ORIGINAL RESEARCH

The Role of Active Vitamin D on Reserved Intestinal Stem Cells

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Inflammatory bowel disease (IBD), a key risk factor for colorectal cancer, affects 2.39 million Americans and has no effective treatment. Current attempts include steroidal immunosuppressants, an inhibitor of immune trafficking to the gut, and therapies that aim to block gut-specific antigens. However, IBD patients often either do not respond or develop tolerance to these treatments. These limitations leave IBD patients in a cruel dilemma: either to pursue more aggressive and taxing therapies or suffer under IBD and risk further intestinal damage or the progression to colorectal cancer. A hopeful alternative to these immunosuppressive treatments is regenerative medicine. When the epithelium fails to regenerate due to inflammation, luminal bacteria infiltrate the epithelial barrier and exacerbate disease. Regenerative therapies aid regeneration to prevent progression of the disease. Recently, a novel therapy with calcitriol (an active metabolite of Vitamin D) has shown promising results for ameliorating symptoms within a murine model of IBD. However, calcitriol is known to promote the differentiation of putative *LGR5+* intestinal stem cells (*LGR5+* ISCs), which are essential to long term-intestinal homeostasis, leaving the broader impact on cell populations unclear. This study focused on the marker genes of reserve ISC populations (*Mex3a, Tert, Hopx, Bmi1*) and mature epithelial cells (*Alpi, Chga, Lyz1, Muc2*). After a meta-analysis of bulk RNA-seq datasets of calcitriol-treated mice, this project found significant upregulation of the reserve ISC markers *Mex3a, Tert*, and *Hopx* and observed a non-significant increase for *Bmi1* after treatment. After independent validation of *Bmi1 in vivo* in mice, an in vitro arm tested dose-dependent effects of calcitriol in intestinal organoids and concluded that high concentrations were needed to activate *Mex3a*. These results on reserve stem cells suggest that calcitriol's ameliorative effects in IBD may rely on reserve stem cells instead of *LGR5+* ISCs. Future research on treatments for IBD could target reserve ISCs instead.

Inflammatory bowel disease (IBD), estimated to affect 2.39 million Americans, is a chronic condition that significantly increases the risk of colorectal cancer (CRC), and it remains without a permanent cure [1–4]. IBD also aggravates established CRC tumors and is implicated in 15% of all CRC-related mortalities in the U.S. during 2020 [5]. Current therapeutic regimes for IBD aim to suppress the abnormal immune response in patients' intestines, but fail to address the repair of pre-existing damage [6,7]. The pathological failure of damage repair characterizes the progression of IBD, allowing a breach of luminal bacteria and toxins into intestinal tissues via a compromised epithelial barrier, further exacerbating the abnormal immune response [8]. This additional inflammation can trigger even more epithelial barrier damage, forming a positive feedback loop that can have disastrous consequences for the patient. As both epithelial barrier damage [9,10] and inflammation [11] can initiate this cycle, implementing epithelial repair treatments may be essential in new interventions that aim to break the cycle and target the root causes of IBD.

Timely repair of damaged intestinal epithelia requires healthy, functional intestinal stem cells (ISCs), which have been shown in previous studies to be either severely compromised or fully ablated in patients suffering from IBD [12–14]. When *Lgr5+* ISCs are experimentally depleted, IBD progression is exacerbated in the colon [15] and regeneration after damage [16] is impaired in the small intestine. The intestines have one of the highest tissue turnover rates in the body; in the gut lining, mature intestinal epithelial cells (IECs) are frequently lost due to homeostatic shedding and injury [17]. To maintain this epithelial lining, ISCs are essential, working to replenish all mature cells in the intestinal epithelium every 3-5 days [18]. IBD manifests when inflammation impairs ISCs' homeostasis of maintaining the intestinal lining [12]. Regenerative therapies aim to enhance the ability of ISCs to heal and finally resolve the inflammatory stimulus.

