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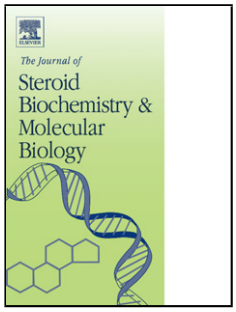

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Vitamin D modulates E-cadherin turnover by regulating TGF-β and Wnt signalings during EMT-mediated myofibroblast differentiation in A459 cells

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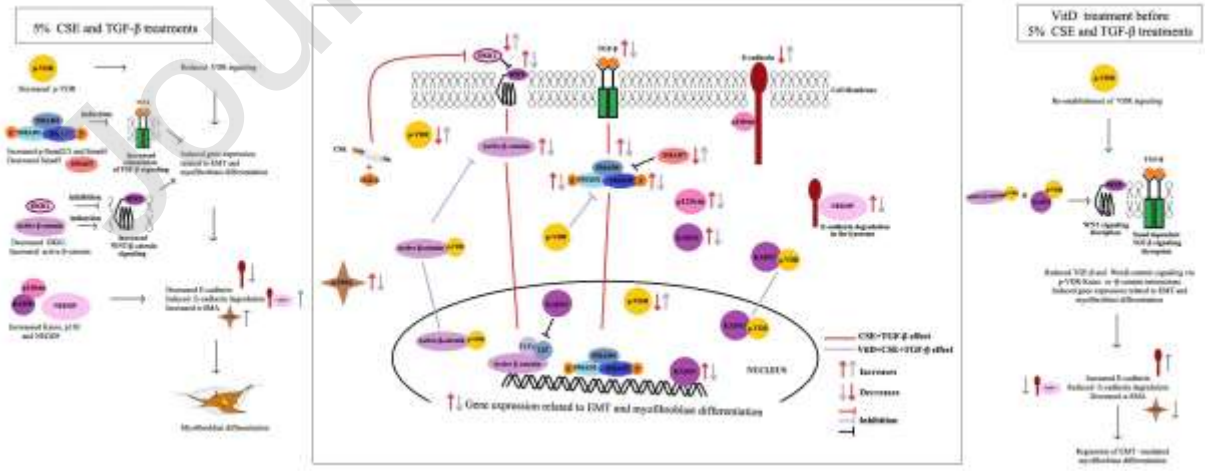
Graphical abstract



Highlights

- VDR signaling decreased by 5% CSE or 5% CSE+TGF-β treatments triggered EMT by activating TGF-β and Wnt/β-catenin signaling pathways and resulted in myofibroblast differentiation.
- The increased levels of p120ctn and NEDD9 led to decrease at the level of E-cadherin by both disrupting the stabilization of E-cadherin in the cell membrane and stimulating lysosomal degradation of E-cadherin
- VDR signaling could regulate the activation of the Wnt/β-catenin pathway in cells by controlling the transcriptional activities of β-catenin and Kaiso.

Abstract

Vitamin D (VitD) has an anti-fibrotic effect on fibrotic lungs. It reduces epithelial-mesenchymal transition (EMT) on tumors. We aimed to investigate target proteins of VitD for the regression of EMT-mediated myofibroblast differentiation. A group of A549 cells were treated with 5% cigarette smoke extract (CSE) and 5%CSE+TGF-β (5ng/ml) to induce EMT. The others were treated with 50 nM VitD 30 min before %5CSE and TGF-β treatments. All cells were collected at 24, 48 and 72 hours following 5%CSE and TGF-β administrations. The expression of p120ctn and NEDD9 proteins acted on E-cadherin turnover in addition to activations of TGF-β and Wnt pathways were examined in these cells and fibrotic human lungs. CSE and TGF-β induced EMT by reducing E-cadherin, p-VDR, SMAD7 and DKK1, increasing α-SMA, p120ctn, Kaiso, NEDD9 and stimulating TGF-β and Wnt/β-catenin signalings in A549 cells. VitD administration reversed these alterations and regressed EMT. Co-immunoprecipitation analysis revealed p-VDR interaction with β-catenin and Kaiso in fibrotic and non-fibrotic human lungs. VitD pre-treatments reduced TGF-β and Wnt/β-catenin signalings by increasing p-VDR, protected from E-cadherin degradation and led to the regression of EMT in A549 cells treated with CSE and TGF-β. Finally, VitD supplementation combined with anti-fibrotic therapeutics can be suggested for treatment of pulmonary fibrosis, which may be developed by smoking, in cases of VitD deficiency.

Abbreviations: **$1,25(OH)_2D_3$:** 1α,25-di hydroxyvitamin D_3, **$25(OH)D_3$:** 25-Hydroxyvitamin D_3, **α-SMA:** Alpha-smooth muscle actin, **A549:** Human lung adenocarcinoma cell line, **BSA:** Bovine serum albumin, **CSE:** Cigarette smoke extract, **ECM:** extracellular matrix, **EMT:** Epithelial-mesenchymal transition, **DKK1:** Dickkopf-related protein 1, **IB:** immunoblotting, **IF:** co-immunoprecipitation, **IPF:** Idiopathic pulmonary fibrosis, **NEDD9:** Developmental down-regulated protein-9 in the neural precursor cells protein, **PBS:** Phosphate-buffered saline, **p120ctn:** p120catenin, **TGF-β:** transforming growth factor **VDR:** Vitamin D receptor, **VitD:** Vitamin D

Keywords: Vitamin D, smoke extract, TGF-β, Wnt signal pathway, EMT, myofibroblast differentiation

1. Introduction

Vitamin D (**VitD**) is a steroid-derived prohormone. It binds to cytoplasmic VitD receptor (VDR), which forms a heterodimer with retinoid X receptor. This complex is then transferred into the nucleus and affects VitD target genes [1]. Although VitD is mainly a molecule responsible for the calcium-phosphate balance for the body, it also regulates more than 200 genes related to cell proliferation, differentiation, and inflammation [2]. It has been reported that the incidence of inflammatory diseases, such as chronic obstructive pulmonary disease, asthma, and allergies, is more prevalent in people with VitD deficiency, especially in children [3-6]. Recently it has also been shown that the VitD deficiency was detected in the serum of a group of patients with idiopathic pulmonary fibrosis (**IPF**) [7]. Although it is known that VitD deficiency can be related to pulmonary fibrosis and impaired lung function, this relationship has not yet been elucidated. Chronic VitD deficiency can destroy lung structure and development in mice and induce the accumulation of extracellular matrix (**ECM**) components in the lung [8, 9]. It has been found that diet with VitD supplementation prevents pulmonary fibrosis by reducing the levels of transforming growth factor (**TGF-β**) and alpha-smooth muscle actin (**α-SMA**) in mice lungs instilled with intratracheal bleomycin [10]. Additionally, it has been shown that VDR signaling can attenuate liver fibrosis by down-regulating fibrotic genes controlled by TGF-β/SMAD3 through the binding of VDR to response elements of SMAD3 target genes [11]. Furthermore, a study performed on VDR-knockout skin cancer mice revealed that VitD may have an anti-carcinogenic effect by reducing Wnt signaling in addition to the anti-fibrotic effect [12].

