary adipocytes and epithelial cells plays a significant role in the modulation of mammary gland development. Loss of VDR from either cellular compartment increases the rate of ductal outgrowth during pubertal mammary gland development through augmented epithelial cell proliferation and decreased apoptosis within TEBs. Moreover, we provide evidence that the acceleration in ductal growth with adipocyte-specific VDR ablation is due to altered adipokine release onto surrounding epithelial cells during pubertal mammary gland development, with IL-6 expression being a key VDR-dependent adipocyte factor. Thus, our results identify multiple cell type-specific VDR-dependent mechanisms required for proper mammary gland development.

MATERIALS AND METHODS

Mice. To selectively eliminate VDR from the mammary epithelium, FVB mice containing the MMTV-Cre transgene (gift from William J. Muller, McGill University) were bred with homozygous Vdr floxed (Vdr<sup>fl/fl</sup>) mice on a C57BL/6 background (gift from Dr. Shigeaki Kato, University of Tokyo) (52). To eliminate VDR in adipocytes, C57BL/6J mice containing the Fabp4-Cre transgene (stock no. 005069; The Jackson Laboratory, Bar Harbor, ME) were paired with Vdr<sup>fl/fl</sup> mice. F1 mice heterozygous for the floxed VDR gene and positively expressing the Cre transgene were back-crossed with Vdr<sup>fl/fl</sup> mice to generate mice containing the cre-recombinase transgene and homozygous for the floxed Vdr allele. Mammary gland development was compared between WT (littermate controls with Cre<sup>-</sup>/Cre<sup>-</sup>/H11003 mice lacking cre-recombinase, Fabp4-Cre expressing, or MMTV-Cre expressing) and cell-type specific loss of VDR mouse models. Mammary glands from nulliparous female mice were harvested at 5, 6, 7, 8, and 10 wk of age. All conditional transgenic mice and control animals were fed a standard rodent chow diet. Global VDR-KO and WT control animals (C57BL/6J) were continuously maintained on a diet high in calcium (2%), phosphorous (1.25%), and lactose (20%) containing 2.2 IU/g vitamin D<sub>3</sub>. (Research Diets, New Brunswick, NJ). All mice were maintained under specific pathogen-free conditions and were treated and euthanized in accord with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Whole mount preparation. Inguinal mammary glands were isolated and processed as described previously (30). Ductal outgrowth was measured on the whole mounts as the distance from middle of the central lymph node to the leading edge of the ductal mass using Axiovision Software by manually counting the number of alveolar buds per tissue. A minimum of eight animals per genotype was evaluated with two sections per animal and three to six TEBs per section.

Western blotting. Ex vivo mammary gland organs, 21-day mammary gland fat pads, or whole mammary glands were homogenized in Laemmli lysis buffer, sonicated, and measured for protein concentration using a BCA protein assay. Immunoblotting was carried out using antibodies targeting VDR-D6 (Santa Cruz Biotechnology, CA), cytokeratin-18 (Epitomics, Burlingame, CA), perilipin-D418 (Cell Signaling Technology, Danvers, MA), ER<sub>A</sub>, and PGR, perilipin, cytokeratin-18, Cyp27b1, or Cyp24a1 mRNA expression in duplicate by real-time PCR analysis using FastStart SYBR Green Master (Roche Diagnostics, Indianapolis, IN). The following forward and reverse mouse primer sets were used: VDR 5'-GAAGCGCAAGGCCCTGTT-3' (forward) and 5'-GGTGCAAGGGTTTCTTATAAGA-3' (reverse); GC 5'-CTTCTATCATGCCGACCT-3' (reverse); ER<sub>A</sub> 5'-GCGGGCTATGCTGGAACTC-3' (forward) and 5'-GCCTTCTATCATGCCGACCT-3' (reverse); PGR 5'-TGTGGGAGCTTCCCAAG-3' (forward) and 5'-GACATCGCCAGCTGGAC-3' (reverse); perilipin 5'-GGAAGGACAATGGAGCTGAA-3' (forward) and 5'-CTTCTTGTGCTGTCCTTGGT-3' (reverse); cytokeratin-18 5'-GAGGCGCAAGGCCCTGTT-3' (forward) and 5'-GGTGCAAGGGTTTCTTATAAGA-3' (reverse); and actin I-19 (Santa Cruz Biotechnology), and actin I-19 (Santa Cruz Biotechnology).

