Heparin-Binding protein 17 (HBp17)/ Fibroblast Growth Factor-Binding Protein-1 (FGFBP-1) Expression is Down-regulated by $1\alpha,25(OH)_2D_3$ through NF-κB Pathway in Oral Squamous Cell Carcinoma Cell Lines

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Chapter II

1α,25(OH)2D3 Down-regulates Heparin-Binding Protein 17/Fibroblast Growth Factor-Binding Protein-1 (HBp17/FGFBP-1) Expression via NF-κB Pathway.

ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	1alpha-dihydroxy vitamin D3 or Calcitriol
BAY	BAY 11-7085 or $CH_3H_{15}NO_{25}$ or (E)3-[(4-t-Butylphenyl)sulfonyl]-2-propenitrile)
CTCF	corrected total cell fluorescence
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
ELISA	Enzyme-linked ImmunoSorbent Assay
EtOH	ethanol
FGF	Fibroblast growth factor
FGF-1	Fibroblast growth factor-1
FGF-2	Fibroblast growth factor-2
FGFBP-1	Fibroblast Growth Factor-Binding Protein-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBp17	Heparin-Binding Protein 17
HSPG	Heparan sulfate proteoglycans
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
kDa	kilo Dalton
mRNA	messenger RNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of Activated B
OSCC	Oral Squamous Cell Carcinoma
P50	NF-κB subunit
P65	NF-κB subunit (RelA)
PBS	phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen

PVDF	polyvinylidene difluoride
qRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
siControl	cells transfected with scrambled siRNA
siRNA	small interfering RNA
VDR	Vitamin D receptor
siVDR	VDR siRNA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 1% Tween-20

Chapter 1

Down-regulation of Heparin-Binding Protein 17/Fibroblast Growth Factor-Binding Protein 1 (HBp17/FGFBP-1) by 1α,25$(OH)_2D_3$ Inhibits FGF-2 Activity in Oral Squamous Cell Cancer Cell Lines

1.0 ABSTRACT

Heparin-Binding Protein 17/Fibroblast Growth Factor-Binding Protein-1 (HBp17/FGFBP-1, GenBank accession no. NP-005121) is prominent for its role as the chaperone for Fibroblast Growth Factor-2 (FGF-2). Together, they play a crucial role in angiogenesis as well as promoting tumor growth. HBp17/FGFBP-1 has also been proposed to be used as a candidate biomarker for a number of cancers for they are frequently found to be elevated in many cancer types including in the tissue and cell lines of oral squamous cell carcinoma (OSCC). In this present study, we utilized the renowned anti-cancer agent, i.e. $1\alpha,25(OH)_2D_3$, to suppress the HBp17/FGFBP-1 expression in OSCC. To date, there are no reports on the probable susceptibility of the HBp17/FGFBP-1 expression in OSCC by $1\alpha,25(OH)_2D_3$. In this present study, we demonstrated that the treatment of OSCC cells with $1\alpha,25(OH)_2D_3$ (40nM) suppressed HBp17/FGFBP-1 expression, and reducing the FGF-2 bio-activation measured in conditioned media. Inhibition of the HBp17/FGFBP-1 expression was analyzed by quantitative RT-PCR, western blotting, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA). In order to understand the involved molecular mechanisms, the effect of $1\alpha,25(OH)_2D_3$ on Vitamin D Receptor (VDR) RNAi-transfected UE cells (siVDR) was investigated. Knocking down of the VDR in the reaction has caused all the above reaction to cease. This result suggests that the suppression of HBp17/FGFBP-1 and FGF-2 by $1\alpha,25(OH)_2D_3$ was a VDR-mediated event. In summary, the ability of $1\alpha,25(OH)_2D_3$ to suppress the expression of HBp17/FGFBP-1 and FGF-2 via VDR represents a novel anti-cancer mechanism of this property.

