Phenotypic Characterization of Adipose-Specific VDR Knockout Mice

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Abstract

Breast cancer is a prominent and lethal disease that currently affects close to three million people in the United States. Each year displays close to 250,000 new cases of breast cancer and around 40,000 deaths in the United States alone. A geographic pattern of breast cancer suggests a higher incidence in more temperate regions. Research has suggested that low Vitamin D levels associated with reduced sun exposure might contribute to increased breast cancer incidence. In recent studies, Vitamin D has been shown to slow the proliferation of breast cancer cells, but the mechanisms involved in vivo are poorly defined. It is known that adipose tissue is involved in the progression of breast cancer. Adipose cells in the mammary tissue are not just for support, they also release signals to the epithelium known as ‘cross talk’. To shed light onto the effects of low vitamin D on adipose signaling in the mammary gland, we studied mice with adipose-specific vitamin D receptor (VDR) deletion. The goal of this project was to study the impact of VDR deletion in adipocytes on mammary gland morphology using histological and whole mount techniques. Tissues of mice with adipose specific VDR deletion (CVF strain) were compared to those of control mice (CN1 strain). By PCR we confirmed that VDR expression was reduced approximately 80% in adipose tissue of CVF mice compared to CN1 mice. Hematoxylin and Eosin Y staining for the mammary gland revealed a slight increase in the number of epithelial ducts in the CVF mice. Further quantitation of the epithelial content of the mammary glands confirmed that glands from CVF mice had higher epithelial tissue density than glands from CN1 mice. Whole mount analysis revealed significantly increased epithelial branching in the mammary glands of the CVF compared to CN1 mice. Collectively, our data indicates that deletion of VDR in adipose tissue results in increased growth of the mammary epithelial tissue. The results imply that VDR activity in the adipose compartment of the mammary gland contributes to the anti-proliferative actions of vitamin D in this tissue.
Acknowledgements

I would like to thank Dr. JoEllen Welsh and Dr. Donald G. Matthews for the support and mentoring that was kindly provided throughout the course of this research project. I would also like to thank Dr. Namita Chatterjee for her guidance and trainings as well as past lab partners Jordan Drelich and Thomas Dunn for their assistance.
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Introduction

Breast cancer is a startlingly common disease in the United States that affects about 1 in 8 women nationwide, second in incidence only to cancers of the skin.\(^1\) Allowed to progress unchecked, survival rates plummet once breast cancer reaches stages 2, 3, and 4, with survival rates past 5 years at 93%, 72%, and 22%, respectively.\(^2\) Higher incidences of breast cancer have been associated with certain conditions, including defective BRCA1 and 2 genes, obesity and age.\(^3\) Past research has revealed an overlap between diagnosed cases of breast cancer in a particular region and the distance of that region from the equator.\(^4\) As distance from the equator increases, the body becomes less able to naturally synthesize Vitamin D in response to sunlight. Consequently, it has been suggested that there is a link between levels of Vitamin D in the blood and frequency of breast cancer diagnoses. In vitro studies revealed that Vitamin D inhibits proliferation and stimulates apoptosis of breast cancer cells.\(^5\) Research by the Welsh lab and others has also demonstrated that the global deletion of the Vitamin D Receptor (VDR) gene in mice escalates oxidative DNA damage, stimulates cell proliferation, and inhibits apoptosis, thereby increasing the likelihood of carcinogenesis.\(^6\)

Vitamin D is naturally synthesized in skin after UVB exposure. Some fish oils and other dietary supplements can also provide Vitamin D. The compound 7-dehydrocholesterol in the skin absorbs ultraviolet rays and is converted into cholecalciferol (Vitamin D), which must be metabolized for biological activity. To activate Vitamin D, it is first hydroxylated to 25-hydroxycholecalciferol in the liver. 25-hydroxycholecalciferol is hydroxylated once again in the kidneys to yield the active 1,25-dihydroxycholecalciferol; more commonly known as 1,25-dihydroxy Vitamin D or calcitriol.\(^7\) This active form produces physiological effects by binding
to the VDR. Some of these effects include regulation of calcium and phosphate homeostasis, stimulation of appropriate immune responses and control of cell proliferation. (8)

