Comparative Medicine
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Vol 70, No 2
April 2020
Pages 120–130

*Original Research*

# Effect of Chronic Vitamin D Deficiency on the Development and Severity of DSS-Induced Colon Cancer in *Smad3*$^{-/-}$ Mice

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**Both human epidemiologic data and animal studies suggest that low serum vitamin D increases the risk of inflammatory bowel disease (IBD) and consequently IBD-associated colorectal cancer. We tested the hypothesis that vitamin D deficiency increases the risk for colitis-associated colon cancer (CAC) by using an established CAC mouse model, 129-*Smad3*$^{tm1Par}$/J (*Smad3*$^{-/-}$) mice, which have defective transforming growth factor β-signaling and develop colitis and CAC after the administration of dextran sodium sulfate (DSS). After determining a dietary regimen that induced chronic vitamin D deficiency in *Smad3*$^{-/-}$ mice, we assessed the effects of vitamin D deficiency on CAC. Decreasing dietary vitamin D from 1 IU/g diet (control diet) to 0.2 IU /g diet did not decrease serum 25-hydroxyvitamin D (25(OH)D) levels in *Smad3*$^{-/-}$ mice. A diet devoid of vitamin D (<0.02 IU/g diet [no added vitamin D]; vitamin D-null) significantly decreased serum 25(OH)D levels in mice after 2 wk (null compared with control diet: 8.4 mg/mL compared with 12.2 ng/mL) and further decreased serum levels to below the detection limit after 9 wk but did not affect weight gain, serum calcium levels, bone histology, or bone mineral density. In light of these results, *Smad3*$^{-/-}$ mice were fed a vitamin D–null diet and given DSS to induce colitis. Unexpectedly, DSS-treated *Smad3*$^{-/-}$ mice fed a vitamin D–null diet had improved survival, decreased colon tumor incidence (8% compared with 36%), and reduced the incidence and severity of colonic dysplasia compared with mice fed the control diet. These effects correlated with increased epithelial cell proliferation and repair early in the disease, perhaps reducing the likelihood of developing chronic colitis and progression to cancer. Our results indicate that vitamin D deficiency is beneficial in some cases of CAC, possibly by promoting intestinal healing.**

**Abbreviations:** 25(OH)D, 25-hydroxyvitamin D; BrdU, bromodeoxyuridine; CAC, colitis-associated colon cancer; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; TGF, transforming growth factor; VDR, vitamin D receptor

**DOI:** 10.30802/AALAS-CM-19-000021

Vitamin D deficiency is increasingly prevalent around the world, in both industrialized and developing countries. Epidemiologic studies suggest that as many as 30% to 50% of children and adults worldwide are at risk of vitamin D deficiency.[35] Humans obtain most of their vitamin D from exposure to sunlight through UVB radiation; however, dietary or oral supplementation is an important means of maintaining adequate serum vitamin D levels in persons who have limited exposure to sunlight. Many factors may contribute to widespread vitamin D deficiency, including inadequate intake of foods rich in vitamin D and insufficient exposure to sunlight due to residing at higher latitudes where UVB exposure is limited or because of increased protection against sun exposure through clothing or sunscreen.[33,52]

Vitamin D is classically known for its role in the regulation of bone metabolism, bone remodeling, and calcium homeostasis.[19,20,32,33] This role is evidenced by the association between prolonged vitamin D deficiency and delayed growth and rickets in children[32] as well as osteoporosis and osteopenia in adults.[31] Recently, vitamin D deficiency has been associated with a wide range of other diseases including asthma,[10] multiple sclerosis,[30] rheumatoid arthritis,[41] type 1 diabetes,[43,57] heart disease,[9,13] inflammatory bowel disease (IBD),[27,63] depression,[5,55] and tuberculosis.[68] In addition, epidemiologic studies suggest that vitamin D deficiency increases the risk of developing and dying from several cancers including colorectal, breast, and prostate cancers.[22,62] This association, along with disagreement regarding what constitutes adequate serum levels of vitamin D,[34,65] has led to an increased interest in studying the effects of both vitamin D supplementation and vitamin D deficiency on human health.

In humans, IBD is a risk factor for the development of colitis-associated colorectal cancer (CAC),[66] and epidemiologic evidence suggests that low serum vitamin D increases the risk for developing both IBD and colorectal cancer.[3,4,22,29] The mechanisms by which vitamin D deficiency influence the risk of CAC are unknown and are difficult to study in humans, because of complicating factors including genetics, varied environments, infrequency of CAC formation in IBD patients, and the long

*Received: 14 Feb 2019. Revision requested: 04 Apr 2019. Accepted: 19 May 2019.*
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time period between IBD onset and CAC development. Consequently, animal models are important tools for studying the effects of vitamin D deficiency in CAC. Therefore to study IBD, investigators used mice lacking the vitamin D receptor (*Vdr*) or the enzyme that generates active vitamin D hormone (*Cy27b1*$^{-/-}$ mice) as well as dietary vitamin D deprivation in susceptible animals. We recently reviewed and summarized many of these IBD studies,[53] but the effects of vitamin D deficiency on CAC are understudied. Because many of the previously published models of vitamin D deficiency were either short term[14,42] or multigenerational studies with vitamin D-deficient diets intended to produce animals with severe calcium imbalances,[14] we tried to identify a dietary regime in which we could induce chronic vitamin D deficiency for as long as 18 wk without causing calcium imbalances or bone density loss to assess whether there is a causal relationship between vitamin D deficiency and CAC using an animal model of inflammation-associated colon cancer, *Smad3*$^{-/-}$ mice.

The TGFβ signaling pathway is frequently altered in patients with IBD and is one of the most commonly deregulated pathways in colorectal cancer.[7,26] *Smad3*$^{-/-}$ (*Smad3*$^{tm1Par}$/J) mice[74] lack the transcription factor SMAD3, resulting in defective TGFβ signaling. These mice develop colitis and subsequent colon adenocarcinomas when infected with *Helicobacter*[49] or when treated with dextran sodium sulfate (DSS).[69] Therefore, *Smad3*$^{-/-}$ mice are a useful tool for mechanistic studies of IBD and CAC. Because vitamin D supplementation in DSS- or azoxymethane-induced IBD and CAC models is protective,[17,21] we hypothesized that vitamin D deficiency in *Smad3*$^{-/-}$ mice treated with DSS would increase the risk of CAC. Surprisingly, we found that a vitamin D-deficient diet protected mice against DSS-induced colon tumors and that these protective effects may be mediated through increased cellular proliferation associated with more rapid healing after epithelial cell damage induced by DSS treatment.

## Materials and Methods

**Mice and diets.** *Smad3*$^{+/-}$ (129-*Smad3*$^{tm1Par}$/J) mice were obtained from The Jackson Laboratory (Bar Harbor ME) and bred at the University of Washington to generate *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ (wild type) mice which were subsequently maintained as separate lines by using homozygous breeding.[49] All mice were group-housed (2 to 5 mice per cage) in autoclaved IVC (Thoren, Hazleton, PA) with autoclaved corncob bedding (The Andersons, Maumee, OH) in an SPF colony free of *Helicobacter* spp. and murine norovirus and screened for pathogens as previously described.[60] Animals were provided with reverse-osmosis purified, acidified, autoclaved water in bottles and were fed irradiated purified rodent diets with either 1 IU (5SRH, AIN93M, control diet), 0.2 IU (5ACE, low vitamin D diet) or less than 0.02 IU vitamin $D_3$ per gram diet (5AV4, vitamin D-null diet). For the vitamin D-null diet, no vitamin D was added. All diets were formulated by Test Diet (St Louis, MO) and based on the AIN93M diet, which was formulated for the maintenance of rodents' health.[64] Studies to determine the optimal dietary regimen to induce chronic vitamin D deficiency were performed using combinations of all the diets described above. Bone disease and IBD–CAC studies were completed with control and vitamin D-null diets. Vitamin D levels in the low and null vitamin D diets were analyzed by liquid chromatography-mass spectrometry (Covance Madison, WI) and were 0.266 IU vitamin $D_3$/g diet and below the limit of detection (<0.02 IU vitamin $D_3$/g diet), respectively. Male and female mice (age, 3 to 14 wk) were used. Animals of different ages and sex were evenly distributed among treatment groups. Animal numbers are described for each experiment in the figure legends. All animal procedures were approved by the University of Washington IACUC.