Smoking can limit the level and effects of VitD in the body. In a study with chronic rhinosinusitis patients, it has been found that the serum level of VitD in patients who smoke was lower than the nonsmoker group. In the same study, human sinonasal epithelial cells exposed to cigarette smoke extract (CSE) were noted to decrease 25-hydroxyVitD$_3$-1α-hydroxylase, an enzyme that enables VitD to gain its active form [13]. Another study showed that short term CSE exposure reduces VDR signaling in A549 cells [14]. To date, studies have demonstrated that fibrosis develops in CSE-exposed mouse lungs, collagen production can be stimulated in lung fibroblasts exposed to CSE, and, myofibroblast differentiation can occur via epithelial-mesenchymal transition (EMT) mediated in CSE-exposed A549 cells [15, 16]. Using VitD as an anti-fibrotic agent resulted in the regression of bleomycin-induced pulmonary fibrosis by decreasing TGF-β and α-SMA levels in mice lungs [10]. VitD deficiency is present in the serum of a group of IPF patients [7]. Also, VitD can reduce EMT in several types of tumors. The present study aimed to investigate possible target proteins of VDR signaling for cross-talk between VitD and TGF-β and Wnt signal pathways, and the regression of EMT-mediated myofibroblast differentiation. Additionally, expression profiles of p120ctn and NEDD9 proteins, which regulate the lysosomal degradation and stabilization of E-cadherin protein inner cell membrane, were investigated for the first time during VitD-mediated regression of EMT. The interaction of VitD with TGF-β and Wnt signal pathways, which modulated pathogenesis of pulmonary fibrosis, was investigated for the first time in these cells and human fibrotic lungs. Co-immunoprecipitation analysis revealed for the first time that VDR interacted physically with β-catenin and Kaiso, but not with p120ctn, in fibrotic and non-fibrotic lungs. Thus, we suggest that VDR signaling regulates EMT through the activation of the Wnt/β-catenin signaling pathway in cells by controlling the transcriptional activity of β-catenin and Kaiso. Data obtained from the present study may help to elucidate the potential

benefits of VitD supplementation, especially in the treatment of pulmonary fibrosis and EMT which may develop due to smoking.

2. Materials and Methods

2.1. Cigarette smoke extracts preparation

CSE was prepared using a modified version of the method used by Aoishba et al. [17]. A commercial cigarette containing 10 mg of tar, 0.8 mg of nicotine and 10 mg of carbon monoxide was aspirated for 3 minutes after removing its filter. The smoke was bubbled through 10 ml of sterile phosphate-buffered saline (PBS, 0.01M) at 37˚C. The pH of the prepared extract was adjusted to 7.4 with 1N NaOH and the extract was passed through a 0.22 µm sterile filter. The obtained extract was accepted to be 100% CSE. In the spectrophotometric measurement, extracts with the absorbance of 2-2.4 OD at 290 nm were used in experiments. Extract was freshly prepared for each experiment and used within 30 min.

2.2. Cell culture and treatments

The human lung adenocarcinoma cell line (A549) was purchased from the American Type Culture Collection. Cells were cultured in *Dulbecco's Modified* Eagle *Medium*/Nutrient Mixture F-12 (DMEM/F12) medium containing 10% fetal bovine serum (FBS), 50 µg/ml streptomycin and 50 U/ml penicillin (Gibco) in *humidity incubator* at *37* °C and *5*% CO_2. Cells were seeded into 6-well culture dishes at 400,000 cells per well. The next day, when cells covered 80% of the wells, the growth medium was replaced with a starvation medium containing 1% FBS. The cells were allowed to adjust to growth medium for one day. Two groups of A549 cells were induced with 5% CSE (CSE experimental group) and 5% CSE+TGF-β (5ng/ml) (CSE+TGF-β experimental group). The cells in groups with VitD supplement (VitD+CSE and VitD+CSE+TGF-β experimental groups) were treated with 50 nM VitD 30 min before CSE and TGF-β treatments. VitD (cholecalciferol) was prepared in dimethyl sulfoxide (DMSO) and PBS mixture (1:9). All cells were collected at 0 (untreated cells), 24, 48 and 72 hours (h) following 5% CSE and TGF-β administrations. Three replicate experiments were evaluated for each analysis.

2.3. Determination of cell viability

To determine cell viability, the 3-(4, *5*-dimethythiazolyl)-2.5-*diphenyl-2H-tetrazolium-bromide* (MTT) assay was used. Briefly, A549 cells (10,000 cells/well) were seeded in 96-well plates 24 h prior to experiments and incubated at 37 °C and 5% CO_2. They were treated with 10 nM, 50 nM and 100 nM VitD 30 min before CSE and TGF-β treatments. They were incubated for 24, 48 and 72 h following 5% CSE and TGF-β administrations. Cell viability testing was performed according to the protocol proposed for kit purchased from iNtRON Biotechnology. The absorbance of each well was measured

at 570 nm by a microplate reader (BioTek µQuant). The cell viability percentages were calculated by the formula: (mean OD of treated cells /mean OD of control cells) ×100.

2.4. Immunoblotting

The cultured A549 cells were carefully scraped from culture dishes and homogenized in a RIPA buffer containing phosphatase and protease inhibitors. After centrifugation of cell lysates at +4°C and 13,000 rpm for 15 min, total protein content in samples was measured using the Bradford method [18]. Next, 40µg protein of each sample was run in 5-10% and 5-8% SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat dried skimmed milk and were incubated with primary antibodies such as anti-p-VDR, (Thermo Fisher), anti-E-cadherin (Santa Cruz) anti-α-SMA (Thermo Fisher), anti-p-SMAD2/3 (Santa Cruz), anti-SMAD4 (Santa Cruz), anti-SMAD7 (Santa Cruz), anti-Dickkopf-related protein 1 (DKK1) (Santa Cruz), active β-catenin (Cell Signaling), anti-Kaiso (Santa Cruz), anti-p120ctn (Santa Cruz) and anti-NEDD9 (Abcam) at +4°C overnight. After washing with PBS containing Tween-20 (PBST), they were incubated with secondary antibodies for 1h at room temperature and reacted with enhanced chemiluminescence detection solution. Results were normalized to GAPDH. The intensity of protein bands was quantified by using Kodak Gel Logic Molecular Imaging Software (Kodak GL 1500, New Haven, CT, USA). Bands were quantified by densitometry in 3 separate experiments.

2.5. Immunofluorescence

A549 cells (60,000 cells/well) were seeded on coverslips placed in 24-well plates 24 h prior to experiments at 37 °C and 5% CO_2. Cells were stimulated with 5% CSE+TGF-β and VitD by the same experimental procedure above. At the end of the experimental time, the medium was removed and cells were fixed with a 1:1 cold acetone-methanol mixture for 10 min. Following fixation, cells were washed with PBS containing 0.1% bovine serum albumin (BSA) for 3 times. After blocking with 5% BSA, they were incubated with rabbit anti-p-VDR (Thermo Fisher) overnight at +4˚C and were then washed with PBST. Next, cells were treated with goat anti-rabbit IgG-FITC-Fab secondary antibody (Millipore) in the dark for 1 h at room temperature. At the end of 1 h, they were washed with PBST containing 0.1% BSA and blocked with 5% BSA for 30 min. After blocking, cells were incubated with rabbit anti-p-SMAD2/3 (Thermo Fisher) overnight at +4 °C. Following incubation, cells were washed with PBST containing 0.1% BSA and treated with goat anti-rabbit IgG-Cy3 (Millipore) in the dark for 1 h. Cells were then washed with PBST containing 0.1% BSA. The nuclei of cells were counterstained with 4′,6-diamidino-2-phenylindole. Cells were visualized and images were obtained using a 40× oil-immersion objective under a fluorescence microscope (Nikon, EclipseT*i*). The same method was used for anti-p-VDR and anti-Kaiso double labeling. The primary and secondary antibodies used were as follows: rabbit anti-p-VDR, goat anti-rabbit IgG-FITC-Fab, mouse anti-Kaiso (Santa Cruz) and goat anti-mouse IgG TexasRed (Santa Cruz).

2.6. p-VDR-proteins co-immunoprecipitation experiments in human lung samples

All the human lung samples were collected according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). Fibrotic (n=3) and non-fibrotic (n=4) lung samples, which were diagnosed as IPF and non-fibrotic at clinical and radiologic-pathologic examinations in a preceding study, were used in the present study (Yildirim et al., unpublished data) These human fibrotic lung samples were characterized by increased α-SMA, collagen1a and decreased E-cadherin protein levels. Lung samples were homogenized in a RIPA buffer containing protease inhibitor. Total protein was measured using the Bradford method. Protein samples (100 µg for each sample) were incubated with anti-p-VDR antibody for 1 h at 4°C and protein A/G plus-agarose beads overnight at 4°C with gentle rotation, respectively. Samples were centrifuged at 4°C for 5 min at 2,500 rpm. Beads were washed with PBS, suspended in sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, and boiled for 10 min at 70°C. Precipitated proteins, which bound to p-VDR, were run in 5-10% SDS-PAGE gel and analyzed by immunoblotting using anti-active β-catenin, -Kaiso and -p120ctn primary antibodies.

