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13. ABSTRACT (Maximum 200 Words) In these studies, we have tested whether VDR ablation enhances sensitivity to oncogenic transformation, using the MMTV-neu transgenic mouse model of breast cancer. Our studies involved crossing VDRKO mice with MMTV-neu mice and monitoring tumor development and mammary gland morphology over time. Tumor development in the bigenic mice was slowed due to the influence of the C57BL6 genetic background, which is known for resistance to mammary tumorigenesis. At 15 months of age, tumor development was less than 40% in bigenic mice, compared to 100% incidence in parental MMTV-neu mice at 8.5 months of age. Histological assessment of MMTV-neu primary tumors indicated no consistent effects of VDR ablation on tumor morphology or neu expression. VDR was highly expressed in neu driven mammary tumors. The delay in tumor development precluded a rigorous test of the effect of VDR ablation on MMTV-neu tumorigenesis. We therefore examined mammary gland morphology at 12 months of age and found increased incidence of pre-neoplastic lesions, as well as dilated and thickened ducts, in MMTV-neu/VDRKO mice compared to MMTV-neu/VDR WT mice. These data suggest that VDR ablation impacts on the morphology of the mammary gland in MMTV-neu mice, and that neu driven mammary tumors, which have high levels of VDR protein, might be responsive to vitamin D based therapeutics that trigger VDR signaling.				
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INTRODUCTION

In these studies, we are testing the hypothesis that the Vitamin D3 Receptor (VDR) represents a nutritionally modulated growth regulatory gene in mammary gland. The VDR is a nuclear receptor that modulates gene expression when complexed with its ligand, 1,25dihydroxyvitamin D3 (1,25D), the active metabolite of vitamin D3. We propose that VDR induces genes which suppress proliferation and maintain differentiation in mammary gland, and consequently, that dysregulation of VDR target genes will predispose mammary epithelial cells to transformation. Extensive preclinical and clinical data indirectly support this concept. In animal models, vitamin D3 negates the effects of dietary fat and chemical carcinogens on mammary tumorigenesis. In clinical studies, low serum 1,25D correlates with increased breast cancer risk and metastasis. Data from the NHANES I epidemiological study suggests that optimal vitamin D3 nutrition affords protection against breast cancer. Genetic studies have identified specific alleles of the VDR which correlate with an increased risk for sporadic breast cancer and with more aggressive metastatic disease. Mechanistic data indicate that 1,25D inhibits proliferation of human breast cancer cells via induction of growth arrest and apoptosis. Despite these consistent data supporting a protective role for vitamin D3 and its receptor in breast cancer, it has been difficult to demonstrate that lack of vitamin D3 directly affects mammary gland *in vivo*. Dietary vitamin D3 deficiency has profound effects on calcium homeostasis which confound interpretation of 1,25D's effect on mammary gland differentiation. In recent studies we have examined the effect of VDR ablation on mammary gland using VDR knockout (VDRKO) mice fed a high calcium "rescue" diet which normalizes calcium homeostasis (Zinser et al, 2002). These studies indicated that lack of VDR accelerates mammary gland development during puberty, supporting the concept that the VDR suppresses hormone driven proliferation in mammary cells. In the studies funded by DAMD17-00-1-0644, we have used VDRKO mice to directly examine if lack of the VDR in mammary gland enhances sensitivity to oncogenic transformation and whether mammary tumors which lack VDR exhibit more aggressive behavior than tumors that express VDR.

BODY OF REPORT

Experimental Design.

To test whether VDR ablation enhances sensitivity to oncogenic transformation, we chose a transgenic mouse model of breast cancer, the MMTV-neu mice (Guy et al, 1992). Our studies involved crossing VDRKO mice with MMTV-neu mice, which overexpress the proto-oncogene neu, a tyrosine kinase growth factor receptor in the EGFR family, and predictably develop metastatic breast cancer. Homozygous MMTV-neu mice are viable and fertile and develop focal mammary tumors beginning at 4 months of age, which are preceded by hyperplastic lesions. This model was chosen for the following reasons: 1) the latency, frequency, histology, and metastatic potential of the mammary lesions is well characterized, 2) the phenotype is not associated with very rapid onset or overly aggressive tumors; thus enabling detection of synergy if it exists; 3) synergistic tumorigenesis has been observed in crosses of MMTV-neu mice and transgenic mice overexpressing TGF α ; demonstrating the feasibility of our approach, and 4) 1,25D has been shown to oppose signaling through the EGF family of receptors; thus, lack of VDR may be expected to amplify tumorigenesis initiated by neu amplification.