**Induction of colitis.** Animals were fed experimental diets for 2 wk prior to induction of colitis with DSS (36,000 to 50,000 MW, MP Biomedicals, Solon, OH). DSS was prepared as a stock solution (40%) in autoclaved distilled water and then diluted further to a 1.5% solution in autoclaved, acidified reverse-osmosis-purified water and placed in autoclaved water bottles. Animals were placed on 1.5% DSS in the drinking water for 3 d, followed by untreated water. This treatment regimen was chosen based on our previous studies,[69] in which a 7-d treatment time was used to induce colitis and approximately 30% cancer incidence. The shorter time frame (3 d compared with 7 d) was used in the studies presented here because of the concern that vitamin D deficiency could exacerbate colitis and reduce survival.[24,42] Animals were weighed weekly, and health checks were performed at least 3 times weekly. Mice were euthanized by $CO_2$ asphyxiation and necropsied at designated endpoints or when they developed signs of severe disease including 20% body weight loss, ulcerated rectal prolapse, diarrhea, hunched posture, and dehydration.

**Serum vitamin D, calcium, and phosphorus and tissue collection.** For repeated blood sampling, submandibular puncture was performed to collect a maximum total volume of less than 1% of body weight in a 2-wk period. Blood draws ranged from 50 to 200 µL blood per mouse per time point. At the termination of each study, blood was obtained by using cardiac puncture immediately after euthanasia by $CO_2$ asphyxiation. Blood was placed directly into serum separator tubes (Serum Separator Tubes–Gold, BD Biosciences, San Jose California) after collection. Serum was separated by centrifugation within 2 h of collection and stored at –80 °C until samples were submitted to Heartland Assays (Ames, IA) for quantification of 25-hydroxyvitamin D (radioimmunoassay) and calcium and phosphorus levels (colorimetric assays). Mesenteric lymph nodes, cecum, colon, and rectum were fixed in 10% phosphate-buffered formalin and processed for routine histologic examination. For short-term studies (9 and 16 d), the colon was opened longitudinally, and feces were removed by gently flushing with PBS. The colon was then cut into thirds (proximal, mid, and distal) and fixed in 10% phosphate-buffered formalin. After fixation, the sections of the colon were cut longitudinally into strips and were embedded on edge. For long-term studies, the colon was prepared by using a 'Swiss roll' technique.[3] Hindlimbs were fixed in 10% phosphate-buffered formalin, decalcified, and stained with hematoxylin and eosin for routine histologic examination.

**Bone mineral density.** Bone density was assessed by densitometry in *Smad3*$^{-/-}$ and wildtype mice after 18 wk of being fed either control or vitamin D-null diet. After euthanasia and abdominal tissue harvest, bone mineral density was measured by using a PIXImus Lunar densitometer (GE Healthcare, Waukesha, WI).

**Pathology.** Cecum and colon were evaluated for inflammation, dysplasia, and neoplasia by a board-certified veterinary pathologist (PT), who was blind to experimental treatment groups. Typhlocolitis scores were assigned by evaluating tissue for severity of mucosal loss, mucosal epithelial changes, degree of inflammation, and extent of pathology, as previously described.[69] Dysplasia was classified according to a previous scale,[16,69] and only frankly invasive adenocarcinoma scored as neoplasia.[48,69] The femur and tibia were evaluated for evidence of abnormal bone remodeling or osteomalacia.

**Evaluation of bromodeoxyuridine (BrdU) incorporation.** To assess cellular proliferation after DSS administration, mice were injected intraperitoneally with BrdU labeling reagent (0.2 mL


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per animal, Invitrogen, Eugene Oregon) 24 h prior to euthanasia. After euthanasia, tissues were collected and processed as described earlier. Paraffin-embedded tissues were submitted to the Histology and Imaging Core at the University of Washington (Seattle, WA) for immunohistochemical staining of BrdU. Immunohistochemical staining was performed on a Bond Max automated immunostainer (Leica Biosystems, Buffalo Grove, IL) using associated reagents. Sections were baked for 30 min at 60 °C and deparaffinized. After antigen retrieval with citrate buffer, pH 6.0 (catalog no. AR9640, Leica) at 100 °C for 10 min and blocking with 10% normal goat serum in Tris-buffered saline for 10 min, the sections were incubated with rat antiBrdU antibody (dilution, 1:2000; catalog no. ab6326; Abcam, Cambridge, MA) or rat IgG 2b isotype control (1:1000; catalog no. 553986, Pharmingen, San Diego, CA); both antibodies were diluted in Bond Primary Antibody Diluent (catalog no. AR9352) and were incubated for 30 min at room temperature. Slides were probed with rabbit antirat IgG secondary antibody (1:300; catalog no. AI-4001, Vector Labs, Olean, NY) for 8 min at room temperature and then incubated with antirabbit polymer–HRP–IgG (BOND Reagent Detection System, catalog no. DS9800, Leica Biosystems) for 8 min at room temperature. Additional blocking for endogenous peroxidase was performed by using a peroxide block (3% $H_2O_2$; catalog no. DS9800, Leica) for 5 min at room temperature, and sections were incubated with 3,3′-diaminobenzidine for 10 min at room temperature (catalog no. DS9800, Bond Mixed Refine DAB Substrate, Leica). Tissues were counterstained with hematoxylin for 4 min followed by 2 rinses in water. Slides were then dehydrated through 100% ethanol, cleared in xylene, and mounted by using synthetic resin mounting medium and a no. 1.5 coverslip. Colonic epithelial cell proliferation was assessed in 3 segments of the colon (proximal, middle, and distal colon). The total numbers of BrdU-positive and -negative epithelial cells were manually counted at 20× magnification from 7 to 10 randomly selected crypts in a full-plane section per colon segment to determine the average percentage of BrdU-positive epithelial cells per crypt.

**Statistical analysis.** Prior to statistical analysis, distribution of data was assessed for normality. When data were not normally distributed, transformation was attempted; when transformation did not normalize the distribution, nonparametric tests were performed. Serum vitamin D, serum calcium, histologic scoring, and bone mineral densities were analyzed by using either unpaired *t* or Mann–Whitney *U* tests. For studies comparing more than 2 groups, either parametric one-way ANOVA followed by Bonferroni posthoc testing or nonparametric Kruskal–Wallis Test followed by Dunn multiple comparison testing was used. Cancer and dysplasia incidences were analyzed by using the Fisher exact test. Survival was assessed by using a log-rank test. All data are presented as mean ± 1 SD. Differences with a *P* value of 0.05 or less were considered significant. All statistical analyses were performed by using Prism software (version 5.04, GraphPad Software, La Jolla, CA).

