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Synergistic effect of vitamin D and low concentration of transforming growth factor beta 1, a potential role in dermal wound healing

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ABSTRACT

Dermal wound healing, in which transforming growth factor beta 1 (TGFβ1) plays an important role, is a complex process. Previous studies suggest that vitamin D has a potential regulatory role in TGFβ1 induced activation in bone formation, and there is cross-talk between their signaling pathways, but research on their effects in other types of wound healing is limited. The authors therefore wanted to explore the role of vitamin D and its interaction with low concentration of TGFβ1 in dermal fibroblast-mediated wound healing through an *in vitro* study.

Human dermal fibroblasts were treated with vitamin D, TGFβ1, both, or vehicle, and then the wound healing functions of dermal fibroblasts were measured. To further explore possible mechanisms explaining the synergistic effect of vitamin D and TGFβ1, targeted gene silencing of the vitamin D receptor was performed.

Compared to either factor alone, treatment of fibroblasts with both vitamin D and low concentration of TGFβ1 increased gene expression of TGFβ1, connective tissue growth factor, and fibronectin 1, and enhanced fibroblast migration, myofibroblast formation, and collagen production. Vitamin D receptor gene silencing blocked this synergistic effect of vitamin D and TGFβ1 on both collagen production and myofibroblast differentiation. Thus a synergistic effect of vitamin D and low TGFβ1 concentration was found in dermal fibroblast-mediated wound healing *in vitro*.

This study suggests that supplementation of vitamin D may be an important step to improve wound healing and regeneration in patients with a vitamin D deficiency.

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Abbreviations: αSMA, alpha smooth muscle actin; COL1, type 1 collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; FN1, fibronectin 1; HPRT, hypoxanthine-guaninephosphoribosyltransferase gene; HTS, hypertrophic scar (s); siRNA, small interfering RNA; TGFβ1, transforming growth factor beta 1; VDR, vitamin D receptor.

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1. Introduction

Vitamin D, particularly its most active metabolite $1,25(OH)_2D_3$, has effects on a wide range of physiological processes [1]. Vitamin D is known to be important in many aspects of biology, including calcium, phosphorus, and bone metabolism, muscle strength, immune homeostasis, and other functions in tissues expressing vitamin D receptors [2,3]. Vitamin D deficiency increases the risk for many pathological conditions, such as poor bone metabolism, chronic liver and kidney diseases, infectious diseases, cancer, metabolic syndrome, autoimmune disorders [3–7,1,8], and fibrosis including liver fibrosis, cystic fibrosis, systemic sclerosis and intestinal fibrosis [9–14].

The transforming growth factor beta (TGFβ) superfamily influences many aspects of cell growth and cell development, as well as playing important roles in the process of wound healing and scar formation [15]. Of the three major mammalian TGFβ isoforms, TGFβ1 has been characterized most extensively. It was first identified in human platelets as a protein with a potential role in wound healing [16] by regulating diverse biological processes such as cellular proliferation and differentiation, immune modulation, and extracellular matrix (ECM) remodeling [17–21]. TGFβ is required for wound healing in part through the stimulation of angiogenesis, proliferation of fibroblasts, differentiation of myofibroblasts, synthesis of collagen, granulation tissue formation and re-epithelialization [22,23]. TGFβ is a powerful chemoattractant that attracts monocytes into the healing wound during inflammation whereas in the proliferative phase it stimulates ECM production, angiogenesis, and epithelialization by directing the function of fibroblasts, endothelial cells, and keratinocytes. In addition, it also induces the formation of myofibroblasts leading to wound contraction in the maturational phase [24]. Philip et al. proposed the application of TGFβ at specific stages of wound healing might provide an effective means of accelerating the healing of many types of injuries including diabetic wounds [25].

Early in 1990s, Takeshida et al. reported a novel regulation by $1,25(OH)_2D_3$ of TGFβ1-induced activation protein-1 activity in osteoblasts *via* genomic action [26], and subsequent studies demonstrate significant cross-talk between the vitamin D3 and TGFβ signaling pathways.

Low vitamin D level exists in adult patients with extensive burns or chronic non-healed ulcers because of prolonged hospitalization and inadequate sunlight exposure. We hypothesized that vitamin D deficiency impacts dermal fibroblast-mediated normal tissue repair stimulated by TGFβ, which may be one of the causes that contribute to delayed wound healing and scar development. Thus, in this study, human dermal fibroblast-mediated wound healing funtions were investigated by modultion with vitamin D and TGFβ1 *in vitro*. This study aims to explore potentially novel and innovative strategies to improve wound healing for patients with chronic non-healed ulcers or large burns.