Histological staining. Formalin-fixed, paraffin-embedded right inguinal mammary glands from 6- and 10-wk-old mice were cut into 5-μm tissue sections and stained with hematoxylin and eosin for gross morphological assessment. Additional 6-wk inguinal mammary gland sections were assessed for cellular proliferation and apoptosis within TEBs. Epithelial cell proliferation was evaluated by immunohistochemical Ki-67 staining. Briefly, antigen retrieval was carried out in citrate buffer, and slides were quenched for endogenous peroxide, blocked in goat serum, and incubated with the primary antibody (1:100; Fisher Scientific, Waltham, MA) at 4°C overnight. Slides were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h prior to avidin conjugation (Vectastain ABC kit standard; Vector Laboratories). Epithelial cell apoptosis within TEBs was determined by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining using ApopTag Plus Peroxidase In Situ Apoptosis Kit (Milipore, Billerica, MA), following the manufacturer’s instructions. Detection of Ki-67 and TUNEL staining was visible following treatment with 3,3′-diaminobenzidine (Sigma). Ki-67 and TUNEL staining were analyzed at ×40 magnification using ImageJ Software (National Institutes of Health, Bethesda, MD). All immunohistochemical staining for cell proliferation and apoptotic index was quantified within the body cell region of TEBs as a percentage, i.e., epithelial cells with positive incorporation over the total number of epithelial cells within each TEB. The range of epithelial cells per TEB was 213–692, with an average of ~356 ± 111 cells counted per TEB. A minimum of eight animals per genotype was evaluated with two sections per animal and three to six TEBs per section.

Quantitative real-time PCR. Total RNA was isolated from mammary organoids, fat pads, or whole mammary glands were homogenized in Laemmli lysis buffer, sonicated, and measured for protein concentration using a BCA protein assay. Immunoblotting was carried out using antibodies targeting VDR-D6 (Santa Cruz Biotechnology, CA), cytokeratin-18 (Epitomics, Burlingame, CA), perilipin-D418 (Cell Signaling Technology, Danvers, MA), ER<sub>A</sub>, and PGR, perilipin, cytokeratin-18, Cyp27b1, or Cyp24a1 mRNA expression in duplicate by real-time PCR analysis using FastStart SYBR Green Master (Roche Diagnostics, Indianapolis, IN). The following forward and reverse mouse primer sets were used: VDR 5'-GAAGCGCAAGGCCCTGTT-3' (forward) and 5'-GGTGCAAGGGTTTCTTATAAGA-3' (reverse); GC 5'-CTTCTATCATGCCGACCT-3' (reverse); PGR 5'-TGTGGGAGCTTCCCAAG-3' (forward) and 5'-GACATCGCCAGCTGGAC-3' (reverse); perilipin 5'-GGAAGGACAATGGAGCTGAA-3' (forward) and 5'-CTTCTTGTGCTGTCCTTGGT-3' (reverse); cytokeratin-18 5'-GAGGCGCAAGGCCCTGTT-3' (forward) and 5'-GGTGCAAGGGTTTCTTATAAGA-3' (reverse); and actin I-19 (Santa Cruz Biotechnology), and actin I-19 (Santa Cruz Biotechnology)
control. Error bars represent the standard error of the mean from three independent experiments.

Steroid hormone pellet implant. Five-week control and experimental mice were surgically implanted with a 21-day-release hormone pellet containing 21 mg of progesterone and 21 μg of estradiol (Innovative Research of America, Sarasota, FL). The control or hormone pellet was implanted subcutaneously on the neck/upper back for 14 days. At 7 wk of age, the mice were euthanized and mammary glands removed for analysis. Left inguinal mammary glands were whole mounted for visualization of growing ducts and the onset of alveolar bud development and side branching in response to the hormone stimulation.