The VDR is a nuclear receptor that binds active 1, 25-dihydroxy Vitamin D. In an active ligand state, VDR modulates gene expression through heterodimerization with the retinoid X-receptor and/or recruitment of various activating and repressing proteins. (9) Recent studies have linked VDR activity to the proper growth and development of mammary glands in mice. Global VDR deletion results in accelerated ductal growth and branching in the mammary gland, suggesting that VDR is involved in negative growth regulation. (10) Abnormal atrophy of the mammary fat pad as well as white adipose tissue depots has also been reported in aged VDR null mice, suggesting VDR has a role in energy metabolism as well as the maintenance of the stromal microenvironment of the mammary gland. (11) In addition, the VDR pathway has been shown to impact metabolic organs, for example Vitamin-D inhibits fibrotic responses in liver, kidney, and pancreas. Global VDR knockout mice exhibit hepatic, pancreatic, renal, and intestinal fibrosis, supporting Vitamin D’s metabolic effects. (12,13,14,15) Global VDR ablation has also been shown to lead to insulin resistance in mice, suggesting Vitamin D has a role in the development of Type 2 Diabetes which is another risk factor of breast cancer. (16) These findings collectively suggest a link between the Vitamin D pathway, adiposity, and breast cancer development or progression.

Adipose tissue is involved in the normal development of the mammary glands by regulating the growth and differentiation of the adjacent epithelial cells. For example, during pregnancy, adipocytes signal the growth of the epithelium to create milk-producing cells. Other work shows that deletion of white adipose tissue in mice prevents mammary gland development. (17) Obesity has long been associated with both Vitamin-D deficiency and breast cancer, but the mechanisms and cross talk underlying this association are largely unknown. (18) Since the
absence of VDR causes increased growth in the mammary glands, we hypothesized that Vitamin D regulates epithelial cells via the mammary adipose tissue.\(^{(19)}\)

In this experiment, we compared mice with deletion of the VDR gene specifically in adipose tissue (CVF strain) to the CN1 control strain from which they were derived. Specifically, we compared body and tissue weights, whole mount morphology and histopathology of the mammary tissue, white adipose tissue, liver, and pancreas from mature CVF and CN1 mice. We also assessed VDR expression in adipose tissue by quantitative reverse transcription PCR to validate the model.

**Methods**

**Mice**

The VDR gene was eliminated from adipose tissue of mice using standard cre-flox knockout procedures. The gene deletion was targeted to the adipose tissue through use of the well-characterized adipose-specific promoter of the fatty acid binding protein 4 (Fapb4) gene. CN1 mice bearing the cre transgene driven by the FABP4 promoter were mated with VDR floxed mice (originally generated by Dr. S. Kato, Tokyo University, Japan) to generate offspring with the adipose-specific VDR knockout, which were termed CVF mice. A limitation of this model is that the FABP4-driven gene deletion occurs in mature adipocytes, but not in mesenchymal stem cells or pre-adipocytes, therefore residual VDR expression in these cell types is expected. Previous studies on mice with global VDR deletion revealed enhanced proliferation of the mammary epithelium during puberty and pregnancy, but severe atrophy of the mammary fat pad with age.\(^{(11)}\) In the studies reported here, CN1 and CVF mice were maintained on high fat diets to promote adipogenesis and enhance the proliferative signaling from the adipocytes to the epithelium. A subset of CN1 and CVF mice were reared on a high calcium, high lactose 'rescue'
diet instead of a high fat diet for comparison to the global VDRKO mice. At time of harvesting, ages of the mice were recorded, as well as body weight, mammary gland weight, and abdominal white adipose tissue weight.