Keywords: 1α,25$(OH)_2D_3$; Vitamin D receptor; HBp17/FGFBP-1; NF-κB; IκBα; Oral squamous cell carcinoma

2.0 INTRODUCTION

Heparin-Binding Protein (HBp17), with a molecular mass of 17kDa, was originally purified from the culture media conditioned by A431 human epidermoid carcinoma cells, with no homology with any known protein at that time [1]. HBp17, also known as Fibroblast Growth Factor-Binding Protein-1 (FGFBP-1), is highly tissue specific and strictly regulated through different promoter elements [2]. It binds to Fibroblast Growth Factors-1 and -2 (FGF-1 and FGF-2) in non-covalent and reversible manner to facilitate their release from the extracellular matrix (ECM) [3-6]. This reaction served as an alternative mode of delivering activated FGFs from an extracellular storage site to its receptor while protecting them from degradation [1]. This event contributes to cell migration, proliferation and differentiation, mechanisms important during embryogenesis, wound healing as well as angiogenesis [6-8]. Therefore, HBp17/FGFBP-1 is frequently found up-regulated in several tumors including squamous cell carcinomas (SCC) [1, 9]. In carcinogenesis, HBp17/FGFBP-1 acts as the paracrine factor where it causes mobilization of FGF-2 [1]. In fact, HBp17/FGFBP-1 was found to be associated with FGF-2 when it was first purified in 1991 [1]. Furthermore, this molecule was noted to be associated with the early stages of tumor formation in which angiogenesis plays a critical role [10, 11]. Since then, this molecule was speculated to be a potential rate-limiting factor in tumor growth [8]. Besides, HBp17/FGFBP-1 has been described to have an aberrant expression in a number of cancers [3, 10, 12-14] and therefore, proposed to be used in the diagnostic screening for the early detection of cancer [12, 14].

Fibroblast Growth Factors (FGFs) consist of broad spectrum mitogens having more than 50% homology in amino acid sequences across species [15]. FGF-2, one of the 22 distinct FGFs

to date, could be found in four different polypeptides ranging from 18kDa to 24kDa (subject to translation) [16], all without a signal sequence for secretion. They are found to be tightly bound to heparan sulfate proteoglycans (HSPG) and only bio-activated when freely soluble [1]. For activation, they have to be transported to the surrounding ECM via three proposed mechanisms; i.e., through heparan sulfate proteoglycan digestion by either proteases (i) or heparanases (ii), or through a carrier protein (secreted HBp17/FGFBP-1) (iii) [17, 18]. Once released, FGF-2 could stimulate angiogenesis [6], wound healing and tissue development [19]. Basically, it acted as an autocrine growth factor on the tumor cell itself, and at the same time, promoting angiogenesis in the surrounding stroma [20]. Overexpression of FGF-2 has been associated with the pathogenesis of cancer [21-23], thus recently, FGF-2 was reported to be a possible salivary biomarker for oral squamous cell carcinoma (OSCC) patients [24].

Several publications have reported the positive modulation of HBp17/FGFBP-1 on FGF-2 [3, 4, 6]. Basically, FGF-2's fate is dependent (at least partly) on the HBp17/FGFBP-1 as a releasing agent from ECM storage where FGF-2 is usually kept latent. Therefore, it is apparent that the expression of HBp17/FGFBP-1 and FGF-2 would be proportional to each other. Taking this into account, we proposed the possibility to suppress the FGF-2 expression in cancer cells by governing the expression of its carrier; i.e., HBp17/FGFBP-1. So far, other than ribozyme targeting [3, 25], the retinoid was one of the well-studied properties proven to have the ability to down-regulate the HBp17/FGFBP-1 gene expression by post-transcriptional and transcriptional mechanisms [26].

1α,25-Dihydroxyvitamin D3 (1α,25$(OH)_2D_3$) is a multifunctional hormone renowned for its role in regulating calcium and skeletal homeostasis [27]. Since 1980, the potential of this compound has been expanded and recognized as an anti-cancer agent. For example,

$1\alpha,25(OH)_2D_3$ has appeared to induce the differentiation and inhibiting proliferation, invasiveness, and metastatic potential of cancer cells [28, 29]. Subsequently, $1\alpha,25(OH)_2D_3$ has been proven to be effective in the therapy of several cancers in conjunction with other conventional therapies [30]. Among the many molecular pathways that have emerged for its anti-cancer activities [31], the inhibition of the NF-κB activation and signaling [32] has captured our attention. To the best of our knowledge, there has been no research discussing the possible effect of $1\alpha,25(OH)_2D_3$ on the expression of HBp17/FGFBP-1 and FGF-2 which are often found overexpressed in cancer cells.