Results: The data generated by these studies include assessment of tumor incidence, tumor morphology, VDR and neu expression, pre-neoplastic lesions and ductal branching in mammary gland whole mounts as described below. All Figures referenced in this section are included in the Appendix.

Generation of MMTV-neu mice with or without VDR ablation.

To generate bigenic mice, female MMTV-neu mice (purchased from Jackson Laboratories) were mated to male VDRKO mice from our breeding colony. Heterozygous offspring were crossed to generate MMTV-neu mice that were VDR WT, VDR heterozygous (VDR HET) or VDR KO. No difficulties in breeding, fertility or pup survival were noted in the crosses, and sufficient numbers of mice to test the hypothesis were generated. Three distinct groups of female mice that carried the MMTV-neu transgene were monitored in our study:

1. *Parental MMTV-neu mice, line #202*, which were those originally obtained from Jackson Labs that were bred to generate the bigenic mice. These mice were wild type for VDR and were multiparous (since they were breeders). There were 20 mice in this group.
2. *The F1 generation mice*, which were derived from crossing parental MMTV-neu mice with VDRKO mice. These mice were heterozygous for the VDR and were also multiparous, since they were used to generate the experimental (F2) mice. There were 19 mice in this group.
3. *The F2 generation mice*, derived from crossing males and females of the F1 generation. These mice differed in VDR expression: either VDR WT (n = 76), VDR HET (n=164) or VDR KO (n=120). These mice were not bred. Only female offspring were used for study.

Mammary Tumor development.

All mice were palpated weekly to detect tumors, and tumor incidence was plotted against time for all three groups of animals. Data on tumor development (expressed as percentage of tumor free mice) is presented in Figure 1.

Group 1. In the parental MMTV-neu females, tumors were detected as early as 5 months of age. In these mice, 50% tumor incidence was observed at 6.5 months of age, and all mice were tumor positive by 8.5 months of age (Figure 1A). These data on tumor development are highly comparable to that reported by Guy et al (1992) for the original strain (line #202) of MMTV-neu mice.

Group 2. The F1 generation mice were obtained by crossing MMTV-neu females (which are on the FVB genetic background) with VDRKO mice (which are on the C57BL6 genetic background). We found that the mixture of genetic backgrounds significantly slowed mammary tumor development. In these F1 MMTV-neu mice, the first tumors were detected at 6.5 months of age, and 50% tumor incidence was observed at 12.5 months of age (Figure 1B). This is not unexpected since the C57BL6 background is considered to be more resistant to mammary tumor development than other genetic backgrounds (Rowse et al, 1998).

Group 3. In F2 MMTV-neu mice, tumor development was further delayed compared to F1 MMTV-neu mice (Figure 1C), as the first tumors were detected at >8 months of age in F2 mice. We thus requested and received a no-cost extension to continue monitoring mice up to 15 months of age. The final tumor development curves were virtually identical for the three VDR genotypes up until 13 months of age. Final tumor incidence (at 15 months) was 31% (in MMTV-neu/VDR WT mice, 22% in MMTV-neu/VDRKO mice and 38% in MMTV-neu/VDR HET mice. Further statistical analysis is underway to determine if MMTV-neu driven mammary tumor development curves are significantly different as a function of VDR genotype. A confounding problem is that aging MMTV-neu/VDRKO mice were not as healthy as MMTV-neu/VDR WT mice or MMTV-neu/VDR HET mice, and this may have adversely impacted long term tumor development indirectly.