## Results

**Effects of vitamin D-null diet on serum 25(OH)D and serum calcium levels in *Smad3*$^{-/-}$ mice.** Chronic severe vitamin D deficiency can result in calcium imbalances and bone weakness.[2,14,32,33] Because colon tumor development in the *Smad3*$^{-/-}$ mice requires approximately 16 wk, we were concerned about inducing severe vitamin D deficiency due to chronically feeding mice a vitamin D-deficient diet. Therefore, we evaluated methods to produce a substantial decrease in serum 25(OH)D levels without causing severe vitamin D deficiency symptoms. *Smad3*$^{-/-}$ breeders were placed on the low vitamin D diet (0.2 IU vitamin D/g diet; containing 80% less vitamin D compared with control) after the birth of a litter through weaning of that litter. The pups were then weaned onto the low vitamin D diet and fed the diet for 6 wk. Unexpectedly, the low vitamin D diet regimen did not result in lower serum vitamin D or serum calcium levels compared with mice fed a control diet for 6 wk (Figure 1 A and B).

Because 0.2 IU vitamin D/g diet did not result in the desired decrease in circulating serum vitamin D levels, *Smad3*$^{-/-}$ mice were then fed a diet formulated without vitamin D (vitamin D-null diet). After 2 wk on the diet, animals fed vitamin D-null diet had lower serum 25(OH)D compared with mice fed the control diet (Figure 1 C, $P < 0.0001$). Serum vitamin D levels continued to decrease from week 2 to week 4 on this diet, demonstrating that a vitamin D-null diet is required to significantly decrease serum vitamin D levels in *Smad3*$^{-/-}$ mice (Figure 1 C). Because our goal was to induce vitamin D deficiency over an extended period without causing hypocalcemia complications, we then switched animals to a low vitamin D diet after feeding the vitamin D-null diet for 4 wk. Surprisingly, serum 25(OH)D levels of mice fed low vitamin D diet rebounded within 3 wk of switching to low vitamin D diet and reached similar levels to those of mice fed a control diet (Figure 1 C). Serum calcium levels remained unchanged on both the vitamin D-null and low vitamin D diets (Figure 1 D). These data demonstrate that a diet deficient in vitamin D is necessary to maintain low serum vitamin D levels, whereas 0.2 IU vitamin D/g diet (1/5 of control level) is insufficient to induce or maintain vitamin D deficiency in *Smad3*$^{-/-}$ mice.

Next we tested whether the vitamin D-null diet can be used for a long-term study without causing severe vitamin D deficiency and associated pathology in mice. We hypothesized that weanling and young adult animals, which are in rapid phases of growth, would be most susceptible to vitamin D deficiency. Therefore, 3-wk-old (weanling) and 6-wk-old (young adult) *Smad3*$^{-/-}$ mice were fed the vitamin D-null diet for 18 wk. Blood samples were collected at 2- to 3-wk intervals to measure serum 25(OH)D and serum calcium. Serum 25(OH)D levels were significantly lower in mice fed the vitamin D-null diet compared with mice fed the control diet after 2 wk on the diets ($P < 0.01$ for adults, $P < 0.001$ for weanlings) and dropped below the limit of detection after 9 wk on the vitamin D-null diet (Figure 2 A). However, the vitamin D-null diet did not cause changes in serum calcium (Figure 2 B) or body weight (Figure 2 C) compared with the control diet throughout the 18-wk experimental period. In addition, serum phosphorus levels, measured after 18 wk of diet feeding, were not different between groups (Figure 2 D). These observations were not specific to *Smad3*$^{-/-}$ mice, given that the vitamin D-null diet also reduced serum 25(OH)D levels ($P < 0.0001$) without causing changes in serum calcium, serum phosphorus, and body weight in wildtype mice (Figure 3).

**Effect of vitamin D-null diet on bone disease in *Smad3*$^{-/-}$ and wildtype mice.** Because vitamin D deficiency can significantly increase bone resorption, resulting in the development of rickets particularly in growing animals, mice were evaluated for evidence of bone disease through bone mineral density scanning and histology. After 18 wk on the vitamin D-null diet, both *Smad3*$^{-/-}$ mice and wildtype mice showed no evidence of cortical bone loss or abnormal bone remodeling according to densitometry (Figure 4 A and B) and no histologic abnormalities (data not shown). These data are consistent with our findings that serum calcium levels do not change in the *Smad3*$^{-/-}$ or wildtype mice fed a vitamin D-null diet, suggesting that calcium homeostasis


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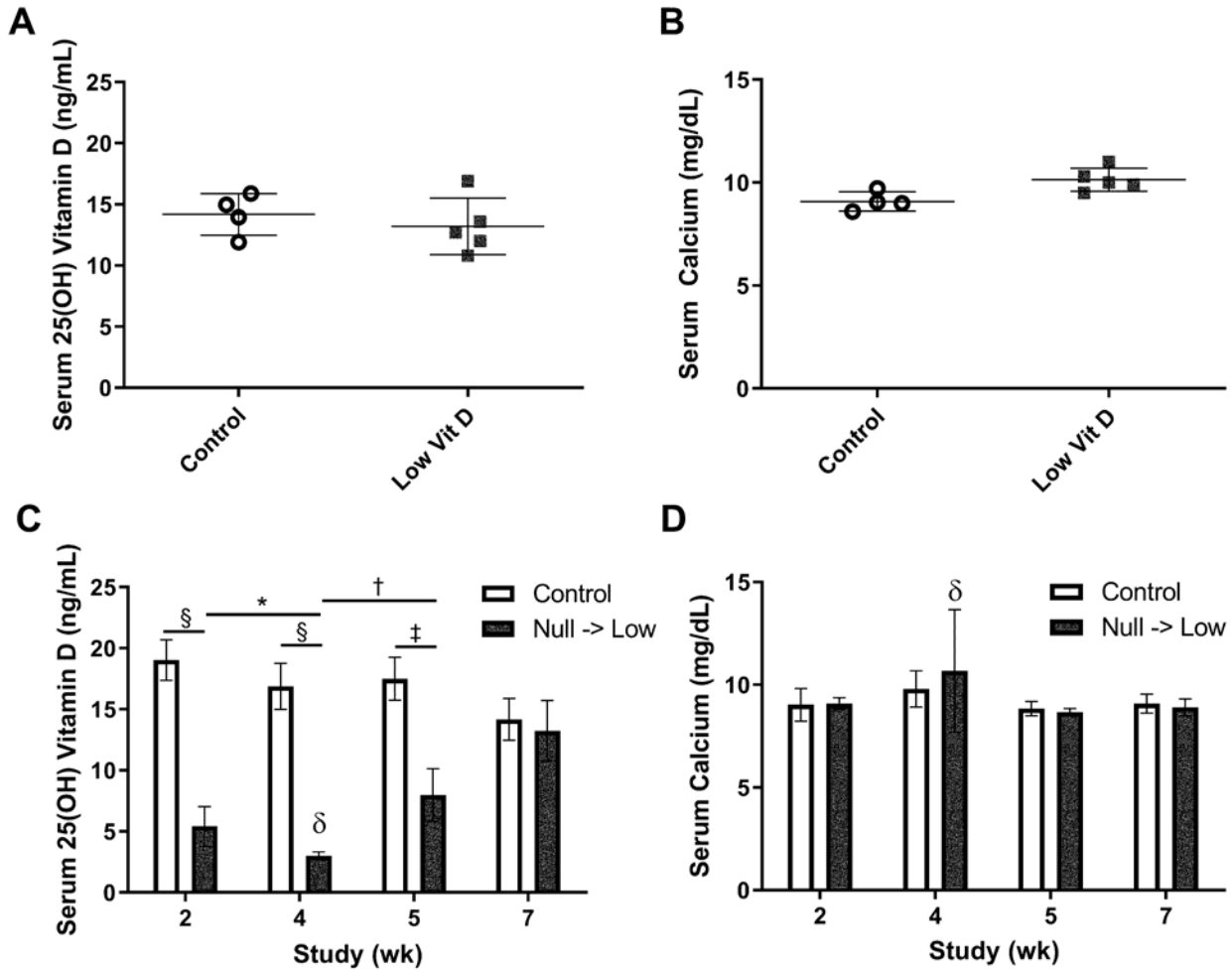


