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Vitamin D Mitigates the Adverse Effects of Obesity on Breast Cancer in Mice

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Abstract

Obesity is an established risk factor for postmenopausal breast cancer (BCa), insulin resistance and vitamin D deficiency, and all contribute to increased synthesis of mammary estrogens, the drivers of estrogen receptor-positive (ER+) BCa growth. Since both dietary vitamin D and calcitriol treatments inhibit breast estrogen synthesis and signaling, we hypothesized that vitamin D would be especially beneficial in mitigating the adverse effects of obesity on ER+ BCa. To assess whether obesity exerted adverse effects on BCa growth and whether vitamin D compounds could reduce these unfavorable effects, we employed a diet-induced obesity (DIO) model in ovariectomized C57BL/6 mice. Breast tumor cells originally from syngeneic *Mmtv-wnt1*

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DECLARATION OF INTEREST

R. L. Horst has an ownership interest in Heartlands Assays LLC. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

transgenic mice were then implanted into the mammary fat pads of lean and obese mice. DIO accelerated the initiation and progression of the mammary tumors. Treatments with either calcitriol or dietary vitamin D reduced the adverse effects of obesity causing a delay in tumor appearance and inhibiting continued tumor growth. Beneficial actions of treatments with vitamin D or calcitriol on BCa and surrounding adipose tissue included: repressed *Er*, aromatase and *Cox-2* expression, decreased tumor derived estrogen and PGE₂ and reduced expression of leptin receptors and increased adiponectin receptors. We demonstrate that vitamin D treatments decreased insulin resistance, reduced leptin and increased adiponectin signaling and also regulated the LKB1/AMPK pathway contributing to an overall decrease in local estrogen synthesis in the obese mice. We conclude that calcitriol and dietary vitamin D, acting by multiple interrelated pathways, mitigate obesity enhanced BCa growth in a postmenopausal setting.

Keywords

breast cancer; vitamin D; obesity; adipokines; aromatase

INTRODUCTION

The incidence of obesity in the general population is rising and becoming widespread worldwide. In the U.S, overweight (BMI = 25–29.9 kg/m²) and obese (BMI ≥ 30 kg/m²) individuals combined constitute almost two-thirds of the population (Wang, et al. 2011). Obesity is a risk factor for breast cancer (BCa), especially postmenopausal estrogen receptor alpha-positive (*ER+*) BCa, and is associated with increased incidence as well as poor prognosis and response to treatment (Gilbert and Slingerland 2013; Iyengar, et al. 2015). The hypothesized mechanisms for the adverse effects of obesity on BCa include increased inflammation in obese adipose tissue with high cytokine and adipokine levels that create a chronic inflammatory microenvironment favoring tumor cell motility, invasion and epithelial-mesenchymal transition (EMT) (Gilbert and Slingerland 2013). Obesity is also frequently associated with systemic insulin resistance and elevated proinflammatory mediators and growth factors, leading to the interplay of local and systemic effects including elevated levels of leptin, circulating insulin and IGF1 (Gilbert and Slingerland 2013). Leptin, a protein synthesized and secreted by adipose cells, circulates at levels proportional to adiposity and has many actions that stimulate breast cancer growth (Grossmann, et al. 2010; Strong, et al. 2015).

In the breast microenvironment there is local estrogen synthesis by adipose stroma and tumor cells, which drive BCa growth (Howe, et al. 2013; Simpson and Brown 2013a). This local estrogen production is especially significant in postmenopausal women whose estrogen synthesis from dormant ovaries is greatly diminished resulting in low circulating levels of estrogens. Additionally, obesity and elevated leptin cause dysregulated metabolism and inhibition of the tumor suppressor AMPK that can constrain cell proliferation by multiple mechanisms including the *LKB1/AMPK* pathway (Simpson and Brown 2013a). It has been shown that *AMPK* negatively regulates the actions of *CREB*-dependent aromatase, the enzyme that catalyzes the synthesis of estrogens in breast adipose tissue, via phosphorylation of *CRTC2*, a transcription factor required for the activation of the aromatase

promoter PII in BCa cells (Luo, et al. 2010; Simpson and Brown 2013a, b; Wang, et al. 2015). Finally, there is evidence linking obesity and associated insulin resistance (Allott and Hursting 2015; Hursting and Berger 2010; Mitri and Pittas 2014) with changes in the breast adipose stromal compartment that result in increased inflammation (Berstein, et al. 2007; Brown and Simpson 2010; Howe et al. 2013; Maccio, et al. 2009), leading to the development of an adverse adipokine milieu (Simpson and Brown 2013a, b) and culminating in increased synthesis of estrogen, which drives postmenopausal BCa progression (Wang et al. 2015).

We and others have shown that supplementation with dietary vitamin D or treatment with its active hormonal form calcitriol [1,25(OH)₂D₃] exhibits multiple actions to inhibit BCa growth as demonstrated in cultured BCa cells and in lean mouse models of BCa (Deeb, et al. 2007; Feldman, et al. 2014; Rossdeutsch, et al. 2015; Shui and Giovannucci 2014; So and Suh 2015; Welsh 2012). These vitamin D actions include the transcriptional repression of aromatase (*Cyp19*) (Krishnan, et al. 2010; Swami, et al. 2011), inhibition of *Er* expression (Swami, et al. 2000; Swami, et al. 2013), and suppression of cyclooxygenase-2 (COX-2) expression (Moreno, et al. 2005) leading to the reduction in the synthesis of pro-inflammatory mediators such as PGE₂ that are major stimulators of aromatase transcription in BCa (Brown and Simpson 2010; Simpson and Brown 2013a). Dietary vitamin D acts equivalently to calcitriol (Swami, et al. 2012) and exerts anti-inflammatory effects (Krishnan and Feldman 2011) and reduces insulin resistance by improving insulin secretion and signaling (Calle, et al. 2008). Since estrogens are the major driver of *ER+* BCa growth (McDaniel, et al. 2013), these multiple vitamin D and calcitriol actions provide a rationale for using vitamin D supplementation to reduce BCa risk and improve outcomes.

Although many studies mentioned above have examined the benefits of vitamin D in cancer using a variety of models, previous reports have not examined whether vitamin D or calcitriol has the capacity to mitigate the negative effects of obesity on breast cancer. In the current study we have examined the ability of calcitriol and dietary vitamin D supplementation to alter the growth of mouse mammary tumor virus *Mmtv-wnt1* mammary tumors in an ovariectomized (OVX), diet-induced obesity (DIO) model and compared the effects in obese mice to lean mice. Our novel findings indicate that DIO promotes BCa initiation and growth and that calcitriol and dietary vitamin D supplementation reduce the adverse effects of obesity on BCa development and progression. We show for the first time that vitamin D compounds mitigate the adverse effects of obesity by multiple mechanisms both systemically as well as directly on the tumor cells and the surrounding adipose tissue leading to improvement in insulin resistance and the suppression of local/breast estrogen synthesis by inhibiting prostaglandin production and altering the adipokine expression profile and signaling. In addition, we discovered that vitamin D compounds enhances pAMPK pathways that suppress local estrogen synthesis in the breast microenvironment that are enhanced by obesity. Our results suggest that calcitriol or dietary vitamin D supplementation would be a particularly beneficial adjunct in the treatment of *ER+* BCa in obese postmenopausal patients where these adverse pathways are substantially enhanced.