2. Materials and methods

2.1. Culture and treatment of human dermal fibroblasts

Normal skin biopsies were collected from three female patients undergoing abdominoplasty surgery (mean age 38.8 ± 5.7 years). The Health Research Ethics Board of the University of Alberta Hospital approved the research protocol, and all participants provided written informed consent before participating in the study.

Dermal fibroblasts were cultured from the skin tissue as previously described [27]. Briefly, dermal specimens were minced into small pieces of less than 0.5 mm in any dimension in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO). The tissue fragments were then washed with medium and distributed into 60 mm × 15 mm Petri dishes. A sterile glass coverslip attached to the dish with a drop of sterile silicone lubricant was used to immobilize the tissue fragments. 3 mL of DMEM containing 10% FBS was added to each dish and they were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity. The medium was replaced every 5 days. After 4 weeks, the fibroblasts were released from the dishes after treatment with trypsin (0.25%, w/v), transferred to 75 cm^2 culture flasks, and sub-cultured for experiments.

Cells were cultured in DMEM media with 2% FBS overnight, before the cells were treated with multi-dose of TGFβ1 (R&D Systems, Minneapolis, MN), $1,25(OH)_2D_3$ (Sigma–Aldrich, St. Louis, MO), or both in the same concentrations in sterile phosphate buffered saline (PBS), or vehicle (PBS) alone for further functional analysis. For simplicity, in the subsequent text, we describe the treatment of cells as TGFβ1, $1,25(OH)_2D_3$, both or vehicle.

2.2. Real time polymerase chain reaction (PCR)

To quantify the expression of target genes, fibroblasts were cultured to 80–100% confluence in 12-well plates and then treated with $1,25(OH)_2D_3$, TGFβ1, both, or vehicle for 48 h. The control wells were treated with vehicle alone. After being washed, cells were collected using TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction using RNeasy Mini Kit (QIAGEN Sciences, ML). 0.5 μg total RNA was used for cDNA synthesis using a cDNA Syntheses Kit (Invitrogen, Carlsbad, CA). Real time PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 25 μL volume containing 5 μL of 1:10 diluted cDNA product and 1 μM of gene-specific primers (Eurofins mwg/operon, Huntsville, AL) for TGFβ1 (forward AGG ACC TCG GCT GGA AGT G; reverse GAG GCA GAA GTT GGC ATG GT), connective tissue growth factor (CTGF) (forward GAG TGT GCA CTG CCA AAG AT; reverse GGC AAG TGC ATT GGT ATT TG), fibronectin 1 (FN1) (forward GCA GCC TGC ATC TGA GTA CA; reverse GGT GGA ATA GAG CTC CCA GG), SMAD2 (forward CTC CAG GTA TCC CAT CGA AAA G; reverse GAT CAG GCC AGC GCC ATA), SMAD3 (forward ACC ACC AGA TGA ACC ACA G; reverse AGG AGA TGG AGC ACC AGA AG), SMAD7 (forward CAG CTC AAT TCG GAC AAC AAG; reverse

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TAC ACC CAC ACA CCA TCC AC), and vitamin D receptor (VDR) (forward AGA TGA CCC TTC TGT GAC C; reverse TCT TAG CAA AGC CAA TGA CC). Human hypoxanthine-guanine phosphoribosyltransferase gene (HPRT) was used as the normalization standard (forward CGG CTT GCT CGA GAT GTG AT; reverse GCA CAC AGA GGG CTA CGA TGT). Amplification and analysis of cDNA were carried out using the StepOnePlus Real time PCR System (AB Applied Biosystems, Foster City, CA). Relative gene expression was measured as cycle threshold (Ct) and normalized with individual HPRT control Ct values.

2.3. Scratch wound healing assay

The scratch wound healing assay was performed by making a scratch wound through the center of confluent cell monolayer in each well using a 1 mL pipette tip. Debris was removed from the wound and the edges smoothened out by rising with PBS. The cells were then treated with 1,25-$(OH)_2D_3$, TGFβ1, both, or vehicle. To assess the progress of would heal, digital photographs were taken at 0 h, 6 h, 12 h, and 24 h after treatment. The wound closure was analyzed by Image J software (NIH, Bethesda, MD) by circling and measuring the unhealed area of each wound in the wound images, and displayed as square millimeters (mm^2).