These data indicate that tumor incidence was lower in all F2 generation mice, regardless of genotype, than in F1 mice. This difference may reflect the nulliparous state of the F2 mice, as compared to F1 mice, which were multiparous, since endogenous hormones can stimulate the MMTV promoter (and thus enhance transgene expression) during pregnancy. However, other factors may also be involved, since Guy et al (1992) have reported that tumor development in line #202 (on FVB background) is not strictly dependent on pregnancy. For example, the importance of pregnancy in susceptibility to tumor development could be modified by the C57BL6 genetic background.

Analysis of mammary tumors.

Tumors were collected from F2 generation mice to assess general pathology and expression of the VDR and the neu transgene. As demonstrated in Figure 2, tumors from MMTV-neu/VDR WT mice (Figure 2A), MMTV-neu/VDR HET mice (Figure 2B), and MMTV-neu/VDRKO mice (Figure 2C) were composed of solid nests of pale intermediate cells characteristic of neu transgene expression (Guy et al, 1992). No consistent differences in tumor pathology were noted as a function of VDR genotype.

A prerequisite for an effect of VDR ablation on MMTV-neu driven tumorigenesis would be that neu tumors express VDR. Since there have been no publications regarding the expression of VDR in MMTV-neu tumors in mice, or neu expressing breast cancers in women, we examined VDR expression by immunohistochemistry in tumors derived from MMTV-neu/VDR WT mice (Figure 3A), MMTV-neu/VDR HET mice (Figure 3C), and MMTV-neu/VDRKO mice (Figure 3E). Nuclear VDR expression was detected in primary tumors derived from MMTV-neu/VDR WT mice and MMTV-neu/VDR HET mice but not in those from MMTV-neu/VDRKO mice. VDR was also detected in lung metastases from MMTV-neu/VDR WT mice and MMTV-neu/VDR HET mice (data not shown). These data indicate that therapeutics that activate VDR signaling, such as vitamin D analogs, might be effective growth inhibitors of tumors that express neu.

To verify expression of the neu transgene in these tumors, sections were subjected to immunohistochemistry with an antibody that recognizes rat c-neu. As demonstrated in Figure 3, tumors from MMTV-neu/VDR WT mice (Figure 3B), MMTV-neu/VDR HET mice (Figure 3D), and MMTV-neu/VDRKO mice (Figure 3F) expressed comparable levels of membrane localized neu protein. These results indicate that VDR ablation did not alter neu expression in the tumors.

Both frozen and paraffin embedded mammary tumor samples from MMTV-neu/VDRKO mice, MMTV-neu/VDR HET mice and MMTV-neu/VDR WT mice have been archived for future studies.

Mammary gland morphology.

The delayed development of mammary tumors in MMTV-neu mice on the C57BL6 background prompted us to examine the effect of VDR ablation on mammary gland morphology in MMTV-neu mice. At 2, 4 and 6 months of age, two-three mammary glands were surgically dissected from MMTV-neu/VDRKO mice and MMTV-neu/VDR WT mice, whole mounted and stained with carmine-alum. No pre-neoplastic or other lesions were detected in mammary glands of mice of either genotype at these ages.

A more extensive examination of mammary gland morphology was conducted in 12 non-tumor bearing F2 MMTV-neu mice of each genotype at 10 months of age. Whole mounts were scored for alveolar growth, ductal thickness and pre-neoplastic lesions by three observers in a double blind fashion. Representative whole mounts are presented in Figure 4. The left panels demonstrate ductal morphology and branching, whereas the right panels demonstrate pre-neoplastic lesions. Top panels (A,B) are from MMTV-neu/VDR WT mice, middle panels (C,D) are from MMTV-neu/VDR HET mice and lower panels (E, F) are from MMTV-neu/VDR KO mice. The data indicated that VDR genotype did not affect the extent of alveolar growth in MMTV-neu mice. However, the incidence of pre-neoplastic lesions tended to be higher in MMTV-neu/VDRKO mice (82%) and MMTV-neu/VDR HET mice (75%) than in MMTV-neu/VDR WT mice (50%). In addition, generalized thickening and dilation of the mammary ducts was present in 92% of MMTV-neu/VDR KO mice and 25% of MMTV-neu/VDR HET mice, compared to 8% of MMTV-neu/VDR WT mice. These findings indicate that VDR ablation impacts on the gross morphology of the aging mammary gland, and future studies to assess histopathology and neu expression in relation to the VDR in mammary gland are warranted.