**Figure 1.** Low vitamin D diet does not decrease serum vitamin D or serum calcium levels in *Smad3*$^{-/-}$ mice. Serum 25(OH)D and calcium were evaluated in 2 dietary regimens involving low vitamin D diet. In one regimen, (A) serum 25(OH) D and (B) serum calcium were measured after animals were fed control ($n$ = 4) or vitamin D low ($n$ = 5) diet for 6 wk starting at weaning. In the other regimen, (C) serum 25(OH)D and (D) serum calcium were measured at weeks 2, 4, 5, and 7 from study initiation in mice that received vitamin D-null diet for 4 wk and then were switched to low vitamin D diet compared with those on control diet for the duration of the study. δ indicates the time point at which mice on vitamin D-null diet in the null→low diet treatment group were switched to the low vitamin D diet. Mean and SD are indicated on dot plots. Error bars on bar graphs are SD. *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$ (Student's $t$ test) between groups are indicated. Comparisons were made between control compared with null→low group and also between null→low diet groups before and after the diet switch at 4 wk.

can be maintained despite feeding of a vitamin D-null diet for a prolonged period (18 wk).

**Effect of vitamin D deficiency on DSS-induced IBD and cancer in *Smad3*$^{-/-}$ mice.** Using the vitamin D-null diet, we sought to determine whether chronic vitamin D deficiency exacerbated DSS-induced colitis and CAC in *Smad3*$^{-/-}$ mice. Mice were placed on either control or vitamin D-null diet for 2 wk prior to treatment with DSS and were followed afterward for 16 wk, which is sufficient time for CAC development in this model.[69] Therefore, at the time mice were treated with DSS, mice should have had 1.5- to 3-fold lower serum 25(OH)D levels compared with mice on control diet (Figures 1 C and 2 A). Unexpectedly, as assessed by survival, dysplasia, and tumor incidence, the severity and incidence of disease were lower in mice fed the vitamin D-null diet compared with mice fed the control diet. Survival was significantly ($P$ = 0.009) improved in animals treated with DSS in drinking water and fed the vitamin D-null diet (Figure 5 A) compared with DSS-treated mice fed the control diet and was not significantly different ($P$ = 0.2337) from vitamin D-deficient *Smad3*$^{-/-}$ animals given water only. Along with improved survival, the incidence of invasive colon carcinoma was significantly lower (4.4-fold, $P$ = 0.0007, Figure 5 B) in DSS-treated mice animals fed a vitamin D-null diet compared with those fed a control diet. In addition, typhlocolitis scores in DSS-treated mice were significantly ($P < 0.001$) lower in mice fed the vitamin D-null diet compared with those fed the control diet (Figure 5 C). Finally, the incidence (31% compared with 61%, $P$ = 0.0014) and severity ($P < 0.01$) of colonic dysplasia in DSS-treated mice were lower in mice fed the vitamin D-null diet compared with those fed the control diet (Figure 5 D).

To determine whether the protective effects of vitamin D-deficiency against DSS-induced colitis and CAC were associated with the roles that vitamin D plays in cell cycle regulation and epithelial cell proliferation,[8,37,61] we examined colonic inflammation and epithelial cell proliferation in DSS-treated *Smad3*$^{-/-}$ mice during the early inflammatory phase after DSS treatment. Two specific time points were chosen for the evaluation of typhlocolitis: 9 d after DSS treatment, when clinical disease peaks, and 16 d


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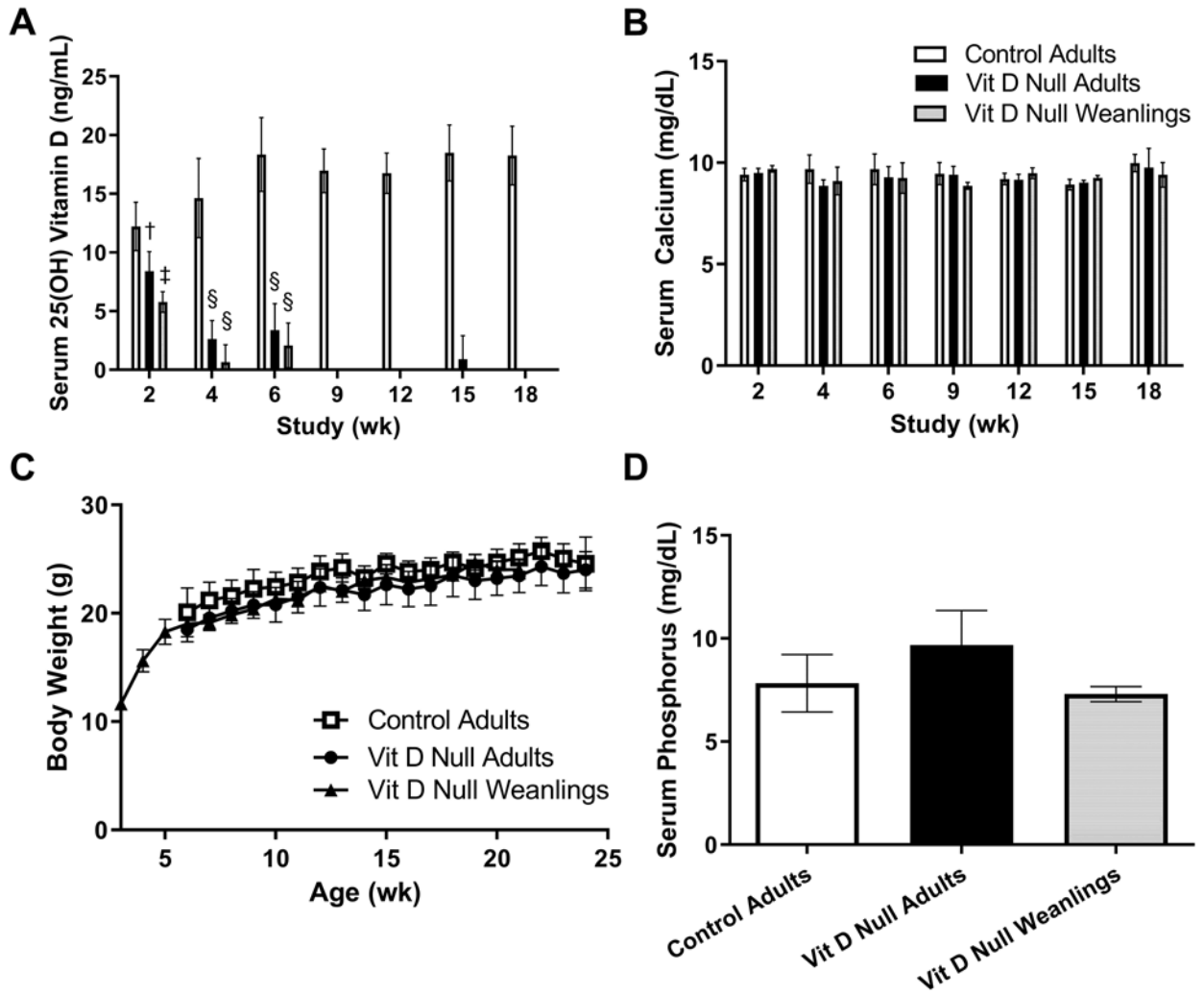