2.4. Liquid chromatography/mass spectrometry

4-Hydroxyproline was determined to evaluate collagen production by fibroblasts in the cell supernatant using liquid chromatography/mass spectrometry. Briefly, before the treatment of the cells, the medium was changed with 1 mL DMEM containing ascorbic acid (50 μg/mL), β-aminopropionitrile (50 μg/mL), proline (0.1 mM/L, 0.0115 mg/mL), and FBS (2%). Cells were then treated with 1,25$(OH)_2D_3$, TGFβ1, both, or vehicle for 48 h, and the supernatant were collected. Total proteins were precipitated from the supernatant using acetonitrile, and then hydrolyzed in 6 N HCl solution at 110 °C to release 4-hydroxyproline from the collagen protein. A known amount of N-methyl-proline was added as an internal standard along with 150 μL of butyl ester reagent for derivatization. After drying the mixture under vacuum, 4-hydroxyproline levels were assessed using an HP1100 liquid chromatography linked to an HP 1100 mass selective detector monitoring the ions 188 (N-butyl ester of 4-hydroxyproline) and 186 (N-butyl ester of N-methyl-proline). Each sample was run in triplicate and compared to a standard curve of 4-hydroxyproline.

2.5. Immunofluorescence staining

To observe myofibroblast differentiation, fibroblasts were seeded on coverslips in 12-well plates and starved overnight. Cells were then treated with 1,25$(OH)_2D_3$, TGFβ1, both, or vehicle for 48 h, washed and fixed using fresh cold 4% formaldehyde in PBS. After permeabilization with 0.5% saponin in PBS, samples were blocked with 10% bovine serum albumin (BSA) in PBS and stained with mouse anti-human αSMA antibody (Dako, Burlington, ON, Canada) and FITC-conjugated goat anti-mouse IgG2aκ as secondary antibody. Coverslips were mounted with ProLong Gold Antifade reagent containing DAPI (Life Technologies, Burlington, ON, Canada). The cells were observed under a fluorescent microscope (Leica TCS SP5, Leica Microsystem, Concord, ON, Canada) using appropriate filters.

2.6. Flow cytometry

In addition, flow cytometry was also performed to quantify myofibroblast formation. Fibroblasts were subjected to 48 h treatment with 1,25$(OH)_2D_3$, or multi-dose TGFβ1, both, or vehicle before being permeabilized with 0.5% saponin in PBS. After blocking Fc receptors with blocking reagent (Miltenyi Biotec, Auburn, CA), the cells were stained with PE mouse anti-human αSMA antibody (R&D Systems, Minneapolis, MN), fixed with 1% paraformaldehyde in PBS and the αSMA-expressing cells were counted using a BD FACSCanto II flow cytometer. Data is presented as percentage of control.

2.7. Gene silencing assay

Gene silencing assay was performed wherein potent inhibition of VDR gene expression was experimentally achieved by the transfection of fibroblasts with small interfering RNA (siRNA). VDR-specific siRNA duplexes were designed using SciTools from Integrated DNA Technologies (Coralville, IA). Three siRNA sequences that target VDR mRNA (accession number in GenBank: NM000376) corresponding to positions 606–630 (5′-CUCCAAUCUGGAUCUGAGUGAAGAA-3′), 797–821 (5′-GUGCCAUUGAGGUCAUCAUGUUGCG-3′) and 1079–1103 (5′-CCAACACACUGCAGACGUACAUCCG-3′) were selected, which were then synthesized by Integrated DNA Technologies. Gene-specific targeting of the selected siRNA sequences was confirmed by BLAST analysis. Non-silencing siRNA served as a negative control. The transfection of siRNA was carried out using TriEFCTin™ reagent from Integrated DNA Technologies according to the manufacturer's instructions. The knockdown efficiency was confirmed by real time PCR in the transfected cells.

2.8. Statistical analysis

Each experiment was performed in the fibroblasts isolated from dermal tissues of three patients in this study. Results are expressed as mean ± standard error of measurement (SEM). Statistical significance for all targets among the four groups, 1,25$(OH)_2D_3$, TGFβ1, both, and the vehicle, was assessed using analysis of variance (ANOVA). A P-value ≤ 0.05 was considered significant.

3. Result

3.1. Vitamin D increased TGFβ1-induced gene expression of wound healing-related factors in dermal fibroblasts

As we mentioned above, TGFβ1, as a growth factor, involves in wound healing *via* stimulating fibroblast proliferation, myofibroblast differentiation, collagen synthesis, ECM remodeling, angiogenesis and reepithelialization in the proliferation phase

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[17–23]. Another key growth factor is CTGF, which serves a pro-reparative role in early wound healing [28]. FN is an adhesive molecule that participates in the ECE formation and therefore plays a critical role in wound healing [29]. To understand the effect of vitamin D, the gene expressions of these wound healing-related factors were studied in the fibroblasts under varying conditions. As compared to stimulation by TGFβ1 (2 ng/mL), $1,25(OH)_2D_3$ (100 nM) alone had no effect on the gene expression of either TGFβ1, CTGF or FN1. However, it significantly enhanced the effect of TGFβ1 in the same gene expression of cells (Fig. 1).