Although recent papers have questioned the boundaries and markers that define actively cycling ISCs (aISCs) and reserve ISCs (rISC), it has been accepted that both rISCs and ISCs have the ability to produce progeny that populate the intestines in times of injury [15,19–30]. The aISC population is believed to be located at the bottom of the intestinal crypts, and is a type of crypt base columnar (CBC) cells [31]. To isolate which cells located at the bottom of the intestinal crypts had aISC function, Barker et al. found that LGR5 (leucine-rich-repeat-containing G-protein-coupled receptor 5) accurately marked CBC cells with aISC function [31]. Lineage tracing revealed that upon exiting the crypt base, they differentiated into progenitor cells that could create more mature epithelial cells [32]. This paper was one of the first to assign a marker gene to describe aISCs in the intestines accurately. However, it is prudent to consider that not all *LGR5+* cells possess aISC activity [24], and not all cells that possess aISC activity express LGR5 [25,28,33].

Separate from *LGR5+* ISCs are reserve ISCs [31]. First described as label-retaining stem cells in the +4 position from above the columnar base, rISCs are damage-resistant and exit quiescence to actively cycle and produce de novo *LGR5+* ISCs after injury [24,34,35]. One marker of rISCs specific to the small intestines is the gene *BMI1* (B cell-specific Moloney murine leukemia virus integration site 1) [32,36]. Beyond the small intestines, another rISC population is a subset of the *LGR5+* ISC population marked by expression of the gene *Mex3a* (mex-3 RNA binding family member A). Notably, unlike *BMI1+* rISCs, these *Mex3a+ LGR5+* rISCs are not entirely quiescent and are instead slowly proliferating, consistently generating multi-lineage progeny and even directly producing actively cycling *LGR5+* ISCs [24]. However, current methods to specifically identify these

rISCs are limited: marker genes are expressed across functionally heterogeneous cell populations [14], marked cells are localized at varying positions [24], and slow cell kinetics make lineage tracing and RNA-velocity studies difficult [37].

One promising drug candidate is calcitriol or 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), the active metabolite of Vitamin D3 [38]. While in the context of IBD, it is mainly studied for its immunoregulatory effects [38,39], it also has effects in situations such as radiation-induced injury models [40], where the immunoregulatory function of calcitriol alone could not fully explain its regenerative effect. The mechanisms by which calcitriol activates to promote intestinal regeneration are still unclear [41,42]. Considering the known effect of calcitriol to induce intestinal stem cell differentiation, it becomes pertinent to determine if calcitriol treatment for IBD would inadvertently lead to ISC depletion in the context of actively cycling putative ISC populations and rISCs [41,43].

Another important consideration is the tight physiological regulation of calcitriol levels and its loose correlation with Vitamin D3 supplementation [38,44]. Due to the risk of hypercalcemia, a potential therapy would be to locally deliver high calcitriol levels without perturbing serum levels. Recently, this was achieved using transgenic inflammation and gut-homing macrophages to locally synthesize calcitriol [39]. This technique will be used within the in vivo arm of this study.

Methods

Bioinformatics

Differential gene expression results or raw counts were accessed from bulk RNA-seq datasets in the Gene Expression Omnibus for in vivo murine models of calcitriol treatment (Table 1).

Accession	Time	Dose	Tissue
GSE69170 [45]	6 h	2ng/g BW IP	SI Duodenum
GSE133949 [46]	4 h	10ng/g BW IP	Colon / SI Duodenum
GSE144978 [47]	4, 24, 48h	1ng/g BW IP	Colon / SI Ileum

Table 1 | Studies chosen to incorporate meta-analysis.

Datasets were selected to be included in the meta-analysis through searching on the Gene Expression Omnibus (GEO), then manually curated to datasets with an accompanying paper relevant to calcitriol treatment of the intestines (Fig. 1).

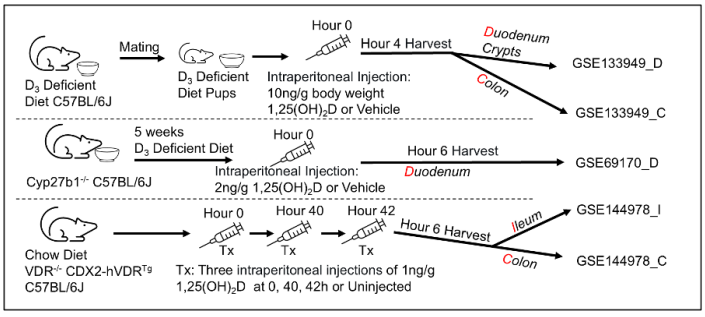
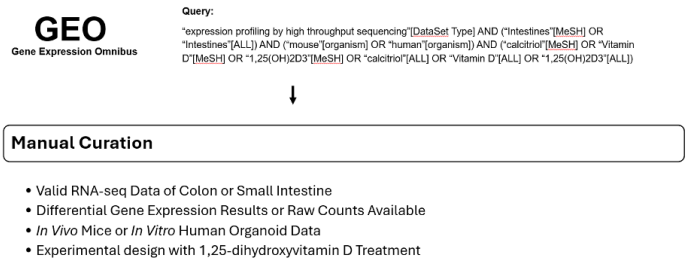