Most functions of $1\alpha,25(OH)_2D_3$ in cells are mediated by the Vitamin D Receptor (VDR) [33]. The interaction between these two molecules is well-known to provide protection from sporadic malignancies [34]. The anti-proliferative effects of $1\alpha,25(OH)_2D_3$ and VDR have been demonstrated in a wide variety of cancer cell lines, but to date, none in oral squamous cell carcinomas (OSCC). Therefore, our study focuses on the possibility of $1\alpha,25(OH)_2D_3$ suppressing the readily elevated expression of HBp17/FGFBP-1 and FGF-2 in OSCC cell lines and discuss the possibility of $1\alpha,25(OH)_2D_3$ in anti-tumor therapy targeting both of these molecules. In the first part of our study, we reported the efficacy of $1\alpha,25(OH)_2D_3$ to suppress the HBp17/FGFBP-1 gene expression in OSCC cell lines. In addition, we proved that the suppression of HBp17/FGFBP-1 occurred via the NF-κB pathway and VDR appeared to facilitate the reaction [35]. In this present study, we report the impact on the biological role of HBp17/FGFBP-1 as a carrier to FGF-2. In regards to the positive correlation between these two molecules, we hypothesize the probability of $1\alpha,25(OH)_2D_3$ as a limiting factor for the FGF-2 protein expression through HBp17/FGFBP-1 modulation.

3.0 MATERIALS AND METHODS

3.1. Cell Cultures

The cell lines used were OSCC cell lines (UE: HO-1-u-1 [36] and Kono (KO) [37], (established from a patient with oral cancer in our laboratory) and epidermoid carcinoma cell line, A431 [38]. Those cell lines were cultured in serum-free medium as follow.

UE was cultured at 2.5 x 10^4 cells/well in 6-well plates (non-collagen coated) (BD Falcon™, California, USA) in RD6F medium [39, 40] which was composed of the basal nutrient medium (RD) supplemented with 6 factors, i.e. insulin (10μg/ml), transferrin (5μg/ml), 2-aminoethanol (10μM), sodium selenite (10nM), 2-mercaptoethanol (10μM), and oleic acid conjugated with acid-free bovine serum albumin (9.4μg/ml) (All chemicals were from Sigma-Aldrich, St. Louis, USA). The RD basal nutrient medium was prepared as follow; RPMI1640 medium (Sigma-Aldrich) mixed in equal part (1:1) with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) dissolved in Milli-Q water (ELIX, Millipore, Billerica, USA) added with 90mg/L of ampicillin sodium (Meiji Seika Kaisha, Ltd, Tokyo, Japan), 90mg/L of kanamycin sulfate (Invitrogen, CA, USA), 20mM of 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulforic acid (HEPES) (Dojindo, Kumamoto, Japan), 110mg of sodium pyruvate and 2g/L of sodium bicarbonate (Sigma-Aldrich), adjusted to pH 7.4 and filtered with 0.2μm membrane filter (Acrocap, Gelman Laboratory, Ann Arbor, USA).

KO and A431 were cultured in DF6F medium which was composed of the basal nutrient medium (DF) 6 factors. The DF basal medium was prepared as follow; Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DF) (Sigma-Aldrich) mixed in Milli-Q water

(ELIX, Millipore). These basal medium was as well supplemented with 6 factors (Sigma-Aldrich).

Growth were allowed at 37℃ in a 5% CO_2 incubator (Thermo Fisher Scientific™, Massachusetts, USA) for 48hours. Subsequently, 40nM 1α,25$(OH)_2D_3$ (Enzo Life Sciences, Inc.©, NY, US) or 2μM BAY 11-7085 ($CH_3H_{15}NO_{25}$/ (E)3-[(4-t-Butylphenyl)sulfonyl]-2-propenitrile) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added and further cultured for a certain amount of time according to the experimental design.