In vitro cell and ex vivo fat explant culture conditions. The murine epithelial cell line EpH4 (ATCC, Manassas, VA), established from the mammary gland of a midpregnant BALB/c mouse (27, 36), was grown in DMEM supplemented with 10% FBS and puromycin. Cells were cultured to 80% confluency. Left and right inguinal mammary fat pads from 21-day-old WT C57BL/6J or global VDR-KO mice were extracted, lymph node and visible blood vessels were removed, and 50-mg (wet wt) pieces were added per well into a 24-well plate. The explant medium contained DMEM-F-12 (1:1) and was supplemented with 1% penicillin-streptomycin and 200 nM adenosine. Vitamin D3, in the form of 1 mg/ml thiazolyl blue tetrazolium bromide (Sigma) in 10% heat-inactivated fetal bovine (Clone MP5–20F3; R&D Systems) was added to the conditioned medium or placed onto EpH4 cells at a final concentration of 10 nM. Confirmation of neutralization was carried out via ELISA. IL-6 levels in conditioned medium were measured by ELISA. In designated experiments, recombinant mouse IL-6 (R & D Systems, Minneapolis, MN) was added to the media cocktail prior to incubation with EpH4 cells at the specified concentration. For IL-6 neutralization, mouse IL-6 monoclonal antibody (Clone MP5–20F3; R & D Systems) was added to the conditioned medium or placed onto EpH4 cells at a final concentration of 10 μg/ml. Confirmation of neutralization was carried out via ELISA analysis (data not shown). After 48 h the medium was aspirated, and 1 mg/ml thiazolyl blue tetrazolium bromide (Sigma) in 1× PBS was added at 100 μl/well for 60 min prior to incubation with DMSO. Absorbance was measured at 570 nm.

Secretome arrays. Left and right inguinal mammary fat pads from four 21-day-old VDR-WT and VDR-KO mice were harvested, cleared of lymph nodes and visible blood vessels, cut into 50-μg (wet wt) pieces and treated with 10 nM 1,25D3 for 24 h. Conditioned medium samples were pooled from eight wells and screened for secretory cytokines and adipokines using proteome profiler mouse antibody arrays (R & D Systems), following the manufacturer’s instructions. Arrays were repeated three times, using conditioned medium from three separate experiments, and each experiment pooled conditioned medium from four mice (8 inguinal mammary glands). Results were quantified using ImageJ Software.

ELISA. IL-6 levels in conditioned medium were measured by ELISA (R & D Systems). The assay was carried out according to the manufacturer’s instructions using a 1:10 dilution of the conditioned medium. Samples were run in duplicate from three separate experiments.

Flow cytometry. EpH4 cells were treated with conditioned medium from VDR-WT or VDR-KO mammary gland fat pads supplemented with 10 nM 1,25D3 or EtOH for 48 h, as described above. Cells were then stained for annexin V/propidium iodide (PI) as per the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ). Cells were analyzed using the FACS Aria machine and FACS Diva software (BD Biosciences) in two separate experiments with 10,000 events per treatment group.

Statistical analyses. Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA). An analysis of variance (ANOVA) test was applied to determine significant differences between groups. Data are expressed as means ± SE. A P value of <0.05 was regarded as significant.

RESULTS

Characterization of VDR loss in murine models. To elucidate the cell-specific role of VDR signaling within epithelial or adipose tissue microenvironments, conditional cre-loxP deletion models were utilized. Cell-type specific loss of VDR was achieved using a floxed Vdr line with loxP sites spanning a 1.1-kb fragment flanking exon 2, which encodes the first zinc finger of the DNA-binding domain (52). Targeted deletion of VDR in adipocytes was achieved using the fatty acid-binding protein 4 (Fabp4) promoter to promote cre recombinase expression. Fapb4 is constitutively expressed in both brown and white mature adipocytes (18). Mice with a selective loss of VDR in adipocytes are subsequently referred to as Fabp4VDRfl/fl mice.

Loss of VDR in the mammary epithelium was performed through the use of the long terminal repeat of the mouse mammary tumor virus (MMTV) promoter (1) to drive cre recombinase crossed into the floxed Vdr line (referred to as MMTVVDRfl/fl mice). The MMTV promoter is expressed in secretory epithelial cells of the mammary gland with the onset of puberty in the presence of sex hormones (34, 49).

Loss of VDR protein and gene expression in mammary fat pads from 21-day-old mice or epithelial organoids from mature mammary glands was confirmed by immunoblotting and quantitative (q)RT-PCR analysis (Fig. 1, A and B). Targeted deletion was specific to the adipose and epithelium of Fabp4cre and MMTV-cre expressing mice, respectively, as 21-day-old mammary gland fat from MMTVVDRfl/fl mice and organoids from Fabp4VDRfl/fl mice did not show reduced VDR protein or mRNA expression. Additionally, local VDR ablation from either cell type did not alter estrogen receptor (ER) or progesterone receptor (PR) expression levels assessed by protein and RNA expression within the mammary gland (Fig. 1, C and D).