Fig. 1

The process of curating these datasets is shown in Fig. 2. For datasets that did not provide pre-processed differential gene expression results, reads were pseudo-aligned with kallisto (0.50.1) according to the Ensmbl GRCm39 reference genome (release 110). In R (4.41), counts were processed into gene-level with tximport (1.30.0) and filtered for low counts in edgeR (4.0.16) before being analyzed for differential expression with DESeq2 (1.42.1). The package RecordTest (2.20) was used to perform Fischer's method for the overall effect, and forest plots were generated through ggplot2 (3.5.1) and ggrepel (0.9.5). See (Sup. Fig. 1) for experimental outline. *(Find supplemental figures at WUJUR.org)*



In Vivo Experiment

Determining the effect of local calcitriol in vivo was carried out through similar methods as previously described [29,39]. Epithelial cells were isolated from small intestinal tissue by inversion followed by inflation and deflation in cell recovery solution [29,39]. Then, the cells were lysed and processed into cDNA for RT-qPCR [29,39]. From the experimental groups of NO DSS, DSS, DSS MAC, and DSS MAC-CYP in the ongoing experiment IACUC: #2022-017 in the lab, this cDNA was provided to the author. On day 1 in IACUC: #2022-017, C57BL/6J mice were split, with NO DSS mice receiving normal water ad libitum while DSS, DSS MAC, and DSS MAC-CYP received water containing 3% 36–50 kDa dextran sulfate sodium (DSS) to induce colitis. Groups of CD11b+Gr1+ macrophages transfected with GFP only (MAC) and with the human *CYP27B1* gene (MAC-CYP) were obtained from the Tang lab. On day 7, 2x106 MAC and MAC-CYP cells were injected into mice in DSS MAC and DSS MAC-CYP groups, respectively; DSS treatment was stopped, and DSS, DSS MAC, and DSS MAC-CYP groups started receiving normal water. On day 12, among the other tissues harvested, small intestinal duodenal tissue was processed into cDNA and protein lysate as previously described [29].

The genes *MSI1*, *LGR*5, *Mex3a*, *Bmi1*, *Hopx*, *Cyp24a1*, and *Fgfbp1* were identified as genes of interest by the meta-analysis and relevance in literature [18,23,24,29,36,48–50] for their role in regeneration. They were then assayed via RT-qPCR as previously described [29]. Primer sequences are in (Sup. Table 1).

In Vitro Experiment

Small intestinal organoids from C57BL/6J mice at the Tang lab were thawed from storage and passaged until 90% viability. Culture media was made with L-WRN conditioned X-Vivo 15 media (Lonza). Cold 150µL Matrigel was prepared for each of 12 wells of the 24-well plate with the suspended organoid solution mixed such that each well was plated with 5 x 104 Cells of Organoids. After the Matrigel solidified at room temperature, 300µL of L-WRN conditioned media was added to each well. The wells were split into 0, 10, 50, and 100nM groups and dosed with calcitriol to reach the desired concentration; the dose would be readministered every other day, with an additional media change on day 4. The same genes of *MSI1*, *LGR5*, *Mex3a*, *Bmi1*, *Hopx*, *Cyp24a1*, and *Fgfbp1* were run for qPCR as previously described [29].

Results

Meta-Analysis

To reconcile the observed effects of calcitriol from individual studies, this project combined publicly available GEO datasets to perform a meta-analysis (Table 1). These results confirmed previous studies that suggested calcitriol induced differentiation of actively cycling LGR5+ ISCs (Figure 2A) [29,41] and upregulation of the cycling cell marker *MSI1* (Figure 2B) [25,42,49].

