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Dietary Vitamin D Exposure Prevents Obesity-induced Increase in Endometrial Cancer in *Pten*$^{+/-}$ Mice

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Abstract

The possibility that dietary VD_3 exposure inhibits endometrial carcinogenesis in an animal model, and modifies the enhanced risk of endometrial carcinoma associated with obesity was investigated. When 4 weeks of age, *Pten*$^{+/-}$ and wildtype mice were each divided into four treatment groups and fed AIN93G control diet, or AIN93G based diet containing either 25K IU of VD_3/kg diet, 58% fat to induce obesity (high fat), or high fat and 25K IU of VD_3/kg diet. Mice were kept on these diets until they were sacrificed at week 28. Although VD_3 did not affect endometrial cancer risk, it inhibited obesity-induced increase in endometrial lesions. Specifically, high fat diet increased focal glandular hyperplasia with atypia and malignant lesions from 58% in the control diet fed *Pten*$^{+/-}$ mice to 78% in obese mice. Dietary VD_3 decreased the incidence of endometrial pathology in obese *Pten*$^{+/-}$ mice to 25% ($p<0.001$). VD_3 altered the endometrial expression of 25-hydroxylase (25-OHase), 1α-OHase and vitamin D receptor in the wildtype and *Pten*$^{+/-}$ mice. Estrogen receptor (ER) -α mRNA levels were higher ($p<0.014$) and progesterone receptor (PR) protein levels in the luminal epithelium were lower ($p<0.04$) in the endometrium of control diet fed *Pten*$^{+/-}$ than wildtype mice, but the expression of these receptors was not affected by the dietary exposures. VD_3 reversed the obesity induced increase in osteopontin ($p<0.001$) and significantly increased E-cadherin expression ($p<0.019$) in the endometrium of obese in *Pten*$^{+/-}$ mice. Our data confirm the known association between obesity and endometrial cancer risk. Dietary exposure to VD_3 inhibited the carcinogenic effect of obesity on the endometrium; this protective effect was linked to a reduction in the expression of osteopontin and increase in E-cadherin.

INTRODUCTION

Endometrial cancer is the fifth most common cancer among women (1), with approximately 41,200 new cases being diagnosed in the U.S. in 2006 (2). Unopposed exposure to high estrogen levels is the main risk factor for this disease (3). Other risk factors for endometrial

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cancer include obesity which elevates the risk by 3–5 -fold (4;5). Vitamin D, in contrast, has been proposed to reduce endometrial cancer risk (6;7) as well as the risk of several other cancers (8;9). Vitamin D (VD_3) is mainly obtained through synthesis by skin exposed to sunlight, but it can also be obtained via diet. VD_3 is converted by 25-hydroxylase (25-OHase) enzyme in the liver to $25(OH)D_3$, and then by 1α-OHase in the kidney to $1,25(OH)_2D_3$, an active form of VD_3. $1,25(OH)_2D_3$ participates in calcium homeostasis and bone metabolism by acting through nuclear vitamin D receptor (VDR). VDR heterodimerizes with retinoid × receptors (RXRs) and this complex potentially mediates the cancer preventive effects of vitamin D (10). Besides the liver and kidney, VD_3 metabolizing enzymes are expressed in several other tissues (11;12), including the endometrium (13), and therefore local conversion of VD_3 to $25(OH)D_3$ or $1,25(OH)_2D_3$ could contribute to the actions of this vitamin. Compared to the normal tissue, 25-OHase, 1α-OHase and VDR are found to be over-expressed in several pre-malignant and well to moderately differentiated malignant tissues (14), also in the endometrium (13), and thus the enzymes or VDR might be useful targets to treat endometrial cancer (14).

The effects of vitamin D in preventing cancer may include cell differentiation and apoptosis (15); expression profiling has identified several targets reflective of these actions (16). It also has been proposed that osteopontin and E-cadherin mediate the effects of VD_3 (17). Osteopontin is an extracellular matrix glycophosphoprotein implicated in metastasis due to inducing anchorage-independent growth and abrogating adhesion (18). Osteopontin is a well-established target of VDR (19) and its expression also is increased by obesity (20). E-cadherin may mediate the growth inhibitory effects of VD_3 by inhibiting β-catenin transcriptional activity (19;21;22), but it is not known whether it's expression is affected by obesity.

The estrogen receptor (ER) might also be involved in mediating the actions of VD_3 on the endometrial cancer. Treatment of MCF-7 human breast cancer cells with $1,25(OH)_2D_3$ reduces ER levels in a dose-dependent manner (23), and suppresses E2-induced increase in progesterone receptor (PR) expression. Further, $1,25(OH)_2D_3$ inhibits MCF-7 cell growth, and decreases the growth-stimulatory effect of 17β-estradiol (E2) on these cells (24). These findings indicate that $1,25(OH)_2D_3$ exerts a direct negative effect on ER gene transcription, and thus the antiproliferative effects of $1,25(OH)_2D_3$ could be partially mediated through their action to down-regulate ER levels and thereby attenuate estrogenic bioresponses (23).

Obesity has an opposite effect on estrogen signaling than VD_3. First, obesity is associated with an increase in systemic estrogen levels due to a high level of aromatization of androgens occurring in adipose tissues (25); this is thought to be the key mechanism mediating the effects of obesity on reproductive system cancers, including endometrial cancer. Second, there is some evidence that obese women exhibit higher levels of ER in the endometrium (26) and breast tumors (27) than lean women. The possibility that vitamin D might interact with the effects of obesity on endometrial cancer risk has not been investigated, but based on the observations that vitamin D and obesity have opposing effects on ER signaling, we sought to test the hypothesis that vitamin D intake may prevent the effects of obesity on endometrial cancer risk and the protective effect might occur through ER.

Heterozygous Phosphatase and Tensin Homologue Deleted on Chromosome 10 ($Pten$)$^{+/-}$ mice were used for this purpose. PTEN is a known tumor suppressor, and frequently mutated or deleted in many cancers, particularly in endometrial cancer (28). Homozygous PTEN deletion is embryonically lethal, but lack of one allele of this gene is sufficient to induce multifocal hyperplasia with atypia and endometrial cancer which is detected between 28 and 52 weeks of age in heterozygous $Pten^{+/-}$ mice (29). It has been proposed that $Pten^{+/-}$

mice represent the most biologically relevant model of human endometrial cancer available (30). Additional benefit of using *Pten*$^{+/-}$ mice here is that loss of PTEN has been found to activate ERα-dependent pathways that are then suggested to be pivotal for the neoplastic process in these mice (31).

Our results indicate that dietary exposure to 25K IU VD_3 for 24 weeks prevented the obesity-induced increase in endometrial pre-malignant and malignant lesions in *Pten*$^{+/-}$ mice. VD_3 also increased bone density, but did not induce any toxicity. The effect of VD_3 against obesity-induced increase in endometrial carcinogenesis may be related to inhibition of obesity-induced increase in osteopontin levels and up-regulation of E-cadherin, but it is unlikely to be explained by changes in endometrial expression of ER-α, ER-β or PR in *Pten*$^{+/-}$ mice.

METHODS

Animals

Heterozygous *Pten*$^{+/-}$ mice (B6.129-^{Ptentm1Rps}), which are at C57BL/6 background and were obtained from MMHCC at NCI (Frederick, MD), were used. The mouse colony was established by breeding wildtype C57BL/6 female mice with heterozygous *Pten*$^{+/-}$ male mice. *Pten*$^{+/-}$ female offspring develop endometrial hyperplasia, some of which progress to adenocarcinomas starting at about 28 weeks of age (32). On the week before weaning at age 21 days, tail samples were obtained and the offspring were genotyped using the primers specified by MMHCC (http://mouse.ncifcrf.gov/protocols.asp?ID=01XH3&pallele=Pten%3Ctm1Rps%3E&prot_no=1).