2.7. Statistical Analysis

MTT and Western blotting findings were evaluated statistically by using One-Way analysis of variance (ANOVA) and Student's T-test in Graphpad Prism 7.0 program and comparisons were made between groups. P-values less than 0.05were accepted as statistically significant.

3. Results

3.1. Cell viability results

When A549 cells were treated with 10nM, 50nM and 100nM VitD 30 min before 5% CSE and 5% CSE+TGF-β treatments, cell viability was not adversely affected at 24 and 48 h. However, 100 nM VitD caused decreased cell viability around 47% ($p<0.001$) and 55% ($p<0.01$), at 72 h in cells treated with 5% CSE (Fig. A.1) and 5% CSE+TGF-β (Fig. B.1), respectively. Since the dose of 50 nM VitD has low cytotoxicity and high effect on regressing EMT, this VitD dose was used for *in vitro* experiments.

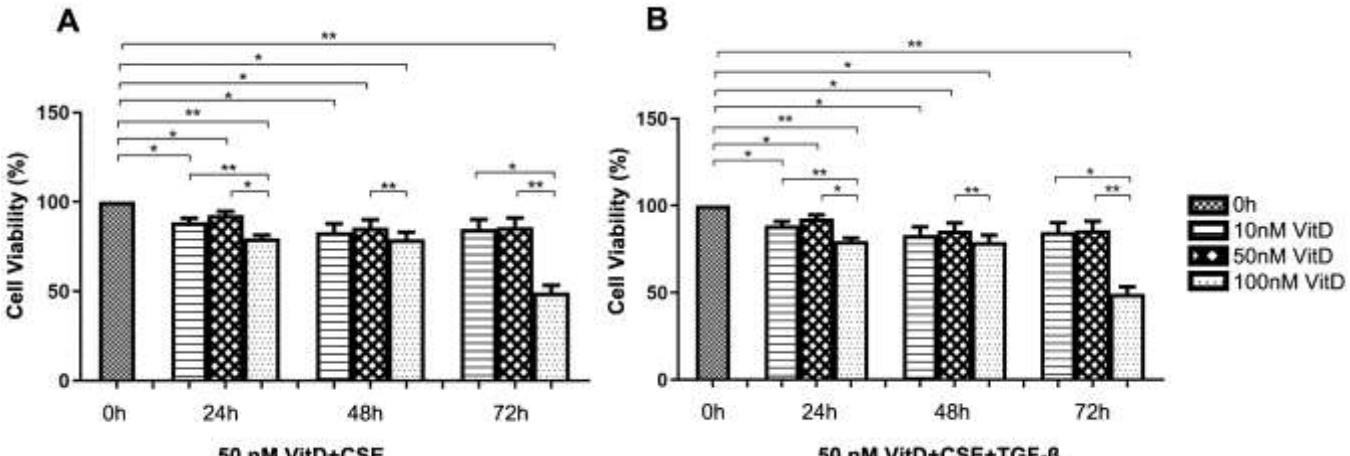


Fig. 1. The effects of different doses of VitD on cell viability of A549 cells treated with 5% CSE (A) and 5% CSE+TGF-β (B). p values: * p <0.05, ** p <0.01 and *** p <0.001.

3.2. VitD treatment led to the induction of p-VDR signaling and regression of EMT in A549 cells treated with 5% CSE and 5% CSE+TGF-β

A549 cells treated with 5%CSE and 5%CSE+TGF-β were characterized by the progressive decreases at p-VDR and E-cadherin levels, and an increase at α-SMA level from 0 h to 72 h. A significant reduction at p-VDR protein levels were observed at 48 and 72 h in cells treated with 5% CSE and 5% CSE+TGF-β ($p < 0.05$ for 48 and 72 h in 5% CSE group; $p<0.05$ for 48 h and $p<0.01$ for 72 h in 5% CSE+TGF-β group). VitD treatments led to an increase at the level of p-VDR in A549 cells exposed to 5% CSE and 5% CSE+TGF-β at 24 ($p < 0.01$ and $p < 0.05$ for 5% CSE and 5% CSE+TGF-β treatments, respectively), 48 and 72 h ($p < 0.05$ for both groups) when compared to 0 h. When 5% CSE group compared to VitD+5% CSE group and 5% CSE+TGF-β group compared to VitD+5% CSE+TGF-β group, we observed that VitD treatment caused a progressive increase at the p-VDR level for both groups (Fig. A.2). While the levels of E-cadherin protein was decreased by the 5% CSE treatment at 48 and 72 h ($p < 0.05$), decrease in E-cadherin level started at 24 h (24 h ($p < 0.05$), and continued at 48 and 72 h ($p < 0.01$)) in 5% CSE+TGF-β treatment group. VitD treatment significantly increased the protein level of E-cadherin only at 24 h ($p < 0.01$) compared to 0 h in A549 cells exposed to 5% CSE. When comparing the 5% CSE group with the VitD+5% CSE group and the 5% CSE+TGF-β group with the VitD+5% CSE+TGF-β group, it was seen that VitD treatment caused a progressive increase of the E-cadherin level for both groups (Fig. B.2). We also observed a significant increase in α-SMA protein levels at 24 ($p < 0.05$), 48 and 72 h ($p < 0.01$) compared to 0 h in cells exposure to 5% CSE and 5% CSE+TGF-β. VitD treatments reduced the levels of α-SMA protein in these cells and brought them closer to normal values at 72 h (Fig. C.2).

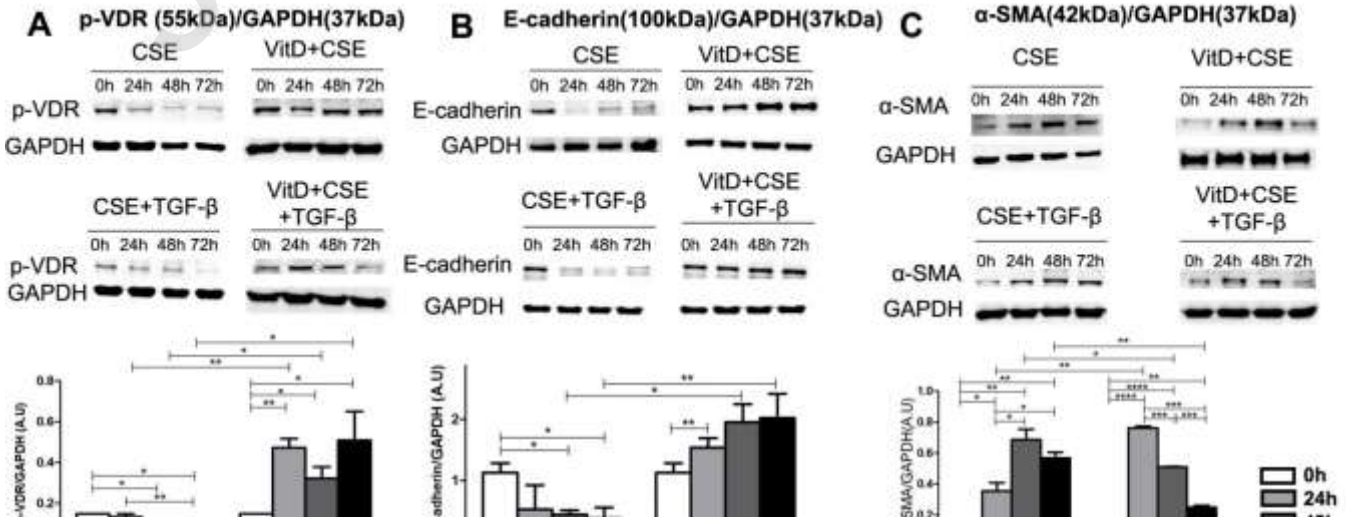


Fig. 2. CSE and TGF-β treatments decreased the level of p-VDR (A) and stimulated EMT (B and C) in A549 cells. VitD pre-treatment regressed EMT, by inducing p-VDR signaling and E-cadherin level and reducing α-SMA level. P values: * p <0.05, ** p <0.01, *** p <0.001, **** p<0.0001.