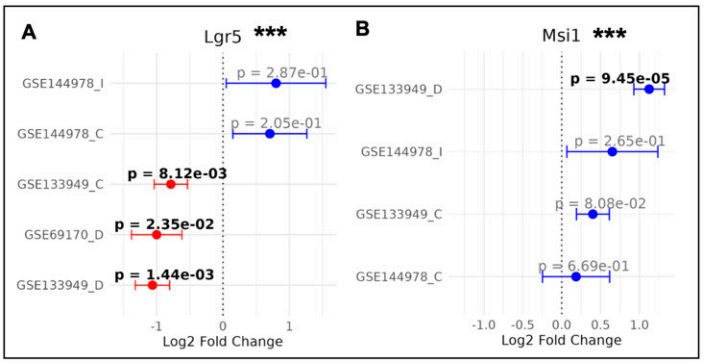


Fig. 2

Following the loss of *LGR5+* ISCs, this project examined rISC markers to determine the overall effect on the stem cell compartment. The *Bmi1* gene, known to mark a duodenal rISC population [36], had a nonsignificant upward trend after calcitriol in all studies analyzed (Figure 3D).

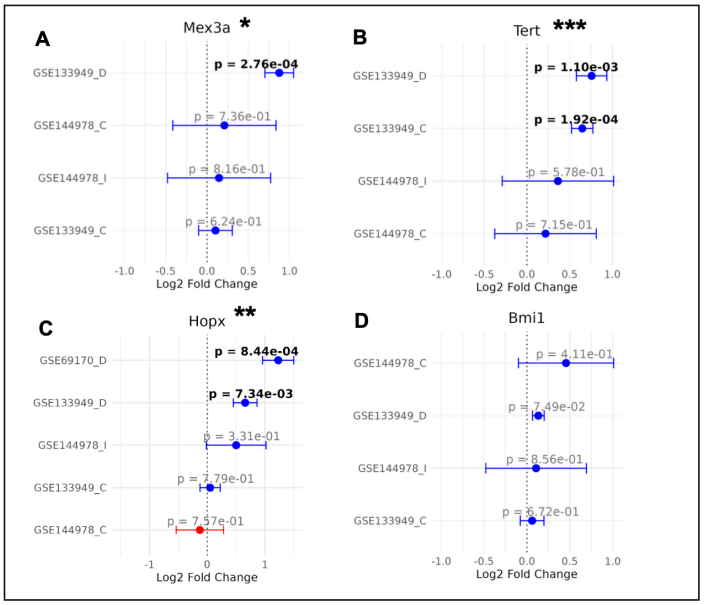


Fig. 3

Additional markers of reserve stem cell populations, *Mex3a* [24], *Tert* [34], and *Hopx* [50], were significantly upregulated in both individual studies and after calculating the overall effect from calcitriol (Figure 3A, B, C). To determine if these reserve stem cells were forming as the main lineages from differentiating *LGR5+* ISCs or from dedifferentiation of mature epithelial cells, this project examined the effect of calcitriol on mature epithelial cell markers.

This meta-analysis did not find any significant overall effects from calcitriol treatment on the mature epithelial markers *Muc2* of goblet cells [51], *Lyz1* of paneth cells [52], *Chga* of enteroendocrine cells [53], or *Alpi* of enterocytes ([54] (Figure 4A, B, C, D).