Summary.

These studies utilized VDR ablated mice to examine the role of the vitamin D signaling pathway on neu driven tumorigenesis in a mouse model. The data indicate that neu driven tumors express the VDR and would therefore likely be amenable to vitamin D based therapeutics. In these studies, VDR ablation did not enhance the development of primary tumors, however, MMTV-neu mice lacking VDR were not as healthy and long lived as littermates expressing VDR. Tumor development may have been slightly enhanced in MMTV mice heterozygous for VDR compared to those wild type for VDR. Mammary gland morphology of MMTV-neu mice lacking VDR was characterized by thickened dilated ducts which were not found in MMTV-neu mice wild type for VDR. VDR ablation also appeared to enhance the development of pre-neoplastic ductal lesions in mammary gland. These studies implicate the vitamin D endocrine system in negative growth regulation of the mammary gland, and support the potential use of vitamin D3 analogs in prevention or treatment of breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Generated MMTV-neu transgenic mice on the VDR KO, VDR WT or VDR Het background.
- Assessed the effect of VDR ablation on neu driven mammary tumorigenesis and pre-neoplastic lesions
- Characterized the effects of VDR ablation on mammary gland morphology of aging MMTV-neu mice
- Demonstrated the presence of the VDR in MMTV-neu tumors
- Archived mammary tumor samples from MMTV-neu/VDRKO mice, MMTV-neu/VDR HET mice and MMTV-neu/VDR WT mice for subsequent gene array, proteomic or histological analyses.

REPORTABLE OUTCOMES

Zinser GM and Welsh JE. Vitamin D receptor and mammary tumorigenesis. Abstract, Era of Hope meeting, to be presented Sept. 2002.

CONCLUSIONS

The concept tested in these studies is that nutritional regulation (dietary vitamin D3) of a putative tumor suppressor gene (the VDR) could modify breast cancer development. During the time frame of the Concept award, we generated MMTV-neu mice on the VDRKO background, and demonstrated that VDR ablation impacted on mammary gland morphology and pre-neoplastic lesion development in MMTV-neu mice. The time course of tumor development in this model did not allow for analysis of the effect of VDR ablation on actual tumor development, since the incidence of tumor formation was low even at 15 month follow-up. However, we demonstrated that tumors that developed in response to the oncogene neu express the VDR, suggesting that vitamin D derivatives that trigger VDR mediated growth inhibition might be useful in treatment of human breast tumors that express neu.

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Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A.* 89:10578-10582, 1992

Rowse GJ, Ritland SR, Gendler SJ. Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. *Cancer Res.* 58:2675-2679, 1998

APPENDICES

Figure 1. Tumor incidence in MMTV-neu mice

Figure 2. MMTV-neu tumor morphology

Figure 3. VDR and neu expression in MMTV-neu tumors

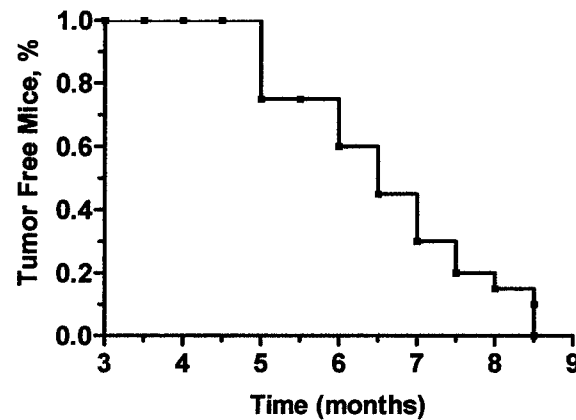
Figure 4. Representative whole mounts from MMTV-neu mice

Abstract to be presented at Era of Hope meeting, September, 2002.