3.3. VitD treatment led to the reduction of TGF-β and Wnt/β-catenin signalings in A549 cells treated with 5% CSE and 5% CSE+TGF-β.

A549 cells exposed to 5% CSE and 5% CSE+TGF-β were characterized by progressive increases in p-SMAD2/3 and SMAD4 levels, and a progressive decrease at SMAD7 level from 0 h to 72 h. Treatments with 5% CSE+TGF-β initiated changes in these protein levels earlier (Fig. 3). Pre-treatment with VitD on these cells reduced p-SMAD2/3 (Fig. A.3) and SMAD4 (Fig. B.3) levels by starting from 48 h in both groups and it normalized their levels at 72 h. While 5% CSE treatment decreased SMAD7 protein level at 72 h (p <0.05), 5% CSE+TGF-β treatment reduced the level of SMAD7 protein at 48 and 72 h (p <0.01). VitD treatment on A549 cells treated with 5% CSE and 5% CSE +TGF-β resulted in an increased SMAD7 level at 72 h and at 48 and 72 h (p<0.05), respectively (Fig. C.3).

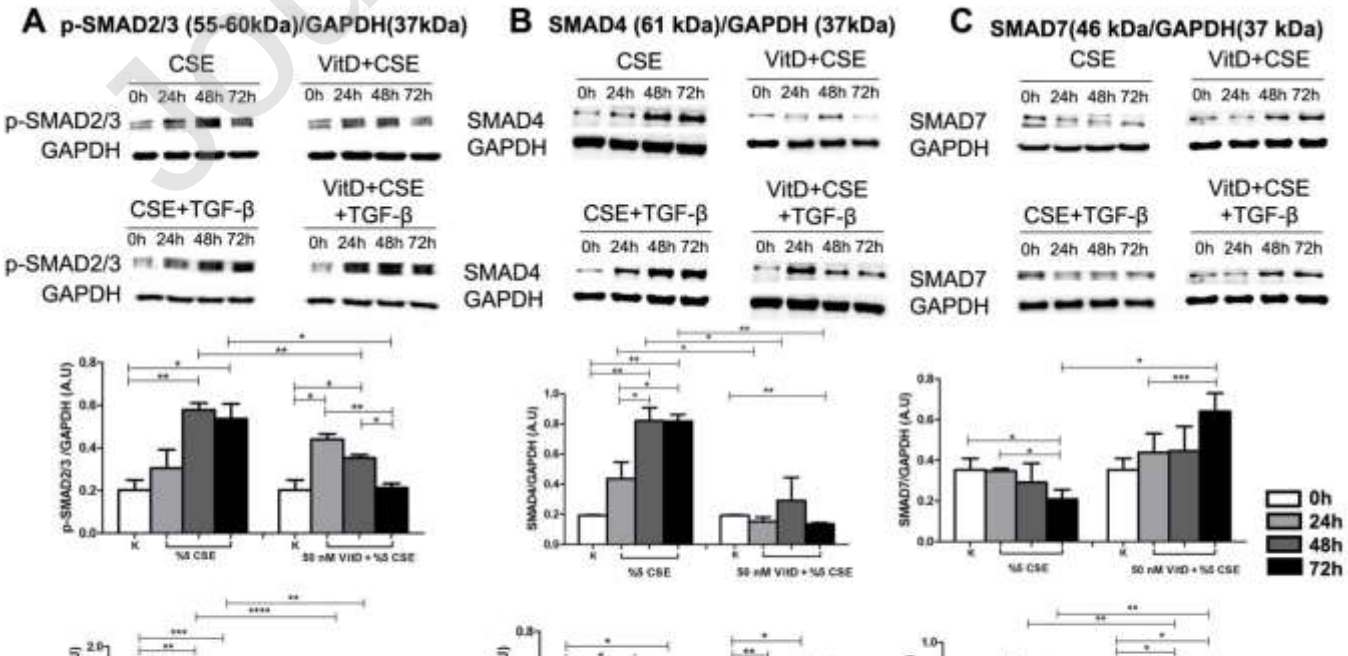


Fig. 3. CSE and TGF-β treatments triggered myofibroblast differentiation via EMT in A549 cells, by activating SMAD2/3-dependent TGF-β signaling. VitD pre-treatment caused the reduction of SMAD2/3 activation (A) and SMAD4 level (B), the induction of SMAD7 level (C) and the regression of EMT. P values: * p <0.05, ** p <0.01, *** p <0.001 and **** p<0.0001.

We observed that a significant increase at the protein levels of active β-catenin at 48 and 72 h (p <0.01) in cells treated with 5% CSE and 5% CSE+TGF-β, by starting from 24 h in cells with 5% CSE+TGF-β (p <0.05). Pre-treatment with VitD decreased the protein levels of active β-catenin in cells of both groups, bringing its level closer to that of 0 h, especially at 72 h (Fig. A.4). 5% CSE treatments significantly reduced DKK1 protein level in A549 cells at 24, 48 and 72 h (p <0.01), whereas 5% CSE+TGF-β decreased DKK1 protein only at 72 h (p <0.01). Pre-treatments with VitD increased the levels of DKK1 in these experimental groups and brought it closer to the level at 0 h (Fig. B.4).