3.2. Quantitative RT- PCR (qRT-PCR) analysis

The total RNA was extracted using the RNeasy total RNA spin Mini RNA Isolation Kit (GE Healthcare UK, Ltd., Buckinghamshire, England). Reverse transcription (RT) was performed using the Super Script first-strand synthesis system (Life Technologies™, NY, USA). Quantitative RT-PCR analyses for HBp17/FGFBP-1, VDR and FGF-2 were performed using the Stratagene Mx3000P™ system (Stratagene, Agilent Technologies, USA) with GAPDH as the internal control. The sequences of primers and TaqMan™ fluorogenic probes for HBp17/FGFBP-1, VDR and FGF-2 were designed according to the ProbeFinder™ software of Roche Universal Probe Library system (Roche Applied Science, N.J., USA): 5'-CGTGTGCTCAGAACAAGGTG-3', 5'- GAGCAGGGTGAGGCTACAGA-3' and #46 fluorescence probe (Roche Diagnostics, N.J. USA) for HBp17/FGFBP-1 (NM__005130); 5'-CTTCTCTGGGGACTCCTCCT-3', 5'-TGGACGAGTCCATCATGTCT-3' and #15 fluorescence probe (Roche Diagnostics) for VDR (NM __ 001017536); 5'-

TTCTTCCTGCGCATCCAC -3', 5'- TGCTTGAAGTTGTAGCTTGATGT-3' and # 7 for FGF-2 (NM_002006.4).

3.3. siRNA transfection

VDR siRNA (5'- AGAUCACUGUAUCACCUCUTTtt-3') and the control scrambled siRNA (5'- AGUUCAACGACCAGUAGUCtt-3') were synthesized at the Ambion siRNA facility (Ambion, Austin, Texas, USA). The UE cells were inoculated at $15x10^4$ cells/well in 6-well plates for 24 hours and then transfected with 1μM VDR or scrambled siRNA using the siPORT^{TM} NeoFX^{TM} Transfection Agent (Ambion) in serum-free medium for 48hours. The cells were then treated with 40nM $1\alpha,25(OH)_2D_3$ (Enzo) or ethanol as the control for 12 hours. The mRNAs were next isolated for gene expression analysis.

3.4 Cell Fractionation

Cells were inoculated at 1.25×10^5 cells/well in a 10cm dish (BD Falcon^{TM}), and cultured for 48 hours in RD6F or DF6F serum-medium. The cells were then treated with 40nM $1\alpha,25(OH)_2D_3$ (Enzo) or ethanol for 0, 3, 6, 12 and 24 hours, collected by scrapping the cells with ice-cold PBS, and centrifuged at 4°C, at 500rpm. The cells were fractionated into cytosol and nuclear fractions using the Nuclear Extract Kit (ACTIVE MOTIF, California, USA). Both fractions were kept at -80°C or immediately used for analysis.

3.5. Western Blot Analysis

The cells were inoculated at $2.5x10^4$ cells/well in 6-well plates (BD Falcon™), and cultured in serum-free medium for 48 hours, then treated with 40nM $1\alpha,25(OH)_2D_3$ (Enzo) or ethanol. The cells were then collected at the time points of 0, 3, 6, 12, and 24 hours by scrapping them out in Ca^{2+} and Mg^{2+} -free Phosphate buffered saline (CMF-PBS). The protein extract was prepared by dissolving cells in RIPA buffer (10mM Tris-HCL, 150mM NaCl, 5mM EDTA, 1%Triton X-100, pH 7.4 [Nacalai Tesque Inc., Kyoto, Japan]) followed by sonication on ice for 30 seconds by Sonicator (Taitec UltraS Homogenizer VP-5S, Taitec, Tokyo, Japan). Quantification of the protein content was performed according to the standard protocol of Bradford Assay (Bio-Rad Laboratories Inc., California, USA). Proteins were then solvilized in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) or kept at -80°C.

The protein extract (2.5µg proteins) was first resolved by SDS-PAGE in an 11% gel gradient (20mA/gel). The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by a semi-dry blotting system (Bio-Rad) (90mA/gel) for 1 hour 30 minutes.