**Figure 2.** Vitamin D-null diet significantly decreases serum vitamin D levels without altering serum calcium, weight gain, or serum phosphorus in *Smad3*$^{-/-}$ mice. (A) Serum 25(OH)D and (B) serum calcium in *Smad3*$^{-/-}$ mice fed control diet ($n = 5$, 6-wk-old mice) or vitamin D-null diet ($n = 5$, 3-wk-old mice [weanling] and $n = 5$, 6-wk-old mice [adult]). Body weight was measured weekly and (C) average body weight (in grams) is plotted for each group. (D) Serum phosphorus was measured after 18 wk on diet ($n = 4$ per group). Error bars are SD. One-way ANOVA (parametric) followed by a Bonferroni post hoc test for multiple comparisons. †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$ compared with control.

afterward, when healing from the DSS-induced damage should be evident.[69] DSS-treated mice demonstrated higher typhlocolitis scores (not significant) compared with animals maintained on no-DSS water regardless of the diet at day 9 after DSS (Figure 6 A). However, at day 16 after DSS treatment, typhlocolitis scores were significantly lower in DSS-treated mice fed vitamin D-null diet compared with DSS-treated control-diet fed animals (Figure 6 B, $P < 0.05$). These data suggest that regardless of diet, mice develop similar degrees of typhlocolitis initially after DSS treatment, but the inflammation resolves more quickly in animals fed the vitamin D-null diet. To determine whether typhlocolitis resolves more quickly due to changes in epithelial cell proliferation, we evaluated epithelial cell proliferation by BrdU uptake. Mice fed vitamin D-null diet had a higher incidence of epithelial cell proliferation, demonstrated by increased BrdU staining at 16 d after DSS treatment (Figure 6 C, $P = 0.0257$) as compared with mice fed control diet. These data suggest that the decreased colitis and cancer in *Smad3*$^{-/-}$ mice fed vitamin D-null diet could be due in part to increased epithelial cell proliferation, resulting in faster reepithelialization of the colon after DSS treatment.

## Discussion

Vitamin D deficiency has been associated with an increased risk for IBD and colorectal cancer in human epidemiologic studies[3,4,22,29] and in animal models.[6,11,38,42] Animal models have been useful for investigating the role of vitamin D in IBD because mechanisms behind the associative studies in humans cannot be assessed precisely under controlled genetic and environmental conditions. Furthermore, in animal models, it is easier to modify and monitor vitamin D intake and to determine whether the changes result in altered vitamin D status by analyzing serum and tissues. This situation allows investigators to determine how variations in systemic vitamin D over time (and under different stages of disease development) can impact disease outcomes.

To investigate the role of chronic vitamin D deficiency on IBD/CAC, we investigated diets containing various levels of vitamin D in *Smad3*$^{-/-}$ mice, a model of IBD–CAC. Our goal was to achieve significantly decreased levels of serum vitamin D without causing hypocalcemia and associated bone disease. For this purpose, we measured 25(OH)D, which is the product of the first of 2 hydroxylation steps needed to generate


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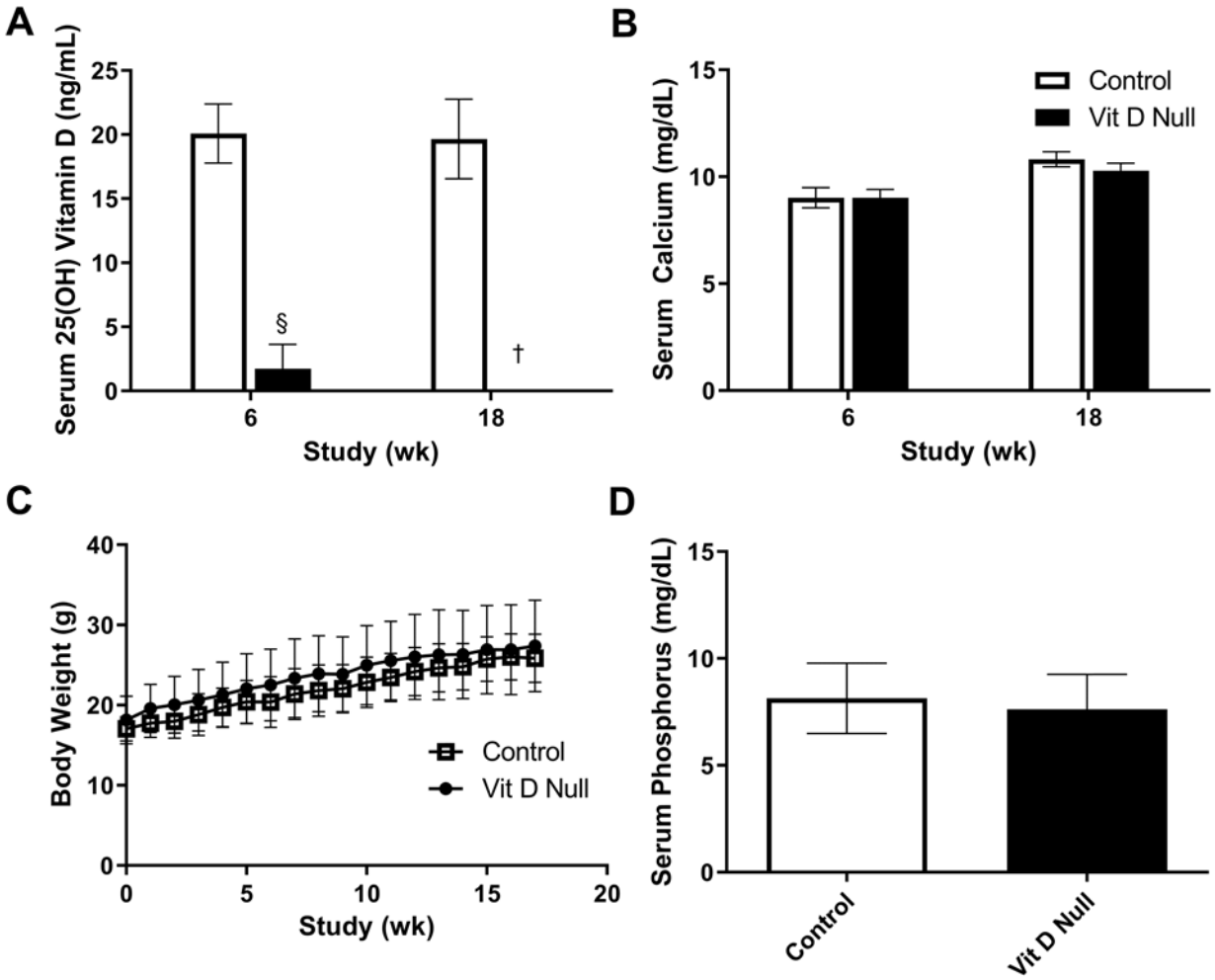