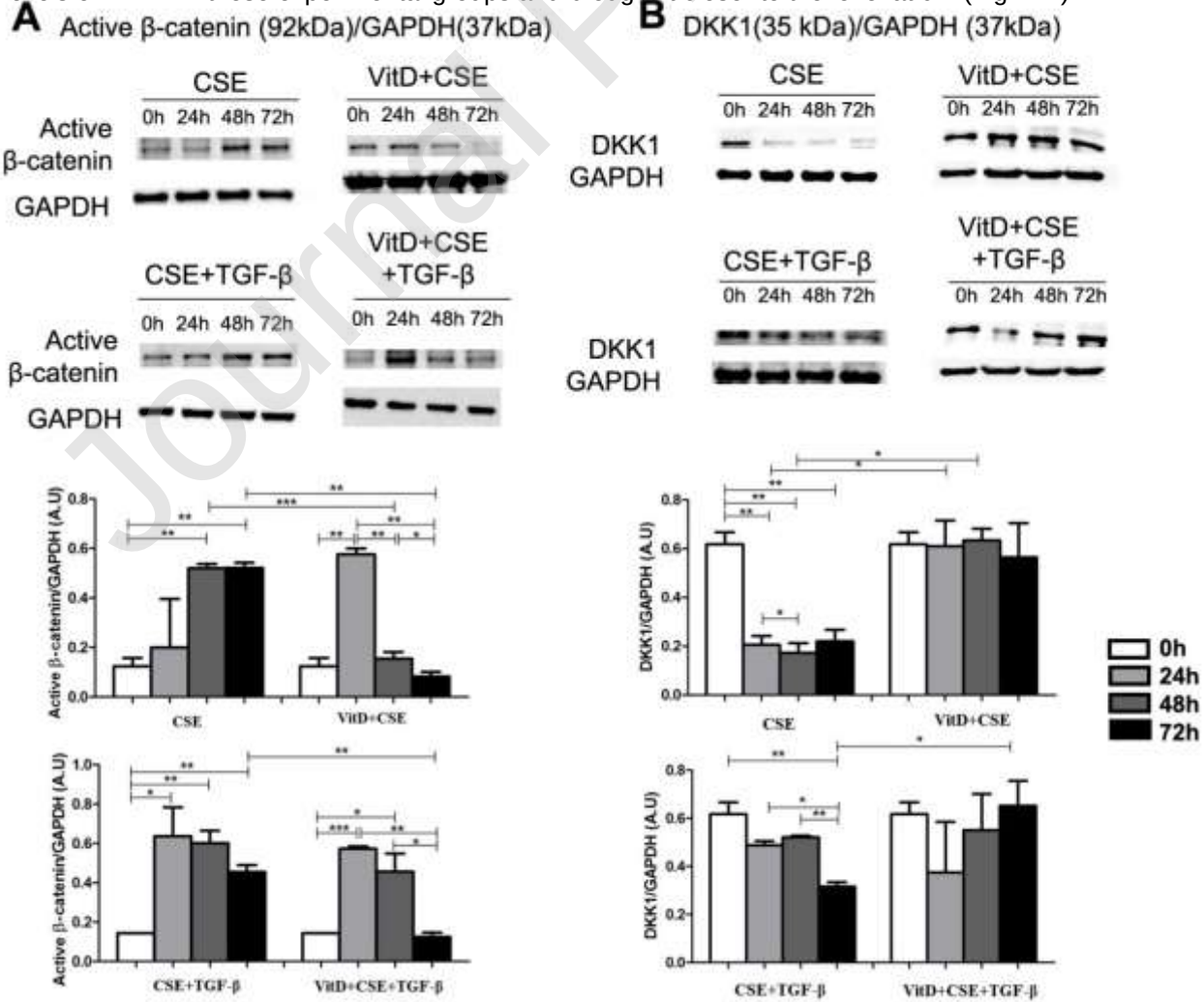


Fig. 4. CSE and TGF-β treatments triggered Wnt /β-catenin signaling in A549 cells. VitD pre-treatment inhibited Wnt signaling by reducing the levels of active β-catenin (A) and increasing the levels of DKK1 (B). P values: * p <0.05, ** p <0.01 and *** p <0.001.

3.4. VitD treatment regressed the induction of effective proteins on degradation and membranal stabilization of E-cadherin in A549 cells treated with 5% CSE and 5% CSE+TGF-β.

Both 5% CSE and 5% CSE+TGF-β treatments generally increased the levels of p120ctn and Kaiso starting from 24 h in A459 cells, while the level of NEDD9 was increased by 5% CSE at 48 h ($p<0.05$), and by 5% CSE+TGF-β at 72 h ($p<0.05$). Pre-treatment with VitD decreased the levels of p120ctn (Fig. A.5) and Kaiso proteins (Fig. B.5) in these cell groups and brought their level at 72 h closer to the level at 0 h. When comparing the 5% CSE group with the VitD+5% CSE group and the 5% CSE+TGF-β group with the VitD+5% CSE+TGF-β group, VitD treatment seemed to cause a decrease at NEDD9 level at 48 h ($p<0.05$) in cells treated with 5% CSE. While VitD could decrease NEDD9 level at 72 h ($p<0.05$) in cells treated with 5% CSE+TGF-β and brought its level closer to that of level at 0 h (Fig. C.5).

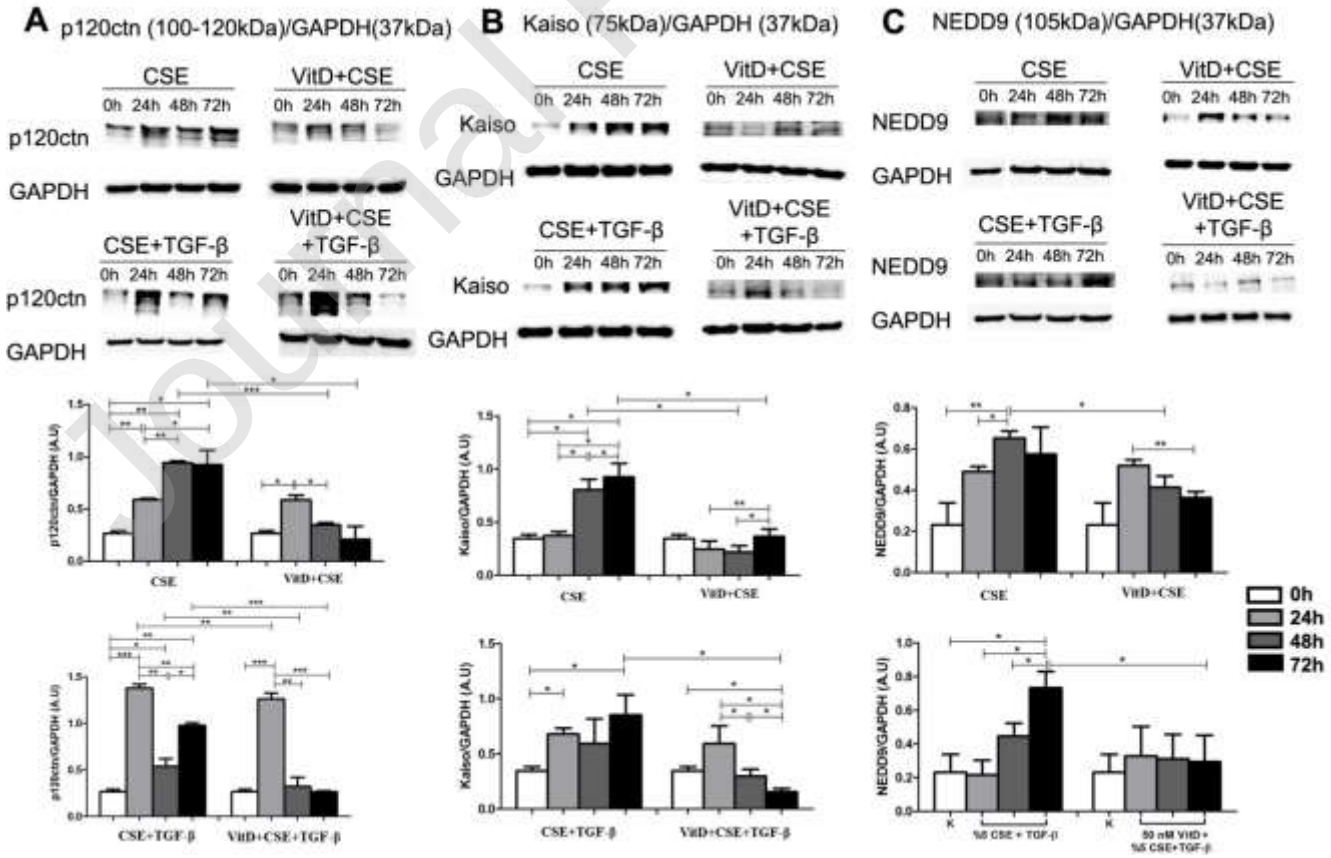


Fig. 5. CSE and TGF-β treatments induced alterations in the effective protein levels on degradation and membranal stabilization of E-cadherin, such as p120ctn (A), Kaiso (B) and NEDD9 (C) in A549 cells. VitD pre-treatment regressed these alterations and improve the degradation and membranal stabilization of E-cadherin. P values: * p <0.05, ** p <0.01 and *** p <0.001.

3.5. Immunofluorescence results

Immunoreactivities of p-VDR, p-SMAD2/3 and Kaiso were observed in both the cytoplasm and nuclei of A549 cells. We determined that immunoreactivity of p-VDR progressively decreased and localized in the cytoplasm of cells treated with 5% CSE+TGF-β, whereas the immunoreactivity of p-SMAD2/3 progressively increased and localized in nuclei (Fig. 6). Kaiso immunoreactivity was progressively increased by the 5% CSE+TGF-β treatment from 24 h to 72h and cytoplasmic Kaiso immunoreactivity was prominent (Fig. 7). Pre-treatment with VitD caused an increase of p-VDR immunoreactivity in A549 cells treated with 5% CSE+TGF-β and its re-localization to nuclei. Immunoreactivities of p-SMAD2/3 and Kaiso were decreased in cells treated with VitD+5% CSE+TGF-β (Figs. 6, 7). Kaiso immunoreactivity was mostly localized in the cytoplasm around the nucleus in these cells.

