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Short communication

Vitamin D receptor is a novel transcriptional regulator for *Axin1*

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ABSTRACT

Background: Axin1 is a scaffold protein in the β-catenin destruction complex, which, if disrupted, contributes to pathogenesis of various human diseases, including colorectal carcinogenesis and inflammatory bowel diseases (IBD). We have previously demonstrated that *Salmonella* infection promotes the degradation and plasma sequestration of Axin1, leading to bacterial invasiveness and inflammatory responses. Vitamin D and the vitamin D receptor (VDR) appear to be important regulators of IBD and colon cancer. Although VDR and Axin1 are all involved in intestinal inflammation, it remains unclear whether these processes are related or function independently. In the current study, we hypothesize that VDR is an important regulator for the maintenance of physiological level of Axin1.
Methods: Using the intestinal epithelial conditional VDR knockout mouse model ($VDR^{\Delta IEC}$) and cultured cell lines, influences of VDR status on the expression of *Axin1* was evaluated by Western blots and real-time PCR. Loss- and gain-of-function assays were used to investigate the regulation of VDR on *Axin1* at the transcriptional and translational levels. Cells were treated with cycloheximide or actinomycin for molecular mechanistic studies. Candidate genomic VDR binding sites for *Axin1* were tested by chromatin immunoprecipitation (ChIP) assay. Physical interactions among VDR, Axin1, and β-catenin were tested by immunoprecipitation. Cellular localization of Axin1 with different VDR status was determined by fractionation and immunohistochemistry.
Results: We found that VDR deletion led to lower protein and mRNA levels of *Axin1*, whereas knockdown of *Axin1* did not change the expression level of VDR protein. Immunoprecipitation data did not support physical interaction between VDR and Axin1. The VDR regulation of *Axin1* was through a VDR genomic binding site for *Axin1* gene on the regulatory region. Fractionation data showed that cytosolic Axin1 was significantly reduced due to VDR deletion, leaving the nuclear fraction unchanged. In ileum, Axin1 was distributed in the cytosol of apical epithelium and crypts.
Conclusion: VDR is important for the maintenance of physiological level of Axin1. The discovery of *Axin1* as a VDR target gene provides novel and fundamental insights into the interactions between the VDR and β-catenin signaling pathways.

1. Introduction

β-Catenin, an adhesion molecule and a component of Wnt signaling, is closely related to development, proliferation, differentiation and carcinogenesis [1–6]. Axin1 is a scaffold protein in the β-catenin destruction complex. The sequence is located on chromosome 17 qA3.3. Its protein consists of an RGS domain in the N terminus, which binds with APC, and a DIX domain in the C terminus important for the homodimerization of the protein [7]. It is supposed to be rate-limiting in the regulation of β-catenin, and restricts the Wnt signaling to basal level, and is expressed constitutively [8]. Deletion of *Axin1* leads to lethality *in utero*, and its mutations have been associated with a wide variety of cancer types including colorectal cancer [8]. Our recent study has demonstrated that *Salmonella* exposure promotes the degradation and plasma sequestration of Axin1, leading to bacterial invasiveness and inflammatory responses [9]. Furthermore, Axin1 also intermediates TGF-β signaling, SAPK signaling, *etc.* [8]. Therefore,

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Axin1 plays a vital role in gene expression and pathway activation, which potentially cause pathogenesis of various diseases upon dysregulation.

VDR plays a vital role in innate/adaptive immunity, and is widely expressed in a variety of organs and tissues. Vitamin D exerts its regulatory effects mainly via VDR, a nuclear receptor, which forms a heterodimer with retinoid-X receptor (RXR), and then binds to genomic VDR binding sites on regulated regions of target genes, triggering expression of target genes. It is known that VDR transcriptionally regulates the expression of cathelicidin antimicrobial peptides (CAMPs), β-defensins autophagy regulator ATG16L1, and tight junction protein Claudin-2 [10–13]. For example, $1,25(OH)_2D_3$ leads to upregulation of CAMPs and the killing of intracellular *Mycobacterium tuberculosis* in human monocytes [14]. The absence of VDR is associated with shifts in the bacterial load and profile [12,15]. In our recent study, both taxonomic assignment and functions of the intestinal microbiota are altered due to VDR status [16]. In the experimental models, VDR knockout increases the risks of infection, inflammation and risks to intestinal disorders. VDR negatively regulates bacteria-induced inflammation through NF-κB pathway by affecting IκBα [17,18].

VDR activation represses signaling and oncogenic effects of β-catenin, which, in reverse, has the potential to enhance $1,25(OH)_2D_3$ initiated transcription [19]. Vitamin D/VDR inhibits β-catenin signaling by competitively replacing it from the LEF/TCF complex [20]. However, the relationship between VDR and Axin1 remains largely unknown. Although VDR and Axin1 are both involved in intestinal inflammation and cancer, it remains unclear whether these processes are related or function independently.

In the current study, we hypothesize that VDR is an important regulator for the maintenance of physiological level of Axin1. The regulation was mainly on transcriptional level. Moreover, we showed that intestinal epithelial conditional VDR knockout in mice altered the expression of Axin1 at the protein and mRNA levels and changed their distribution in intestine. Our study provides novel insights into the regulatory role of VDR, supplementing a novel link in the delicate Wnt/β-catenin signaling network.