MATERIALS AND METHODS

MATERIALS

Calcitriol was from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Rodent diets were from Research Diets Inc. (New Brunswick, NJ). Tissue culture media, supplements and fetal bovine serum (FBS) were from GIBCO BRL (Grand Island, NY) and Mediatech Inc. (Herndon, VA).

METHODS

Mice—All animal procedures were performed in compliance with the guidelines approved by Stanford University Administrative Panels on Laboratory Animal Care (APLAC). Six week-old female, OVX C57BL/6Ncr mice were from the Jackson Laboratory (Bar Harbor, Maine). Mice were housed in the Research Animal Facility, Stanford University School of Medicine in a designated pathogen-free area.

Animal Studies and treatments—The OVX mice were randomized to receive either a standard diet AIN76 (STD – 11% fat, 24% protein and 64% carbohydrate-derived calories) or an AIN76 high-fat diet (HFD), (60% fat, 20% protein and 20% carbohydrate-derived calories). The source of fat in the diets was from lard. Body weights were assessed weekly to monitor weight gain. After 10 weeks, mammary tumors were established in all mice by orthotopic implantation of *Mmtv-wnt1* tumor cells into their mammary fat pads. The tumor cell preparations were made from spontaneously developing mammary tumors in *Mmtv-wnt1* transgenic mice on a C57BL/NCr background (a gift from Dr. Stephen Hursting, University of North Carolina, Chapel Hill, NC) that are *Er+* and growth inhibited by tamoxifen (Nunez, et al. 2008; Yue, et al. 2005). Tumor cells (~ 150,000) suspended in a small volume of a 1:1 mixture of culture medium (RPMI + 10% fetal bovine serum) and matrigel were inoculated into the fourth mammary fat pad of each mouse. The procedure was performed under APLAC approved conditions with carprofen (5 mg/kg) for analgesia. The mice were then randomly assigned to the various treatment groups and diets were continued for the following 6 weeks (Figure 1). Both STD and HFD diets contained 530 IU of vitamin D₃/kg. The vitamin D-supplemented (VD) STD and HFD diets contained 5300 IU of vitamin D₃/kg. Calcitriol (Cal), diluted in sterile PBS was administered at a dose of 25 ng given by i.p. injections 3 times a week (Swami et al. 2011). Control mice (STD diet and HFD diet) received i.p. injections of 0.1% ethanol in sterile PBS.

Tumor sizes were measured weekly and tumor volumes were calculated from two tumor diameter measurements with a digital caliper using the formula: tumor volume = (length × width²)/2. At study end, 14 hours after the final calcitriol injections, mice were euthanized according to APLAC guidelines using CO₂. Blood was collected by cardiac puncture, serum separated and frozen. Tumors and surrounding adipose tissue were harvested and snap frozen in liquid nitrogen for subsequent analysis.

Measurement of serum calcium and vitamin D metabolites—Serum calcium was measured with an ELISA kit (Cayman Chemical Company, Ann Arbor, MI). Serum 25-hydroxyvitamin D (25(OH)D) and 1,25(OH)₂D levels were measured at Heartland Assays

(Ames, IA) by methods previously described (Hollis, et al. 1996; Hollis, et al. 1993). The assays did not distinguish between vitamin D₂ or D₃ metabolites.

Evaluation of insulin resistance—Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews, et al. 1985) using the formula [fasting serum insulin (μU/ml) × fasting blood glucose (mmol/L)] / 22.5. Pancreatic B-cell function was assessed by the homeostasis model assessment of B cell function (HOMA-B) using the formula [20 × fasting serum insulin concentration (μU/ml)] / [fasting blood glucose (mmol/L)-3.5] (Matthews et al. 1985).

Quantitative reverse transcriptase-PCR—Total RNA was isolated from tumor and mammary adipose tissue using Trizol (Invitrogen, Carlsbad, CA) (Moreno et al. 2005). Five μg of RNA were subjected to reverse transcription using the SuperScript III first strand synthesis kit (Invitrogen) (Krishnan et al. 2010). Relative changes in mRNA levels were assessed using gene specific primers (Table 1) by the comparative C_T (C_T) method and were normalized to that of TATA box-binding protein (TBP) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

Measurement of hormones and adipokines—One week prior to the experimental end-point, an APLAC-approved saphenous vein blood draw procedure was employed to obtain fasting blood samples from a subset of all mice after a 6-hour fast. Fasting blood glucose levels were measured using a glucometer (Free Style, Abbott Diabetes Care Inc, Alameda, CA). Fasting serum samples were assayed for insulin (EMD Millipore Corporation, Billerica, MA) and triglycerides (Cayman Chemical Company) using ELISAs. Protein extracts from tumor and the surrounding mammary fat were prepared in Tris-EDTA buffer containing high salt (Zhao, et al. 1999) and protein concentration determined by the method of Bradford (Bradford 1976). ELISA kits were used to measure estradiol (E₂) (Cayman Chemical Company), estrone (E₁) (ALPCO Immuno Assays, Salem, NH) and PGE₂ (Cayman Chemical Company). Serum leptin and adiponectin were measured in mammary fat extracts and in the end of study serum samples by ELISA (EMD Millipore Corporation).

LKB1 promoter activity in MCF-7 breast cancer cells—MCF-7 human BCa cells were routinely cultured in DMEM:F12 medium supplemented with 10% FBS and maintained at 37°C and 5% CO₂ in a humidified chamber. A ~ 3Kb LKB1 promoter (a kind gift from Dr. E. Simpson and K. Brown, Monash University, Australia) inserted into a pGL3-Basic reporter vector was transiently transfected into MCF-7 cells using Lipofectamine 2000 reagent (Life Technologies/Invitrogen) according to manufacturer's instructions. Following transfections the cells were treated with vehicle (0.1% ethanol) or varying concentrations (0.1 to 10 nM) of calcitriol for 24 h. In a separate set of experiments the LKB1 promoter construct was co-transfected with human vitamin D receptor (VDR) expression plasmid into MCF-7 cells and treated with 10 nM calcitriol as described above. Luciferase activity was measured using the Luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

pAMPK in tumor and MCF-7 breast cancer cells—MCF-7 cell cultures grown to ~75% confluence were treated with vehicle (0.1% ethanol), calcitriol (10 nM), leptin (100 ng/ml) or adiponectin (500 ng/ml) for 24 hours. Protein extracts from tumor and surrounding mammary fat were processed simultaneously. Extracts from tumors or cells were used to measure total AMPK using the DuoSet IC ELISA (R&D Systems, Minneapolis, MN). Phosphorylation of AMPK was assayed using the PathScan Phospho-AMPKa (Thr172) Sandwich ELISA (Cell Signaling Technology, Boston, MA), a solid phase ELISA that detects endogenous levels of AMPKa when phosphorylated at Thr172.