**Figure 3.** Vitamin D-null diet significantly decreases serum vitamin D levels without altering serum calcium, weight gain, or serum phosphorus in wildtype mice. (A) Serum 25(OH)D and (B) serum calcium in wildtype mice fed control ($n$ = 5, 5- to 8-wk-old mice) or vitamin D-null diet ($n$ = 6, 5- to 8-wk-old mice). Body weight was measured weekly, and (C) average body weight (in grams) is plotted for each group. (D) Serum phosphorus was measured at 18 wk after diet initiation. Error bars indicate 1 SD. †, $P < 0.01$ (Mann–Whitney $U$ test); §, $P < 0.0001$ (Student $t$ test) compared with control.

the biologically active form of vitamin D, 1,25-dihydroxyvitamin D.[12,73] The active form is not suitable as a predictor of overall vitamin D nutritional status as its production is tightly regulated[20] and its half-life is relatively short (4 to 20 hr). We found that decreasing the dietary vitamin D level to 1/5 that of the control diet, AIN93M, did not change serum vitamin D levels in mice. AIN93M diet, which has been determined to be sufficient to support growth and maintenance of health of rodents,[64] contains 1 IU vitamin D/g diet. Therefore, we next tested a vitamin D-null diet that was identical in formulation to AIN93M except that vitamin D was removed from the vitamin mixture added to the diet. This vitamin D-null diet decreased serum vitamin D levels in *Smad3*$^{-/-}$ mice without causing deleterious effects involving bone and calcium homeostasis up to 18 wk.

We were surprised to find that in our hands, the low vitamin D diet (0.2 IU vitamin D/g) was not effective at inducing vitamin D deficiency despite similar regimens being used in other models.[23,51] One group has demonstrated that feeding adult FVB mice diets containing either 0.25 or 0.05 IU vitamin D/g were effective at significantly reducing serum 25(OH)D levels by 4 wk on the diet compared with mice fed a control level of vitamin D (1.5 IU/g), with serum 25(OH)D levels remaining steady from approximately 1 mo after diet change until as long as 4 mo.[51] Similar results were observed in C57BL/6 mice.[23] In contrast, we did not observe decreased serum 25(OH)D levels in mice fed a 0.2 IU vitamin D/g diet even after 6 wk on diet. Furthermore, our 'low vitamin D' diet increased serum 25(OH)D in mice that had previously been fed a null diet to levels similar to those of control diet-fed mice (Figure 1 C). Several differences might explain the discrepancies between our studies and others.[23,51] Although all of the diets were custom-made, purified diets, they were all prepared by different manufacturers with slightly different nutrient compositions. In addition, the analysis of the dietary vitamin D concentration as well as the serum 25(OH)D concentrations were performed by using different methodologies, thus making direct comparisons between the studies difficult. Also, our studies used mice on a 129 background whereas the other studies used mice on FVB and C57BL/6 backgrounds. There are few data comparing vitamin D absorption and metabolism across different strains of mice; however, it is known that differences in mouse strain can affect serum analytes even in age- and sex-matched healthy mice.[58] Together, our data emphasize the importance of verifying vitamin D concentration


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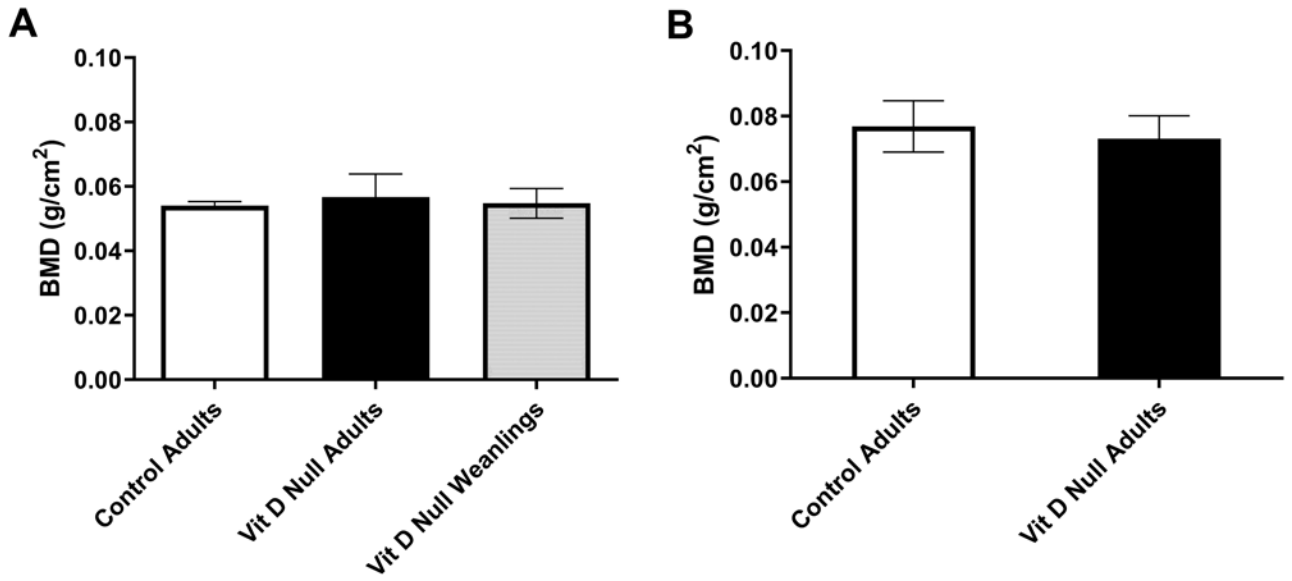


**Figure 4.** Vitamin D-null diet does not alter bone density in *Smad3*$^{-/-}$ or wildtype mice. Total body bone mineral density (BMD) scanning was performed after euthanasia of *Smad3*$^{-/-}$ and wildtype mice at 18 wk after diet initiation. (A) *Smad3*$^{-/-}$ mice fed control diet ($n$ = 5, 6-wk-old mice) or vitamin D-null diet ($n$ = 5, 3-wk-old mice [weanling] and $n$ = 5, 6-wk-old mice [adult]) and (B) wildtype mice fed control ($n$ = 5, 5- to 8-wk-old mice) or vitamin D-null diet ($n$ = 6, 5- to 8-wk-old mice). Error bars indicate 1 SD. No significant differences according to Kruskal–Wallis testing.

in the diet as well as verifying serum vitamin D status in each animal model to ensure that the intended effects of the dietary treatment are reached within a study.

Other published studies using vitamin D-deficient diets are either short-term[14,42] or multigenerational studies with vitamin D deficient diets intended to produce animals with severe calcium imbalances.[14] In our studies, we found that a vitamin D-null diet can be used to induce chronic vitamin D deficiency for as long as 18 wk without causing calcium imbalances or bone density loss. It is likely that sufficient dietary calcium (0.5%) in our diet compensated for the decreased calcium absorption typically seen with decreased serum vitamin D levels, because it has been shown that providing increased calcium in the diet can maintain normal calcium levels in mice lacking vitamin D receptor signaling in the gastrointestinal tract.[45] Our data agree with the findings in a recent report that a vitamin D-null diet could be used to induce chronic vitamin D deficiency in geriatric mice without causing serum calcium imbalances or osteomalacia.[71] In that report, the authors suggest that their findings in geriatric mice might differ depending on the age and growth rate of the mouse; however, our data in young mice showed that a vitamin D-null diet can be used to induce chronic vitamin D deficiency in this age group of mice.