Mice were housed at the Georgetown University Comparative Medicine Research Facility at appropriate temperature and a standard 12hr light-dark cycle. When not otherwise specified, they were fed pelleted semi-purified American Institute of Nutrition (AIN) 93G diet. All the studies were approved by the Institutional Animal Care and Use Committee.

Post-weaning dietary exposures

Four-week-old female *Pten*$^{+/-}$ and wildtype mice were each divided to four treatment groups (n=8–12 per group) and fed AIN93G based diet containing either (1) 18% energy from fat and 1K international units (IU) of cholecalciferol/kg diet (=standard AIN93G diet; in this paper this diet is called control diet and cholecalciferol is called VD_3), (2) 18% fat and 25K IU of VD_3/kg diet, (3) 58% fat to induce obesity (obesity-inducing AIN93G based diet, OID) containing 1.8K IU of VD_3/kg diet , and (4) 58% fat and 25K IU of VD_3/kg diet. OID contains more VD_3 than AIN93G diet, because of an excessive deposition of VD_3 in body fat (33) resulting obese individuals to have lower 25(OH)D_3 levels (34). The daily adequate allowance of VD_3 in humans is 0.4 IU; however, it is not clear what is the recommended daily allowance (RDA) or upper limit (UL) for VD_3 (http://ods.od.nih.gov/factsheets/vitamind.asp). Some studies suggest that it is as high as 10,000 IU/day (35). The dose of VD_3 used in our study – 25K IU – is 2.5 times higher that the highest dose recommended for humans (35). However, due to metabolic differences between the two species (36), higher VD_3 exposure levels in mice are required to achieve the same biological effects seen in humans.

The mice were kept on these diets until they were sacrificed at 28 weeks of age; i.e., a total of 24 weeks. The diets were prepared by Harlan Teklad (Madison, WI). The fat content of the diets were slightly modified from AIN93G diets; all the diets contained 50 g/kg soybean oil (the sole oil in AIN93G diet) and either 20 g/kg (AIN93G) or 300 g/kg feed lard (OID). VD_3 was added to fat modified diets.

All mice were weighed once per week using a digital scale to determine changes in body weight development from weaning to 28 weeks of age.

End-points determined at 28 weeks of age

When mice were 28 weeks of age, they were sacrificed to determine the presence of pathological changes in the endometrium. Thus, endometrium was collected and the middle sections of each uterine horn were removed and processed for paraffin blocks for immunohistochemistry and histopathology. The remaining tissue of the two horns were stored in −80 °C for Western blot and real time PCR assays.

Endometrial mRNA expression of 25-hydroxylase (25-OHase), 1α-OHase, 24-OHase, vitamin D receptor (VDR), estrogen receptor (ER)-α, ER-β and progesterone receptor (PR) was determined by real time PCR. Immunohistochemical analysis was used to measure ER-α and PR protein levels separately in the luminal or glandular epithelium and in the endometrial stroma. Pten protein levels were measured using Western blot.

Bone mineral density (BMD) and bone mineral content (BMC) were determined from the carcass using DXA.

Pre-malignant and malignant changes in the endometrium

Changes in endometrial morphology were assessed from histopathological sections processed as paraffin blocks, following the guidelines set by Fyles et al (30). Transverse sections of the uterine horns, and longitudinal sections of the uterocervical junction and ovaries, were examined by a board-certified veterinary pathologist (JMC) blinded to treatment group and genotype. Complex hyperplastic lesions were identified by glandular proliferation and crowding. Cellular atypia was noted in some lesions, consisting of glandular epithelial cell enlargement, loss of normal cellular polarity, and altered nuclear features (dispersed chromatin and prominent nucleoli). Adenocarcinoma was identified by invasion with disruption of the glandular basement membrane.

Endometrial mRNA expression of 25-OHase, 1α-OHase, 24-OHase, VDR, osteopontin, E-cadherin, ER-α, ER-β and PR

Total RNA was extracted from the endometrium of 4–8 *Pten*$^{+/-}$ and wildtype mice per group, kept on the four different diets. RNA was then cDNA reverse-transcribed from 100 μg/ml of total input RNA using Taqman Reverse Transcription Reagents as described by the manufacturer (Applied Biosystems, Foster City, CA). Next, PCR products were generated from the cDNA samples using the Taqman Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand (Applied Biosystems) for the appropriate target gene. The 18S Assay-on-Demand (Applied Biosystems) was used as an internal control. All assays were run on 384 well plates so that the cDNA sample from each endometrium was run in triplicate for the target gene and the endogenous control. Real time PCR was performed on an ABI Prism 7900 Sequence Detection System and the results assessed by relative quantitation of gene expression using the $\Delta\Delta C_T$ method.

Immunohistochemistry to determine ER-α and PR protein levels in the epithelial and stromal compartments of the endometrium

Five μm paraffin sections, cut transverse, were deparaffinized and rehydrated from xylene through a graded series of ethanol. Antigen retrieval was carried out in a high pH Target retrieval solution (pH 9, Dako S2368, Carpinteria, CA, USA) in a pressure cooker for 20 min, followed by 2 hours cool down in RT. After blocking of endogeneous peroxidases the sections were incubated with monoclonal mouse anti-human ERα (M7047, Dako, Carpinteria, CA, USA, 1:35 dilution) and polyclonal rabbit anti-human PR (A0098, 1:400

dilution) primary antibodies. For negative controls a corresponding IgG was used. The slides were incubated with the primary antibody in +4°C overnight, followed by a secondary antibody and detection using Dako's EnVison™ Dual Link System HRP DAB+ (K4065, Carpinteria, CA, USA), as instructed by the manufacturer, and counterstained with Harris Hematoxylin (Fisher Scientific, Kalamazoo, MI, USA). To quantify the immunohistochemical staining for ERα and PR, the sections were scored both for the number of positive cells and the intensity of the staining separately in the luminal or glandular epithelium and uterine stroma using a score modified from Allred *et al.* (37).

Western blot to determine Pten protein levels

The uterine tissue was homogenized, centrifuged and the protein extract collected from the supernatant. 50 μg of protein extract was loaded onto a NuPAGE 12% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA), and gels were run at 150 V. Membranes were then washed with TBST and blocked in 5% milk in TBST for 30 min at room temperature. After blocking, membranes were incubated with antibodies against Pten (1:500 dilution, Cell Signaling Technology, Danvers, MA) overnight at 4°C. Next, membranes were incubated with secondary anti-rabbit IgG or mouse IgG horseradish peroxidase antibodies (1:5000 dilution, Amersham Pharmacia Biotech, Piscataway, NJ) and developed using Super Signal (Pierce, Rockford, IL). Fold differences were calculated by normalization against beta-actin.

Bone density

Bone mineral density (BMD) and bone mineral content (BMC) were determined using dual-energy X-ray absorptiometry (DXA) (GE Lunar Piximus II, Madison, WI). This instrument has been validated for measures of body composition and bone density in mice (38;39). Necropsied carcasses were placed on the specimen tray and scanned a single time.

Data Analysis

Diet-induced changes in body weight were determined using repeated measures analysis of variance (ANOVA). Where appropriate, between-group comparisons were done using Fisher's LSD method. To determine whether endometrial changes in *Pten*$^{+/-}$ mice were modified by dietary VD_3 and/or high fat exposures, Chi2 test was used. Two-way ANOVA was used to assess treatment effects on Pten expression, VD_3 metabolic enzymes, osteopontin, E-cadherin, hormone receptors, body composition and bone characteristics. When the data were not normally distributed, the results were log transformed prior to analysis. Correlations among (a) ER-α, ER-β and PR, and (b) VD_3 metabolic enzymes and VDR and endocrine histopathology were assessed using Spearman rank order correlation. Analysis of Covariance ANCOVA was used to determine body weight-independent effects of treatments on bone end-points (40). The analyses were performed using SigmaStat Version 3.0 or SAS JMP Version 5.0. The differences were considered significant if the p-value was less than 0.05. All probabilities were two-tailed.