Loss of VDR signaling in mammary epithelial cells or adipocytes leads to accelerated ductal outgrowth during pubertal development. The VDR plays an important role in regulating the growth of the mammary epithelium during development. Global VDR-KO mice have heavier pubescent mammary glands than age-matched WT controls (55). To assess the significance of VDR signaling within different cell types of the mammary gland during pubertal development, mice with a conditional loss of VDR (Fabp4VDRfl/fl and MMTVVDRfl/fl) mice) and control mice were euthanized between 5 and 10 wk of age. With the onset and conclusion of pubertal mammary gland development, no significant differences in ductal length were apparent at 5 or 10 wk of age between groups. However, Fabp4VDRfl/fl and MMTVVDRfl/fl mice displayed significantly increased ductal extension at 6, 7, and 8 wk of age compared with WT controls (Fig. 2, A and B). These time points encompass the peak of pubertal mammary gland development, where the most rapid growth is exhibited. Similarly to previous data using global VDR-KO mice (55), the greatest difference in ductal outgrowth for the cell type-specific VDR-ablated ani-
mammary glands had no significant differences in TEB number or branching morphogenesis during pubertal development (Fig. 2C).

**Loss of VDR in both the mammary epithelium and adipocytes led to reduced TEB clearing with increased epithelial cell proliferation and decreased apoptosis.** Further analysis conducted on 6-wk-old Fabp4\textsuperscript{VDR \text{-} fl/fl} and MMTV\textsuperscript{VDR \text{-} fl/fl} mammary glands revealed altered TEB morphology with poor epithelial cell clearing compared with VDR-WT glands (Fig. 3A, top). By 10 wk of age, ductal morphology was analogous to age-matched VDR-WT controls as assessed by hematoxylin and eosin staining (Fig. 3A, upper middle). These data are consistent with earlier studies demonstrating that TEB differentiation into terminal end ducts is VDR independent (43, 55). Importantly, postpubertal mammary gland functionality in Fabp4\textsuperscript{VDR \text{-} fl/fl} and MMTV\textsuperscript{VDR \text{-} fl/fl} parous females was indistinguishable from age-matched VDR-WT mice (data not shown), consistent with previous studies in global VDR-KO mice where dams were able to nurse to term and maintain healthy litters with similar pup size and weight (56).

To further evaluate the epithelial cell-dense TEB phenotype in Fabp4\textsuperscript{VDR \text{-} fl/fl} and MMTV\textsuperscript{VDR \text{-} fl/fl} mice, mammary sections from 6-wk-old mice were stained for Ki-67 to assess epithelial cell proliferation. Similarly, the apoptotic potential was determined via TUNEL analysis (Fig. 3A, lower middle and bottom). The percentage of Ki-67-positive epithelial cells within TEBs of Fabp4\textsuperscript{VDR \text{-} fl/fl} and MMTV\textsuperscript{VDR \text{-} fl/fl} mammary glands was significantly higher than the age-matched VDR-WT controls (Fig. 3B). TEBs from Fabp4\textsuperscript{VDR \text{-} fl/fl} and MMTV\textsuperscript{VDR \text{-} fl/fl} mammary glands also exhibited significantly less epithelial cell death (Fig. 3C).