Statistical Analysis—Statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Data were evaluated by ANOVA with Scheffe's F test as the post-hoc analysis.

RESULTS

Effects of diets and treatments on body weight, serum vitamin D metabolites and serum calcium

Ingestion of the HFD over 10 weeks caused significant obesity compared to mice fed the STD diet (Fig 2A). Tumor cell inoculation and VD and Cal treatments began at week 10. Calcitriol treatment did not alter the body weights in the lean mice (STD+Cal). However, a decrease in the body weight in the obese HFD+Cal group was observed starting at week 13 and continuing through the end of the experiment, a sign of calcitriol toxicity causing hypercalcemia (Fig 2B). There were no significant changes in body weights compared to controls in the lean and obese mice receiving VD.

We measured the concentrations of 25(OH)D and 1,25(OH)₂D in the serum from all mice. While, mice fed a STD+VD or HFD+VD diet had significant elevations in 25(OH)D, the circulating form of vitamin D (Fig 2B) no changes were detected in the levels of 25(OH)D with just HFD alone. This was contrary to the findings in literature where obesity is associated with vitamin D deficiency. This unexpected finding could be attributed to the presence of lard in the diet. Lard is good source of vitamin D and we suspect that this contributed to the observed levels of 25(OH)D in the mice fed HFD obviating the relationship between vitamin D deficiency and obesity. As expected calcitriol treatment caused significant decreases in serum 25(OH)D levels in both lean and obese mice from increased catabolism of 25(OH)D due to induction of the degradative enzyme CYP24A1. Interestingly, vitamin D supplemented diets caused increases in serum 1,25(OH)₂D levels (Fig 2B), a phenomenon we have previously described in other mouse BCa models following the administration of a vitamin D-supplemented diet. We attributed this effect to increased local 1,25(OH)₂D synthesis and secretion by the tumors in response to elevated serum 25(OH)D substrate since a rise in serum 1,25(OH)₂D was not seen in supplemented mice without tumors (Swami et al. 2012). Serum calcium levels remained unchanged except for modest but statistically significant elevations in serum calcium levels in the obese mice treated with calcitriol (Fig 2B). The mild hypercalcemia indicated that the calcitriol dose was a maximal dose that could be administered with limited toxicity.

Effects of DIO and treatments on tumor appearance and growth

Within one week of tumor cell inoculation, palpable tumors were evident in 50% of the obese mice (HFD) whereas 50% tumor appearance in the lean mice (STD) was detected 2 weeks later (Fig 2C). The administration of calcitriol or VD to obese mice (HFD) caused a delay in tumor appearance with 50% of the obese mice showing palpable tumors by 1 and 2 weeks after tumor cell inoculation, respectively compared to week 1 for the non-treated obese mice (Fig 2C). Calcitriol or VD administered to the lean mice (STD) delayed tumor appearance with 50% of the treated lean mice exhibiting palpable tumors by week 4 compared to week 3 for the mice fed the STD diet alone. Comparing the time until the appearance of palpable tumors in 50% of mice, there was a 3-week delay between the obese mice receiving no treatments and the lean mice receiving calcitriol or VD (Fig 1C). Since the interval from inoculation to experiment termination was 6 weeks, the 3-week difference in tumor appearance was quite substantial ($p < 0.01$).

We next compared the end-point tumor volumes. As shown in Fig 2D, the tumor volume in the obese mice was significantly greater than in the lean mice (~74% increase) indicating an increased rate of tumor growth in the obese mice. In the lean mice, calcitriol and VD treatments resulted in decreased mean tumor volumes (by ~46% and ~40% respectively) compared to STD diet alone. However, calcitriol and VD treatment of the obese mice caused even more significant decreases in the mean tumor volumes compared to untreated mice on the HFD, (~47% decrease in HFD+Cal and ~67% decrease in HFD+VD compared to HFD). These results demonstrate that both vitamin D treatments inhibited tumor growth and that treatment with vitamin D was sufficient to mitigate the adverse impact of obesity on tumor growth in these mice.

Effects of DIO and treatments on aromatase expression, estrogen synthesis and PGE₂ production

In order to understand the mechanisms by which vitamin D treatment mitigates the effects of obesity, we next examined the expression of aromatase, in both the tumors and surrounding mammary fat. Very significant elevations in aromatase mRNA expression were seen both in the mammary fat (Fig 3A) and tumor tissue (Fig 3B) in the obese mice. Calcitriol and VD decreased aromatase mRNA expression in both lean and obese mice, with the decreases being more pronounced in the obese mice. Measurement of estradiol (E₂) concentration in extracts of tumors and surrounding mammary fat also revealed decreases similar to those seen in aromatase mRNA. Considerable elevations in E₂ levels in tumor and mammary fat were seen in the obese mice, which were substantially reduced following treatments with calcitriol and VD (Figs 3C & D). Estrone (E₁) concentrations in the mammary fat extracts were also elevated in the obese mice and the levels were reduced by the treatments (Figs 3E & F). Although there was a trend towards increased E₁ in the tumors of the obese mice, these changes were not statistically significant.

We also examined the changes in the expression of *Cox2* and PGE₂ concentration in breast tumors. A considerable increase in *Cox2* mRNA was evident in the tumors from obese mice (Fig 3G). Calcitriol and VD treatments significantly decreased *Cox2* mRNA in both lean and obese mice, with the decreases being more pronounced in the obese mice. The obese mice

showed a modest increase in tumor PGE₂ levels. Administration of calcitriol to both lean and obese mice significantly decreased tumor PGE₂ levels, while the decrease due to dietary VD was only evident in the obese mice (Fig 3H).

Obesity substantially elevated the expression of *Er*, the mediator of estrogen's mitogenic signaling (Fig 3I). Both calcitriol and VD caused large reductions in tumor *Er* mRNA expression in both lean and obese mice, but again, the decreases were more pronounced in the obese mice, which had 4-fold higher levels of tumor *Er* mRNA compared to the lean mice.

Effects of DIO and treatments on systemic measures of insulin resistance

The weight gain seen in the obese mice was associated with expected changes in metabolic parameters measured in fasting serum samples. As shown in Table 2, there were statistically significant increases in fasting serum insulin and triglycerides in obese mice compared to lean mice. Fasting glucose levels also showed an increasing trend, which however did not achieve statistical significance. HOMA-IR was statistically significantly elevated in the obese mice indicating increased insulin resistance explained by the higher insulin concentration required to maintain a similar level of glucose. Calcitriol improved most of these metabolic parameters but the improvement was more pronounced in the obese mice. For example, while calcitriol treatment significantly reduced fasting insulin concentrations in both lean and obese mice, the decreases in glucose and triglyceride levels and HOMA-IR due to calcitriol were statistically significant only in the obese mice. VD similarly showed decreasing trends in insulin levels and HOMA-IR with the decrease in HOMA-IR in the obese mice reaching statistical significance. There were no statistically significant changes in HOMA-B (Table 2). Overall, calcitriol seemed more effective than vitamin D supplementation. We hypothesize that while local conversion to 1,25(OH)₂D₃ plays an important role for the effects of VD on the tumors, this might be less evident in the multiple sites that regulate the insulin resistance and homeostasis.