After CO2 euthanasia, mammary glands, including the entire mammary fat pad, were dissected from the CN1 and CVF mice. One inguinal gland from each animal was weighed, formalin-fixed, paraffin embedded and sectioned. The contralateral inguinal gland was used for whole mounting. One thoracic gland from each animal was quick-frozen in liquid nitrogen for RNA isolation. Visceral white adipose tissue collected from the abdominal cavity was also archived for histological and molecular analysis.

**Analysis of mammary gland morphology**

*Sectioning and H&E staining.* Using a microtome, 5-micrometer slices were cut and plated to charged slides, to ensure tissue adhesion. These tissues were then stained with both Hematoxylin and Eosin Y. This allowed for a general morphological analysis, which includes the overall density of cell clusters, size of cells, and ductal growth. Images were taken of the stained sections at 25x magnification and 100x magnification. A representative area adjacent to the lymph node was chosen for quantitative analysis, which was performed with Image J software to estimate the epithelial density. Pixel counts were used to determine the area of epithelium present in relation to the total glandular area. Regions containing gaps, holes, or cuts in the tissue were removed from the sample, and thus from the total pixel count to provide the pixel count of the relevant tissue. Epithelial cell pixels were then divided by the tissue pixel count to yield the epithelial to mammary gland ratio which was expressed as a percentage.
**Whole mount analysis**

Dissected inguinal glands were stretched onto glass slides and fixed with Carnoy’s solution. The glands were then stained with carmine alum to stain the epithelium, and subsequently dehydrated and mounted. This allowed for the visualization of ductal morphogenesis and outgrowth across the entire gland. Quantitation of the epithelial density was conducted with Image J on images taken at 10x magnification. Images were converted to black and white and the light/dark threshold was adjusted to outline epithelial branches. The pixel count of the outlined epithelial branches was divided by the total pixel count of the image to yield a ratio of epithelium to the rest of the gland which was expressed as a percentage.

**Analysis of white adipose tissue morphology**

Abdominal white adipose was dissected and subsequently sectioned and stained with both Hematoxylin and Eosin Y. Gross morphological analysis was performed to observe changes in adipocyte size and density. Quantitation with Image J was performed to measure the number of adipocytes per field at 25x magnification.

**Analysis of liver morphology and adiposity**

Liver sections were obtained in a similar fashion as noted above and stained with Hemotoxylin and Eosin Y. Images were taken at 25x magnification. A representative area of each tissue was chosen such to avoid cuts or ducts. Quantitative analysis of adipose content was performed using Image J. Images were converted to black and white and the light/dark threshold was adjusted to fill in the area of white fat globules vs. the rest of the tissue. Pixel counts were then used to determine the total pixel area of adipose globules. Adipose globule pixels were then divided by the total tissue pixel count to yield the adipose to liver ratio which was expressed as a percentage.
Analysis of Pancreas Morphology

Pancreas sections were obtained through similar processes as previously described and stained with Hematoxylin and Eosin Y. Gross morphological analyses was conducted with special consideration given to the pancreatic islets and ducts.

Analysis of liver and pancreatic fibrotic response

Liver and pancreas sections were stained with Masson’s trichrome to allow for evaluation of fibrosis. Gross morphological analysis was performed to search for evidence of fibrosis within the sections.

RNA isolation and qRT-PCR

To verify that the VDR gene was deleted in adipose tissue, RNA was isolated from visceral white adipose tissue removed from CN1 and CVF mice. Briefly, 100-150mg of tissue was subjected to TRIzol extraction and isolated RNA was reverse transcribed into cDNA. Samples were analyzed for VDR expression using Invitrogen platinum mix (Life Technologies, Grand Island, NY). Concurrently, 18S RNA expression was determined for normalization purposes. qRT-PCR was run for 40 cycles, and data for VDR expression was expressed relative to that of 18S RNA and reported as relative gene expression with CN1 values set to 1.