3.2. *Treatment of both vitamin D and TGFβ1 accelerated wound closure in the scratch wound model*

Traditionally as the inflammatory phase of wound healing is ending, fibroblasts begin entering the wound site from the adjacent uninjured cutaneous tissue [30] utilizing fibrin cross-linking fibers to migrate across the wound, and subsequently adhering to fibronectin [31]. This process of fibroblast migration during wound healing can be simulated by the scratch wound healing assay *in vitro*. Cells were treated in different conditions and wound closure was observed by digital photography under a microscope at multiple time points over 24 h. Minimal cell migration was seen in the wound of cells treated with vehicle, whereas TGFβ1 (2 ng/mL) or $1,25(OH)_2D_3$ (100 nM) treatment induced cell migration and closed the wounds to some degree. However, the scratch wounds treated with both $1,25(OH)_2D_3$ and TGFβ1 healed significantly over time such that by 24 h after scratching, the wounds were almost completely closed by migration from the edges (Fig. 2).

3.3. *Vitamin D promoted TGFβ1-induced collagen production in dermal fibroblasts*

Collagen is a major protein of connective tissue produced by fibroblasts during the proliferation phase of wound healing. In this study, TGFβ1 simulated COL1 gene expression and collagen protein released by fibroblasts. Although $1,25(OH)_2D_3$ (100 nM) alone had minimal effect, the treatment of fibroblasts with both $1,25(OH)_2D_3$ and TGFβ1 (2 ng/mL) had a significant and synergistic increase in collagen protein synthesis (Fig. 3).

3.4. *Vitamin D improved TGFβ1-induced myofibroblast formation in dermal fibroblasts*

In the proliferative phase of wound healing, fibroblasts stimulated by growth factors differentiate into αSMA-expressing myofibroblasts and play a key role in wound contraction and closure. Myofibroblast differentiation was examined by analyzing αSMA expression in treated fibroblasts using flow cytometry and immunofluorescence staining. Dermal fibroblasts were treated with multi-dose of TGFβ1 with or without $1,25(OH)_2D_3$ (100 nM). 2 ng/mL of TGFβ1 caused a rapid increase in αSMA-expressing fibroblasts, and then there were no significant changes when TGFβ1 increased to 5 and 10 ng/mL (Fig. 4). Although the same effects of vitamin D or low concentration of TGFβ1 alone were found in αSMA expression, vitamin D significantly up-regulated low dose TGF β1-induced αSMA expression in dermal fibroblasts (Fig. 5).