**Mammary epithelial-specific VDR signaling modulates alveolar precursors during pubertal development.** Despite similarities in the numbers of TEBs as well as in secondary and tertiary branching (Fig. 2C), precocious alveolar budding was suspected in nulliparous MMTV\textsuperscript{VDR \text{-} fl/fl} mammary glands compared with age-matched VDR-WT controls throughout pubertal development (Fig. 4A). Alveolar buds are precursors to mature lobuloalveoli and are composed of epithelial and myoepithelial cells that eventually direct milk production and transport for proper lactation (17). Alveolar budding in the mammary gland occurs predominantly during pregnancy and lactation, and it is not commonly found in the developing mammary gland. However, previous work has demonstrated that global VDR-KO mice are more sensitive to hormonally induced mammary alveologenesis in vivo and ex vivo (55). Figure 4B depicts the average number of alveolar precursors per area and shows that the MMTV\textsuperscript{VDR \text{-} fl/fl} mammary glands exhibit a significantly higher number at 5, 6, 7, 8, and 10 wk compared with age-matched Fabp4\textsuperscript{VDR \text{-} fl/fl} and VDR-WT controls. To further elucidate the cell type-specific contribution of VDR to hormonal hypersensitivity, estrogen and progesterone pellets were implanted subcutaneously into pubescent mice for 2 wk. Hormone exposure stimulated secondary and tertiary branching morphogenesis, stunted ductal outgrowth, and promoted precocious alveolar budding in developing mammary glands of all genotypes (Fig. 4C). However, the number of alveolar...
precursors per gland was significantly higher in hormone-stimulated MMTV/VDR fl/fl mice compared with VDR WT controls and Fapb4/VDR fl/fl mammary glands (Fig. 4D).

Vitamin D3-dependent VDR signaling in mammary adipose tissue modulates mammary epithelial cell turnover through promotion of cell death. Given the novel data documenting the role of VDR signaling in adipose tissue on epithelial ductal extension in the mammary gland, we sought to examine VDR-modulated cross-talk between these two cell types in vitro. EpH4 cells, a nontransformed murine mammary epithelial cell line, were utilized as the source of mammary epithelial cells. EpH4 cells express mRNA levels of Vdr and Cyp27b1 similar to mammary epithelial organoids from VDR-WT mice (Fig. 5A) and are responsive to vitamin D3 treatment, as judged by the upregulation of Cyp24a1 mRNA (a VDR target gene) in a dose-dependent manner (Fig. 5B). To examine the effect of VDR signaling in adipocytes on mammary epithelial cell growth, mammary fat pad explants from 21-day-old VDR-WT mice were cultured ex vivo, and conditioned medium was applied to EpH4 cells. After 48 h, the change in EpH4 cell number was calculated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays, and values were normalized to the control medium, which was set at 1. As shown in Fig. 5C, conditioned medium from fat was able to significantly augment EpH4 cell number in the absence of exogenous vitamin D3. However, conditioned medium from VDR-WT fat explants treated with 1,25D3 dose-dependently reduced EpH4 cell number to a greater extent than direct treatment of cells with 1,25D3 alone (Fig. 5D), suggesting that epithelial cell growth inhibition occurs through vitamin D3-mediated paracrine signaling from adipose tissue. At 10 nM 1,25D3, conditioned medium from VDR-WT mammary fat pads significantly reduced EpH4 cell number by roughly 60% compared with the ethanol-treated (0 nM 1,25D3) VDR-WT fat explant conditioned medium control (Fig. 5E). In sharp contrast, conditioned medium from VDR-KO fat explants had a minimal effect on EpH4 cell number regardless of 1,25D3 supplementation. Heat inactivation of the VDR-WT conditioned medium rescued EpH4 viability, suggesting that a heat-labile protein produced by the fat in response to 1,25D3 was responsible for the growth inhibition of the mammary epithelial cells (Fig. 5E). Analysis of EpH4 cells by flow cytometry showed that conditioned medium from VDR-WT mammary fat pads treated with vitamin D3 induced death in ~40% of the cells (Fig. 5F).

Fig. 2. Increased mammary ductal extension with VDR ablation in adipose tissue or the mammary epithelium. A: representative whole mounts from 6-wk-old Fapb4/VDR fl/fl and MMTV/VDR fl/fl transgenic mice showing enhanced ductal outgrowth compared with age-matched controls. Scale bar, 2 mm. B: average ductal extension in mammary glands from 5-, 6-, 7-, 8-, and 10-wk-old transgenic mice compared with VDR-WT controls. VDR ablation from either cell type results in significantly augmented epithelial outgrowth at 6, 7, and 8 wk compared with controls. C: average no. of terminal end buds (TEBs), secondary branch points, and tertiary branch points in mammary glands from conditional mutant mice compared with age-matched VDR-WT controls. Data represent means ± SE from 8 to 40 inguinal mammary glands per group at each time point. *P < 0.05.
5F), whereas cell proliferation measured by EDU incorporation was not altered with any treatment (data not shown).