Alternative method of RNA isolation

The isolation of RNA from the white adipose tissue posed several issues. Since adipose tissue is high in lipid and low in cellular density, total RNA yields were very low. The low yields made it very difficult to purify enough RNA for PCR. We therefore modified the protocol to include Lithium Chloride precipitation as an additional step after the isopropanol precipitation. LiCl is a very specific for precipitating RNA, as it will not efficiently precipitate DNA, protein, or carbohydrates. We expected that the inclusion of the
LiCL step would avoid subsequent steps to enhance RNA purity which tend to decrease yield. Purity of samples isolated with and without the LiCL step was compared on an Agilent Bioanalyzer which calculated the 28S/18S ration and the RNA Integrity Number (RIN) values.

**Statistical analyses**

All data in graphs depict the mean values derived from CN1 or CVF mice accompanied with a standard error bar. Three mice from each strain were utilized for analysis of VDR in adipose tissue. For Hematoxylin and Eosin Y quantitation, four mice from each strain were analyzed. Whole mount analysis involved seven CN1 mice and eight for CVF. Two outliers were removed from the H+E staining results (CN1 151 and CVF 200) based on Grubbs’ test.

Significance was tested with an unpaired t test for each data set.

**Results**

**RNA Isolation and qRT-PCR**

RNA from white adipose tissue was isolated, reverse transcribed, and analyzed by qPCR for VDR expression which was normalized in each sample against 18S rRNA expression. The data comparing VDR expression in adipose tissue from age-matched CVF adipose-specific VDR knockout mice and CN1 control mice is displayed in Figure 1. Compared to the endogenous level of VDR in adipose tissue detected in CN1 mice, tissue from CVF mice exhibited a mean reduction in VDR expression of approximately 80%. Despite the small number of samples utilized (n=3), the difference was statistically significant, indicating successful VDR deletion in adipose tissue. Residual VDR expression in the CVF adipose tissue likely represents expression of the gene in cells other than mature adipocytes, such as mesenchymal stem cells or pre-adipocytes.
Mice Characterization

Overall body weights of the mice as well as the inguinal mammary gland were measured. Both CN1 and CVF mice fed a high fat diet demonstrated increased body weight gain compared to the lower fat, high calcium and lactose rescue diet (Figure 2). Interestingly, female CVF mice gained weight at an accelerated rate compared to CN1 mice on both types of diet. Caloric intakes between CVF females compared to their CN1 counterparts were not significantly different (data not shown). However, there were no significant differences in weight gain patterns in male mice on either diet. This could indicate that VDR’s function in adipose tissue is gender specific.

Figure 3 represents the weight of the mammary gland as the percent of the total body weight for the subset of CN1 and CVF mice fed high fat diets. The weight of the mammary glands from CVF mice were slightly reduced compared to those of CN1 mice, however this difference was not statistically significant (p=0.0552).
Figure 3: Mammary gland weights in CN1 and CVF mice fed high fat diets. Whole inguinal mammary glands were weighed after dissection. Results were expressed as mammary gland weight in relation to total body weight. Bars indicate the mean ± standard error for each data set.

Figure 2: Post weaning body weight of CN1 and CVF mice. Temporal body weight gain of CN1 and CVF mice weaned on high calcium/lactose rescue diets (left) or high fat diets (right). Points and error bars represent mean standard deviation of 4-12 mice per datapoint. Statistical significance at p<0.05 was determined by linear regression slope.
Whole Mounts
Whole mounts were used to observe the entirety of the mammary gland morphology including its ductal system. Representative images of glands from 2 CN1 and 2 CVF mice are shown in Figure 4 at 3.5x magnification. These low power stereoscope images suggest that glands from the CVF mice have more extensive epithelial branching than glands from CN1 mice. At higher magnification (10x, Figure 5) ductal structure appears normal in both strains but higher density is evident in CVF mice. Figure 6 represents the quantitation of the 10x magnification whole mount images of the mammary gland, which confirmed increased epithelial density in the CVF mice.

a. CN1 150
b. CN1 165
c. CVF 200
d. CVF 213

Figure 4: Representative images of mammary gland whole mounts from CN1 and CVF mice. Two representative images for each strain are shown CN1 (a,b) are control mice whereas CVF (c,d) are mice with adipose-specific VDR deletion. Images were captured at 3.5x magnification.
Figure 5: High magnification images of mammary gland whole mounts from CN1 and CVF mice. Two representative images of each strain are shown. CN1 (a,b) are control mice whereas CVF (c,d) are mice with adipose-specific VDR deletion. Images were captured at 10x magnification.