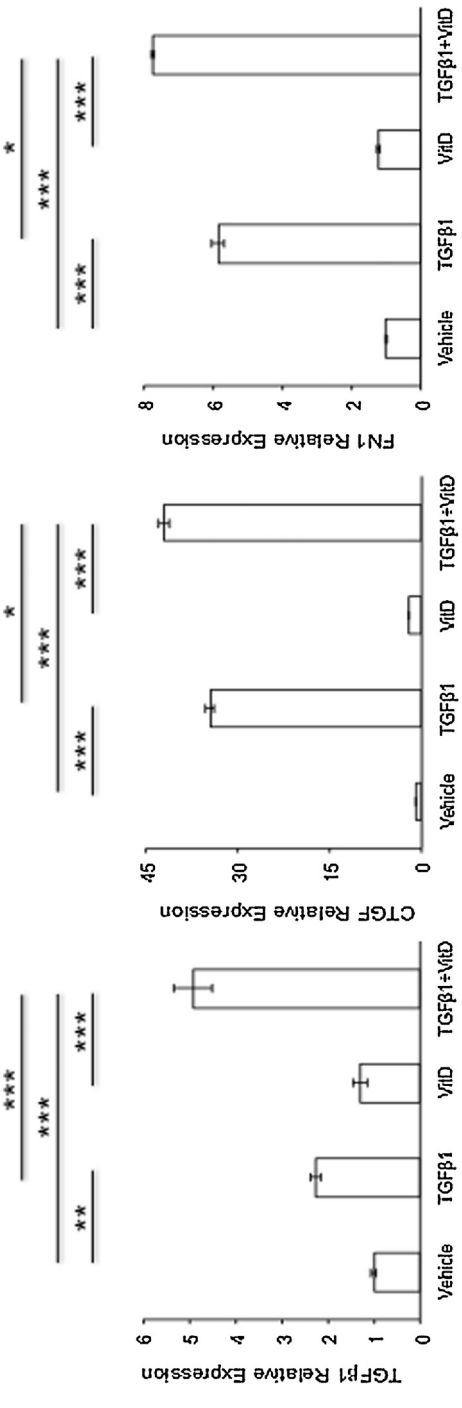

Fig. 1 – The effect of $1,25-(OH)_2D_3$ (VitD) and transforming growth factor beta 1 (TGFβ1) on the gene expressions of growth factors and extracellular matrix molecule in dermal fibroblasts. Cells were cultured to 80–100% confluence and then treated with VitD, TGFβ1, both, or vehicle. After washing, the cells were collected for RNA extraction and cDNA synthesis. Real time PCR was conducted for the gene expressions of TGFβ1, connective tissue growth factor (CTGF) and fibronectin 1 (FN1). N = 3. $^{*}p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

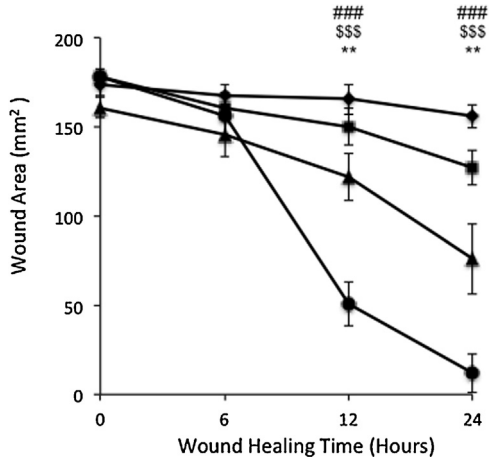

Fig. 2 – The effect of 1,25-$(OH)_2D_3$ (VitD) and transforming growth factor beta 1 (TGFβ1) on dermal fibroblast migration. Cells were cultured to a confluent cell monolayer and then a scratch wound was created in each well using a sterilized plastic tip. After a treatment of cells with VitD, TGFβ1, both, or vehicle, the wound healing was recorded by digitally photographing at multi time points. The time-dependent wound healing was quantified by measuring unhealed wound area using Image J software. # TGFβ1 + VitD *vs.* vehicle; $ TGFβ1 + VitD *vs.* TGFβ1; * TGFβ1 + VitD *vs.* VitD. $^{**}p < 0.01$. $^{\#\#\#}$ or $^{\$\$\$}p \leq 0.001$. N = 3.

3.5. *Vitamin D enhanced the effect of TGFβ1 through the activation of vitamin D receptor (VDR)*

To further explore the mechanism by which vitamin D enhanced the effect of TGFβ1 on fibroblast-mediated wound healing functions, the role of vitamin D in the expression of

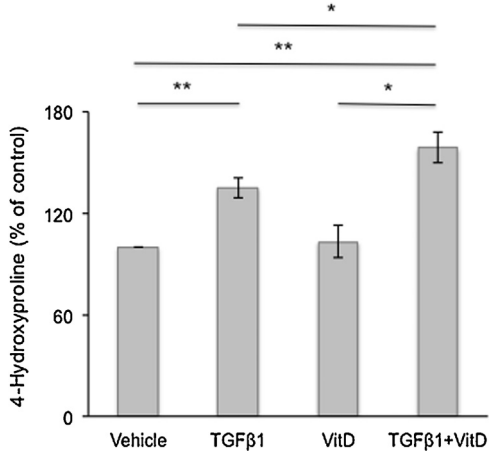

Fig. 3 – The effect of 1,25-$(OH)_2$-D_3 (VitD) and transforming growth factor beta 1 (TGFβ1) on collagen production by dermal fibroblasts. To determine the collagen protein release, the supernatant from fibroblasts treated by VitD, TGFβ1, both, or vehicle were collected from the treated cells for quantification of 4-hydroxyproline. The results are displayed as percentage of control. N = 3. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

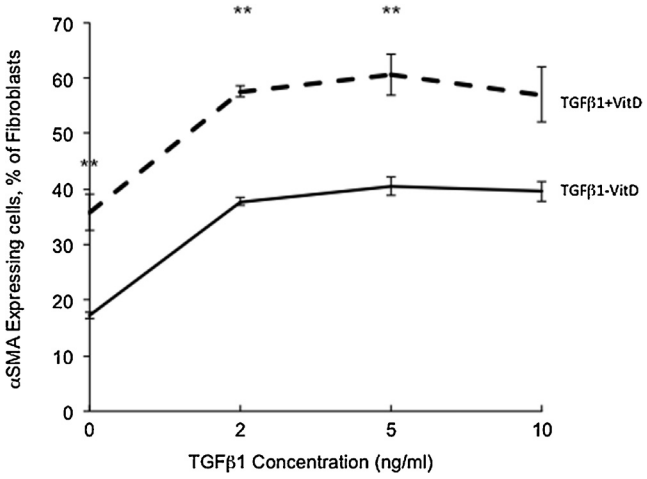

Fig. 4 – TGFβ1 dose-dependent effects on αSMA protein expression. After a treatment of fibroblasts with multi-dose of TGFβ1 with or without 1,25-$(OH)_2$-D_3 (VitD), or vehicle, the aSMA-expressing cells were counted by flow cytometry. N = 3. $^{**}p \leq 0.01$.