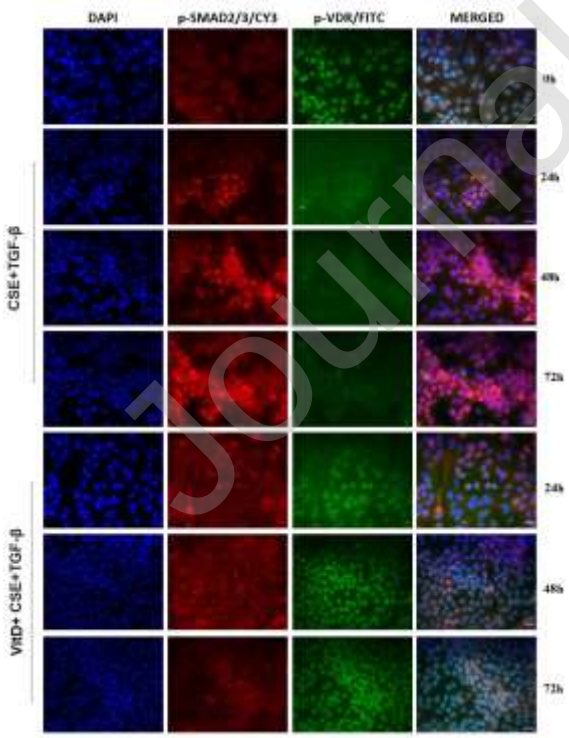


Fig. 6. Immunoreactivity of p-VDR (FITC) decreased and localized in the cytoplasm of A549 cells treated with 5% CSE+TGF-β, whereas the immunoreactivity of p-SMAD2/3 (CY3) increased and localized in nuclei. VitD pre-treatment mediated the re-localization of p-VDR to nuclei. Thus, it

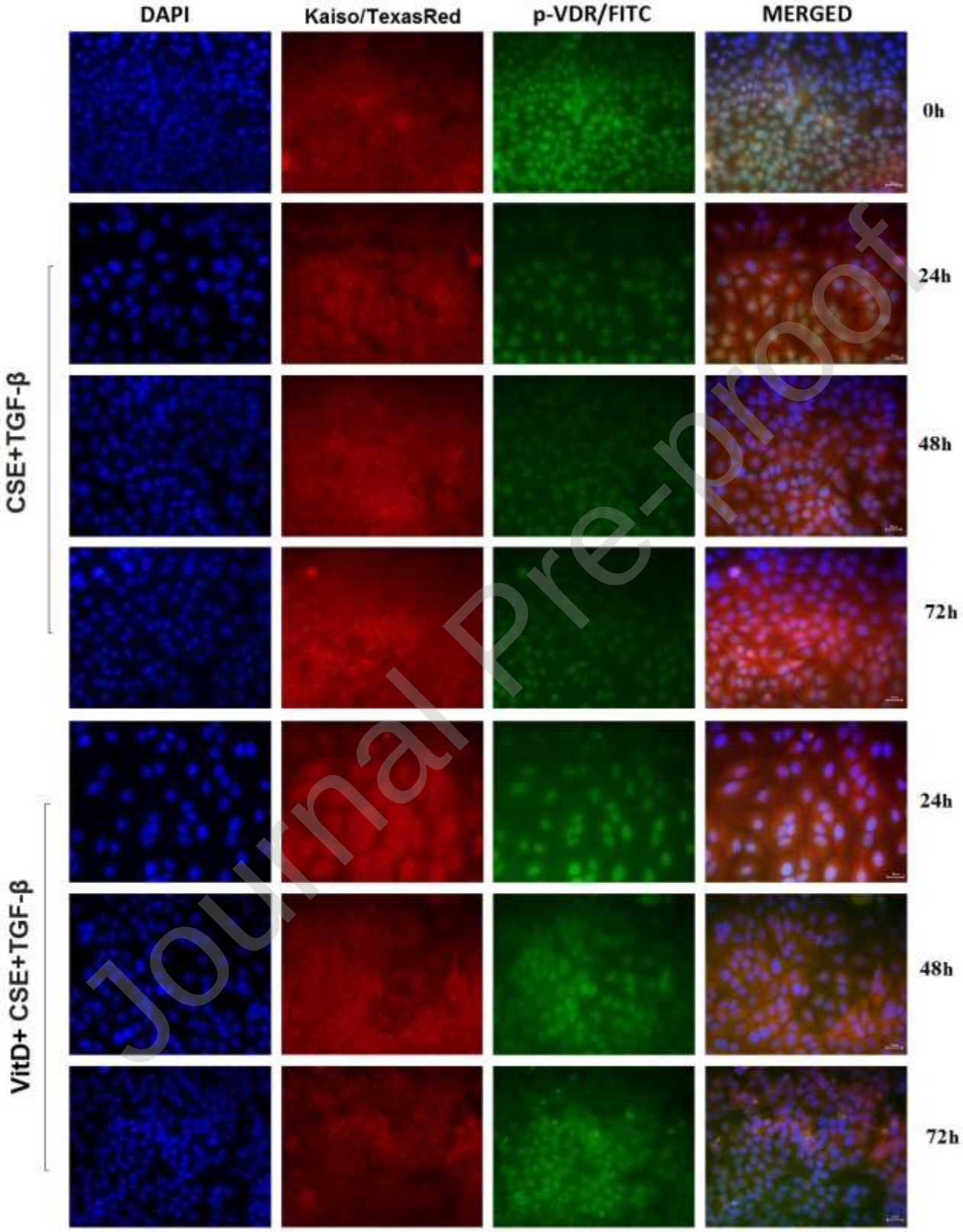


Fig. 7. 5% CSE+TGF-β treatment increased cytoplasmic Kaiso immunoreactivity (texas red) and decreased p-VDR immunoreactivity (FITC). VitD pre-treatment decreased Kaiso immunoreactivity and caused its localization around the nucleus, whereas VitD resulted in an increase in the nuclear p-VDR immunoreactivity. Bar size: 100µm.

3.6. p-VDR levels and precipitated proteins with p-VDR in fibrotic and non-fibrotic human lungs

Although the levels of p-VDR in fibrotic lungs were less than that of non-fibrotic lungs, these reductions did not reach statistical significance (Fig. A.8). Co-immunoprecipitation experiments revealed that active β-catenin and Kaiso proteins interacted with p-VDR, whereas p120ctn did not interact with p-VDR (Fig. B.8).

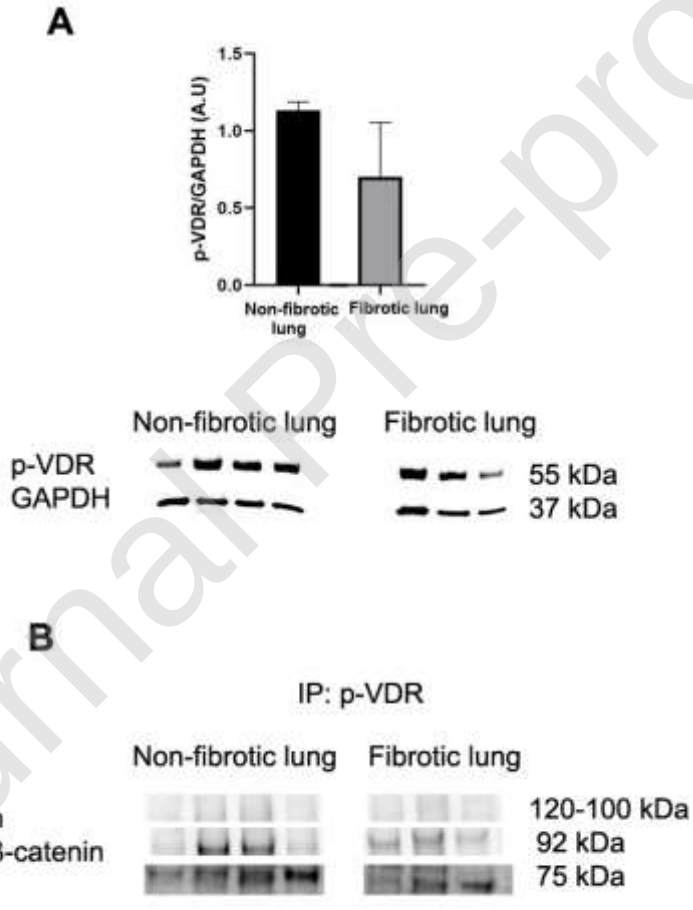


Fig. 8. The p-VDR immunoblotting (**IB**) (A) and co-immunoprecipitation (**IP**) analysis (B) of protein extracts from fibrotic and non-fibrotic human lungs. IP was performed with the anti-p-VDR antibody.