Figure 6: Quantitation of mammary epithelial branching on whole mounts from CN1 and CVF mice. Image J was used to quantitate the amount of epithelial branching present in the mammary gland whole mounts. Images at 10x magnification were analyzed. Bars indicate mean ± standard error for each data set. *p< 0.05, as determined by t test.
Histological Assessment of Mammary Gland Morphology in High Fat Diet fed Mice

Mammary gland sections were taken at 5 microns for the CVF and CN1 strains and stained with Hematoxylin and Eosin Y allowing for routine morphological analysis. Initial examination indicated that the development of the mammary fat pad was not compromised by deletion of VDR from mature adipocytes (Figure 7). There were no discernable differences in adipocyte size or lipid content between the strains, although further quantitative analysis will be necessary to confirm this initial observation. With respect to the epithelial compartment, tissue from CVF mice exhibited an increase in epithelial ductal density compared to that of CN1 mice (Figure 7). Quantitation of the epithelial density by Image J confirmed significantly higher amounts of epithelial tissue in the CVF mice (Figure 8).

Histological Assessment of Mammary Gland Morphology in Rescue Diet fed Mice

Similarly to the high fat diet fed mice, mammary gland sections were taken at 5 microns for CVF and CN1 strains fed high calcium/lactose rescue diet. Sections were stained with Hematoxylin and Eosin Y for routine morphological analysis. Figure 7 represents images taken at 25x magnification. Initial qualitative analysis indicated no obvious discernable differences in adipocyte density, but confirmed the finding that CVF mice exhibit increased epithelial content. Quantitation of epithelial density by Image J confirmed significantly higher amounts of epithelial tissue in the CVF mice. Of note, the rescue diet fed mice exhibited increased epithelial content compared to adipose content compared to the high fat diet, regardless of strain.
Figure 7: Hematoxylin and eosin (H&E) stained mammary sections from CN1 and CVF mice on high fat diet. Representative sections of formalin fixed, paraffin embedded tissue were stained with H&E and photographed at 25x magnification. A, B: CN1 mice; C, D: CVF mice with adipose specific VDR deletion.

Figure 8: Comparison of mammary gland histology in CVF and CN1 mice. Hematoxylin and Eosin Y stains were performed on mammary gland sections. Images were gathered at 25x magnification. Image J was used to quantitate epithelial content. CVF mice contained increased amount of epithelial tissue within the mammary gland than the CN1 mice on both high fat and rescue diet. Bars indicate the mean ± standard error for each data set. *p< 0.05, as determined by t test.
White Adipose Tissue (WAT) Morphology

The CVF female mice exhibited significant increase in abdominal WAT mass compared to CN1 mice on both rescue and high fat diets. In CN1 and CVF males, WAT mass was not significantly increased in response to diet or genotype (Data not shown).

WAT sections were taken at 5 microns for the CN1 and CVF strains and stained similarly as the mammary gland samples. Figure 9A compares CN1 and CVF WAT sections on high fat and rescue diets. This data suggests larger adipocytes in CVF mice versus the CN1 mice fed rescue diets, but this was not observed in the high fat diet. Further quantitative analysis confirmed that there were significantly fewer cells per image in CVF mice on rescue diet and high fat mice of both genotypes when compared to CN1 mice on rescue diet (Figure 9B).