SMAD and VDR genes was investigated. 1,25$(OH)_2D_3$ significantly up-regulated VDR gene expression in the fibroblasts as compared to SMAD genes (Fig. 6A). Based on preliminary experiments, the effective inhibition of VDR gene was achieved after 48 h of transfection by a combination of three siRNA sequences at 10 nM each. Thereafter VDR gene-silencing was performed to confirm the role of 1,25$(OH)_2D_3$ in low dose TGFβ1-induced fibroblast functions. As illustrated in Fig. 6, siRNA treatment blocked the TGFβ1-mediated increase in collagen production and myofibroblast differentiation (Fig. 6B and C).

4. Discussion

In this study, we investigated the role of vitamin D in dermal fibroblasts-mediated wound healing and the interaction between vitamin D and TGFβ1 *in vitro*. Our data suggest that, although vitamin D alone has minimal effects, vitamin D enhanced the effect of low TGFβ1 concentrations on various dermal fibroblast-mediated wound healing functions. Vitamin D receptor gene silencing blocked this synergistic effect of vitamin D and TGFβ1 in collagen production and myofibroblast differentiation, suggesting positive regulation by vitamin D of low dose TGFβ1-induced dermal fibroblasts *via* the VDR signaling pathway.

Signal transduction in response to TGFβ is initiated following ligand binding to TGFβ specific receptors, which are high-affinity heteromeric complexes on the cell surface. Intracellular responses are then transduced by a set of second messengers known as SMADs [32]. Specifically, the regulatory SMAD proteins, SMAD2 and SMAD3 form complexes with SMAD4 which then translocate to the cell nucleus to regulate gene transcription. In contrast, SMAD6 and SMAD7, known as inhibitory SMADS or anti-SMADS, function as negative feedback inhibitors for a number of molecules in the TGFβ

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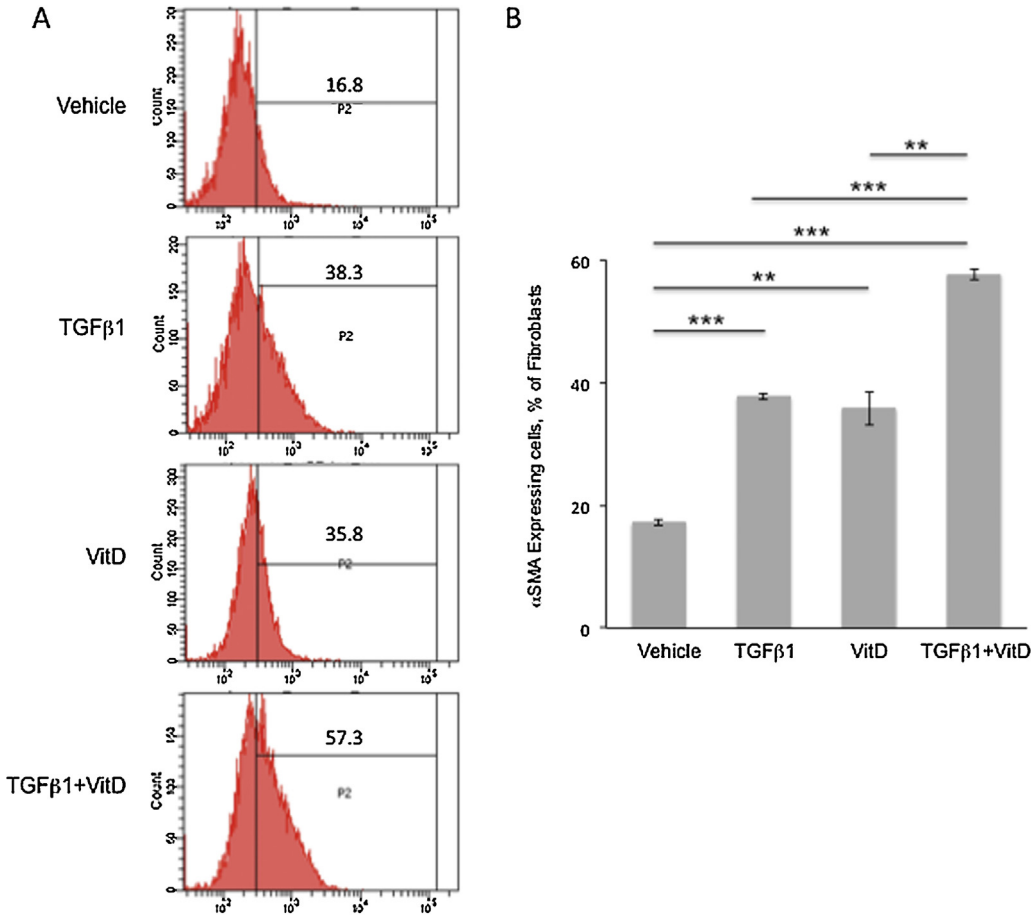