Ligand-dependent VDR signaling in mammary adipose tissue inhibits the growth of mammary epithelial cells through secretion of IL-6. To identify factor(s) differentially secreted from the VDR-WT vs. VDR-KO mammary adipose tissue, cytokine and adipokine arrays were probed with conditioned medium from VDR-WT and -KO mammary fat explants treated with 1,25D3 ex vivo. Whereas many proteins were similarly expressed, including TNFα, VEGF, Pref-1, CCL5/RANTES, and FGF-1 (data not shown), a number of differentially expressed proteins were identified, namely C5α, IL-6, macrophage colony-stimulating factor, IGF-binding protein-1, leptin, and LIF (Fig. 6A). Of particular interest was IL-6, as this cytokine has been shown to inhibit growth of various mammary epithelial cell lines (2, 4, 46). Secretion of IL-6 from fat explants was consistently reduced in the conditioned medium from VDR-KO mice compared with WT controls (Fig. 6A). In addition, IL-6 mRNA expression was significantly reduced in 21-day-old mammary gland fat pads of VDR-KO and Fapb4<sup>VDR fl/fl</sup> mice compared with age-matched VDR-WT controls by roughly threefold, consistent with the cytokine and adipokine arrays on fat explant conditioned medium (Fig. 6B). ELISA analyses demonstrated a significant increase in IL-6 (~1.6-fold) in the VDR-WT conditioned medium when fat explants were treated with 1,25D3, suggesting that IL-6 production is vitamin D3 responsive (Fig. 6C). Secretion of IL-6 from VDR-KO fat explants was not affected by exogenous 1,25D3, indicating that vitamin D3 treatment augments IL-6 production through a VDR-dependent mechanism. The difference in
the concentrations of IL-6 between the VDR-WT and -KO conditioned medium treated with 1,25D_3 was ~4 ng/ml (Fig. 6C).

To define the growth inhibitory potential of IL-6, EpH4 cells were grown in conditioned medium supplemented with a neutralizing mouse IL-6 antibody (Fig. 6D). Addition of the IL-6-neutralizing antibody to both VDR-WT and VDR-KO conditioned medium augmented EpH4 cell number, demonstrating the role of IL-6 as an inhibitory growth factor (Fig. 6D). Immunoneutralization of IL-6 in the VDR-WT conditioned medium treated with 1,25D_3 increased the EpH4 cell number by roughly 40% compared with VDR-WT conditioned medium with 1,25D_3. Although robust, this augmentation only partially rescued EpH4 cell number compared with the non-1,25D_3-treated VDR WT control, suggestive of additional paracrine growth-regulatory factors. Contrastingly, EpH4 cells cultured in VDR-KO conditioned medium and supplemented with the IL-6-neutralizing antibody were not affected by 1,25D_3 treatment to the mammary gland fat pad, further suggesting that IL-6 production from the mammary adipose tissue is VDR dependent.

To further elucidate the growth inhibitory potential of IL-6, EpH4 cells were grown in conditioned medium supplemented with recombinant mouse IL-6 using physiologically relevant concentrations determined from the ELISA analyses. Addition of IL-6 to EpH4 cells in VDR-WT conditioned medium treated with 1,25D_3 failed to further reduce EpH4 cell number (Fig. 6E). However, when IL-6 was exogenously administered at 4 ng/ml to EpH4 cells in vitamin D_3-treated VDR-KO fat pad conditioned medium, EpH4 cell number was reduced by roughly 30% (Fig. 6E). Although this reduction was not analogous to EpH4 cells cultured with vitamin D_3-treated VDR-WT conditioned medium, the data suggest that VDR-dependent adipose-specific IL-6 production is a partial contributor to epithelial growth inhibition by VDR. Collectively, these data support the hypothesis that mammary adipose tissue produces and secretes IL-6 in response to 1,25D_3, which then inhibits the growth of surrounding epithelial cells, thus modulating ductal morphogenesis during mammary gland development.

