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Effect of Vitamin D_3 receptor ablation on murine mammary gland development and tumorigenesis☆

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1. Introduction

Over the past several years, our laboratory has examined the role of the Vitamin D_3 endocrine system in regulation of proliferation, differentiation, and apoptosis in transformed cells of the mammary gland [1–3]. The purpose of this review is to highlight the role of the Vitamin D_3 receptor (VDR) and its ligand, 1,25-dihydroxyvitamin D_3 ($1,25D_3$) in normal mammary gland and to propose mechanisms by which the VDR may participate in suppression of mammary gland transformation. Based on collective data from a number of laboratories, we have hypothesized [4] that the $1,25D_3$–VDR complex induces a program of genes which suppresses proliferation and maintains differentiation in normal mammary gland, and that dysregulation of VDR signaling could predispose mammary epithelial cells to transformation. In this review, we discuss supportive evidence for this hypothesis generated from our laboratory using the VDR knockout (VDRKO) mouse model.

2. Breast cancer overview

Adenocarcinoma of the breast arises from the epithelial cells present in the mammary ducts or alveoli. Considerable evidence indicates that estrogen, which drives mammary epithelial cell proliferation, is intricately involved in the etiology of human breast cancer. Anti-estrogens such as tamoxifen are effective for both treatment and prevention of estrogen dependent breast cancer. Since only one third of breast tumors are estrogen-dependent, however, there is a need for alternative therapies that target estrogen independent cells and that minimize the progression of estrogen responsive disease to hormone independence. Other nuclear receptors present in mammary cells, such as the progesterone receptor, retinoid receptors, and the VDR, have emerged as promising therapeutic targets. Based on the importance of nuclear receptors in mediating expression of genes involved in proliferation, differentiation and apoptosis, synthetic structural analogs of nuclear receptor ligands which exhibit biological properties distinct from the natural ligands have been developed. Many of these synthetic analogs exhibit desirable therapeutic profiles, and some are in clinical trials for various indications, including cancer. However, further development of synthetic ligands for either treatment or prevention of breast cancer requires more accurate understanding of the role(s) of their cognate nuclear receptors in the biology of the normal mammary gland.

3. Expression and role of VDR in mammary cells

The VDR is present in over 80% of breast cancers, does not necessarily colocalize with ER, and has been shown to act as a negative growth regulator of ER positive and ER negative breast cancer cells [1,3,5–7]. In fact, Vitamin D analogs have proven to be as effective as tamoxifen in inhibiting growth of estrogen responsive breast cancer both in vitro and in vivo. Furthermore, both epidemiological and pre-clinical studies suggest that Vitamin D_3 may play a role in prevention of breast cancer (for review, see [8]). To gain insight into the mechanisms by which the Vitamin D_3 signaling pathway may impact on breast cancer development, we have utilized mouse models to examine the role of the VDR in normal mammary gland proliferation, differentiation and apoptosis.

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Mammary gland development occurs primarily after birth and is dependent on complex stromal–epithelial interactions involving various hormones, growth factors, and proteases. Studies from genetically engineered mice have provided evidence that dysregulation or ablation of numerous hormones, growth factors, receptors, or proteases can impair mammary gland morphogenesis, and in many cases, alter the sensitivity of mammary epithelial cells to transformation. The VDR has been identified in human, rabbit and rodent mammary glands [9–12] and we have recently examined the particular cell types within the mouse mammary gland that express VDR, as well as its developmental regulation [13]. Our data indicate that the VDR is present in all of the major cell types in the gland and that its expression is not temporally or spatially uniform. During puberty, VDR is expressed at low levels in the proliferative cap cell population but is highly expressed in the differentiated basal and luminal epithelial cells of the ducts. During pregnancy, lactation, and involution (periods of rapid tissue remodeling), VDR expression in the mammary gland is also elevated. We have also detected VDR in the stromal cell compartment, although here the expression is at low levels and does not appear to be developmentally regulated. In vitro studies have shown that VDR expression can be regulated by estrogen, lactogenic hormones, and growth factors, however, the specific factors that govern cell type specific VDR expression in the mammary gland in vivo are unknown. Regardless, the developmental changes in VDR expression suggest that $1,25D_3$ and the VDR participate in the control of proliferation or differentiation of the normal mammary gland. This suggestion is supported by studies that have demonstrated that $1,25D_3$ alters calcium transport, casein expression and branching morphogenesis in mouse mammary gland organ culture [13–15].