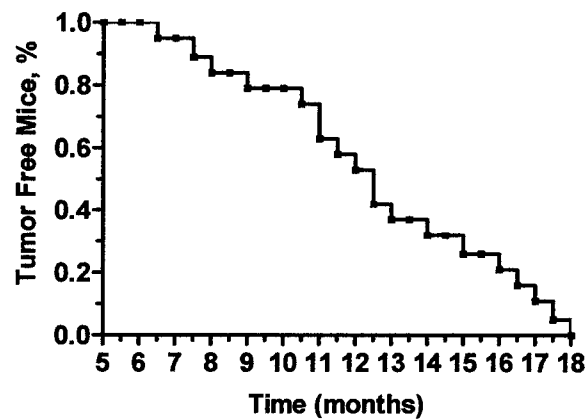
Zinser GM and Welsh JE. Vitamin D receptor and mammary tumorigenesis.

Figure 1. Tumor incidence vs. time in parental (A), F1 generation (B) and F2 generation (C) MMTV-neu mice.

A. Parental MMTV-neu mice (VDR WT)



B. F1 generation MMTV-neu mice (VDR Het)



C. F2 generation MMTV-neu mice (VDR WT, VDR Het and VDRKO)

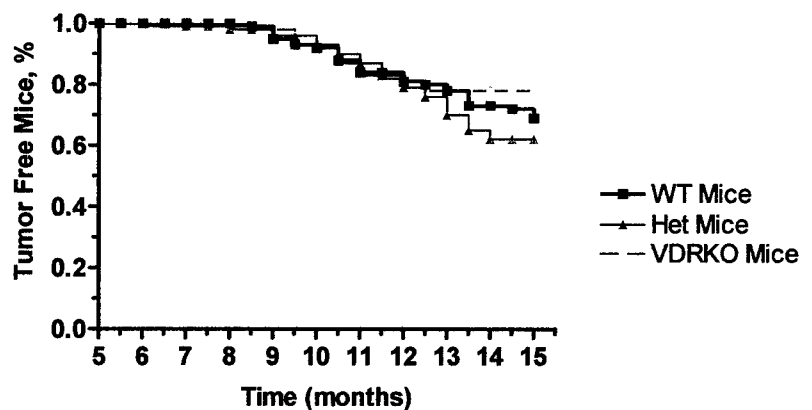


Figure 2. Pathology of mammary tumors from F2 generation mice. H&E staining was performed on tumor sections from F2 generation MMTV-neu mice. A: VDR WT; B: VDR Het; C: VDR KO