The presence of active β-catenin and Kaiso proteins that interacted with p-VDR was determined by IB. We did not observe any band belonging to p120ctn in the membranes.

4. Discussion

The present study provides the first data regarding both the interaction of VitD with TGF-β and Wnt/β-catenin pathways, and the expression profiles of p120ctn, Kaiso and NEDD9 proteins, which regulate E-cadherin turnover, during VitD-mediated regression of EMT in A549 cells with treated with 5% CSE and 5% CSE+TGF-β. The prominent findings of the study were as follows: (I) VDR signaling that was decreased by 5% CSE or 5% CSE+TGF-β treatments triggered EMT by activating TGF-β and Wnt/β-catenin signaling pathways and resulted in myofibroblast differentiation in A549 cells; (II) These cells were characterized by increased p120ctn, Kaiso, and NEDD9 protein levels; (III) The increased levels of p120ctn and NEDD9 led to decrease at the level of E-cadherin by both disrupting the stabilization of E-cadherin in the cell membrane and stimulating lysosomal degradation of E-cadherin; (IV) The p-VDR/β-catenin, p-VDR/p120ctn, and p-VDR/Kaiso co-immunoprecipitation experiments from the lungs of IPF patients showed that VDR interacted physically with β-catenin and Kaiso, lending us to say that VDR signaling could regulate the activation of the Wnt/β-catenin pathway in cells by controlling the transcriptional activities of β-catenin and Kaiso; (V) Treatment of 50 nM VitD 30 min before CSE and TGF-β treatments in A549 cells caused re-establishment of normal VDR signaling. Increased VDR signaling protected A549 cells from EMT by reducing the activation of TGF-β and Wnt/β-catenin pathways through p120ctn, Kaiso, DKK1 and active β-catenin proteins. Based on these results, we suggest overactivation of TGF-β signaling accompanied by smoking may lead to decreased p-VDR-mediated EMT and myofibroblast differentiation. Additionally, VitD supplementation combined with anti-fibrotic therapeutics can be suggested for the treatment of pulmonary fibrosis, which was caused by smoking, in cases of VitD deficiency.

Cigarette smoke reduces VitD levels in the blood and limited VDR signaling in fibrotic organs [8, 13, 14, 19-22]. It was stated that VitD deficiency was present in the serum samples of IPF patients [7]. It has been reported that mouse lungs with VitD deficiency are damaged due to the accumulation of collagen and fibronectin, and increased levels of α-SMA [8]. It has been shown that VitD treatment decreased TGF-β and α-SMA protein levels in the bleomycin-induced pulmonary fibrosis mouse model and regressed fibrosis [10]. Additionally, VitD decreased E-cadherin levels in rat RLE-6TN cells, which exhibit characteristic properties of alveolar type II cells [23]. The protein levels of the snail, N-cadherin and vimentin in addition to mRNA levels of fibronectin and collagen-1 were increased by TGF-β in A549 cells and were reduced by $1.25(OH)_2D_3$ treatment. Thus, EMT was regressed by $1.25(OH)_2D_3$, by inducing E-cadherin protein synthesis in these cells [24]. Similar to the results outlined above, in the present study, A549 cells treated with 5% CSE and 5% CSE+TGF-β were characterized by a progressive decrease in the p-VDR and E-cadherin levels, and an increase in α-SMA level from 0 h to 72 h after stimulation. Moreover, VitD treatment 30 min before CSE and TGF-β treatments protected A549 cells from EMT stimulation by increasing the level of E-cadherin, decreasing the α-SMA level,

and stimulating the VDR signaling. These results support that VDR signaling reduced by cigarettes or cigarette use may be an important risk factor in smoking-related pulmonary fibrosis. Tzilas et al. [7] also reported that IPF patients had VitD deficiency in their serum and suggested that VitD may be a potential therapeutic target in the treatment of IPF. In the current study, we did not detect any significant difference at levels of p-VDR between fibrotic (n=3) and non-fibrotic (n=4) lung tissue examples. However, we think that our result might suffer from the low number of patient samples and suggest replication in a larger cohort.

The treatment of VitD in mice with cardiac and liver fibrosis leads to regression of fibrosis by reducing TGF-β and p-SMAD2/3 levels [25, 26]. Another study revealed that VDR competed with SMAD3 and suppressed TGF-β-induced pro-fibrotic genes by binding target genes of SMAD3 in the fibrotic liver [11]. Additionally, $1,25(OH)_2D_3$ attenuated high glucose-induced EMT in human peritoneal mesothelial cells via the TGF-β/SMAD3 pathway by reducing SMAD3 and p-SMAD3 protein levels [27]. Studies performed on various non-lung cell lines show that VitD treatments can control TGF-β signaling via SMAD4 and SMAD7 [28-31]. Similar data were obtained by the present study: 5% CSE and 5% CSE+TGF-β treatments resulted in the induction of TGF-β signaling by increased protein levels of p-SMAD2/3 and SMAD4, and decreased protein levels of SMAD7 in A549 cells. The SMAD2/3 dependent TGF-β signaling was significantly reduced by VitD pre-treatment 30 min before CSE and TGF-β treatments at 48 and 72 h. Furthermore, these results were confirmed by p-VDR/p-SMAD2/3 immunofluorescent double staining. It has been observed that the 5% CSE and 5% CSE+TGF-β-stimulated A549 cells showed low p-VDR and high nuclear p-SMAD2/3 immunoreactivities over time. VitD pre-treatment before 5% CSE and 5% CSE+TGF-β exposure in A549 cells again increased VDR signaling and p-VDR immunoreactivity reduced by CSE and TGF-β. Increased VDR signaling caused the reduction of SMAD2/3-dependent TGF-β signaling and the regression of EMT in these cells.

Wnt/β-catenin signaling modulates EMT [32]. VDR binds to β-catenin and blocks the Wnt signaling by inhibiting the binding of β-catenin to TCF4/LEF complex in colon cancer cells and epidermal keratinocytes [33, 34]. A study with 16HBE and A549 cells showed that CSE treatment increased β-catenin levels. Treatment with $1,25(OH)_2$-D_3 induced β-catenin levels were reversed in these cells [24, 35]. The data from the aforementioned studies indicate that VitD could regulate Wnt/β-catenin signaling by reducing the levels of β-catenin or by controlling the cytoplasmic and nuclear localization of β-catenin. Similarly, the present study showed that β-catenin levels induced by 5% CSE and TGF-β treatments were reduced with VitD pre-treatments and were brought closer to that of the level at 0 h. Moreover, the results of p-VDR-β-catenin co-immunoprecipitation experiments showed that p-VDR can bind to β-catenin in fibrotic (patients with IPF) and non-fibrotic human lungs. It has been found that VitD treatments increased DKK1 levels, an inhibitor of Wnt signaling, in colorectal and colon cancer cells [36, 37]. In the present study, the reduction of DKK1 levels by 5% CSE or 5% CSE+TGF-β treatments were normalized by pre-treatment with VitD. All these data verify that CSE and TGF-β treatments stimulate Wnt signaling by increasing β-catenin levels and reducing DKK1

levels in A549 cells. We suggest that VitD pre-treatments 30 min before CSE and TGF-β treatments can inhibit Wnt signaling by reducing the high levels of β-catenin, increasing the low levels of DKK1, and controlling the transcriptional activity of β-catenin in CSE and TGF-β exposed A549 cells. VitD pre-treatments caused the reduction of SMAD2/3 dependent TGF-β and Wnt/β-catenin signalings by increasing p-VDR levels and thus it led to the regression of EMT in A549 cells treated with CSE and TGF-β.