Fig. 5 – The effect of 1,25-$(OH)_2$-D_3 (VitD) and transforming growth factor beta 1 (TGFβ1) on myofibroblast formation from dermal fibroblasts. (A) Representative images of αSMA-expressing cell numbers after treatment and (B) the αSMA-expressing cells were counted by flow cytometry displayed in P2 area. $N = 3$. $^{**}p < 0.01$; $^{***}p < 0.001$.

molecule superfamily [19]. VDR is a vitamin D-regulated nuclear receptor that functions with co-activators of the steroid receptor co-activator 1/transcriptional intermediary factor 2 family [19]. Mice lacking VDR exhibit impaired bone formation, uterine hypoplasia, and growth retardation after weaning [33], all of which suggest that vitamin D is necessary for proper growth and development. In 1999, Yanagisawa et al. found that SMAD3 mediated cross-talk between vitamin D and TGFβ signaling pathways, establishing a possible molecular basis for the cross-talk between the TGFβ and vitamin D signaling pathways [34]. Further work on SMAD3 cross-talk by Subramaniam et al. demonstrated that for certain genes, such as osteocalcin, TGFβ and vitamin D have a synergistic upregulatory effect [35], which is supported by a number of articles in the literature. Liu et al. found that although alkaline phosphatase activity was inhibited by TGFβ and increased by $1,25(OH)_2D_3$ in human bone marrow stromal cells, co-treatment with TGFβ and $1,25(OH)_2D_3$ caused maximal alkaline phosphatase activity demonstrating synergistic activation [36]. Others have demonstrated that vitamin D deficiency impairs wound healing in a rat rotator cuff injury model [37], and that vitamin D can upregulate TGFβ in certain contexts [38]. Suggesting that increased vitamin D is important in improving wound healing. This stands in contrast to the body of literature, which suggests that vitamin D reduces fibrosis. Some authors have found that vitamin D deficiency increases tubulointerstitial damage and fibrosis in an acute kidney injury model [39], and that lower serum vitamin D concentrations are associated with increased fibrosis in patients with systemic sclerosis [14]. While other studies have found that vitamin D downregulates various gene targets of TGFβ, appearing to reduce fibrosis in several *in vitro* fibroblast models. Ding et al. [40] reported that VDR ligands inhibit hepatic stellate cell activation by TGFβ1, and repressed 39 TGFβ1-induced genes central to hepatic fibrogenesis. And both Zerr et al. [41], and Ramirez et al. [42] demonstrated an antifibrotic effect of vitamin D analogs, including reduced fibroblast proliferation, myofibroblast transformation, and collagen expression. With similar effects of vitamin D on profibrotic keloid fibroblasts [43], and in other types of fibrosis such as has been reported in airway subepithelial fibrosis, intestinal fibrosis, renal fibrosis, and myocardial fibrosis [44–49].

We believe these contrasting bodies of literature, and our experiments, demonstrate a dichotomous interaction between vitamin D and TGFβ based on the preexisting wound healing environment and TGFβ concentrations present. While TGFβ is considered a prototypic profibrotic cytokine, this view