Figure 9: Characterization of WAT from CN1 and CVF mice on rescue diet vs. high fat diet. (A) Representative sections of formalin fixed, paraffin embedded WAT were stained with H&E and photographed at 25x and 100x magnification. CN1 and CVF mice fed rescue diet are shown on the left, with CN1 and CVF mice fed high fat diet are shown on the right. (B) Quantitation of adipocyte density was measured on H&E stained sections of WAT imaged at 100x.
**Histological assessment of liver morphology and lipid content**

Liver sections were taken at 5 microns for the CVF and CN1 strains and stained with Hematoxylin and Eosin Y. Initial examination indicated an increase in hepatic lipid accumulation in CVF vs CN1 mice as evidenced by abundant fat globules. This qualitative observation was displayed by both the high fat diet and rescue diets, though mice fed the high fat diet appear to have increased overall liver lipids versus the rescue diet fed mice. (Figure 10) Quantitation of lipid content with Image J confirmed that there are significantly higher amounts of adipose tissue within the liver of CVF mice versus CN1 mice for each respective diet group. More specifically on the high fat diet, CVF knockout mice displayed a 1.5-fold increase in liver lipid content when compared to CN1. Rescue diet CVF knockout mice displayed a 2-fold increase in lipid content when compared to their CN1 counterparts. (Figure 11)

a. CN1 170 Rescue  
c. CVF 218 Rescue

b. CN1 157 High Fat  
d. CVF 210 High Fat

*Figure 10: Characterization of liver from CN1 and CVF mice on rescue diet vs. high fat diet.* Representative sections of formalin fixed, paraffin embedded liver tissues were stained with H&E and photographed at 100x magnification. CN1 and CVF mice fed rescue diet are shown on the top, with CN1 and CVF mice fed high fat diet are shown on the bottom.
Histological assessment of pancreas morphology and islets

Pancreas sections were taken at 5 microns and stained with Hematoxylin and Eosin Y. Qualitative analysis discovered an increased number and size of the islets of Langerhans in the CVF knockout mice fed rescue diets when compared to their CN1 counterparts (Figure 12). No other obvious morphological differences were observed.

Figure 11: Comparison of liver lipid content in CVF and CN1 mice. Hematoxylin and Eosin Y stains were performed on liver sections. Images were gathered at 100x magnification. Image J was used to quantitate adipose content vs background tissue. Bars indicate the mean ± standard error for each data set. *pp< 0.05, as determined by t test.

Figure 12: Characterization of pancreas from CN1 and CVF mice on rescue diet. Representative sections of formalin fixed, paraffin embedded pancreas tissues were stained with H&E and photographed at 25x magnification. CVF knockout mice are compared to their CN1 counterparts fed rescue diets. The above arrows depict location of islets.
Masson’s trichrome analysis of pancreatic and liver collagen and fibrosis

Masson’s trichrome stain was utilized to analysis collagen content and fibrosis in pancreas and liver sections. Qualitative analysis did not reveal any evidence of liver or pancreatic fibrosis across all mice regardless of strain or diet. Further observation also did not indicate any increase in overall collagen content in sections nor increased collagen deposits around ducts. (Figure 13)

Figure 13: Characterization of liver and pancreas with Masson’s trichrome stain. Representative sections of formalin fixed, paraffin embedded liver tissues were stained with Masson’s trichrome stain and photographed at 25x magnification. CN1/CVF liver sections are shown at the top. Pancreas sections are shown at the bottom. Collagen is stained blue as depicted above.
**LiCl RNA Isolation Results**

Lithium chloride was used as an additional isolation step after isopropanol precipitation.

Samples were gathered from white adipose tissue and analyzed with a Bioanalyzer for quality.

With the exception of CN1 157 and CVF 203, all samples had sufficient yield to be used for cDNA synthesis, and had pure, intact RNA (Figure 14). Graphs for CN1 150 and CVF 212 are included in Figure 15. A clear advantage of the additional isolation step of LiCl is that cleaning the RNA post-isolation is no longer necessary, allowing preservation of yield.