Having demonstrated developmental regulation of the VDR in murine mammary gland, we initiated studies to examine the consequences of VDR ablation on glandular development and function throughout the reproductive cycle. Mammary glands were compared in age-matched VDRKO mice and their wild-type (WT) counterparts fed a high calcium rescue diet to normalize growth, bone development and extracellular calcium. Our data indicates that lack of VDR results in accelerated pubertal mammary gland development. Specifically, glands from VDRKO mice are heavier, exhibit increased ductal extension and branching morphogenesis in vivo, and enhanced sensitivity to estrogen and progesterone stimulation in vitro. During pregnancy, glands from VDRKO mice display precocious alveolar development and premature casein gene expression compared to WT mice. During lactation, VDRKO mice release a higher volume of milk than WT mice, but neither the casein composition nor calcium content of the milk is altered by VDR ablation. Further studies indicated that VDR ablation delays the involution of the mammary gland following weaning, a process driven by apoptosis. Collectively, these in vivo studies indicate that the VDR participates in the regulation of glandular development during puberty, pregnancy and lactation, but does not appear to be required for casein production or calcium transport into milk.

Table 1
Effect of VDR ablation on murine mammary gland development

Puberty	Increased terminal end bud number Enhanced ductal outgrowth and branching Higher gland:body weight ratio
Pregnancy	Precocious lobuloalveolar development Premature casein gene expression
Lactation	Increased milk volume Normal milk calcium content Normal milk casein composition
Involution	Delayed regression Persistent casein expression

Although this data (summarized in Table 1) provides conclusive evidence that the VDR plays a functional role in normal mammary gland development, the process by which VDR mediates these effects remains unclear. Mechanistically, the effects of VDR ablation may result from lack of functional VDR itself (i.e., ligand independent) or from loss of $1,25D_3$ triggered genomic or non-genomic signaling; additional studies are necessary to distinguish between these possibilities. Furthermore, our studies have not clarified the critical target cells for VDR action. The expression of the VDR in both epithelial and stromal compartments of the mammary gland suggests direct effects of VDR, which is consistent with observations that glands from VDR KO mice display differences even when studied in organ culture [13]. Indirect, systemic effects of VDR ablation may also contribute to altered mammary gland development. Under the conditions utilized in our studies, no differences in serum calcium, estrogen or progesterone were detected between WT and VDRKO mice; however, further work is needed to determine whether VDR ablation impacts on other hormones or growth factors important in mammary gland development. Most likely, VDR acts on multiple levels to regulate mammary gland development.

4. Impact of VDR ablation on transformation in mammary gland

We have utilized two distinct mouse models of breast cancer to test the hypothesis that lack of VDR signaling in mammary cells might alter the sensitivity of the gland to tumorigenesis. Since previous work has shown that Vitamin D_3 compounds can prevent carcinogen induced preneoplastic lesions in organ culture and tumorigenesis in whole animal models [16–18], our first studies involved induction of mammary tumors in WT and VDRKO mice with the chemical carcinogen dimethylbenzanthracene (DMBA). Immunohistochemical studies indicated that the VDR is highly expressed in DMBA induced mammary tumors that develop in WT mice in response to the protocol used. With