RESULTS

Effects of OID and VD_3 on body weight

Repeated measures ANOVA revealed a significant increase in body weight over time ($p<0.001$) and differences in the amount of weight gain among different dietary groups ($p<0.001$). Exposure to an OID doubled body weights in wildtype mice ($p<0.001$) (Fig. 1a), and increased them in *Pten*$^{+/-}$ mice by 36% ($p<0.001$) (Fig. 1b). Feeding mice a control diet supplemented with 25K IU VD_3 increased body weight by 38% in the wildtype ($p<0.008$) and 17% in the *Pten*$^{+/-}$ mice (not significant), when compared to the control diet fed mice. Vitamin D_3 supplementation did not modify the effects of OID on body weight. No

significant differences in weight gain between wildtype and *Pten*$^{+/-}$ mice were seen, although *Pten*$^{+/-}$ mice on the AIN93G diet were slightly heavier than wildtype mice throughout the study.

Since VD_3 has been reported to interact with Pten expression (41;42), and such changes could explain the effect of VD_3 on endometrial carcinogenesis in the *Pten*$^{+/-}$ mice, we determined Pten protein levels. Mammary tissues were used for this analysis, because they exhibit less histopathological changes than the endometrium in 28-week-old *Pten*$^{+/-}$ mice (32); transformed tissue may respond differently to VD_3 than normal tissue does (13). As expected, Pten protein expression was significantly lower in the *Pten*$^{+/-}$ mice than in the wildtype mice ($p<0.001$). VD_3 diet did not impact Pten levels in the *Pten*$^{+/-}$ or wildtype mice (Fig. 1c), but OID significantly increased the expression of this gene in both genetic backgrounds ($p<0.047$).

Histopathological changes in the endometrium

The uterine endometrium was examined histologically for evidence of hyperplasia or malignancy. The morphology of lesions was as described previously for this model (30). The findings were characterized as normal, multifocal glandular hyperplasia, (multi)focal glandular hyperplasia with atypia, and endometrial adenocarcinoma. Endometrial hyperplasia with cytologic atypia represents a much greater risk for progression to endometrial cancer than do hyperplasias without cytologic atypia (43). For example, over 50% of women who have atypical hyperplasia at biopsy or curettage are diagnosed with adenocarcinoma in subsequent hysterectomy (44).

None of the wildtype mice developed pre-malignant lesions, defined as focal or multifocal glandular hyperplasia with atypia, whilst 58% of *Pten*$^{+/-}$ mice did. Feeding *Pten*$^{+/-}$ mice an OID increased the pre-malignant and malignant lesions to 78%, with one mouse exhibiting endometrial adenocarcinoma. Dietary exposure to VD_3 significantly decreased the incidence of these endometrial lesions in *Pten*$^{+/-}$ mice fed OID to 25% (Chi^2-test: $p<0.001$) (Table 1a). Figure 2 shows (a) endometrium with multifocal glandular hyperplasia in control diet fed *Pten*$^{+/-}$ mice, (b) endometrium with (multi)focal glandular hyperplasia and atypia in VD_3 supplemented *Pten*$^{+/-}$ mice, (c) endometrial adenocarcinoma in OID fed *Pten*$^{+/-}$ mice, and (d) normal endometrial tissue in obese *Pten*$^{+/-}$ mice supplemented with 25K IU of VD_3.

Effects on the expression of 25-OHase, 1α-OHase, 24-OHase and VDR

25-OHase expression was significantly higher in the *Pten*$^{+/-}$ than wildtype mice ($p<0.002$) (Fig. 3a). Dietary exposures affected 25-OHase expression in the endometrium ($p<0.043$). VD_3 increased 25-OHase expression in the obese wildtype mice ($p<0.027$). Obese *Pten*$^{+/-}$ mice supplemented with VD_3 expressed higher levels of 25-OHase than normal weight VD_3 supplemented mice ($p<0.036$); however, no significant differences were seen among the control diet fed, VD_3 supplemented normal weight and obese *Pten*$^{+/-}$ mice. (Fig. 3a).

1α-OHase expression was not significantly different between the wildtype and *Pten*$^{+/-}$ mice (Fig. 3b). However, 1α-OHase expression was increased by VD_3 in the normal weight wildtype ($p<0.003$) but not *Pten*$^{+/-}$ mice (interaction: $p<0.032$) (Fig. 3b). No other significant changes were seen.

24-OHase expression was not different between the genotypes or among different dietary groups (Fig. 3c).

VDR expression was higher in the *Pten*$^{+/-}$ mice than the wildtype mice ($p<0.006$). VD_3 supplementation did not have any effect on endometrial VDR expression in the wildtype

mice, but it reduced the expression of this receptor in the normal weight *Pten*$^{+/-}$ mice ($p<0.016$) (interaction: $p<0.023$).

Histopathological changes in the endometrium and expression of VD_3 metabolic enzymes or VDR. We also determined whether the presence of benign, pre-malignant or malignant changes in the endometrium of *Pten*$^{+/-}$ mice affected the expression of VD_3 metabolic enzymes or VDR. No significant differences were found in the expression of 25-OHase, 1α-OHase, 24-OHase or VDR among normal, benign lesion, hyperplasia with atypia and cancer (Table 1b), and neither did the expression of VD_3 metabolic enzymes or VDR correlate with the degree of transformation of the endometrial tissue.

Effects on the expression of E-cadherin and osteopontin mRNA

Both E-cadherin ($p<0.004$) and osteopontin ($p<0.001$) levels were significantly higher in the *Pten*$^{+/-}$ than wildtype mice (Fig. 4). Vitamin D or obesity did not have significant effects on E-cadherin expression in either wildtype or *Pten*$^{+/-}$ mice, but in both genotypes E-cadherin was significantly higher in VD_3 supplemented obese mice than in the control diet fed mice ($p<0.019$) (Fig. 4a and b).

Dietary exposures affected the expression of osteopontin ($p<0.003$); however, the effects were different in wildtype and *Pten*$^{+/-}$ mice (p for interaction<0.002). Osteopontin levels were significantly elevated by VD_3 in the wildtype mice ($p<0.007$), but not in the *Pten*$^{+/-}$ mice (Fig. 4c and d). Obesity significantly increased osteopontin levels in the *Pten*$^{+/-}$ mice ($p<0.001$), but the difference failed to reach significance in the wildtype mice ($p<0.11$). In both obese wildtype ($p<0.049$) and *Pten*$^{+/-}$ mice ($p<0.001$), VD_3 reversed the increase in osteopontin levels.

Effects on the expression of ER-α, ER-β and PR mRNA

Since *Pten*$^{+/-}$ mice have been previously reported to express higher levels of ER-α than wildtype mice (30), we first compared the levels of expression in the endometrium between these two groups of mice kept on the control diet. The data indicated that the endometrium of *Pten*$^{+/-}$ mice expressed significantly elevated levels of ER-α ($p<0.014$). However, this difference disappeared when mice were fed OID or supplemented with VD_3 (Fig. 5Aa). Further, neither ER-β (Fig. 5Ab) nor PR (Fig. 5Ac) mRNA expression was altered among different dietary exposure groups. We also determined ER-α/ ER-β ratio, and it was not affected (Fig. 5Ad)

The level of expression of ER-α and PR is known to be strongly linked to each other, and therefore we compared the expression of the three receptors to each other. Highly significant correlations emerged between ER-α and ER-β ($p<0.0001$) or PR ($p<0.0001$), and between PR and ER-β ($p<0.0001$).