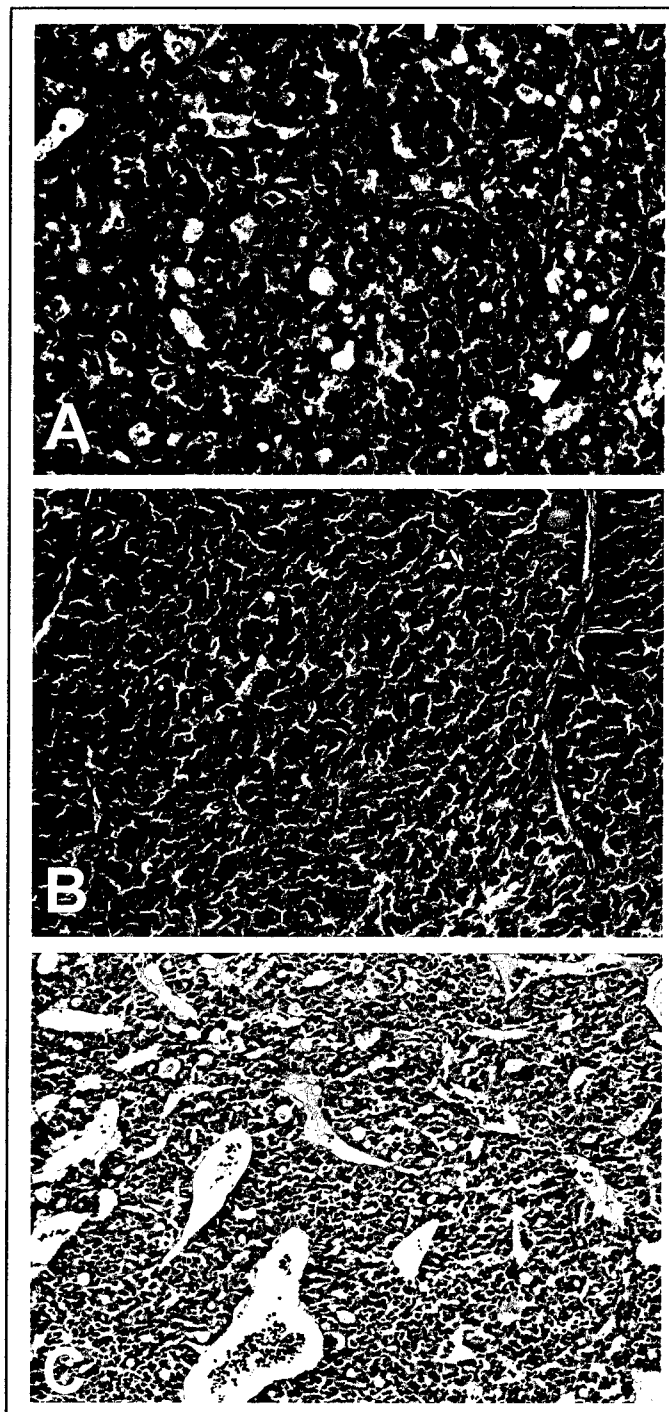


Figure 3. VDR and Neu expression in mammary tumors from F2 generation mice. Immunohistochemistry for VDR (A,C,E) and c-Neu (B,D,F) was performed on tumor sections from F2 generation MMTV-neu mice. A,B: VDR WT; C,D: VDR Het; E,F: VDR KO