<table>
<thead>
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<th>Strain</th>
<th>Yield (ng/μl)</th>
<th>28S/18S</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN1 150</td>
<td>231</td>
<td>1.8</td>
<td>7.9</td>
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<td>1.7</td>
<td>9.2</td>
</tr>
<tr>
<td>CVF 203</td>
<td>78</td>
<td>2.0</td>
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</tr>
<tr>
<td>CVF 209</td>
<td>183</td>
<td>1.5</td>
<td>6.9</td>
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<tr>
<td>CVF 212</td>
<td>281</td>
<td>1.7</td>
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</tr>
</tbody>
</table>

**Figure 14: Alternative Method for RNA Isolation QC Results.** An Agilent Bioanalyzer was used to measure the 28S/18S and RIN values for white adipose tissue samples. Optimal 28S/18S values are above 1.7 and RIN values above 7.

**Figure 15: Alternative Method for RNA Isolation QC Graphs.** An Agilent Bioanalyzer was used to measure the 28S/18S and RIN values for white adipose tissue samples. Optimal 28S/18S values are above 1.7 and RIN values above 7. These graphs were chosen to be representative of all of the samples.
**Discussion**

Our studies on mice with adipose specific VDR deletion indicate that vitamin D signaling in the mammary adipose tissue impacts growth of the adjacent mammary epithelium.

RNA was isolated from white adipose tissue samples from CN1 and CVF mice, and qRT-PCR was run to verify the VDR knockout model. Results showed a significantly lower expression of VDR in adipose tissue from the CVF mice as compared to the control CN1 mice. As expected, VDR was detected in adipose tissue of the CVF strain, since the FABP4-cre activity will only delete VDR in mature adipocytes. Residual VDR expression in adipose tissue from CVF mice likely represents VDR present in non-differentiated cells including stem cells and pre-adipocytes. However, the 80% reduction in VDR expression we observed indicated that the transgene was effective at VDR deletion in the majority of cells within the adipose tissue. Other studies employing the FaBP4-driven gene deletion model of VDR revealed similar results through qPCR and western blots of residual VDR expression. (13) Further studies to analyze the extent of VDR deletion in mammary adipose tissue are warranted.

To provide insight into the impact of VDR signaling in adipocytes on body weight homeostasis, we compared growth of CVF and CN1 transgenic mice reared on high fat or rescue diets. As expected, mice fed high fat diets demonstrated increased weight gain compared to those fed the lower fat rescue diet. Females with adipose-specific VDR ablation exhibited accelerated body weight gain compared to their control counterparts on both diets. Of note, this weight gain was a gradual process over a long period of time post-weaning rather than an effect of birth weight. In the females there were no significant effects of genotype on caloric intake, indicating that the enhanced weight gain in CVF females was not associated with increased food consumption suggesting enhanced feed efficiency (data not shown). Strangely, there were no
significant differences in weight gain patterns of male mice on either diet. This indicates that VDR’s function in adipose tissue could be gender-specific.

Interestingly, neither the weight of the mammary gland nor the histological appearance of the fat pad itself was altered in CVF mice. Thus, vitamin D signaling within mature adipocytes is not critical for maintenance of adipose tissue integrity. However, using three different methods, we detected an increase in the amount of adjacent epithelial tissue in the mammary gland of mice with adipose specific VDR deletion.

Hematoxylin and Eosin Y staining revealed slightly increased levels of ductal growth in CVF mice compared to their control CN1 counterparts. Quantitation of the epithelium present in the glands using Image J confirmed this observation, and revealed a significantly greater amount of epithelium in CVF mice compared to CN1 mice. Whole mounts of the mammary gland also revealed an increase in primary, secondary, and tertiary branching in the CVF mice, verifying data from the H+E stains. Quantitation of the epithelium with Image J confirmed a significantly greater amount of epithelium in CVF mice versus the CN1 mice.

Collectively, these findings indicate that in the absence of VDR, signals from mammary adipocytes upregulate growth of the adjacent epithelial cells and ducts. Therefore, the vitamin D pathway is critical for regulation of the cross-talk between the adipose and epithelial compartments within the mammary gland. In normal mice, the presence of VDR in the adipose tissue suppresses the growth of epithelial tissue within the ducts. These findings predict that when this pathway becomes disrupted, for example due to vitamin D deficiency, accelerated growth in the epithelium will occur. Our findings are consistent with previous studies that reported an increase in epithelial branching and ductal growth in the pubertal mammary gland of mice with global VDR deletion. Our data indicate that at least some of the effects of VDR to
inhibit epithelial growth are mediated via the adipose tissue.