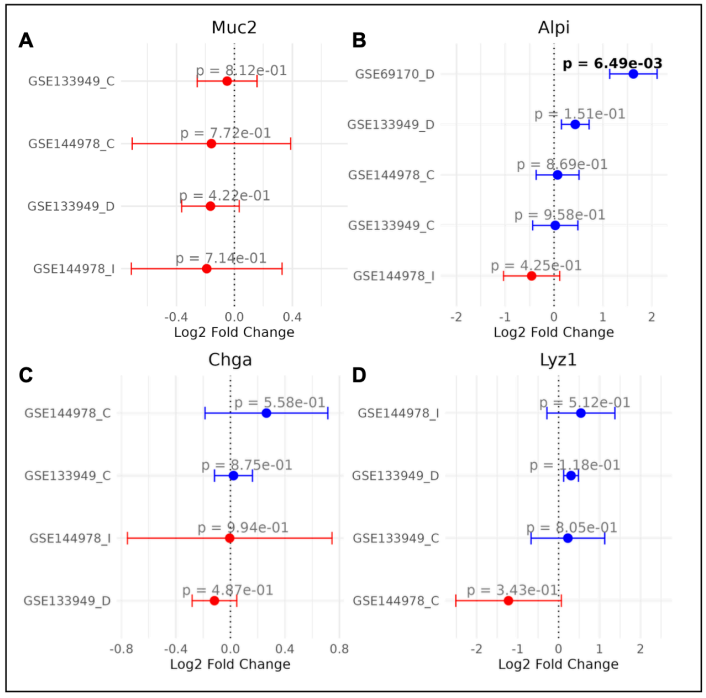


Fig. 4

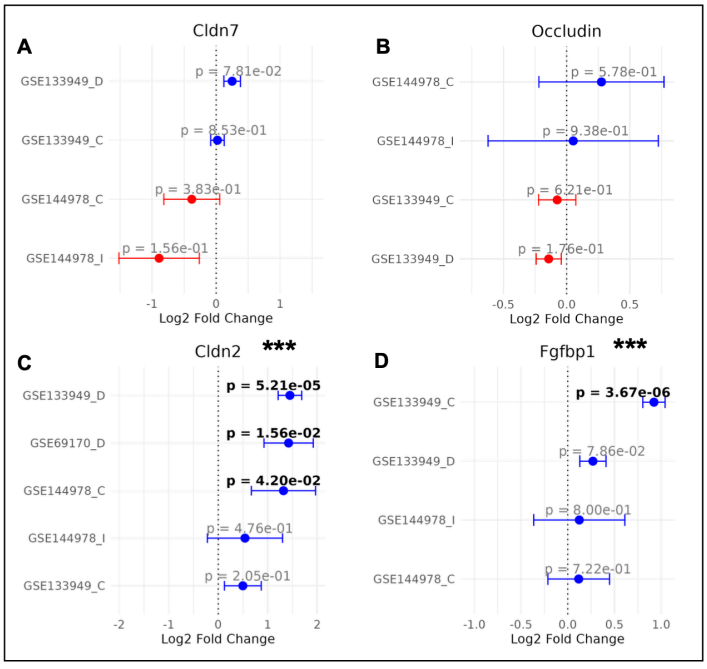


Fig. 5

Beyond mature epithelial cells themselves, their tight junction proteins are essential to barrier integrity. Of which, *Cldn2*, a known target of calcitriol [55,56], was observed to be upregulated (Figure 5C). Moreover, *Fgfbp1*, a gene implicated in the blood-brain barrier [57] and as a maker of a newly proposed stem cell population in the upper crypt [23], was also significantly upregulated overall (Figure 5D).

Outcome from Murine Small Intestinal Duodenal Samples

Quantitative PCR from duodenal sample cDNA revealed a

significant increase in gene *Bmi1* in the presence of calcitriol compared to both the WT macrophage control (MAC-CYP) and the colitis control (DSS) (Figure 6C).

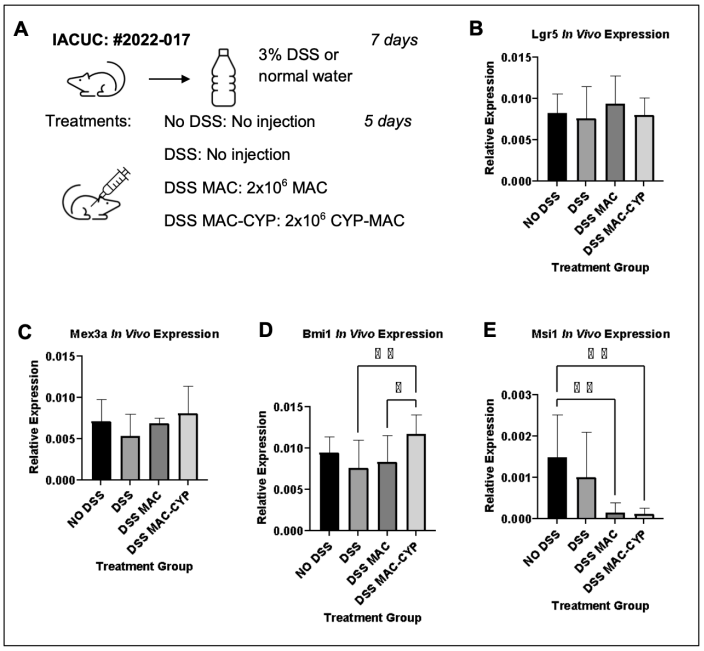


Fig. 6

However, *Hopx* was under the detection range in samples by qPCR, and results for *LGR5* and *Mex3a* showed no significant differences between groups (Figure 6A, B). For *MSI1*, there was a significant downregulation of transcripts in the MAC and MAC-CYP groups compared to DSS alone (Figure 6B).