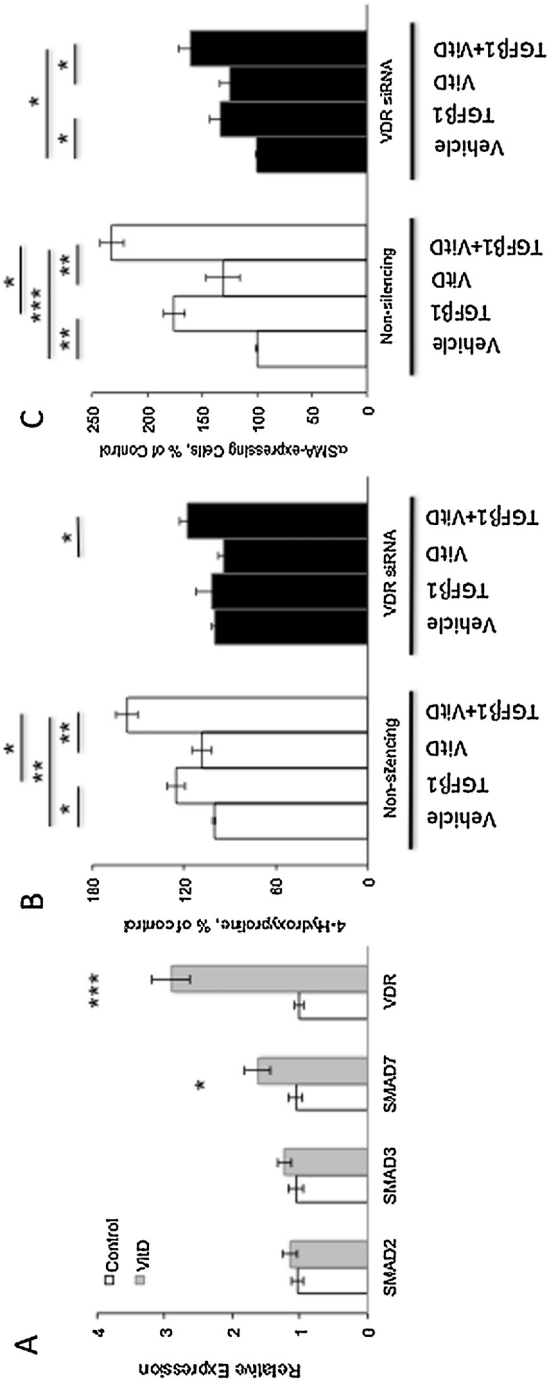

Fig. 6 – The effect of vitamin D receptor (VDR) on transforming growth factor beta 1 (TGFβ1)-induced fibroblast functions. Gene expression of SMADs and VDR were determined by real time PCR in dermal fibroblasts treated with 1,25-$(OH)_2$-D_3 (VitD) or PBS (control) (A). Dermal fibroblasts were transfected with a combination of siRNA sequences targeting to VDR before the treatment with VitD, low dose TGFβ1, both, or vehicle. The supernatant was collected to quantify 4-hydroxyproline by LC/MS (B). αSMA-expressing cells were counted by flow cytometry (C). Non-silencing RNA was used to treat the cells as negative controls. N = 3. $^{*}p \leq 0.05$; $^{**}p < 0.01$; $^{***}p \leq 0.001$.

is likely overly simplistic, with many authors recognizing that a balance is required, such that impaired wounds have improved healing after TGFβ administration [50,51], and high levels leading to fibrosis [32]. In our experiments a low concentration of TGFβ1 (2 ng/mL) analogous to an impaired wound healing state was used for stimulation of fibroblasts, and in this context vitamin D enhanced various measures of wound healing. In contrast, the *in vitro* experiments of Zerr [41], Ramirez [42], and Zhang [43] used higher TGFβ1 concentrations (10 ng/mL) analogous to a profibrotic state, and found that vitamin D had an antifibrotic effect in this profibrotic context. As can be seen in the literature, these results are not mutually exclusive, with both responses having supportive *in vitro* experiments, and corresponding *in vivo* clinical correlates. A situation, which is mirrored by existing work on the biphasic effects of TGFβ itself on fibroblast behavior [52,53]. We feel that this apparently dichotomous picture is a reflection of the underlying wound healing environment and related TGFβ concentrations.

The findings that vitamin D synergistically accelerated TGFβ1-induced fibroblast functions such as collagen production and myofibroblast differentiation support the use of vitamin D supplementation to improve wound healing in patients with vitamin D deficiency.

5. Conclusion

Based on this study, we speculate that low vitamin D levels may lead to impaired wound healing in pediatric burn population, adults with large burns, and patients with chronic non-healing ulcers. There would appear to be an important balance between vitamin D and TGFβ1 levels, where high vitamin D levels in concert with low TGFβ1 levels improve wound healing, whereas high vitamin D levels in concert with high TGFβ1 levels reduce fibrosis. This suggests that vitamin D modulates the wound healing effects of TGFβ1 differentially, depending on the TGFβ1 concentration and underlying wound healing state, whether this is impaired wound healing or fibrosis. Therefore, vitamin D supplementation may be an effective therapy for improving wound healing and also reducing fibrosis, a finding that is certainly worthy of further investigation.

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Conflict of interest

The authors declare no conflict of interest related to this work.

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