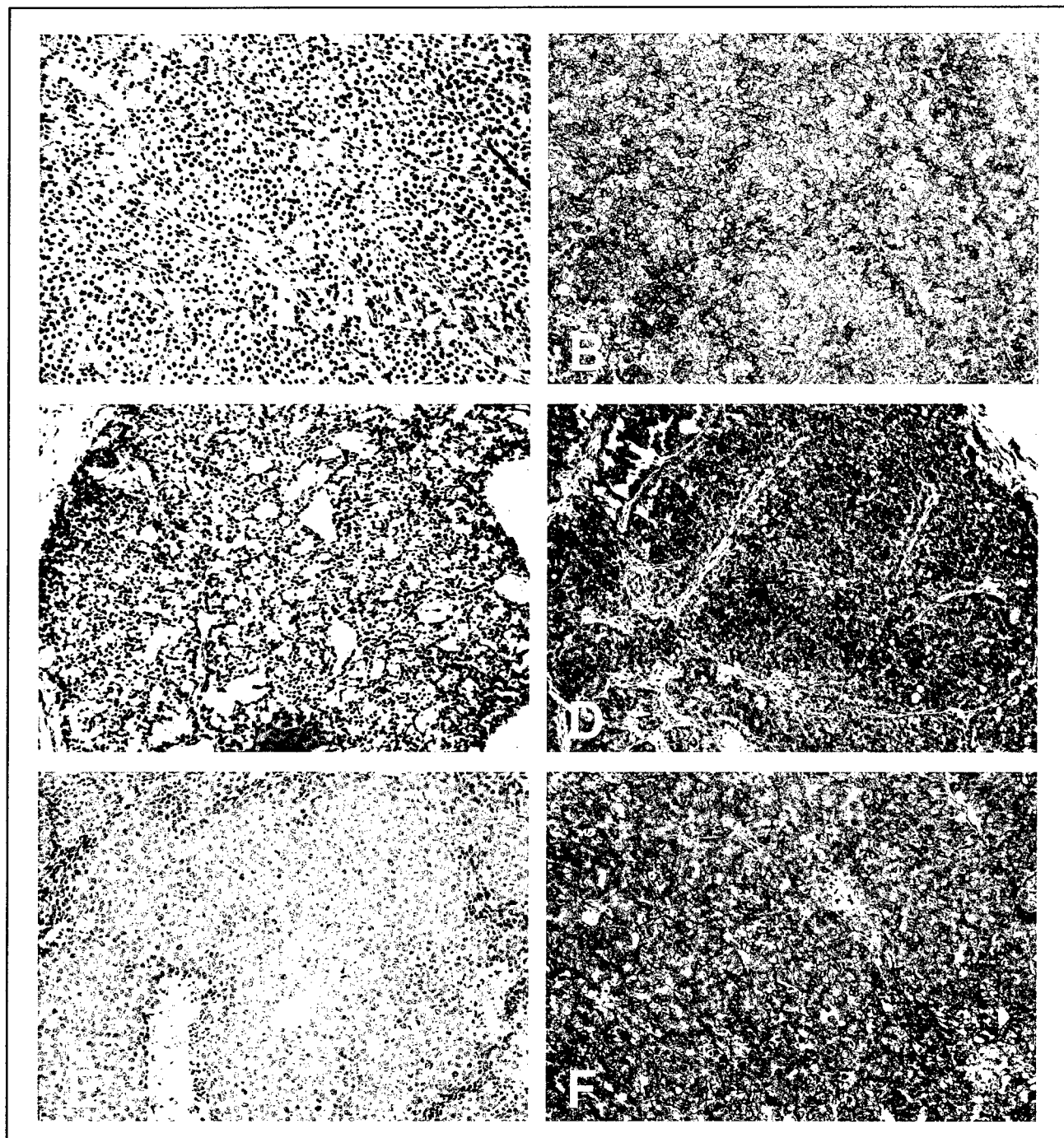
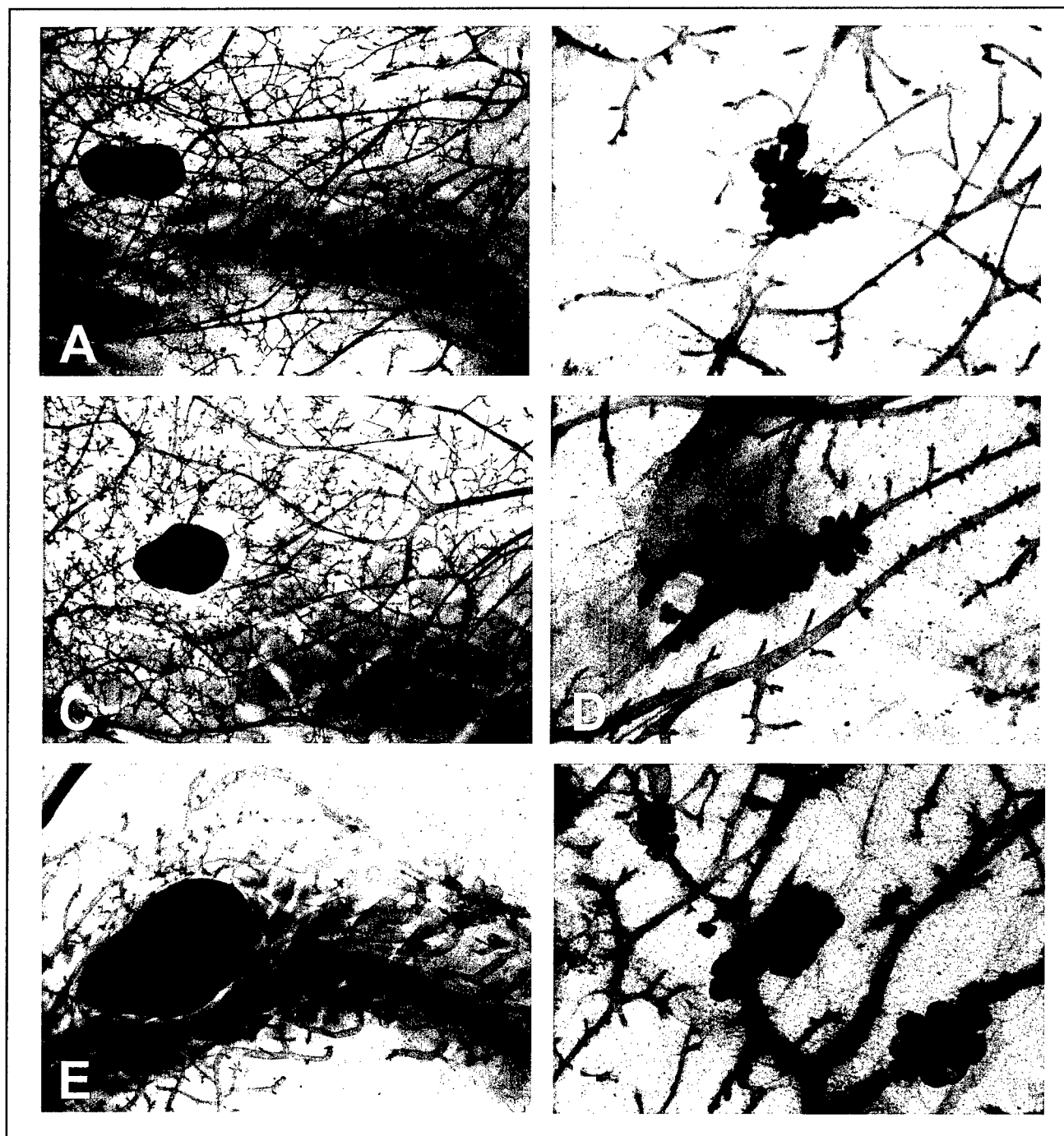