The antibodies used in the experiments were biotinylated anti-human HBp17/FGFBP-1 (R&D Systems, Minneapolis, USA), anti-VDR mouse monoclonal IgG (Santa Cruz Biotechnology Inc., California, USA), anti-IκBα rabbit polyclonal IgG (Santa Cruz Biotechnology Inc.) and anti-FGF-2 rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc.). All antibodies were diluted accordingly in 1% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) (Nacalai Tesque Inc.). After subsequent blocking, the membrane was incubated with the primary antibody for 2 hours at room temperature followed by incubation

with the HRP-conjugated secondary antibody for 1 hour at room temperature. The secondary antibodies used were Streptavidin-HRP (R&D Systems), Anti-mouse IgG HRP-linked (Invitrogen, California, USA), and goat anti-rabbit IgG (Bio-Rad). The reaction was developed with chemiluminescence reagent (GE Healthcare) for generation of the light signal. The light signal was captured by a ChemiDoc XRS Imaging System (Bio-Rad Laboratories, Inc.). Reprobing was used for detection of the internal control antibodies, i.e. Monoclonal anti-β-actin antibody (Sigma-Aldrich) or Monoclonal mouse anti-proliferating Cell Nuclear Antigen Clone PC10 (PCNA) (DakoCytomation, Glostrup, Denmark). All experiments were performed in triplicate or more.

Image analysis was performed using Image Processing and Analysis in Java software (Image J software) (National Institute of Health, NIH, Maryland, USA). Values of each band were expressed as percent of adjusted volume, and the final results were calculated as mean$\pm$SD. The student *t*-test was used to evaluate the differential significance between two variables.

3.6. ELISA

An Enzyme-linked Immunosorbent Assay (ELISA) was performed using the Quantikine® ELISA kit (R&D Systems-Inc.). This kit was used for the quantitative determination of the HBp17/FGFBP-1 and FGF-2 protein contents in conditioned medium.

3.7. Immunofluoresence

A total of $8x10^3$ cells/chamber (LAB-TEK®, Roskilde, Denmark) was cultured in serum-free medium for 48 hours before adding 40nM $1\alpha,25(OH)_2D_3$ (Enzo) or ethanol (EtOH). Staining of the cells was performed at the time points of 12 and 24 hours. The antibodies used were mouse monoclonal anti-human FGF-BP (R&D Systems) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). The DAPI (4',6-Diamidino-2-phenylindole) (Invitrogen) in a dilution of 1:1000 was also included in the secondary antibody solution.

Images of the cells stained with the molecule of interest were captured in two and three dimensions with the aid of a laser scanning confocal microscope, LSM700 (Carl Zeiss, Jena, Germany). The captured images were quantified and analyzed as the corrected total cell fluorescence (CTCF) using the Image J software (NIH).

3.8. Statistical Analyses

Data are presented as mean values $\pm$ standard deviation (S.D.). The student's *t*-test was used for group analysis and *p*-values <0.05 were considered significant. Analysis was performed using data analysis software in Microsoft's Office EXCEL 2010 (Microsoft Corporation, USA). All the experiments were independently repeated at least three times and performed in triplicate assay or more.

4.0 RESULTS

4.1. 1α,25(OH)$_2$D$_3$ (40nM) significantly suppressed HBp17/FGFBP-1 expression in UE cells while sustaining viability.

Determination of the optimum concentration of 1α,25(OH)$_2$D$_3$ to be used in the study was achieved by examining the effect of a wide range of concentrations of this substance on the viability of UE cells cultured for 6 days. At the end, cells demonstrated an optimum growth at concentration of 40nM (Figure 1A).

Next, growth assay was performed to determine for any possible toxicity of 1α,25(OH)$_2$D$_3$ on cell lines. Assay was performed according to standard procedure until day 6. Figure 1B shows exemplary growth curve of (i) UE (OSCC cell line), (ii) A431 (epidermoid carcinoma cell line). Cells showed viable in equivalent to control condition.