Effects of DIO and treatments on circulating and local adipokines

Since obesity-induced changes in adipokines have been linked to increased BCa risk and worse prognosis, serum leptin and adiponectin concentrations were measured in all mice at the time of sacrifice (Figs 4A & B). The obese mice showed a ~3-fold increase in the mean serum leptin a reflection of their increased obesity. Calcitriol and vitamin D significantly reduced serum leptin levels in both lean and obese mice showing systemic as well as local beneficial effects. No significant change was observed in serum adiponectin concentrations in the obese compared to lean mice. However, calcitriol treatment caused modest but statistically significant increases in serum adiponectin in both lean and obese mice whereas vitamin D produced a very modest but also statistically significant increase in serum adiponectin only in the lean mice.

Next we examined the expression of leptin and adiponectin in the mammary fat surrounding the tumors to determine whether the systemic effects of obesity were also occurring locally in the breast adipose tissue. As shown in Fig 4C, a substantial increase in *leptin* mRNA expression was seen in the mammary fat of obese mice when compared to the lean mice.

Administration of calcitriol and vitamin D caused significant decreases in *leptin* mRNA expression in both lean and obese mice. Modest decreases in *adiponectin* mRNA (Fig 4D) expression in the mammary fat were seen in obese mice compared to lean mice, which were not statistically significant. Administration of calcitriol and VD significantly increased mammary fat *adiponectin* mRNA expression in the obese mice while the increase in lean mice did not achieve significance. ELISA measurements for leptin and adiponectin protein showed that the inhibitory changes in mammary fat leptin mirrored the *leptin* mRNA changes (Fig 4E). Mammary fat adiponectin protein was reduced in obese mice but changes after vitamin D treatments were not significant (Fig 4F). We also determined leptin receptor (*Ob-r*) and adiponectin receptor (*Adipo-r1* and *Adipo-r2*) mRNA expression in the tumors. The obese mice showed a significant increase in tumor *Ob-r* mRNA (Fig 4G) and a modest increase in *Adipo-r1* mRNA compared to the lean mice (Fig 4H). *Adipo-r2* mRNA expression in the tumors was negligible. Both calcitriol and VD significantly decreased *Ob-r* mRNA in the obese mice (Fig 4G) while further increasing *Adipo-r1* mRNA levels in the tumors from obese mice (Fig 4H). The effects of calcitriol and VD were however, minimal in the lean mice. Overall these changes indicate a particularly important role for vitamin D treatments to inhibit the elevated leptin signaling and stimulate adiponectin signaling in the tumors of the obese mice.

Effects of DIO and calcitriol treatment on AMPK activation

The LKB1/AMPK pathway has been shown to play an important role in the downstream effects of prostaglandins and adipokines in BCa associated with obesity (Simpson and Brown 2013a, b; Wang et al. 2015). Since *Mmtv-wnt1* cells do not grow well in culture, we used cultured human MCF-7 BCa cells to address the role of calcitriol on the regulation of LKB1 and pAMPK. A dose-dependent increase in the basal LKB1 luciferase promoter activity was seen (Fig 5A) with calcitriol treatment. Cotransfection of *hVDR* expression plasmid elicited a 1.5 fold increase in the basal promoter activity and this was further enhanced with calcitriol treatment (Fig 5B) suggesting a direct action of calcitriol via the *VDR* on the LKB1 promoter. We next examined the effects of calcitriol on the activation of AMPK, a direct target for the actions of LKB1. MCF-7 cells were treated with calcitriol, leptin or adiponectin for 24 hours. No significant changes were observed in the levels of total AMPK, but both adiponectin and calcitriol treatments significantly increased the levels of pAMPK (Figs 5C and D). Leptin did not alter the levels of AMPK or pAMPK at the dose tested. We next investigated whether calcitriol regulated pAMPK levels in the tumors and surrounding mammary fat in both lean and obese mice. No changes were observed in the levels of total AMPK in either the tumor (Fig 5E) or the surrounding mammary fat (Fig 5G). Basal pAMPK levels were slightly lower in both the tumor and surrounding mammary fat in the obese mice compared to the lean mice and a significant increase in pAMPK levels were seen in both the lean and obese mice with calcitriol treatment (Figs 5F and H).

DISCUSSION

Previous studies from our laboratory using non-obese models have identified several important vitamin D actions that inhibit the progression of postmenopausal *ER+* BCa (Feldman et al. 2014; Krishnan, et al. 2012). Because obesity is associated with both vitamin

D deficiency (Boonchaya-anant, et al. 2014; Liel, et al. 1988) and increased BCa risk (Ahn, et al. 2007; Gilbert and Slingerland 2013; Iyengar et al. 2015), we hypothesized that vitamin D supplementation might be an especially useful adjunctive therapy in obese BCa patients. Our current study examined the adverse endocrine, metabolic and inflammatory environment of BCa exacerbated by diet-induced obesity compared to lean mice and the improvement in multiple BCa risk factors as a result of supplementation with VD or treatment with calcitriol.

As reported previously (Nunez et al. 2008), we observed that tumors appeared earlier and attained larger volumes in the obese mice, showing that obesity accelerated initial mammary tumor growth as well as progression. Our new findings reveal that the beneficial effects of calcitriol and VD are particularly effective at mitigating the adverse factors for BCa growth that are enhanced in obesity. In particular, we discovered that vitamin D and calcitriol, acting on both the tumor and the breast adipose stroma, reduced the obesity enhanced pathways including: elevated estrogen and prostaglandin synthesis, up-regulated *Er* and *Cox2* expression, and the adverse changes in the adipokine profile and adipokine receptor expression patterns associated with obesity. Vitamin D actions to increase pAMPK, in both the tumor and surrounding mammary fat and regulate LKB1 expression in MCF7 BCa cells, adds additional novel pathways by which vitamin D retards the rate of tumor growth in the obese mice. Our findings are summarized in the model shown in Fig 6. Vitamin D and calcitriol treatment, acting systemically, also improved overall insulin resistance in the obese mice.