Protein

It is possible that the failure to observe any diet-induced differences in ER or PR expression was due to assessing these receptors in the mRNA obtained from the whole endometrial tissue. To address this possibility, we determined ER-α and PR protein levels by immunohistochemistry which allowed quantitation of these nuclear receptors in the luminal epithelium, glandular epithelium and stroma. ER-α protein levels were not different between the wildtype and *Pten*$^{+/-}$ mice (Fig. 5Ba). The levels of PR in the luminal epithelium were significantly lower in the *Pten*$^{+/-}$ mice, in all dietary exposure groups, compared to wildtype mice ($p<0.041$). No genotype –specific changes were seen in the glandular epithelium (Fig. 5Bb), but in the stroma, PR levels were non-significantly higher in the *Pten*$^{+/-}$ than wildtype

mice (Fig. 5Bc). The latter might explain why PR mRNA levels, determined in the whole uterus, were not altered (Fig. 5Ac).

Bone density

Mice fed VD_3 supplemented diet had higher bone mineral density (BMD) than other mice, regardless of genotype ($p<0.0015$). Adjustment for body weight using analysis of covariance did not alter this result. We also noted that BMD was strongly correlated with body mass in the control diet fed mice ($p<0.0001$). However, no such correlation was seen in the obese mice ($p<0.36$); i.e., the increase in body weight in these mice reflected an increase in adipose depot size, while an increase in body weight in control diet fed mice resulted from an increase in lean mass and to a small extent in bone mass. There was a small, but statistically significant interaction between genotype and diet on bone mineral content (BMC). Obese wildtype mice had greater BMC than *Pten*$^{+/-}$ mice; this difference was not seen in mice fed the control diet (Table 2).

DISCUSSION

Similarly to epidemiological studies in obese women showing an increased endometrial cancer risk (4;5), we found that obesity increased the risk of development of endometrial premalignant and malignant lesions in the *Pten*$^{+/-}$ mouse model. Dietary supplementation with VD_3 inhibited the carcinogenic effect of obesity on the endometrium. The protective effect of VD_3 against endometrial cancer in humans remains controversial, although the interactions among VD_3, obesity and endometrial cancer have not been studied. Some evidence suggests that women exposed to high levels of VD_3 are at a reduced risk of developing endometrial cancer (6;7), but some other studies question the existence of an association (45;46). In our study, VD_3 supplementation did not reduce the incidence of pre-malignant endometrial changes in normal weight *Pten*$^{+/-}$ mice. However, had we assessed the effect of vitamin D at a later time point when more endometrial tumors are present, the findings might have been different. Additional studies on VD_3 and endometrial cancer are needed, including animal studies.

Pten$^{+/-}$ mice are an excellent animal model of human endometrial cancer (30), partly because a loss of PTEN is a common event in endometrial cancer in women (28). In the present study, we found that *Pten*$^{+/-}$ mice exhibit an increased expression of ER-α mRNA in the endometrium, and reduced expression of PR protein in the luminal epithelium. These observations are consistent with the data obtained in women. High ER-α expression in the endometrium is associated with increased endometrial cancer risk (47) and progression (47–50). Further, PR expression, either PR-A or PR-B, is lower in hyperplastic and malignant endometrial tissues than in normal endometrial tissue (51;52). Since ER-α is the predominant ER form in the uterus, ER-β may not play a role in uterine cancer (48;49).

Regardless of the similarity of the changes in ER-α and PR expression in the endometrium of *Pten*$^{+/-}$ mice and women at high risk of endometrial cancer, there is some controversy as to whether endometrial carcinogenesis in *Pten*$^{+/-}$ mice is dependent on ER-α and PR. Vilgelm et al. (31) proposed that a loss of Pten results in the activation of ER-α-dependent pathways that are then pivotal for the neoplastic processes occurring in the endometrium of *Pten*$^{+/-}$ mice. However, Fyles et al. (30) reported that neither ovariectomy nor an exposure to progestin modifies endometrial cancer risk in *Pten*$^{+/-}$ mice. In accordance with these data, we found no evidence that VD_3 intake or an exposure to obesity-inducing OID affected the gene or protein expression of ER-α, ER-β and PR in the *Pten*$^{+/-}$ or wildtype mice. The reason why OID did not affect steroid receptors may be because this diet does not increase circulating estradiol levels, but it increases leptin levels (53). Thus, our data do not support a

role for the three receptors in mediating the endometrial cancer risk increasing effect of an OID or the protective effects of VD_3 in obese *Pten*$^{+/-}$ mice.

Vitamin D up-regulates both osteopontin (19), which promotes anchorage-independent growth, and E-cadherin (19;21;22), which inhibits cell proliferation and invasion. This dual effect may explain why vitamin D has been reported to reduce the incidence and growth of some cancers and possibly increase some (17;19). *Pten*$^{+/-}$ mice expressed significantly higher levels of both genes than the wildtype mice did. In the uterus of ***lean*** wildtype and *Pten*$^{+/-}$ mice, VD_3 did not affect E-cadherin expression, however, in ***obese*** mice, VD_3 increased the expression of this gene. As expected, VD_3 increased osteopontin levels in the wildtype mice; this effect was not seen in the *Pten*$^{+/-}$ mice. However, VD_3 supplementation inhibited the increase in osteopontin mRNA levels in both wildtype and *Pten*$^{+/-}$ mice. These findings suggest that VD_3 may prevent obesity-induced increase in endometrial cancer by up-regulating E-cadherin and down-regulating osteopontin expression.

In accordance with previous studies (54), an OID significantly increased the body weight, and this was seen both in the wildtype and *Pten*$^{+/-}$ mice. However, the *Pten*$^{+/-}$ mice gained less weight than the wildtype mice did. One possible explanation for these findings is the role of PTEN in insulin signaling. Insulin controls glucose and lipid metabolism through phosphatidylinositol 3-kinase (PI3K) and serine-threonine kinase AKT. Since PTEN is a negative regulator of the PI3K/AKT pathway, it also inhibits the metabolic effects of insulin (28;55). Down-regulation of PTEN, in turn, reverses insulin resistance in diabetic mice (56). Further, *Pten*$^{+/-}$ mice, or mice with adipose-tissue specific loss of Pten, exhibit improved systemic glucose tolerance and insulin sensitivity, and decreased fasting insulin levels (57), but no changes in body weight or adiposity (57;58). Thus, the adverse effects of OID on lipid metabolism may have been less severe in the *Pten*$^{+/-}$ mice than in the wildtype mice. Nevertheless, the increase in body weight in OID fed *Pten*$^{+/-}$ mice was sufficient to lead to an increased endometrial carcinogenesis.

Vitamin D is reported to up-regulate PTEN expression in cancer cells (42), and therefore VD_3 supplementation in our study might have reduced endometrial cancer risk in obese *Pten*$^{+/-}$ mice by increasing the expression of the remaining Pten allele. PTEN gene also participates in mediating the growth inhibitory actions of vitamin D on cancer cells (41). Consequently, *Pten*$^{+/-}$ mice might be less sensitive for the effects of VD_3 than mice which have both alleles of this gene. In our study, dietary VD_3 did not modify Pten expression in the wildtype or *Pten*$^{+/-}$ mice. However, OID increased Pten protein levels in both genetic backgrounds. This effect is consistent with obesity leading to insulin resistance (59), and high PTEN levels being related to insulin insensitivity (57;60). *Pten*$^{+/-}$ mice are responsive to vitamin D. In the previous study, the prostates of male *Pten*$^{+/-}$ mice exposed to $1,25(OH)_2D_3$ via a subcutaneous pump exhibited less high-grade PIN with invasions than mice receiving a placebo (61). However, because PTEN participates in mediating the actions of vitamin D (41), it is possible that the chemopreventive effect of VD_3 were underestimated in studies which utilize *Pten*$^{+/-}$ mice.