After optimum concentration of 1α,25(OH)$_2$D$_3$ was decided, its effect on the expression of HBp17/FGFBP-1 in UE cells was examined. This experiment is also to demonstrate the comparable potential of 1α,25(OH)$_2$D$_3$ and BAY 11-7085, a well-known NF-κB inhibitor at their optimum concentration. For this experiment, cells were cultured in serum-free medium before treatment with 1α,25(OH)$_2$D$_3$, or BAY11-7085 or EtOH (control). RNAs were extracted from the cells 12 hour after the treatment, followed by quantitative RT-PCR. As a result, both 1α,25(OH)$_2$D$_3$ and BAY11-7085 proved to cause significant down-regulation of HBp17/FGFBP-1 mRNA expression (Figure 1C). Furthermore, the effect of 1α,25(OH)$_2$D$_3$ was comparable with BAY11-7085 (2μM). This experiment was repeated using cell lines KO (Kono) and A431 and results were similar to those obtained with UE (Figure 1C).

4.2. HBp17/FGFBP-1 expression is down-regulated by 1α,25(OH)$_2$D$_3$ (40nM) in UE cells.

The effect of 1α, 25(OH)$_2$D$_3$ on the mRNA and protein expression of HBp17/FGFBP-1 in OSCC cells (UE) was studied using the optimized concentration of 1α, 25(OH)$_2$D$_3$, i.e., 40nM, at different time points. This experiment represents our first step in the pharmacodynamic study of 1α,25(OH)$_2$D$_3$ targeting HBp17/FGFBP-1 in particular. In this experiment, 3x10^4 cells/well (6-well plate: BD Falcon^{TM}) were seeded in serum-free medium, and further cultured for 3 days. The cells were periodically treated (0, 3, 6, 12 and 24 hours) with 1α,25(OH)$_2$D$_3$. At the end of every time point, the cells were collected for RNA and protein preparation for analysis. As shown by the qRT-PCR analysis (Figure 2A), the HBp17/FGFBP-1 expression was significantly down-regulated with the highest degree of inhibition (over 60%) observed after a 12-hour treatment period. Down-regulation was observed as early as 6 hours and continued for at least 24 hours.

Consequently, this experiment was repeated for the FGF-2. However, the mRNA and protein level of FGF-2 were unaffected by the treatment of 1α,25(OH)$_2$D$_3$ (Figure 2B). These results could be interpreted as either 1α,25(OH)$_2$D$_3$ does not have a direct influence on the FGF-2 synthesis, or the down-regulation of HBp17/FGFBP-1 in cells does not have influence on the intracellular expression of FGF-2.

Apart from that, the VDR expression in the UE cells does not affected by the addition of 1α,25(OH)$_2$D$_3$, although a slight increase of the expression was observed after treatment (Figure 2C).

4.3. HBp17/FGFBP-1 inhibition by 1α,25(OH)$_2$D$_3$ is diminished in siVDR-transfected UE cells.

It has been reported that fibroblast lacking the VDR exhibited an increase in the NF-κB activity [41]. In their study, it appears that cells lacking VDR led to a down-regulation of IκBα causing more p65 to enter the nucleus, making the NF-κB pathway to be more active in synthesizing the subsequent inflammatory cytokines [41]. In order to provide a condition lacking of VDR, we transfected the UE cells with VDR siRNA, allowing us to generate VDR knock-down cells (siVDR) with 80% suppression of the VDR expression (Figure 3A). The mRNA and protein expression of HBp17/FGFBP-1 in siVDR was studied by qRT-PCR [35] and western blotting, and their expression was compared to those of the wild type (siControl) (Figure 3B.). As a result, the HBp17/FGFBP-1 expression was unchanged in the siVDR. These data indicated that VDR does not have a direct effect on the regulation of HBp17/FGFBP-1. On the other hand, the IκBα expression was found to be significantly suppressed by siVDR, as reported previously [41].