Local estrogen synthesis in the breast microenvironment is a critical factor increasing the risk of developing breast cancer and driving its growth in postmenopausal women (Bulun, et al. 1993). We now show that calcitriol or vitamin D treatments are beneficial in mitigating the adverse effects of obesity on estrogen synthesis and signaling that are exacerbated in obesity. Our data demonstrate that BCa in obese mice expressed elevated levels of *Er* and that the local estrogen synthesis was increased in both the tumor microenvironment as well as the surrounding adipose stroma in the obese mice. The increase in *Er* and local estrogen synthesis in the obese mice likely contributes to the acceleration in tumor appearance and the augmented tumor growth seen in the obese mice. The vitamin D compounds reduced local estrogen synthesis in the obese mice via suppression of aromatase expression in the breast adipose tissue. Vitamin D treatments also inhibited the elevated expression of *Cox2*, resulting in decreased prostaglandin synthesis, which we observed in both calcitriol- and VD-treated obese mice. These actions contribute to the inhibition of aromatase and reduced local estrogen synthesis since PGE₂ produced by the mammary tumors is the main stimulator of aromatase promoter pII in the surrounding adipose stroma (Simpson and Brown 2013a). We, and others have reported (Simboli-Campbell, et al. 1997; Swami et al. 2000; Swami et al. 2013) that vitamin D and calcitriol also down-regulate *Er* expression in BCa cells and tumors of lean mice. Here we showed that obese mice have very elevated *Er* expression and vitamin D treatments substantially inhibited *Er* expression thereby interfering with estrogen signaling. The well-established benefit of treating human ER+ BCa with aromatase inhibitors or ER blocking drugs (McDaniel et al. 2013) suggests that these benefits of vitamin D and calcitriol in mice may play a role in human BCa.

Obesity and insulin resistance are associated with changes in the breast adipose-stromal compartment, which result in increased inflammation and the development of an unfavorable adipokine expression profile of increased leptin and decreased adiponectin (Brown, et al. 2010; Simpson and Brown 2013a). Vitamin D treatments reduced the elevated levels of leptin mRNA and protein in both the serum and the breast adipose stroma. Additionally, vitamin D compounds significantly reduced the elevated *Ob-r* mRNA levels while increasing *Adipo-r1* mRNA levels in the mammary tumors of obese mice. While these effects are also found in the lean mice, the changes are more prominent in the obese mice where the leptin levels are much higher than in the lean mice. Overall, our findings indicate that calcitriol and dietary VD mitigate the adverse changes in the adipokine expression pattern seen in the obese mice. These changes also contribute to the increased activation of AMPK and downstream repression of aromatase promoter pII in the breast adipose tissue.

The regulatory effects of adiponectin and leptin on AMPK, a master regulator of energy homeostasis, contribute to the association between obesity and BCa (Brown et al. 2010; Wang et al. 2015). Adiponectin, the most abundant adipokine secreted by the adipose tissue, has insulin-sensitizing effects (Ouchi, et al. 2011) and by increasing the expression and activity of the downstream kinase LKB1, it causes the phosphorylation and activation of AMPK (Brown, et al. 2009). In the presence of pAMPK, CRTCs are phosphorylated and translocate to the nucleus to bind CREB-target genes such as aromatase that is thereby inhibited leading to reduced transcription (Simpson, et al. 2002). Leptin on the other hand inhibits insulin signaling, activating insulin resistance (Grossmann et al. 2010) and has the reverse effects on the pAMPK pathway resulting in increased aromatase transcription (Brown et al. 2009). Thus the altered adipokine milieu associated with obesity and insulin resistance provides a critical link between obesity and postmenopausal BCa in which local production of estrogens in the breast adipose tissue is a major driving force for BCa growth. We demonstrate that calcitriol directly stimulates expression of an LKB1-luc construct transfected into in MCF-7 BCa cells, an effect enhanced by co-transfection of *VDR* indicating a direct effect on the LKB1 promoter. Calcitriol and vitamin D also enhance activation of AMPK to pAMPK, in both the tumor and mammary fat. These actions add additional pathways by which vitamin D inhibits aromatase expression and mitigates obesity enhanced adverse effects on BCa. (Fig 6).

In summary, obesity accelerated the progression of *Mmtv-wnt1* mammary tumors in recipient OVX mice. Treatment with calcitriol or VD reduced the adverse effects of obesity on tumor development and growth. Vitamin D or calcitriol showed diverse actions in both the tumor and the adipose stroma to mitigate the adverse effects of obesity including: inhibition of aromatase, *Cox2* and *Er* expression resulting in diminished estrogen and PGE₂ synthesis; improved insulin resistance; diminished unfavorable profile of adipokine expression by reducing leptin and leptin receptor expression and increasing adiponectin receptor expression; increasing LKB1 promoter activity and increased tumor suppressor pAMPK levels, all contributing to repressed aromatase and limited local estrogen synthesis in the tumor and surrounding breast adipose tissue (Fig 6). We conclude that calcitriol or VD supplementation exerts multiple beneficial effects specifically relevant to ER+ BCa enhanced by obesity since these treatments, acting through several interrelated local and systemic mechanisms, cause a significant inhibition of the synthesis of and signaling by

local estrogens, which drive the growth of BCa in the postmenopausal setting. We speculate that these changes, found in OVX lean and obese mice, are also relevant to postmenopausal women. Vitamin D supplementation could be an especially useful adjunctive measure in obese women with BCa and suggest that a randomized controlled trial to test this hypothesis is warranted.

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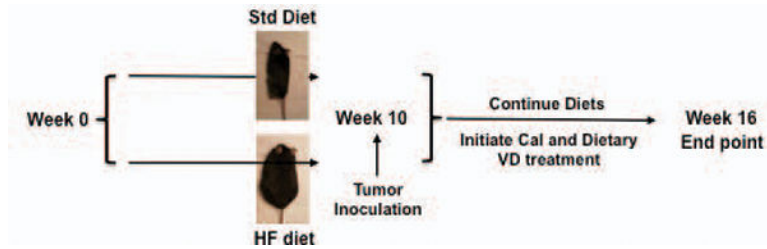


Figure 1.