We addressed the sensitivity of the *Pten*$^{+/-}$ mice to VD_3 by measuring the expression of enzymes which metabolize VD_3 to its biologically active form, $1,25(OH)_2D_3$. If wildtype mice are more sensitive to the actions of VD_3 than *Pten*$^{+/-}$ mice, they are expected to express more significant changes in the expression of VD_3 metabolizing enzymes and VDR. Previous studies indicate that vitamin D up-regulates 25-hydroxylase (25-OHase) in the liver where it converts VD_3 to $25(OH)D_3$, but down-regulates 1α -OHase in the kidney where this enzyme converts $25(OH)D_3$ to $1,25(OH)_2D_3$ (62). VDR is also reported to be down-regulated by VD_3 (62). However, vitamin D may not induce similar changes in all the tissues where these enzymes are expressed (63).

We found that both wildtype and *Pten*$^{+/-}$ mice were affected by VD_3, although the responses were slightly different. VD_3 increased the expression of 25-OHase in the wildtype and obese *Pten*$^{+/-}$ mice. This is consistent with the reported effect of VD3 on 25-OHase in the liver (62) and other tissues (64). The expression of 1α-OHase was increased by VD_3 in the endometrium of wildtype mice, which is opposite to down-regulation reported in the kidney (62). Consistent with previous findings (62), VD_3 reduced the expression of VDR, but this was seen only in the *Pten*$^{+/-}$ mice. The differences between the wildtype and *Pten*$^{+/-}$ mice may originate from interactions between VD_3 and PTEN (41;42), but they are not reflective of *Pten*$^{+/-}$ mice being less sensitive to VD_3 than the wildtype mice were. Alternatively, the differences may be related to carcinogenic process taking place in the endometrium of the *Pten*$^{+/-}$ mice. In humans, expression of 25-OHase, 1α-OHase and VDR are found to be higher in several pre-malignant and well to moderately differentiated malignant tissues (14), including the endometrium (13), when compared to the corresponding normal tissue. Reduced expression is seen in poorly differentiated cancers (14). Consistent with these reports, we found that the expression of 25-OHase and VDR were higher in the endometrium of the *Pten*$^{+/-}$ than wildtype mice. However, when we compared the expression of VDR and vitamin D metabolic enzymes in the normal, benign and pre-malignant/malignant endometrial tissues within *Pten*$^{+/-}$ mice, no significant differences were observed. These results suggest that although the endometrium of some *Pten*$^{+/-}$ mice have not undergone histopathological changes by week 28, the fact that it eventually will (32;65) is sufficient to increase the expression of vitamin D metabolic enzymes and VDR. It is possible that changes in vitamin D signaling in the endometrium are predictive of increased endometrial cancer.

A major limitation in using the biologically active form of vitamin D, $1,25(OH)_2D_3$, is its toxicity due to hypercalcemia (66). In contrast, chronic administration of relatively high doses of VD_3/cholecalciferol (up to 20K international units, IU) appears safe (67). In our study, there was no indication that dietary exposure to 25K IU VD_3 induced toxicity; i.e., weight loss or early death. In fact, we found that an intake of a diet containing 25K IU cholecalciferol for 24 weeks resulted in a higher bone mineral density (BMD), when compared to mice fed a control diet. Obese wildtype mice exhibited the highest bone mineral content (BMC), whilst obese *Pten*$^{+/-}$ mice exhibited lowest BMC. In *Pten*$^{+/-}$ mice, BMC was highest in the VD_3 supplemented mice. Since BMD and BMC were similar in wildtype and *Pten*$^{+/-}$ mice fed AIN93G diet, it is not clear why genotype affected the response of consuming VD_3 supplemented diet or OID on the bone. Possible explanations include the role of PTEN in insulin signaling (57;60), and interactions between PTEN and vitamin D (41;42) which were already discussed above.

In conclusion, we found that dietary exposure to 25K IU of VD_3 prevented the obesity-induced increase in premalignant and malignant endometrial lesions in *Pten*$^{+/-}$ mice. VD_3 did not have any notable hypercalcemic effects, and it increased bone mineral density. Dietary VD_3 exposure affected the expression of VD_3 metabolic enzymes and VDR in the endometrium, but the effects were different in the wildtype and *Pten*$^{+/-}$ mice, possibly reflecting the role of these enzymes and VDR as putative treatment targets in the malignant tissue (14) or alternatively caused by the reported interactions between VD_3 and PTEN (41;42). However, VD_3 did not alter Pten expression in the present study. Although the cancer-risk reducing effects of VD_3 might occur via inhibition of ER-α signaling (23;24), and although *Pten*$^{+/-}$ mice exhibited increased levels of ER-α and reduced levels of PR in the endometrium, there was no evidence of the involvement of these receptors in mediating the protective effects of VD_3. Our study suggests that down-regulation of osteopontin and an increase in E-cadherin levels by VD_3 in obese *Pten*$^{+/-}$ mice may explain how this vitamin reduces obesity-promoted endometrial cancer.

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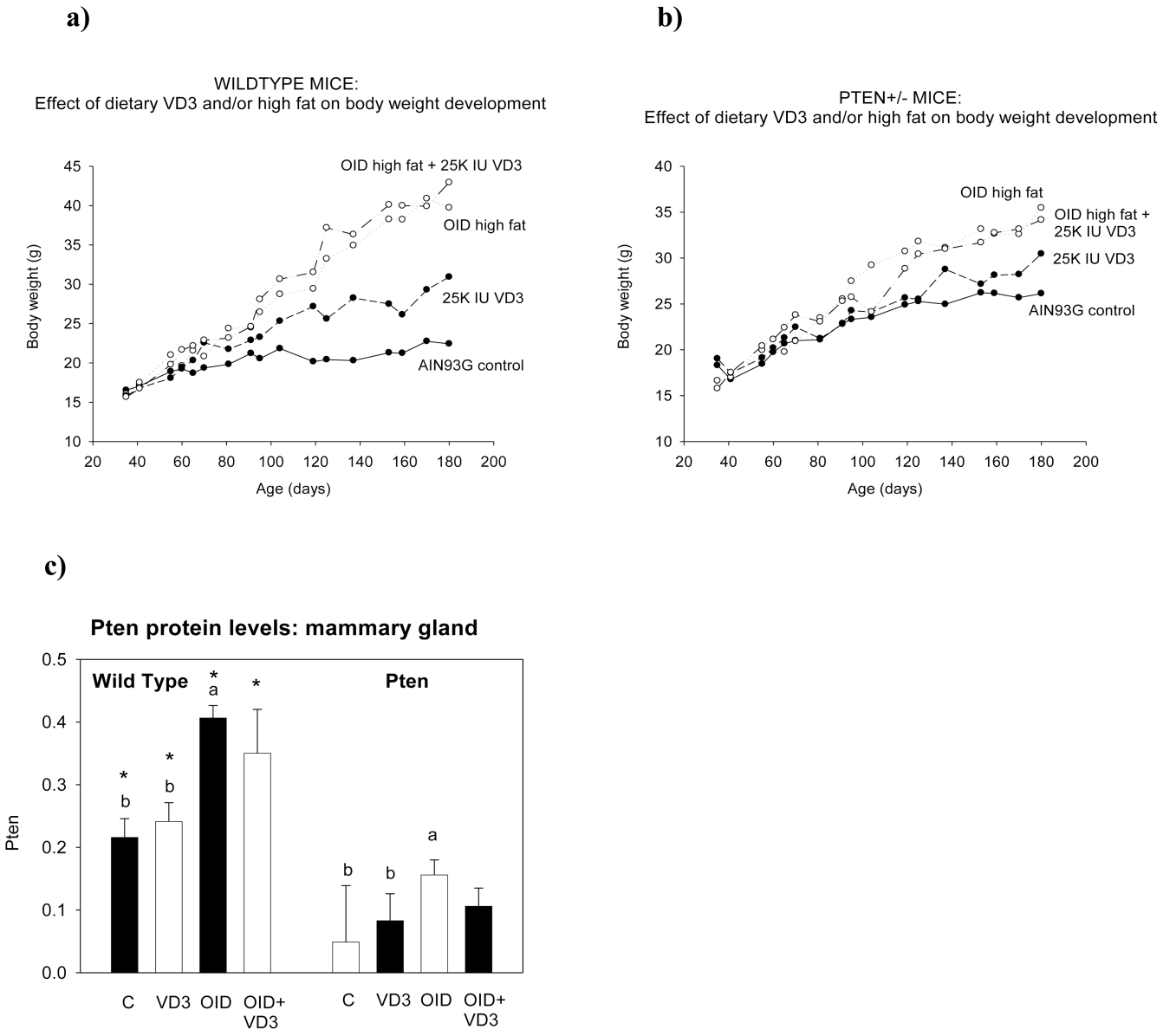

Figure 1.