During the course of this study, our collaborators at the University of Cincinnati evaluated early mammary gland development in the CVF strain and reported that deletion of VDR in adipose compartment enhances proliferation of the epithelium during pubertal development. \(^{(13)}\) Our study extends these findings to demonstrate that the enhanced branching and ductal density persists through adulthood, at least when mice are fed a high fat diet. Collectively, these data strongly indicate that the vitamin D/VDR complex in adipose tissue transmits growth inhibitory signals to the epithelium throughout development. Further studies are needed to identify the specific mediators of this vitamin D-regulated cross talk between the mammary fat pad and the epithelium.

Abdominal WAT weights were measured and sectioned to allow for morphological analysis. Mice with VDR ablation exhibited significantly increased abdominal WAT mass on both rescue and high fat diets when compared to their CN1 counterparts. In fact, the increased weight of WAT attributed for the majority of the accelerated body weight gain observed as previously mentioned. Additionally, Hematoxylin and Eosin Y stains revealed significantly larger adipocytes in CVF knockout mice versus CN1 mice fed a rescue diet. In high fat diets the difference between adipose tissue morphology was minimal, which was a likely result due to the nature of the diet itself. Of note, the males did not display any significant differences in WAT weights or morphology, matching the prior observation that VDR function in adipose tissue appears to be gender specific. Our results therefore display that Vitamin-D signaling in the adipose tissue is involved in regulation of WAT mass and morphology.

Strangely, our adipose specific VDR knockout mice do not mimic the global knockouts with respect to adiposity. Global VDR deletion results in a lean phenotype characterized by
reduced adipose tissue and adipocyte size. This is in direct contrast to the adipose specific VDR knockout mice that displayed increased body weights and increased white adipose depots.

Characterization of liver morphology with Hematoxylin and Eosin Y displayed a significant increase in adipose content of the liver in CVF mice on both diets. This finding sheds light onto the metabolic effects of VDR ablation in the adipose tissue within the liver itself. Fatty liver has been linked to cirrhosis, fibrosis, and diabetes, which may be accelerated by Vitamin D deficiency.

Hematoxylin and Eosin Y staining revealed an increase in the size and number of pancreatic islets of Langerhans in mice with VDR deletion. Prior research has shown global VDR knockout mice to be insulin resistant. (16) As such, it is possible islet hyperplasia may be a compensatory response to resistance. Since the islets are responsible for insulin production and secretion, an increased number and size of the islets could represent an attempt to elevate blood-insulin levels to overcome resistance caused by VDR ablation. These observations lend further insight into the adipose-specific function of Vitamin-D and diabetes, but further research is warranted to measure blood-insulin and blood-glucose levels in adipose-specific VDR knockouts.

Vitamin-D is known to negatively regulate collagen synthesis. Global VDR knockout mice have been shown to develop hepatic and pancreatic fibrosis. (12,15) By contrast, these tissues from adipose-specific VDR knockout mice did not exhibit evidence of fibrosis. Additionally, we saw no qualitative evidence of increased collagen synthesis or deposits around ducts within the liver and pancreas. This suggests that VDR deletion specifically in the adipose tissue is not sufficient to lead to fibrosis or overall increased collagen synthesis. Further studies are warranted including western blotting to accurately quantify collagen content and hence fibrosis.
Quality control results for the use of LiCl as an additional isolation step for RNA proved to be promising. Past issues in isolating RNA from adipose tissues included low purity, and low yields prevented the cleaning of samples. Incorporating LiCl as a purifying step after isopropanol precipitation allowed us to attain pure samples. As expected, RNA yields were still relatively low, but since they do not require additional steps to enhance purify, the yields were sufficient for use in cDNA synthesis and qPCR. Further studies comparing PCR results for RNA isolated with the LiCl protocol will be needed to determine if it is a suitable addition to our protocols.
References