Figure 4. Whole mounts of F2 generation mice. Ductal architecture (A,C,E) and pre-neoplastic lesions (B,D,F) of F2 generation MMTV-neu mice. A,B: VDR WT; C,D: VDR Het; E,F: VDR KO



VITAMIN D RECEPTOR AND MAMMARY TUMORIGENESIS

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In these studies, we are using the vitamin D3 receptor knockout (VDR KO) mouse to test the hypothesis that the VDR represents a tumor suppressor gene in mammary gland. In VDR KO mice, we have observed accelerated mammary gland development during puberty, suggesting that the VDR suppresses hormone driven proliferation in mammary cells. To test whether VDR ablation enhances sensitivity to oncogenic transformation, VDR KO males were mated with MMTV-neu females, and heterozygous offspring were crossed to generate MMTV-neu mice that were VDR WT, VDR heterozygous (VDR HET) or VDR KO. Tumor development was followed in multiparous parental MMTV-neu mice (VDR WT; n = 20), multiparous F1 MMTV-neu mice (VDR HET, n = 19) and in virgin F2 MMTV-neu mice [VDR WT (n = 76), VDR HET (n=164) or VDR KO (n=120)]. In parental MMTV-neu females, 50% tumor incidence was observed at 6.5 months of age. Crossing MMTV-neu females (on FVB background) with VDR KO mice (on C57BL6 background) slowed tumor development, with 50% tumor incidence observed in F1 MMTV-neu mice at 12 months of age. In F2 MMTV-neu nulliparous mice, tumor development was further delayed, with less than 20% incidence for all genotypes at 11 months of age (continued follow-up is in progress). The delayed development of mammary tumors on the C57BL6 background prompted us to examine mammary gland morphology in 12 virgin F2 MMTV-neu mice of each genotype at 10 months of age. We found that although VDR genotype did not affect alveolar growth, the incidence of pre-neoplastic lesions tended to be higher in VDR KO mice (82%) and VDR HET mice (75%) than in VDR WT mice (50%). In addition, generalized thickening and dilation of the mammary ducts was present in 92% of VDR KO mice and 25% of VDR HET mice, compared to 8% of VDR WT mice. These findings indicate that VDR ablation impacts on the gross morphology of the aging mammary gland, and further studies to assess histopathology and neu expression in relation to the VDR are in progress. In summary, our studies in VDR ablated mice implicate the vitamin D3 endocrine system in negative growth regulation of the mammary gland, and support the potential use of vitamin D3 analogs in prevention or treatment of breast cancer.



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