Changes in body weight between postnatal weeks 4 and 28 in the **(a)** wildtype and **(b)** *Pten*$^{+/-}$ mice fed AIN93G based **control** diet containing either 18% energy from fat and 1K international units (IU) of cholecalciferol (VD_3)/kg feed; **vitamin D** supplemented control diet containing 25K IU of VD_3/kg diet; **OID** containing 58% fat and 1.8K UI VD_3/kg diet; and **vitamin D supplemented OID**. When compared to control diet fed mice, OID significantly increased body weights in wildtype and *Pten*$^{+/-}$ mice ($p<0.001$). Body weights were also elevated in wildtype mice fed VD_3 diet ($p<0.008$) or VD_3 supplemented OID ($p<0.001$). Mean ± SEM of 8–12 mice per group are shown. **(c)** Pten protein levels, assessed using Western blot, in the mammary gland of 28-week-old wildtype and *Pten*$^{+/-}$ mice. Bars marked with a different letter are statistically significantly different from each other. Mean ± SEM of 5–7 mice per group are shown.

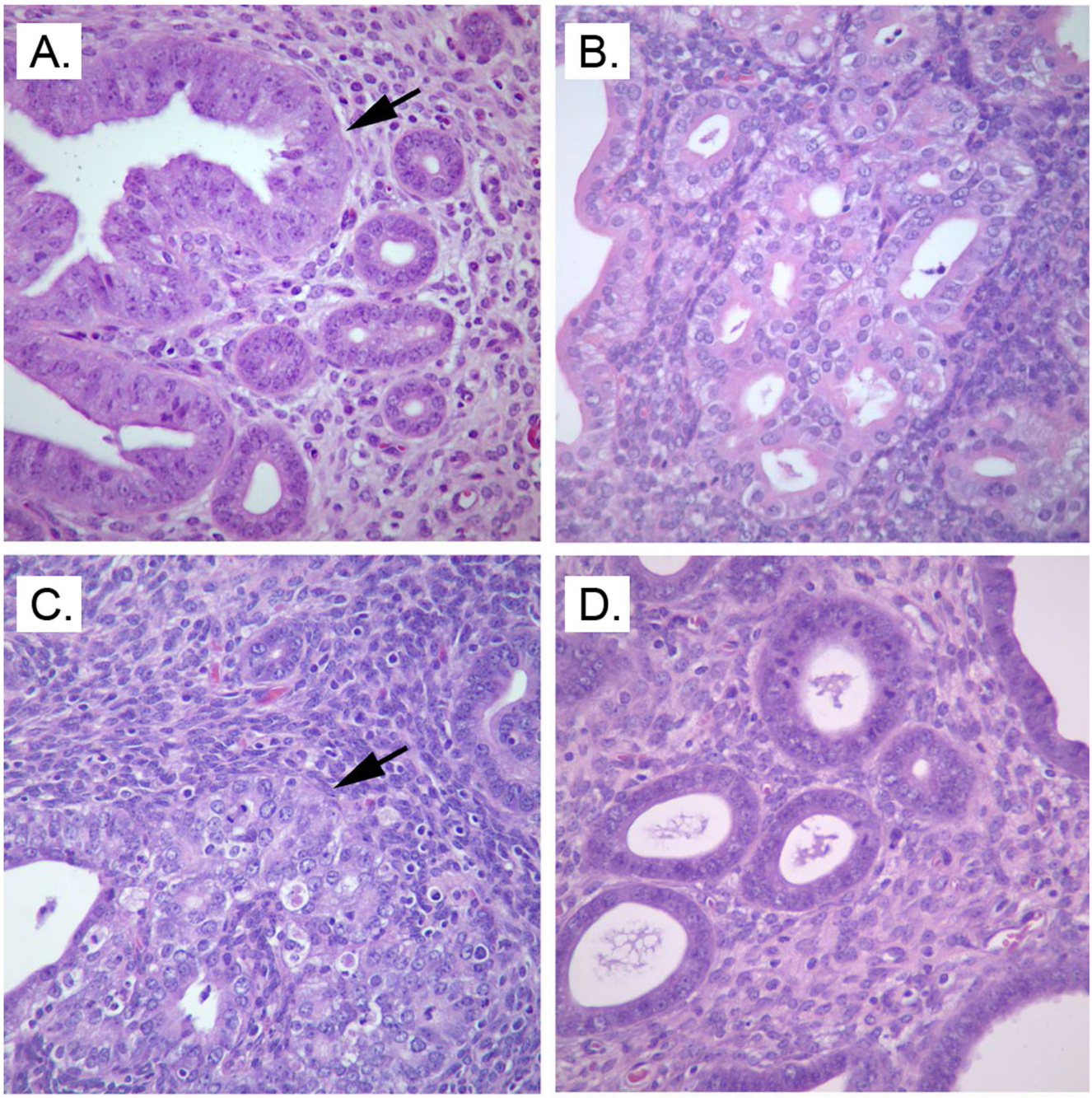

Figure 2.

Mouse endometrium showing **(A)** endometrium with glandular hyperplasia and atypia in *Pten*$^{+/-}$ mouse fed the control diet, **(B)** endometrium with glandular hyperplasia in *Pten*$^{+/-}$ mouse fed VD_3 supplemented diet, **(C)** endometrial adenocarcinoma in *Pten*$^{+/-}$ mouse fed the OID, and **(D)** normal glandular morphology, as in wildtype mice, in *Pten*$^{+/-}$ mouse fed the OID supplemented with VD_3. Endometria were obtained from 28-week-old mice, and sections were stained with hematoxylin and eosin, with 40×objective magnification.