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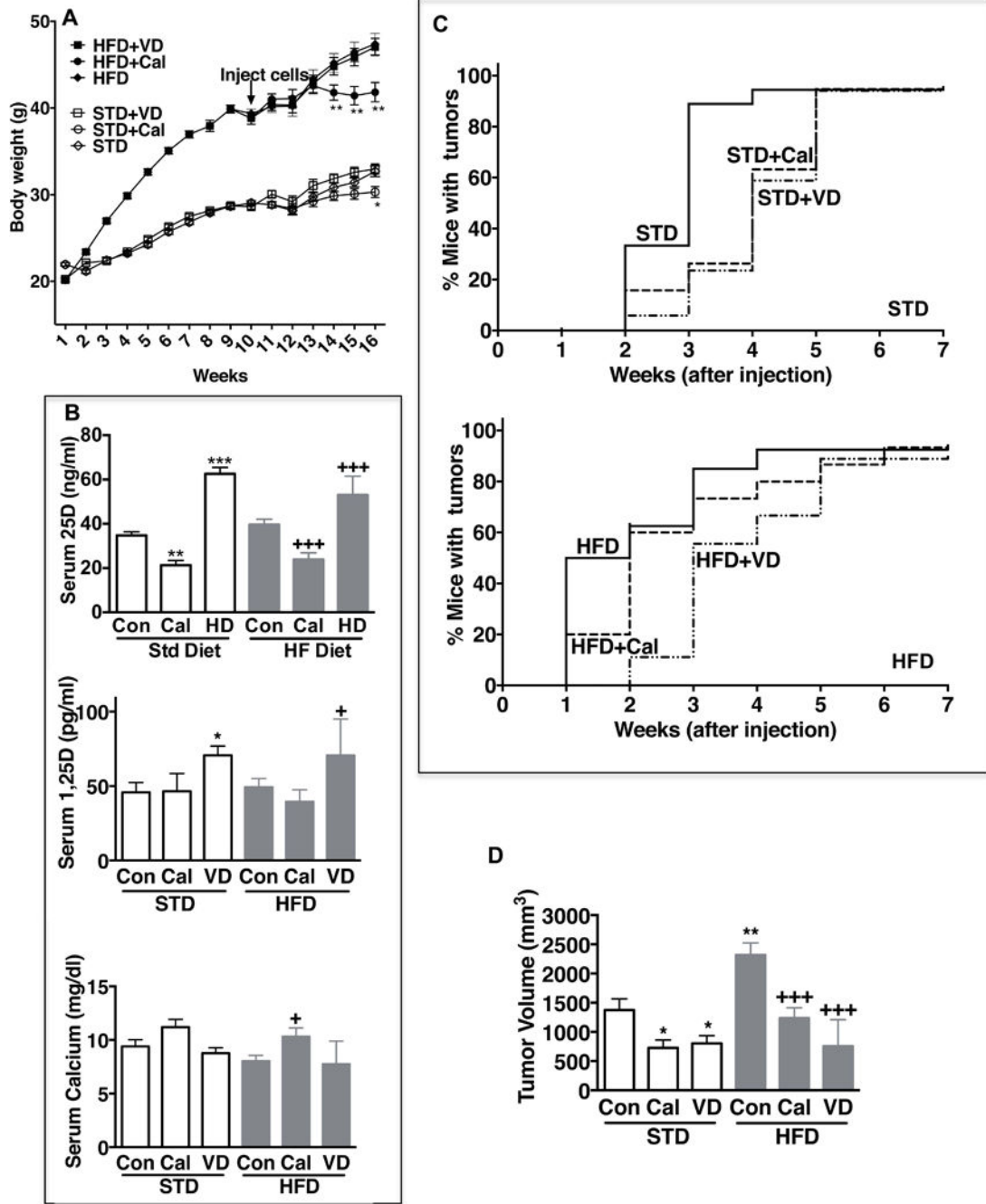


Figure 2.

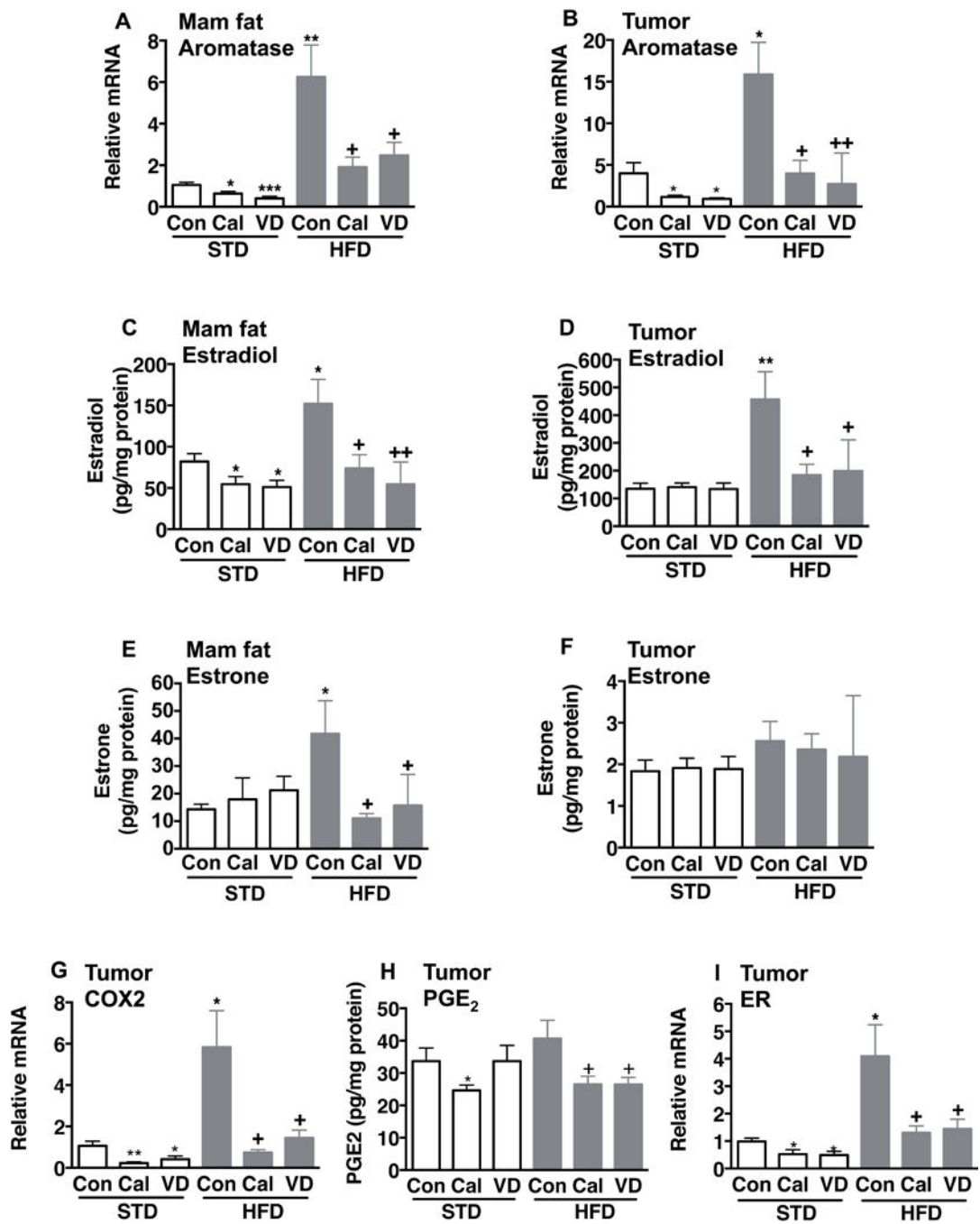


Figure 3.