DISCUSSION
In the present study, cell type-specific conditional deletion in mice was utilized to study the significance of VDR signaling in
mammary adipocytes and epithelial cells during pubertal mammary gland development. Both cell types were found to play important VDR-dependent roles in regulating the growth of the ductal epithelium, as VDR ablation in both adipocytes and mammary epithelial cells accelerated ductal elongation during puberty. Previous studies have provided evidence that the forward movement of TEBs is due to an internal force of dividing epithelial cells within TEBs that channel the cells toward the leading edge (20). We hypothesized that the growth inhibitory effect of vitamin D₃ through its receptor works both directly through epithelial VDR stimulation and indirectly through adipocyte VDR activation. The latter mechanism subsequently releases paracrine factors to inhibit the growth and/or promote the death or clearance of epithelial cells within TEBs. Consistent with this hypothesis, a change in the rate of epithelial cell turnover was visible in Fapb4^{VDR fl/fl} and MMTV^{VDR fl/fl} mammary glands. Compared with controls, TEBs in Fapb4^{VDR fl/fl} and MMTV^{VDR fl/fl} glands exhibited increases in proliferating epithelial cells and decreases in apoptosis (Fig. 3, A–C). TEBs filled with epithelial cells and/or the products of these cells may be a driving force behind the rapid ductal expansion observed between 6 and 8 wk of development. Interestingly, global VDR-KO mice lack differences in epithelial cell turnover compared with age-matched wild-type glands despite elongated ducts (55), suggesting that VDR-dependent extracellular matrix (ECM) components may be regulating...
mammary extension. Similarly contrasting to global VDR-KO mice (55), TEB number as well as size were not altered in mice with a mammary- or adipocyte-specific loss of VDR (Fig. 2, A and C). Again, these data suggest that multiple VDR-dependent cell types contribute to TEB development and progression in vivo. However, additional studies on VDR-modulated ECM deposition, composition, and organization as well as other cell types of the mammary gland that contribute to the ECM, such as fibroblasts, are needed to further identify the precise mechanism by which VDR modulates mammary gland ductal extension.

Pubertal mammary gland development is governed by a complex system of signaling pathways involving numerous hormones, such as estrogen and progesterone, that guide mammary morphogenesis (3). Moreover, the synergistic actions of these hormones are cell type specific. For instance, progesterone induces side-branching and alveologenesis through epithelial PGR activation (6, 29), but the actions of estrogen on the growth of the epithelium during mammary development are dependent largely upon stromal ERα (53). Previous studies in VDR-KO mice demonstrated enhanced sensitivity to lactogenic hormones. Therefore, from studies performed in VDR-KO mice we cannot determine which cell types are contributing to the VDR-diminished response to lactogenic hormones. We show here that loss of VDR signaling from the mammary epithelium but not adipocytes results in an increased incidence of alveolar precursors and a higher trend of secondary branch points (albeit not significant) throughout mammary gland development (Figs. 2 C and 4, A and B), indicating that hormonal hypersensitivity to precocious alveolar development is antagonized by epithelial VDR signaling. Unexpectedly, exogenously administered hormones failed to promote alveolar development when mammary adipocytes lacked VDR signaling (Fig. 4 D), suggesting that hormone-directed epithelial alveologenesis may require a VDR-dependent signal from surrounding adipocytes. Collectively, our results imply that epithelial VDR signaling is alveolar growth inhibiting, and the delicate cross-talk between adipocytes and epithelial cells modulates alveologenesis during development. However, further work is needed to examine the cross-talk in this process.

Although the cell type-specific VDR-targeted mouse models herein fail to differentiate between the ligand-dependent and
-independent effects of VDR on mammary development, data from the ex vivo and in vitro studies support the notion that vitamin D₃, through its receptor, is an integral modulating hormone in mammary epithelial cell growth inhibition through both direct (epithelial) and indirect (adipose) actions. Conditioned medium from VDR-WT mammary fat pads treated with vitamin D₃ decreased EpH4 cell number by roughly 60% (Fig. 5E), with nearly 40% attributable to cell death (Fig. 5F). This finding, which was not observed in EpH4 cells cultured in VDR-KO conditioned medium treated with vitamin D₃, further supports our hypothesis that ligand-dependent VDR signaling in the adipose tissue modulates epithelial cell turnover.