EMT is characterized by the loss of E-cadherin [38]. The p120ctn acts on E-cadherin turnover and stabilization of E-cadherin in the cell membrane, thereby regulating E-cadherin levels [39, 40]. The p120ctn knockdown by siRNA expression resulted in the elimination of E-cadherin and complete loss of cell-cell adhesion [41]. An abnormal decrease in E-cadherin levels was observed due to overexpressed p120ctn isoforms in the cytoplasm of adenocarcinoma cells in the lung [42]. Additionally, p120ctn regulates the Wnt/β-catenin signaling pathway. It blocks both endocytosis of E-cadherin and transfer of β-catenin to the nucleus when p120ctn interacts with E-cadherin and thus it induces Wnt signaling [43- 45]. In the present study, p120ctn levels were significantly increased at 24, 48 and 72 h compared to 0 h in 5% CSE-exposed A549 cells. Considering the increased level of β-catenin, we think that the increased levels of p120ctn might be effective on the induction of EMT in A549 cells by causing the destruction of E-cadherin and stimulation of Wnt signaling. Additionally, it is known that there is a relationship between p120ctn and TGF-β signaling [46]. There was no change in the expression of p120ctn in TGF-β-stimulated colorectal carcinoma cells, but the cytoplasmic localization of p120ctn was directly related to the loss of E-cadherin and could promote EMT [46]. It has been reported that the prevention of SMAD3/2 phosphorylation was regulated by p120ctn bound to E-cadherin. Also, p120ctn facilitates liver fibrosis by promoting the expression of TGF-β target genes and the loss of E-cadherin in the rat liver and hepatic stellate cells [47]. In the present study, we determined that p120ctn increased when E-cadherin was decreased and SMAD2/3 dependent TGF-β and Wnt/β-catenin signaling were induced by 5% CSE and TGF-β stimulation of A549 cells. We believe that the increase in the level of p120ctn occurs as a response to the loss of E-cadherin. Furthermore, the breakdown of the p120ctn-E-cadherin complex may induce the stimulation of TGF-β and Wnt/β-catenin signals in A459 cells. On the other hand, the relationship between p120ctn and VDR signaling has not been known yet. The p-VDR-p120ctn co-immunoprecipitation experiments of the present study on fibrotic and non-fibrotic human lung samples showed that there was no physical interaction between these two molecules. The present study also revealed that VitD pre-treatment normalized the levels of p120ctn, by decreasing the high-level of p120ctn induced by 5% CSE and 5% CSE+TGF-β in A549 cells. Accordingly, we think that treatments with VitD may be effective to reduce EMT stimulation modulated by increased p120ctn.

Kaiso binds directly to the methylated sequences in the promoter of the E-cadherin gene, thereby reducing E-cadherin gene expression and causing EMT [48-50]. We detected the decreased level of E-cadherin protein and increased level of Kaiso protein when EMT was stimulated by CSE and TGF-β treatments in A549 cells. Kaiso could regulate to activation of Wnt/β-catenin signaling. Healthy

HBE cells exposed to the cigarette-concentrated medium increased Kaiso synthesis. It was found that Kaiso interacted with p120ctn accumulated in the cytoplasm due to Kaiso's displacement from the nucleus to the cytoplasm. EMT might develop as a result of Kaiso accumulation in the cytoplasm and the inability of Kaiso to bind DNA/ promoters of the β-catenin's target genes [51]. Similar findings were obtained from the present study. In the 5% CSE and 5% CSE+TGF-β exposed A549 cells, an increase at the level of Kaiso was accompanied by EMT induction and activation of TGF-β and Wnt signaling. And also, Kaiso immunoreactivity was present, especially in cytoplasm around nucleus, when Wnt/β-catenin signaling and EMT were stimulated in these cells. We suggest that CSE and TGF-β increase the protein level of Kaiso in A549 cells and that increased level of cytoplasmic Kaiso contributes to Wnt/β-catenin mediated EMT stimulation. It has been reported that TGF-βR1 and SMAD2 levels were decreased, and EMT was regressed when gene expression of Kaiso was knockdown in breast cancer cells [52]. It can be concluded that Kaiso can control EMT by both silencing the expression of the E-cadherin gene via both binding to the promoter of E-cadherin and the regulation of Smad-dependentTGF-β and Wnt/β-catenin signalings. We suggest that increased TGF-β signaling may have led to an increase in the protein level of Kaiso in A549 cells treated with 5% CSE and TGF-β. And also, we propose that increased levels of cytoplasmic Kaiso around the nucleus might allow the transcription of the target genes of Wnt/β-catenin signaling. For this reason, EMT was induced by both TGF-β and Wnt/β-catenin signalings in A549 cells treated with 5% CSE and 5% CSE+TGF-β. On the other hand, the relationship between Kaiso and VDR signaling is not yet known. The p-VDR-Kaiso co-immunoprecipitation experiments performed on fibrotic and non-fibrotic human lung samples showed that there was a physical interaction between these two molecules in the present study. VitD treatments 30 min before 5% CSE and 5% CSE+TGF-β exposure, decreased and then normalized Kaiso level in A549 cells. The p-VDR/Kaiso double immunofluorescent staining showed that pre-treatment with VitD reduced the increased level of Kaiso which was induced by 5% CSE and 5% CSE+TGF-β treatments, and Kaiso immunoreactivity was also present mostly in the cytoplasm around the nucleus in A549 cells. These data reveal that both the intracellular localization of the Kaiso and its amount in the cell are effective in the determination of Kaiso activity. Additionally, co-immunoprecipitation analysis from human lung samples showed that Kaiso activity could be controlled by p-VDR as well as p120ctn. We think that the increased level of p-VDR can control the Kaiso's activity in A549 cells treated with VitD+5% CSE and VitD+5% CSE+TGF-β because the level of p120ctn was reduced in these cells. In cases p-VDR signaling enhanced, the control of Kaiso activity via p-VDR will be very important to regulate the expression of E-cadherin genes and target genes of Wnt/β-catenin signaling.

The E-cadherin level is increased, when the NEDD9 level is suppressed in A549 cells [53]. The overexpression of NEDD9 adversely affected lung cancer prognosis by decreasing the membrane-bound E-cadherin and β-catenin amounts and increasing N-cadherin levels [54]. It was suggested that the levels of E-cadherin were decreased by NEDD9, which induced its lysosomal degradation, in emphysematous regions of the human lungs [55]. NEDD9 expression can be regulated by Smad-dependentTGF-β signaling [56]. When prostate cancer cells were stimulated with TGF-β,

NEDD9 protein levels were increased, as well as EMT stimulation. However, when the expression of NEDD9 protein was silenced, tumor invasion was decreased and EMT was regressed by causing low TGF-β level [57]. The effect of smoking on NEDD9 protein was not known before. The present study revealed that 5% CSE and 5% CSE+TGF-β treatments on A549 cells resulted in a significant increase in NEDD9 protein levels. Increased NEDD9 protein levels were accompanied by decreased levels of E-cadherin protein and the induction of the Smad-dependentTGF-β signaling in these cells. Thus, we suggest that CSE and TGF-β treatments on A549 cells may induce the degradation of E-cadherin by increasing the levels of NEDD9 induced by TGF-β and cause EMT. Pre-treatment with VitD in A549 cells treated with CSE and TGF-β increased the VDR signaling. Thus, it leads to the normalization of NEDD9 levels and decreasing Smad-dependent TGF-β signaling. As a result, VitD attenuates EMT induced by CSE and TGF-β treatments by increasing the E-cadherin level.

Author statement

I have resubmitted the revised manuscript "**Vitamin D modulates E-cadherin turnover by regulating TGF-β and Wnt signalings during EMT-mediated myofibroblast differentiation in A459 cells "** to **The Journal of Steroid Biochemistry and Molecular Biology.** The present manuscript was written by Ezgi Sari, Fusun Oztay, and Ahmet Erdal Tasci. No conflicts of interest are declared by the authors. The revised manuscript was edited by all authors. This manuscript or a very similar manuscript has not been published, nor is under consideration by any other journal.

6. Declaration of competing interests

No conflicts of interest are declared by the authors.

7. Authors'contributions

F.O. obtained research funding. F.O. and E.S. were involved in conception and study design. F.O. contributed to study supervision. The experiments were performed and analyzed by E.S. F.O. and E.S were responsible for data interpretation. A.E.T. contributed to collect human lung samples. F.O. drafted the manuscript, and F.O and E.S wrote the final version. All authors approved the final version of the manuscript.

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