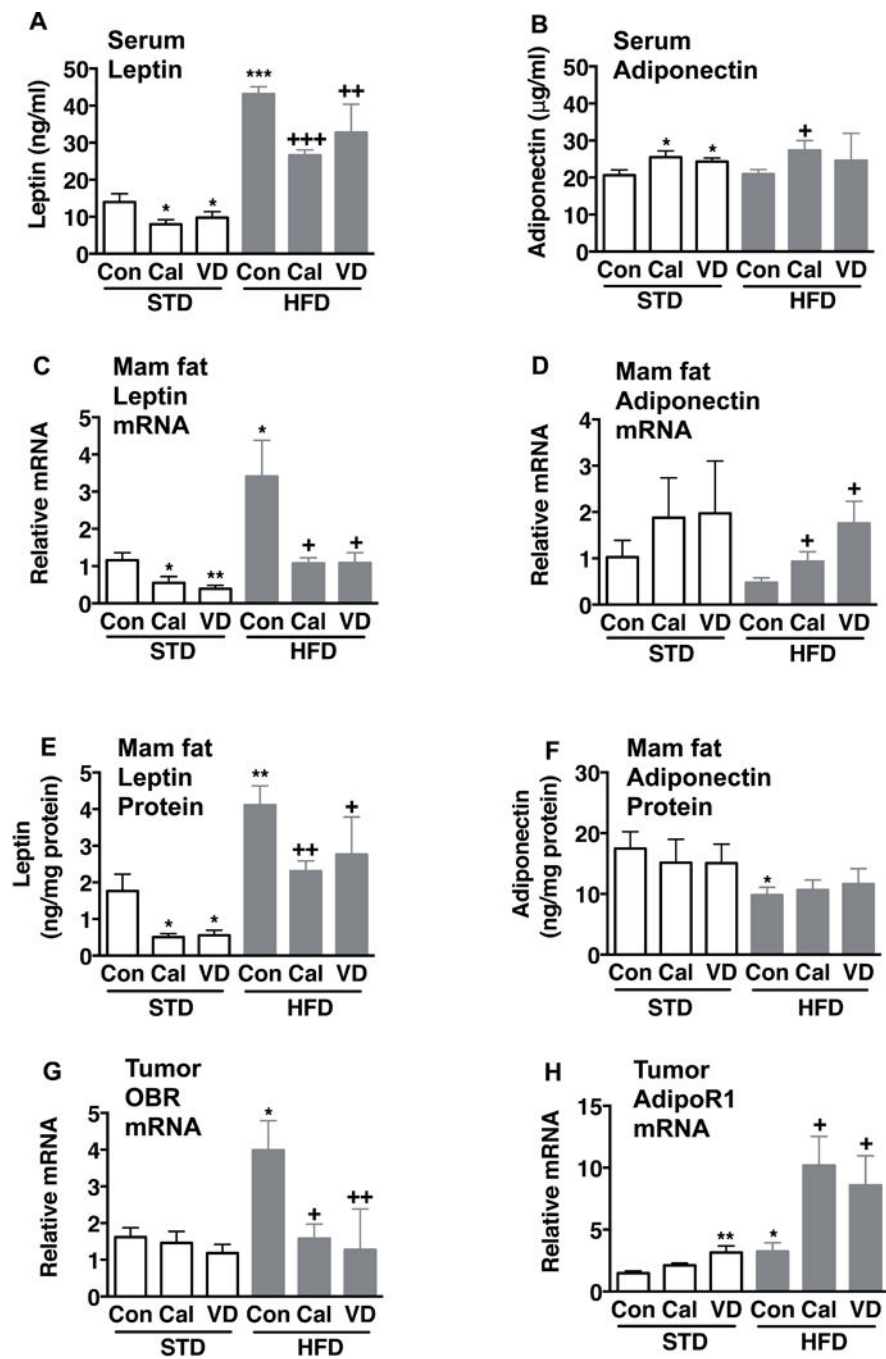


Figure 4.