2. Methods and materials

2.1. Statement of ethics

All animal work was approved by the Rush University Medical Center Committee on Animal Resources and the Animal Care Committee (ACC) at the University of Illinois at Chicago.

2.2. Animal models

Conditional VDR knockout ($VDR^{\Delta IEC}$) mice [12] were obtained by crossing the $VDR^{flox/flox}$ mice [21] with villin-cre mice (Jackson Laboratory, 004586). Tail snips were collected 4 weeks after the mice were born. Six- to eight-week-old littermates for each group were chosen and co-housed till experiments.

2.3. Cell culture

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos bred from $VDR^{+/-} \times VDR^{+/-}$ as was described previously [22]. Genomic DNA extracted from the cells was analyzed by PCR genotyping. After 15 passages, when the $VDR^{-/-}$ and $VDR^{+/-}$ MEFs were immortalized, they were used in subsequent experiments. HCT116 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) (Hyclone, SH30243.01) supplemented with 10% (v/v) fetal bovine serum (GEMINI, 900–108), 50 μg/mL streptomycin and 50 U/mL penicillin (Mediatech, Inc., 30-002CI) as previously described [12].

2.4. Immunohistochemistry and quantification of images

Ileum tissues were freshly isolated, fixed with 10% neutral buffered formalin and paraffin embedded. Immunohistochemistry (IHC) was performed using 4-μm paraffin sections as previously described [23]. The samples were permeabilized for 1 h with 0.2% Triton X-100 and rinsed with Hanks. The coverslips were incubated for 1 h in block buffer (5% BSA + 0.1% Tween 20 in Hanks), primary antibody (1:50 dilution for Axin1 (Cell Signal, Beverly, MA, USA) in block buffer) at 4 °C overnight, washed three times with 0.1% Tween in PBS, and then incubated with biotin-conjugated secondary antibody at room temperature for 1 h, washed, incubated with ABC reagent at RT for 1 h (Vector lab PK-6100 standard), washed, visualized with DAB kit (Vector lab SK-4100) and counterstained with hematoxylin. The areas of positive staining were quantified using Image J2x (2.1.4.7 version, Wayne Rasband, National Institutes of Health, USA).

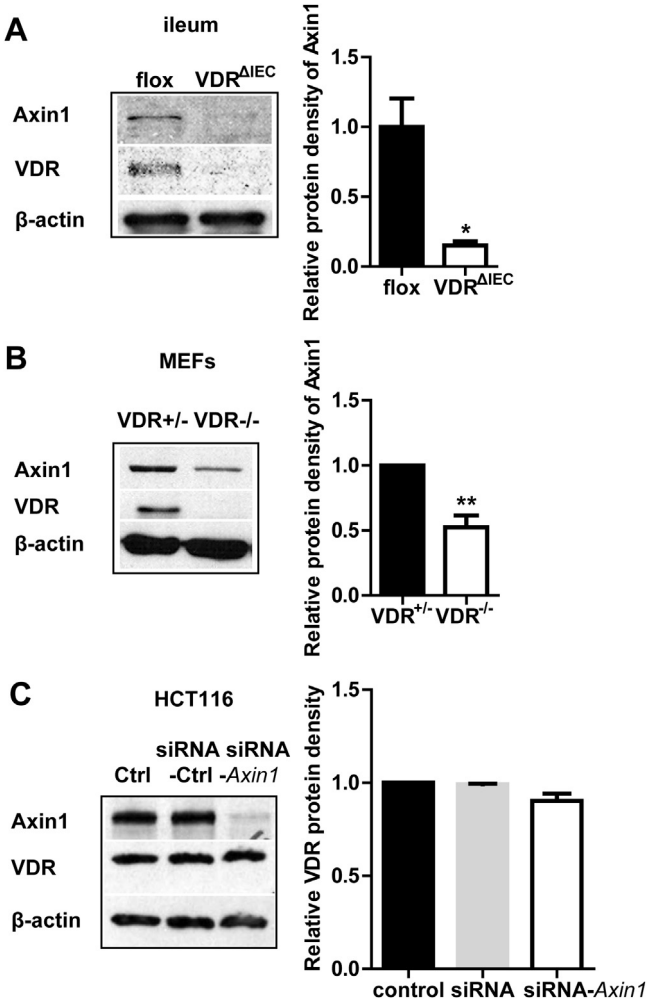

Fig. 1. VDR regulates Axin1 expression at protein level both *in vitro* and *in vivo*. The expression of Axin1 in (A) epithelium of ileum from $VDR^{flox/flox}$ and $VDR^{\Delta IEC}$ mice; and in (B) $VDR^{-/-}$ and $VDR^{+/-}$ MEFs; and (C) the expression of VDR in HCT116 cells with Axin1 knockout by siRNA. VDR knockout reduced the protein level of Axin1 both *in vitro* and *in vivo*, whereas Axin1 knockout did not interfere with VDR expression. Data were expressed as mean ± SD. $^{*}P < 0.05$, and $^{**}P < 0.01$.