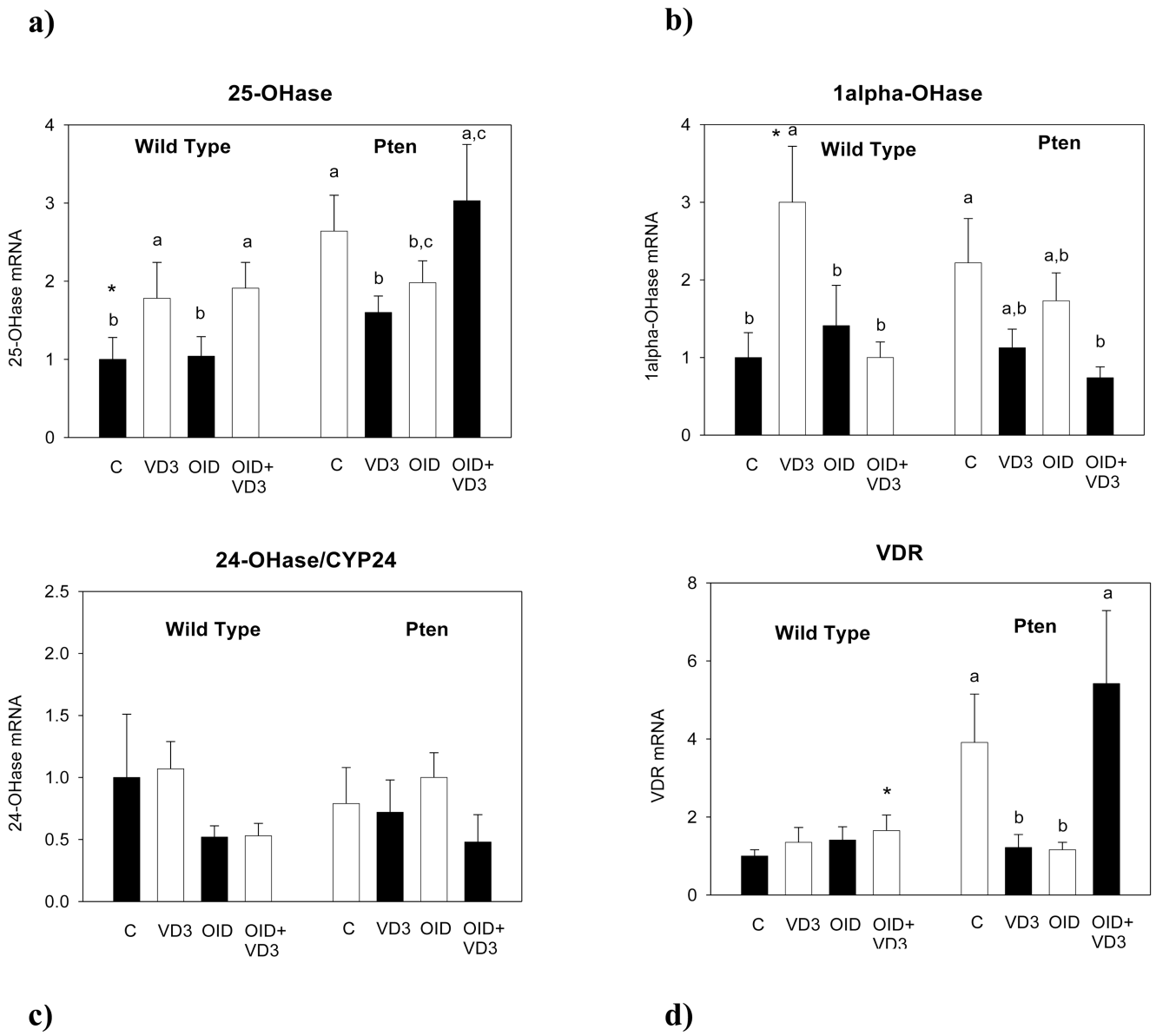

Figure 3.

mRNA expression of VD_3 metabolic enzymes **(a)** 25-hydroxylase (25-OHase), **(b)** 1α-OHase, **(c)** 24-OHase, and **(d)** vitamin D receptor (VDR) in the endometrium of 28-week-old wildtype and *Pten*$^{+/-}$ mice fed control diet (C), VD_3 supplemented control diet (VD_3), obesity-inducing diet (OID), or VD_3 supplemented OID (OID+ VD_3) for 24 weeks. Bars marked with a different letter are statistically significantly different from each other. RT-PCR was used, and data were quantitated using the $\Delta\Delta C_T$method and normalized to the control diet fed wildtype group. Mean ± SEM of 5–7 mice per group are shown.

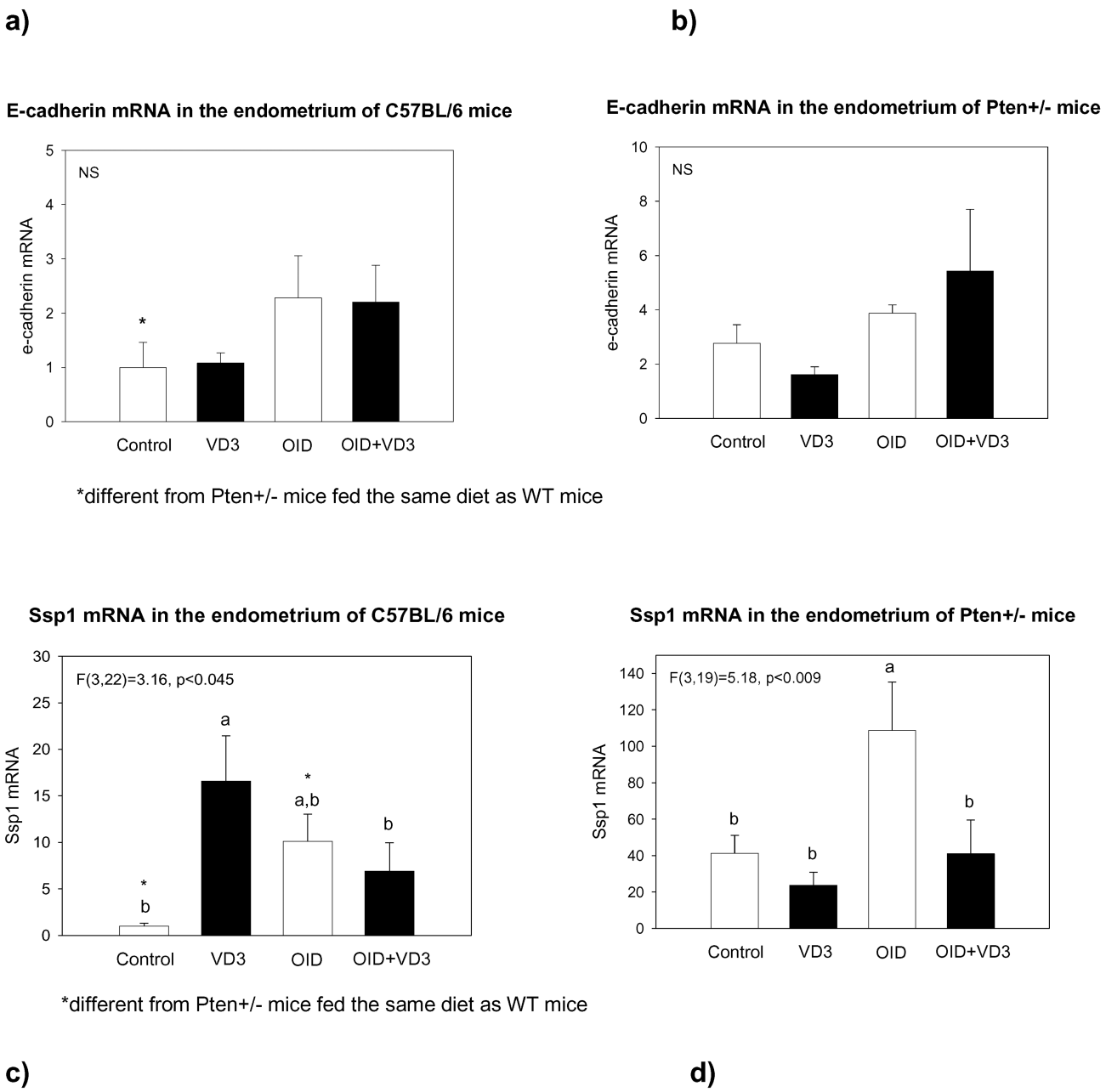

Figure 4.

mRNA expression in the endometrium of E-cadherin in **(a)** wildtype and **(b)** *Pten*$^{+/-}$ mice, and osteopontin in **(c)** wildtype and **(d)** *Pten*$^{+/-}$ mice which were fed control diet, VD_3 supplemented control diet, OID, or VD_3 supplemented OID for 24 weeks. E-cadherin levels were higher in the *Pten*$^{+/-}$ than wildtype mice ($p<0.004$), and VD_3 increased the expression in obese mice ($p<0.019$). Osteopontin (Ssp1) levels also were significantly higher in *Pten*$^{+/-}$ and wildtype mice ($p<0.001$), and VD_3 reversed the increase seen in obese mice ($p<0.003$). RT-PCR was used, and data were quantified using the $\Delta\Delta C_T$method and normalized to the control diet fed wildtype group. Bars marked with a different letter are statistically significantly different from each other. Mean ± SEM of 4–8 mice per group are shown.