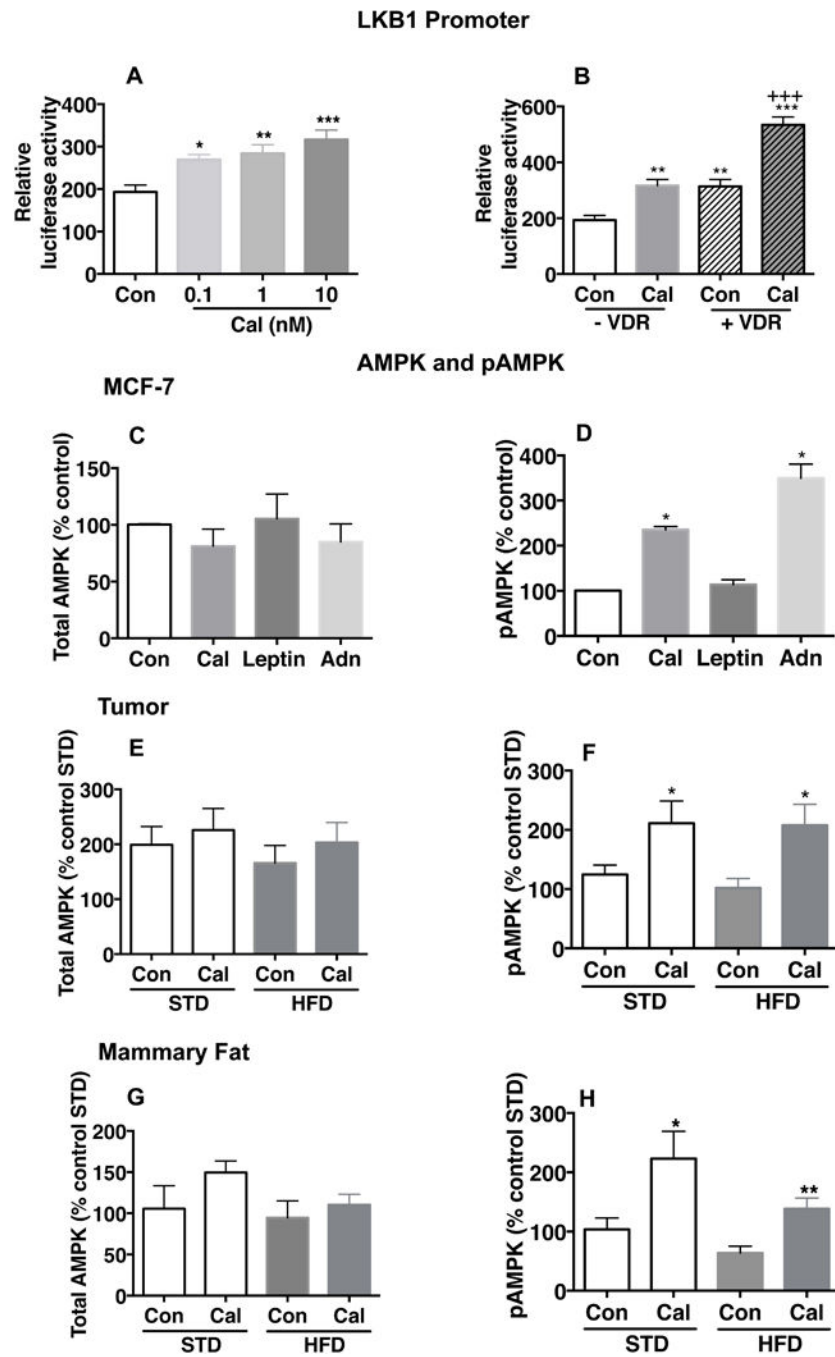


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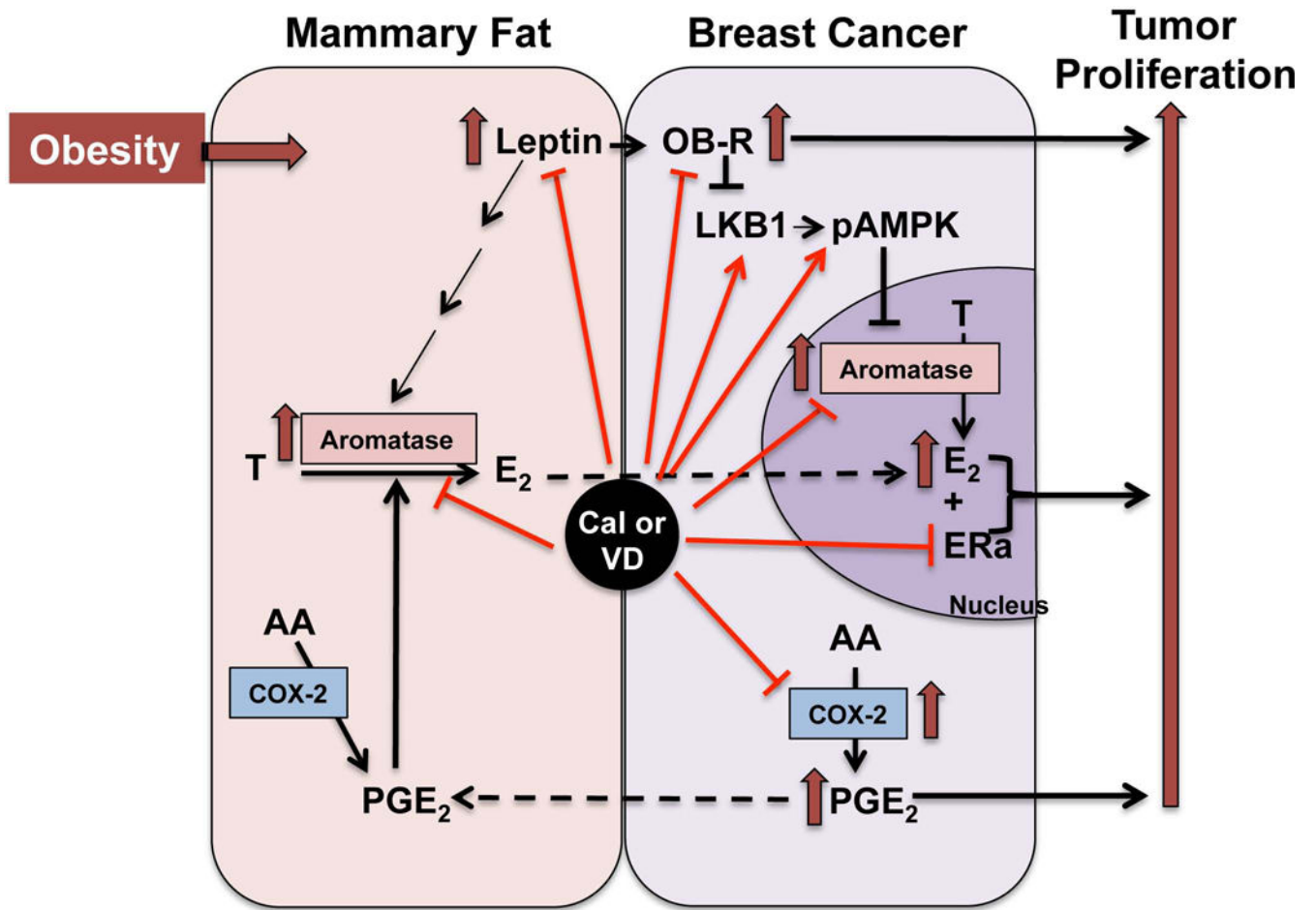


Figure 6.

Table 1

Mouse primer sequences

Gene	Primer Sequences
TBP	For 5'- tcatgagaataagagaccacg Rev 5'- atagggaacttcacatcacagc
GAPDH	For 5'- tcaccatctccaggagcg Rev 5'- ctgcttcaccacctcttga
Leptin	For 5'- caggaggaaaatgtgctggag Rev 5'- ccgactgcgtgtgaaatgct
Adiponectin	For 5'- agagaaggagagaaaggagatgc Rev 5'- tgagcgatacacataagcggc
OB-R	For 5'- actgaaggaagactgg Rev 5'- gcgtagttgagttggct
AdipoR1	For 5'- tacctgctacatggccacagac Rev 5'- cagacgatggagagtagatgag
CYP19(aromatase)	For 5'- cggagaatgcacaggctcgag Rev 5'- cgatgtactcccagcacagc
COX-2	For 5'- ggctcgtgagcttctc Rev 5'- ctttcagtcagggtcaa
ERalpha	For 5'- cacattcgtatcaactttgtatcca Rev 5'- ctgctacataagattgtctcattcaa

Table 2

Fasting Serum Measurements in lean (STD diet) and obese (HFD) fed mice

Groups	Glucose (mg/dl)	Triglycerides (mg/dl)	Insulin (μ IU/ml)	HOMA-IR	HOMA-beta
STD diets					
STD	124.6 \pm 18.0	66.0 \pm 9.1	37.4 \pm 3.4	11.6 \pm 2.2	210 \pm 48
STD+Cal	136.8 \pm 21.8	60.9 \pm 6.2	23.4 \pm 3.8 ^a	9.2 \pm 1.3	356 \pm 243
STD+VD	130.5 \pm 14.4	65.3 \pm 7.8	32.5 \pm 6.7	7.7 \pm 0.7	248 \pm 44
HFD diets					
HFD	155.0 \pm 15.5	89.2 \pm 4.9 ^a	67.7 \pm 10 ^b	27.0 \pm 5.6 ^{aa}	221 \pm 35
HFD+Cal	114.2 \pm 7.9 ^b	74.1 \pm 4.1 ^b	39.5 \pm 4.5 ^b	11.1 1.5 ^{bb}	309 \pm 66
HFD+VD	130.2 \pm 8.9	75.9 \pm 1.2 ^b	52.7 \pm 7.0	13.2 \pm 1.2 ^b	333 20

Abbreviations: STD – Standard diet; HFD – High fat diet; Cal – Calcitriol; VD – Dietary vitamin D supplementation (5300 IU/kg diet). Values represent Mean \pm SE of 5–8 determinations.^a = p<0.05,^{aa} = p<0.01 as compared to STD;^b = p<0.05,^{bb} = p<0.01 as compared to HFD.