1α,25(OH)$_2$D$_3$ is well known to exert its biological effects through regulation of the gene expression mediated by VDR [42]. Therefore, we hypothesized that the effect of 1α,25(OH)$_2$D$_3$ on HBp17/FGFBP-1 is mediated by VDR. In order to speculate on the requirement of VDR in the reaction whereby 1α,25(OH)$_2$D$_3$ inhibits the HBp17/FGFBP-1 expression, the effect of 1α,25(OH)$_2$D$_3$ on cells with the siVDR cells was investigated. siVDR cells and siControl cells were treated with 1α,25(OH)$_2$D$_3$ (40nM) or EtOH for 12 hours, then the HBp17/FGFBP-1 expression was analyzed by western blotting. As a result, a significant suppression of the HBp17/FGFBP-1 expression by 1α,25(OH)$_2$D$_3$ was only observed in the siControl cells, whereas the HBp17/FGFBP-1 expression in siVDR cells did not change (Figure 2B). These

results suggested that the inhibition of HBp17/FGFBP-1 by 1α,25$(OH)_2D_3$ was mediated by VDR.

4.4. HBp17/FGFBP-1 expression is down-regulated by 1α,25$(OH)_2D_3$ (40nM) in the nucleus and cytosol of UE cells.

Our next approach was to observe the distribution of HBp17/FGFBP-1 in UE cells following treatment with 1α,25$(OH)_2D_3$ (40nM) using immunofluorescence staining. The UE cells were cultured in minichambers (LAB-TEK®) and then treated with 1α,25$(OH)_2D_3$ (40nM) or EtOH at various time points before staining with a fluorescent reagent. Images captured by the confocal microscope were analyzed by Image J software and plotted as the corrected total cell fluorescence (CTCF). As a result, at 12 hours, the HBp17/FGFBP-1 expression was reduced in cells treated with 1α,25$(OH)_2D_3$ (Figure 4A). From the image and analysis by the Image J, the down-regulation of HBp17/FGFBP-1 in UE cells was observed in both the nucleus and cytosol region. These data were verified by western blot analysis of the UE cell fraction (Figure 3).

This experiment was extended until time point of 24 hour. As revealed by immunofluorescence image and interpretation by Image J software, HBp17/FGFBP-1 expression was still down-regulated as a whole (Figure 4B). In spite of this, UE cells were observed to start recover HBp17/FGFBP-1 expression in the nucleus. Western blot analysis then verified HBp17/FGFBP-1 recovery in the nucleus of UE cells, while cytosolic HBp17/FGFBP-1 was still down-regulated at this time point (Figure 4B).

4.5. Down-regulation of HBp17/FGFBP-1 by 1α,25(OH)$_2$D$_3$ reduced FGF-2 expression in conditioned medium.

HBp17/FGFBP-1 was first discovered to be associated with FGF-2 in a serum-free culture conditioned by highly tumorigenic A431-AJC human epidermoid carcinoma cells [1]. Following this discovery, HBp17/FGFBP-1 was reported as being significantly expressed in tumor cells as the releasing agent for FGF-2 [1]. Since HBp17/FGFBP-1 works to assist with the FGF-2 release/activation from the extracellular matrix, we hypothesized that the regulation of HBp17/FGFBP-1 by 1α,25(OH)$_2$D$_3$ in the cells may have some effects on the soluble, bio-activated FGF-2 which might work as a stimulant for angiogenesis [19]. To prove our hypothesis, UE cells were cultured and treated with 1α,25(OH)$_2$D$_3$ (40nM) or EtOH for 24, 48 and 72 hours. The amount of soluble HBp17/FGFBP-1 and FGF-2 in the conditioned medium was measured by ELISA. As shown in Figures 5A and 5B, both the secreted HBp17/FGFBP-1 and FGF-2 were significantly reduced by 1α,25(OH)$_2$D$_3$, although the concentration of FGF-2 was traceable in picograms (pg) only after 72 hours (Figure 5B).

Earlier, we examined the expression of FGF-2 mRNA and protein in UE cells (Figure 2B). The result showed no significant difference in the FGF-2 expression despite suppression on its chaperone, HBp17/FGFBP-1. Taken together, these data suggested that 1α,25(OH)$_2$D$_3$ does not have a direct effect on the FGF-2 expression, but rather indirectly, through its chaperone. As reported previously [6], HBp17/FGFBP-1 positively modulates the biological activity of FGF-2. Therefore, these data show the impact of 1α,25(OH)$_2$D$_3$ on the biological role of HBp17/FGFBP-1; i.e. 1α,25(OH)$_2$D$_3$ caused indirect suppression of the bio-activated FGF-2 availability in a cancer microenvironment.