Figure 5A

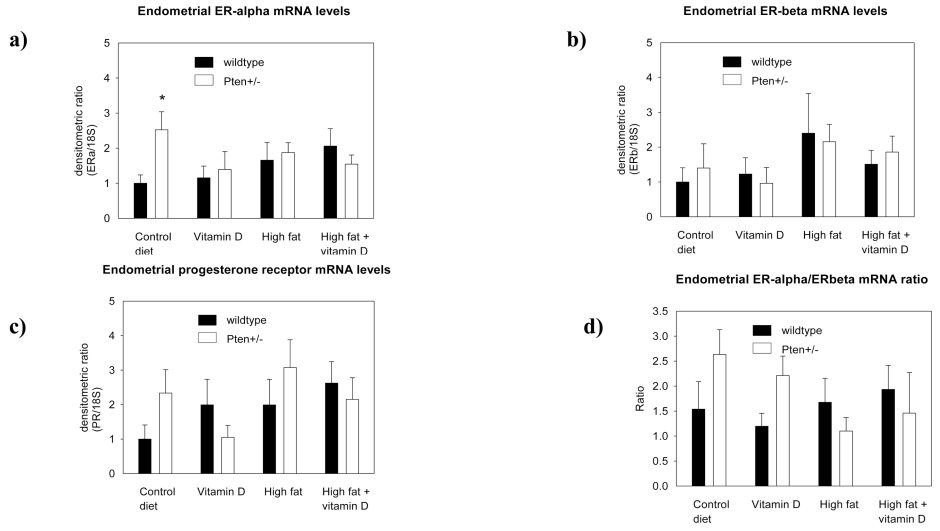

Figure 5B

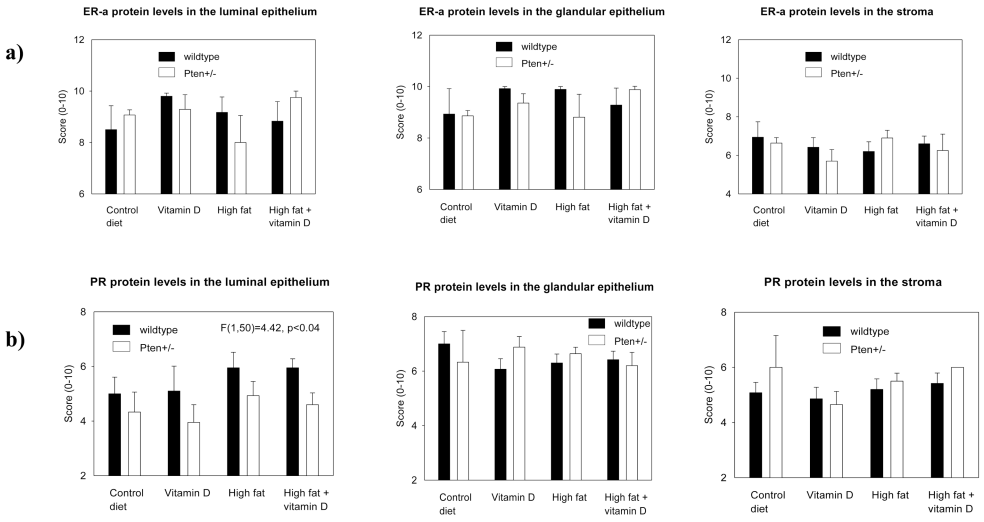

Figure 5.

Figure 5A. mRNA expression of **(a)** ER-α, **(b)** ER-β, **(c)** PR and **(d)** ER-α/ER-β ratio in the endometrium of 28-week-old wildtype and *Pten*$^{+/-}$ mice fed control diet, VD_3 supplemented control diet, OID, or VD_3 supplemented OID for 24 weeks. ER-α mRNA levels were significantly higher in *Pten*$^{+/-}$ mice fed the control diet than in the wildtype mice; *p<0.014. No changes among different dietary exposures were seen. Mean ± SEM of 5–7 mice per group are shown.

Figure 5B. Endometrial protein levels of **(a)** ER-α and **(b)** PR in the luminal and glandular epithelium and stroma of 28-week-old wildtype and *Pten*$^{+/-}$ mice. PR expression was

significantly lower in the luminal epithelium of *Pten*$^{+/-}$ mice than of the wildtype mice ($p<0.04$). Mean ± SEM of 3–10 mice per group are shown.

Table 1

Table 1a. Effects of VD_3 supplementation on pre-malignant and malignant endometrial changes in 28-week-old normal weight and obese *Pten*$^{+/-}$ and wildtype mice.

	N mice per group	Normal	Multifocal glandular hyperplasia (MFGH)	MFGH & atypia [focal/multifocal]	Endometrial adenocarcinoma
Genotype					
WT Control	11	11 (100%)	0	0	0
+ VD_3	8	8 (100%)	0	0	0
High fat	11	11 (100%)	0	0	0
+ VD_3	10	10 (100%)	0	0	0
Pten$^{+/-}$ Control	12	5 (42%)	0	7 (58%) [0/7]	0
+ VD_3	10	3 (30%)	1 (10%)	6 (60%) [4/2]	0
High fat	9	2 (22%)	0	6 (67%) [3/3]	1 (11%)
+ VD_3	8	3 (37.5%)	3 (37.5)	2 (25%) [0/2]	0

Table 1b. Expression of vitamin D metabolic enzymes and VDR mRNA in the histopathologically normal endometrium or endometrium containing benign or pre-malignant and malignant changes in 28-week-old *Pten*$^{+/-}$ mice. Mean and SEM of 3–14 mice per group are shown.

Changes in the endometrium	25-OHase	1α-OHase	24-OHase	VDR
Normal	1.59+0.30	1.40+0.45	0.93+0.23	4.33+1.85
Benign lesions	2.04+0.23	1.03+0.46	0.31+0.08	2.21+0.68
Pre-malignant and malignant lesions	2.48+0.32	1.60+0.36	1.39+0.46	2.36+0.71

Pten$^{+/-}$ mice: Chi2=111.737, df=6, p<0.001

Table 2

Diet and PTEN effects on weight and bone characteristics of C57BL6 mice.

		N	Mass[1] (g) Mean (S.E)	BMD[2] 1000*(g/cm^2) Mean (S.E.)	BMC (g)/10 Mean (S.E.)
Genotype					
WT	Control	6	24.5 (1.9)	50.07 (0.9)	4.3 (0.3)
	w/Vit. D	5	29.0 (1.9)	55.52 (0.9)	4.4 (0.3)
	High Fat	5	47.9 (1.9)	51.32 (0.9)	5.3 (0.3)
	w/Vit. D.	7	41.3 (1.6)	50.00 (0.9)	5.3 (0.3)
HET	Control	6	25.1 (1.7)	51.25 (0.9)	4.5 (0.3)
	w/Vit. D	2	27.3 (2.9)	53.50 (1.1)	5.1 (0.3)
	High Fat	5	39.5 (1.9)	49.58 (0.9)	4.1 (0.3)
	w/Vit. D.	5	37.5 (1.9)	50.06 (0.9)	4.4 (0.3)
P (**Genotype**)			0.0257	0.3750	0.1961
P (**Diet**)			<0.0001	0.0014	0.3857
P (**Genotype*Diet**)			0.1304	0.3120	0.027

[1]Weight after Necropsy, Means are unadjusted.

[2]BMD and BMC reported from GE Lunar Piximus Dual-Energy X-ray Absorptiometer output.

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