Table 2
Effect of VDR ablation on transformation of murine mammary gland

Chemical carcinogenesis (DMBA administration)	DMBA-induced mammary tumors in WT mice express VDR Increased pre-neoplastic lesions in VDRKO mice vs. WT mice Altered tumor pathology Estrogen deficiency in VDRKO mice
Oncogenic model (MMTV-neu × VDRKO mice)	Neu-driven tumors in WT mice express VDR Increased ductal thickening and pre-neoplastic lesions in VDRKO and VDRHet mice vs. WT mice expressing neu transgene Increased incidence of neu-driven mammary tumors in VDRHet mice vs. WT mice Estrogen deficiency, weight loss and poor survival in VDRKO mice expressing neu transgene

this model, we observed an increased percentage of DMBA induced pre-neoplastic mammary lesions in glands from VDRKO mice compared to WT mice. At 8 months of age (the end point of the study), the overall mammary tumor incidence was similar in WT and VDRKO mice, however, circulating estrogen was significantly decreased in the VDRKO mice compared to WT mice. The estrogen deficiency in the VDRKO mice likely limited the outgrowth of pre-neoplastic lesions, precluding firm conclusions regarding the role of VDR in sensitizing glands to DMBA. Interestingly, the histopathology of the tumors that developed in VDRKO mice (primarily pilar tumors) was different than that of WT mice (primarily myoepithelial tumors); this difference could be related to estrogen deficiency in the VDRKO mice. Further studies are necessary to determine whether VDR ablation alters mammary tumor incidence and/or pathology in response to DMBA under conditions where estrogen levels are comparable between WT and VDRKO mice.

Since DMBA treated mice developed numerous non-mammary tumors that complicated the interpretation of our data, we initiated additional studies with the MMTV-neu transgenic mouse model of breast cancer to test whether VDR ablation enhances sensitivity to oncogenic transformation. Using immunohistochemistry, we determined that VDR is highly expressed in neu-driven mouse mammary tumors as well as in pulmonary metastatic foci. Our studies involved crossing VDRKO mice with MMTV-neu mice and monitoring mammary gland morphology and tumor development as a function of VDR gene dosage over time. At 10.5 months of age, we detected an increased incidence of pre-neoplastic lesions and ductal thickening in MMTV-neu/VDRKO and MMTV-neu/VDR heterozygote (Het) mice compared to MMTV-neu/VDR WT mice. During the extended time course of tumor development in this model (18 months), MMTV-neu/VDRKO mice exhibited weight loss, estrogen deficiency and poor survival, thus precluding an assessment of long term tumor incidence as a function of complete VDR ablation. Since MMTV-neu/VDR Het mice did not exhibit weight loss, estrogen deficiency or reduced survival, we assessed whether loss of one copy of the VDR altered neu-driven tumorigenesis. The data indicate that mammary tumor latency was shorter, and final tumor incidence was increased, in the MMTV-neu/VDR Het mice compared to MMTV-neu/WT mice. These data suggest that haploinsufficiency of the VDR is associated with mammary gland pathology and sensitization of the gland to transformation in response to altered growth factor signaling. This finding is consistent with recent studies which have shown that tumor suppressor genes such as p53 and p27 can act in a haploinsufficent manner to enhance tumorigenesis [19].

The data we have generated in these two distinct in vivo models of mammary carcinogenesis are summarized in Table 2. Overall, these studies support a role of the VDR in suppression of mammary tumorigenesis, and provide ample rationale for further studies to identify the specific cellular compartments affected by VDR ablation and the role of the ligand, 1,25D_3. Such knowledge is critical in development of strategies to target the Vitamin D_3 endocrine system in attempts to modify breast cancer risk.

5. Summary and conclusions

Our work has shown that the VDR is a physiologically important negative growth regulatory factor in the mammary gland. The consequences of VDR ablation include an increased number of undifferentiated terminal end buds and accelerated ductal branching during puberty, precocious lobuloalveolar development during pregnancy and impaired regression following weaning. These data suggest a role for VDR signaling in the attenuation of proliferative signals triggered by hormones or growth factors in vivo. Since tamoxifen, an estrogen receptor ligand which inhibits estrogen signaling, has been shown to prevent breast cancer in human populations, further attention to the possibility that VDR ligands may also be able to prevent breast cancer is warranted. Our studies showing that VDR expression impacts on the development of preneoplastic lesions, as well as the incidence and pathology of mammary tumors in two distinct mouse models of mammary carcinogenesis certainly lend credence to this possibility.

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