Outcome from Murine Small Intestinal Organoid Cultures

An organoid model was chosen to validate the results on *Bmi1* and determine if the trend for *Mex3a* would be clearer under more direct treatment. qPCR RNA qualification revealed a significant increase of *Bmi1* and *Mex3a* at the highest dose of 100nM but not at any other treatment level (Figure 7A, B). However, *Hopx* and *MSI1* were under the detection range in samples by qPCR, and results for *LGR5* (Figure 7C) showed no significant differences.

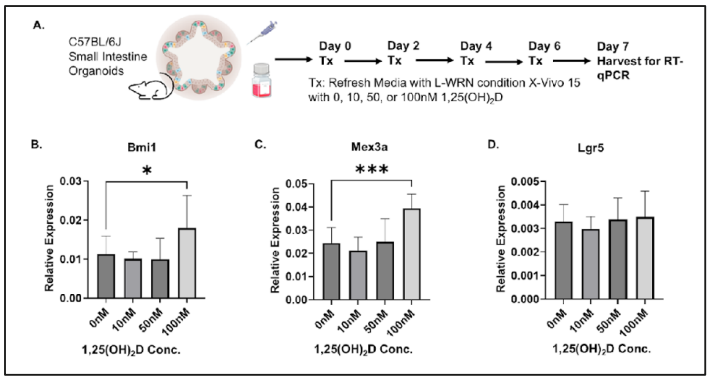


Fig. 7

Discussion (Meta-Analysis)

This project's meta-analysis shows differential gene regulation in murine datasets, specifically the markers of reserve stem cells *Mex3a*, *Hopx*, and *Tert*. In contrast with meta-analysis results, *MSI1*, a well-known direct VDR target [4, 5], was not significantly regulated in vivo and in vitro. However, the upregulation of *MSI1* is seen in several other human studies [42,44]. These results raise the question of whether calcitriol induces differential transcriptional regulation through differential binding of ligand-activated VDR or if the same cellular signals regulate differing species-dependent populations [30]. *Mex3a*, *Tert*, and *Hopx* are known to associate with VDR in CHIP-seq data in murine duodenal tissue, but only *Mex3a* and *Tert* have a similar relation in an existing human dataset of any cell type (leukocytes) [58,59]. Future analysis of human duodenal or colon CHIP-seq for VDR target sites could elucidate the source of this differential regulation.

Discussion (MAC-CYP)

With the delivery vector of CD11b+Gr1+ macrophages, there was no significant difference between MAC and MAC-CYP groups, suggesting the need for further investigation between the effects of CD11b+Gr1+ macrophages alone and the potential of calcitriol regeneration-specific effects versus homeostasis in a manner similar to cancer [42].

Despite existing studies of similar cultures of intestinal organoids in media with calcitriol [47,60,61], this design utilizes longer-term treatments and variable concentrations of calcitriol. With the most prominent in vitro activity from 100nM calcitriol, the exploration of methods to deliver sufficiently high doses of calcitriol locally without inducing hypercalcemia needs future study [39]. The in vivo results demonstrate the utility of previously developed engineered macrophage systems [39]. Previous concerns regarding exhaustion of the ISC pool from long-term calcitriol therapy induced ISC differentiation [18,29,41] could be addressed through the expansion of rISCs, which were revealed to have their markers, including *Mex3a* and *Bmi1*, upregulated in the in vivo and in vitro models in this study.

Discussion (Translational Applications)

While this project focuses on rISC in the intestines and calcitriol, research has shown the role of calcitriol in microglia with neurodegeneration [62], *Mex3a* and neurogenesis [63], and *Msi1* in neurons interacting with the same $p21^{Waf1/Cip1}$ as *Bmi1* rISC activation [64–66]. Transgenic Treg cells could similarly deliver calcitriol past the blood-brain barrier to promote neuron function in Alzheimer's disease [67]. Future research is needed to investigate how secondary effects from calcitriol regulation affect initial VDR target genes in a species and tissue-dependent manner before the transition to human studies. Moreover, validation of these results on the protein level through Western blot or novel single-cell resolution data would be invaluable for incorporating these results into recent data [23,48] and known regulatory pathways [43,49,66,68].

Acknowledgements

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References

For a full list of references, visit WUJUR.org.