DSS is commonly used to model IBD, because it effectively induces colitis in a variety of mouse strains[50] by directly damaging the colonic epithelium resulting in mucosal erosions and loss of epithelial cell barrier function.[15,18,69] Our previous work demonstrated that *Smad3*$^{-/-}$ mice are more susceptible to DSS-induced colitis compared with wild type mice, likely due in part to impaired mucosal healing and abnormal epithelial proliferation.[59,69] This situation, combined with immune abnormalities in *Smad3*$^{-/-}$ mice,[49] likely leads to the development of chronic inflammation, predisposing these animals to develop subsequent inflammation-associated colon cancer.[69] Unexpectedly, we found that vitamin D-deficient *Smad3*$^{-/-}$ mice treated with DSS had improved survival, decreased dysplasia, and significantly fewer colon tumors compared with control diet-fed animals, thus contrasting with previously published studies in other strains of mice. Others have demonstrated that vitamin D deficiency exacerbates DSS colitis in C57BL/6[6,42] and *Il10*$^{-/-}$ mice.[11] In addition, vitamin D receptor (*Vdr*)$^{-/-}$ mice are more susceptible to DSS-induced colitis,[25,40] whereas expression of transgenic VDR in epithelial cells of *Vdr*$^{-/-}$ mice partially rescued the mice due to decreased rates of epithelial cell apoptosis.[46] In a study evaluating colitis and CAC, *Vdr*$^{-/-}$ mice developed increased colon tumor burdens compared with wildtype controls after treatment with azoxymethane combined with multiple rounds of DSS.[17] However, not all studies demonstrate an exacerbating effect associated with vitamin D deficiency. Recent work[28] indicates no differences in histologic or endoscopic disease between vitamin D sufficient or deficient C67BL/6 mice after treatment with DSS. The differences between our findings and those just outlined could be due to differences in the background strain of the mice used, DSS concentration, duration of treatment, or the use of the *Smad3*$^{-/-}$ model with dysregulated TGFβ signaling that can intersect with VDR signaling. Compared with these other studies, we used a lower concentration of DSS for a shorter duration because we have found that *Smad3*$^{-/-}$ mice have high susceptibility and mortality to DSS-induced damage.[69] In addition, vitamin D deficiency may influence IBD–CAC variably even in the same mouse model when different inflammatory triggers, such as DSS compared with *Helicobacter* infection, are used for colitis induction. We previously used a vitamin D-null diet in the *Helicobacter*-induced colitis and CAC in *Smad3*$^{-/-}$ mice and observed that disease neither exacerbated or improved by vitamin D deficiency.[54] Interestingly, recent work in a spontaneous colon cancer model, F344-*Apc*$^{Pirc/+}$ rats, found protective effects associated with vitamin D deficiency, noting a decreased incidence of colon tumors in vitamin D-deficient rats.[36] However, vitamin D deficiency had no effect on tumor incidence in ACI × F344-*Apc*$^{Pirc/+}$ rats.[36] Future studies are needed to help understand mechanisms behind the variable effects of vitamin D deficiency on IBD/CAC by closely evaluating the systemic


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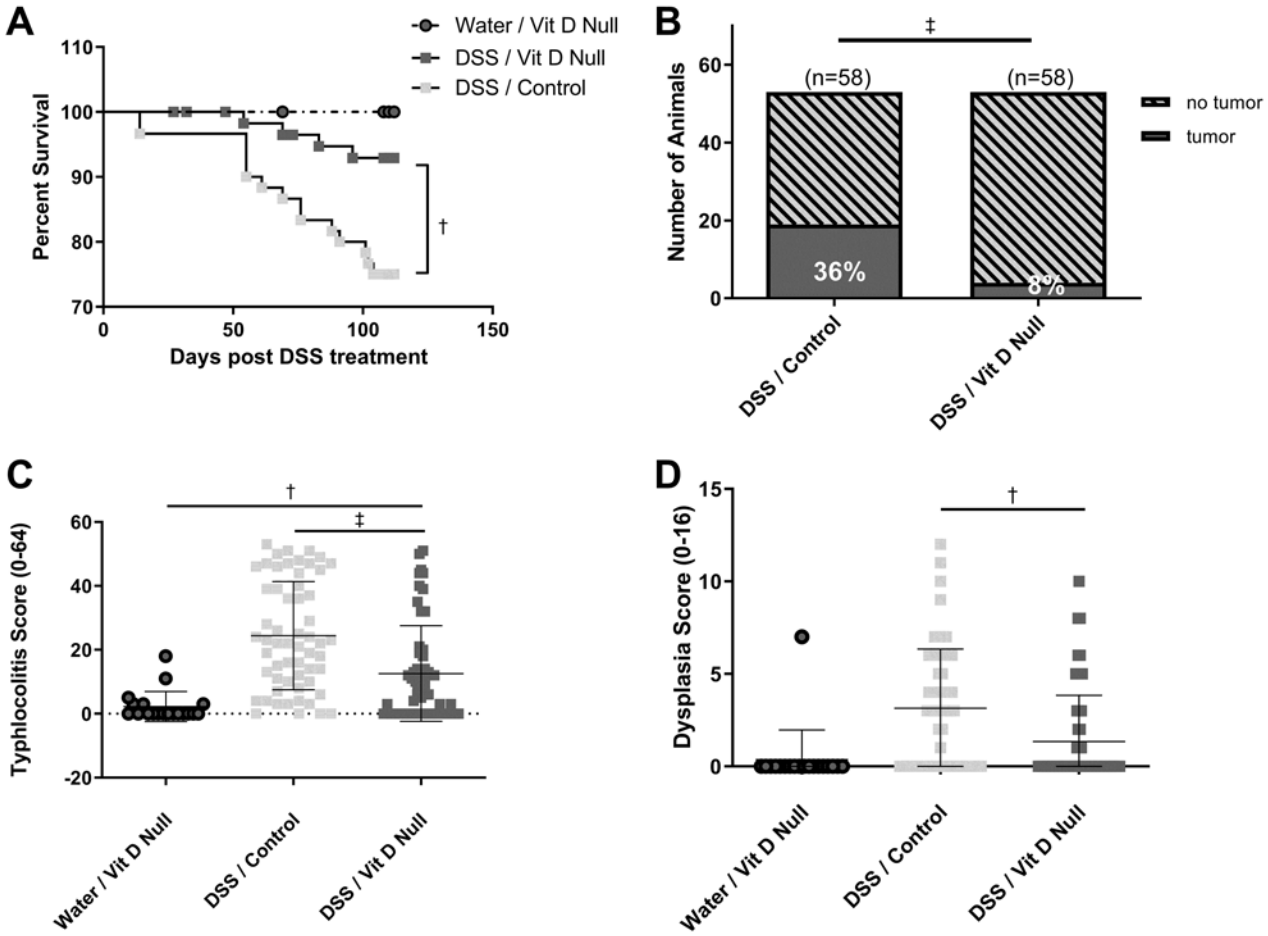


**Figure 5.** Vitamin D-null diet protects against DSS-induced colitis and colon cancer in *Smad3*$^{-/-}$ mice. *Smad3*$^{-/-}$ mice were fed vitamin D-null or control diet and treated with DSS in drinking water ($n$ = 58 mice per group) or with untreated water ($n$ = 20) and followed for disease development until 16 wk after initial DSS treatment, a duration sufficient for CAC development in this model. (A) Survival was compared through log-rank testing. Cecum and colon were assessed histologically at endpoint for evidence of (B) neoplasia, (C) typhlocolitis, and (D) dysplasia. Typhlocolitis and dysplasia scores were compared through Kruskal–Wallis testing followed by Dunn posthoc testing for pairwise comparisons. Means are indicated on dot plots; error bars on bar graphs indicate 1 SD. Dysplasia incidence and neoplasia incidence were compared through Fisher Exact testing. †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$.

compared with localized effects of dietary vitamin D insufficiency over time.