Because our study demonstrated that the VDR-dependent paracrine inhibitory factor(s) is heat labile (Fig. 5E), we utilized cytokine array technology to search selectively for differentially expressed proteins secreted by mammmary fat explants. Examination of cytokine and adipokine profiles from adipocyte VDR-WT and VDR-KO conditioned medium identified many protein candidates for VDR-dependent paracrine growth inhibition (Fig. 6A). Because of the well-known role for IL-6 in growth regulation, the cell growth inhibitory potential of IL-6 was investigated further. Mature adipocytes secrete varying amounts of IL-6 (10, 14, 31), although to our knowledge there are no studies to date on the modulation of mammary gland development by locally produced IL-6 from the surrounding fat pad. IL-6 has been studied extensively in breast cancer (11, 16, 24). Although many reports have been conflicting, IL-6 administration to ER-positive breast cancer cells in vitro has been shown to be growth inhibitory (11). IL-6 mRNA and secreted protein expression were consistently lower in VDR-KO and Fabp4VDRfl/fl fat pads compared with WT controls (Fig. 6, A–C), suggesting a growth inhibitory role on surrounding epithelial cells during mammmary development in vivo. Exogenously administered IL-6 dose-dependently reduced EpH4 cell number when cultured in conditioned medium from VDR-KO fat (Fig. 6E), whereas neutralization in VDR-WT and VDR-KO conditioned medium augmented EpH4 cell number (Fig. 6D). Both findings are suggestive of the role of IL-6 in epithelial cell growth inhibition. However, because administration of recombinant IL-6 failed to reduce EpH4 cell number to levels analogous to VDR-WT conditioned medium treated with 1,25D₃ (Fig. 6E), and neutralization of IL-6 in the VDR-WT conditioned medium from mammary fat pads treated with vitamin D₃ failed to fully rescue EpH4 cell number (Fig. 6D), we recognize that IL-6 may not be the only VDR-mediated paracrine inhibitory adipokine. Our results suggest that IL-6, among other vitamin D₃/VDR-induced proteins secreted from adipocytes, may play a growth inhibitory role in mammary development. However, further analysis is required to verify this modulation in growth in vivo during pubertal development of the mammary gland.

Deficiency in circulating levels of vitamin D₃ is recognized as a worldwide problem due to inadequate synthesis based on many limiting factors within populations such as location (trajectory from the sun), season of year, time of day, and melanin pigmentation (21). Thus, storage of vitamin D₃ and signaling via the breast adipose is likely reduced in human populations. Augmentation of epithelial cell number with conditioned medium from VDR-WT mice in the absence of vitamin D₃ (Fig. 5C) may suggest that vitamin D₃ deficiency in human populations with a functional VDR are at potential risk of aberrant growth during critical times of development or structural rearrangement such as puberty, pregnancy, lactation, involution, or tumorigenesis. This finding is consistent with a recent meta-analysis on vitamin D₃ sufficiency that was found to be associated with lower mortality rates in breast cancer, whereas low levels correlate with poor disease prognosis (32). Understanding the role of VDR signaling in the different cell types of the mammary gland during development is a critical key to understanding the mechanisms by which vitamin D₃ can protect against breast cancer. Here, we have provided the first in vivo evidence for cell-type-specific VDR-mediated modulation of pubertal mammary gland morphogenesis. Our study has demonstrated the cell type-specific role of VDR in the negative modulation of ductal elongation and epithelial cell proliferation while mediating epithelial cell apoptosis within TEBs. In addition, we have shed light upon the specificity of the VDR-dependent stromal-epithelial cross-talk involved in lactogenic hormone-induced alveolar growth. Our results directly show the VDR-dependent actions of vitamin D₃ on the adipose tissue’s modulation of epithelial cell number while providing new leads on the potential paracrine inhibitory factors involved, including IL-6. Future work is needed to identify the role of VDR-stimulated, adipose-derived IL-6 in pubertal mammary gland morphogenesis and later-life breast function and disease susceptibility and whether vitamin D₃ intervention during or after puberty can impede the progression and improve the prognosis of breast cancer. Collectively, our findings have illuminated the individual epithelial- and adipocyte-specific roles of VDR in the negative growth modulation of pubertal mammary gland development.

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DISCLOSURES

The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

A.L.J., G.M.Z., and S.E.W. conception and design of research; A.L.J. and G.M.Z. performed experiments; A.L.J., G.M.Z., and S.E.W. analyzed data; A.L.J., G.M.Z., and S.E.W. wrote the manuscript. The authors declare no conflicts of interest, financial or otherwise.

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