Our data suggest that decreased dietary vitamin D leads to an increase in colonic epithelial cell proliferation after DSS injury that compensates for the impaired mucosal healing present in the *Smad3*$^{-/-}$ mice and provides protection from future CAC in this setting. Similarly, others have shown that *Vdr*$^{-/-}$ mice have increased colonic epithelial cell proliferation,[37] and increased epithelial proliferation promotes wound repair after DSS colitis.[39,72] In addition, in vitro assays have demonstrated that increased concentrations of 1,25-dihydroxyvitamin D are effective at decreasing the proliferation of multiple cell types, including epithelial cells, fibroblasts, keratinocytes, and prostate cells.[8,61] Therefore, it may be possible that colonic epithelial cells in vitamin D-deficient *Smad3*$^{-/-}$ mice proliferate faster after DSS treatment due to reduced levels of active vitamin D. Although vitamin D has been shown to directly regulate genes involved in cell cycle regulation and cell proliferation, including p21waf1, p27, and p53,[67] other factors could interact with or act independently of vitamin D signaling to influence cell division rates in colonic epithelium, such as response to calcium levels,[1] response to epithelial damage or wound repair or barrier defects,[47,70] increased apoptosis,[56] and increased exposure to cytokines that encourage or discourage epithelial cell proliferation.[44,56] Future studies comparing the presence of apoptotic cells compared with actively dividing cells and analyzing the expression of tight junction proteins and inflammatory mediators in response to vitamin D deficiency at early time points during disease development would aid in understanding how vitamin D deficiency could influence colitis and CAC in our model.

Although the protective effect of decreased vitamin D and increased proliferation of the epithelium associated with decreased tumor incidence may be specific to *Smad3*$^{-/-}$ mice, it does raise intriguing possibilities of targeting vitamin D metabolic pathways or VDR signaling to modulate IBD and CAC, given that the TGFβ pathway is commonly mutated in patients with IBD as well as colon cancer.[7] Our findings suggest that targeted or localized vitamin D deficiency by blocking VDR signal or preventing synthesis of active vitamin D in the colon could potentially be useful to prevent or treat inflammation-associated colon cancer. Identification of the protective mechanisms at play in vitamin D deficiency in our model could potentially provide new therapeutic targets for IBD and CAC.


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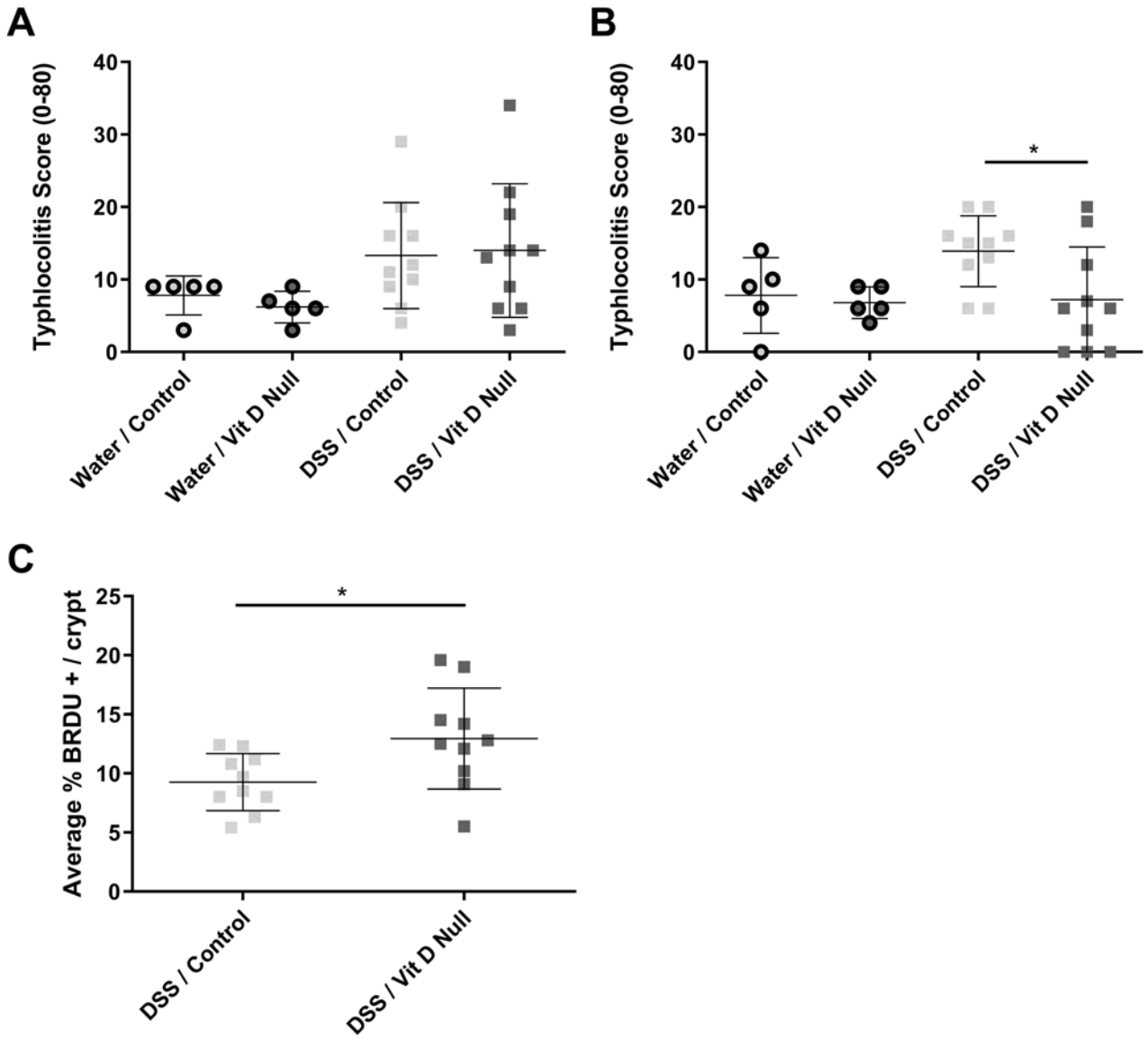


**Figure 6.** Vitamin D-null diet is associated with increased cell proliferation following DSS-treatment in *Smad3*$^{-/-}$ mice during the early inflammatory phase of disease. *Smad3*$^{-/-}$ mice were fed vitamin D-null or control diet and treated with DSS ($n$ = 10 mice per group per time point) or untreated water ($n$ = 5 mice per group per time point). Animals were euthanized at (A) 9 d and (B) 16 d after initiation of DSS treatment, and typhlocolitis scores were generated on the basis of histologic examination of cecal and colonic tissue. Groups were compared by one-way ANOVA (parametric), and pairwise comparisons between groups on the same diet in addition to comparisons between groups in the same treatment (DSS or water) were performed through Bonferroni posthoc testing. (C) BrdU was used to quantify actively proliferating colonic epithelial cells during the healing phase of disease after DSS treatment (16 d after DSS) and compared by using the Mann–Whitney $U$ test. Means (error bars, 1 SD) are indicated on dot plots. *, $P < 0.05$.

## Acknowledgments

This work was supported by the American Institute of Cancer Research (grant no. 09A136-Rev) and the National Institutes of Health (grants nos. R21 CA149995-01A1, T32 DK 007742-17, and K01 OD 021420). We thank Brian Johnson (Histology and Imaging Core, University of Washington) for his careful work with our tissue samples to perform immunohistochemistry.

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