5.0 DISCUSSION

Many provocative studies providing clues that vitamin D may play an important role in cancer therapy have been published in recent years. The study on the anti-neoplastic potential of vitamin D analogs has been reported in several studies discussing its ability to inhibit the replication of cancer cells, induce apoptosis and even inhibit angiogenesis [43]. Although some studies have successfully employed vitamin D analogues as an anti-cancer agent, their studies have often been limited to just one or two cancer types. To date, vitamin D based therapies have been associated in a number of cancers, the frequent were of breast [34], skin [28], colorectal and prostate [13]. Thus far, every evaluated tumor type showed a biochemically distinctive response to vitamin D as well as its anti-cancer capability. Therefore, we presented this study as an attempt to establish the anti-cancer effect of $1\alpha,25(OH)_2D_3$ on OSCC cells, targeting HBp17/FGFBP-1, which is known for its role to release FGF-2 from ECM. Furthermore, this molecule was found to be highly expressed in oral squamous cell carcinomas [9].

The concentration whereby $1\alpha,25(OH)_2D_3$ is safe and comparable to a well-known NF-κB inhibitor (BAY11-7085) was first established. $1\alpha,25(OH)_2D_3$ at the concentration of 40nM was subsequently found to be optimal for cancer cell lines used in this experiment (data not shown).

Our research was based on the known biological role of HBp17/FGFBP-1 as a releasing agent to FGF-2 from its latent state [4]. In this regard, we hypothesized the possible approach to suppress the FGF-2 expression by inhibiting the expression of its carrier, HBp17/FGFBP-1. While FGF-2 is renowned for its role in cancer pathogenesis, $1\alpha,25(OH)_2D_3$, on the other hand, is a relatively new anti-cancer agent of which few fundamental studies have been carried out. To

the best of our knowledge, 1α,25$(OH)_2D_3$ has proven to exhibit an anti-cancer effect on all cancer models used. Following that, many pathways has been identified in the involved reactions including its highly regarded role as an NF-κB inhibitor [44]. Based on the considerable amount of evidence for the anti-cancer ability of 1α,25$(OH)_2D_3$, we decided to investigate the ability of 1α,25$(OH)_2D_3$ to regulate the expression of HBp17/FGFBP-1 and eventually FGF-2 in the OSCC cell line (UE). In addition, this study would show the efficacy of 1α,25$(OH)_2D_3$ to be used as an anti-cancer therapy in OSCC management.

In all of the experiments, the cells were cultured in a defined serum-free media [39, 40], therefore, other possible factors that might affect the experiments were eliminated. Soon after the optimum dose of 1α,25$(OH)_2D_3$ was determined, an assay to determine the effect of 1α,25$(OH)_2D_3$ on HBp17/FGFBP-1 was performed. As a result, the HBp17/FGFBP-1 expression in OSCC cell lines was significantly suppressed by 1α,25$(OH)_2D_3$ (Figure 1). To understand this result, we then referred to the deduced sequence of HBp17/FGFBP-1.

From the deduced promoter sequence of HBp17/FGFBP-1 [45], there are a few binding sites which can modulate the transcription of HBp17/FGFBP-1 when activated. One of them is the NF-κB binding site which is located at the upstream nucleotide of 185 to 176. However, this binding site had never been functionally proven. Recently, 1α,25$(OH)_2D_3$ was reported to decrease the DNA binding of NF-κB by increasing the IκBα level [35, 42, 44]. Thus, 1α,25$(OH)_2D_3$ was then used to confirm the hypothesis that the HBp17/FGFBP-1 transcription could be modulated through this binding site, hence, we speculated that the down-regulation of HBp17/FGFBP-1 is occurring via the NF-κB pathway.

To verify that $1\alpha,25(OH)_2D_3$ had possibly inhibited NF-κB activity, which then resulted in the down-regulation of the HBp17/FGFBP-1 expression, we utilized a well-known NF-κB inhibitor; i.e. Bay 11-7085 (BAY), as the positive control of the NF-κB inhibitor. BAY, a soluble NF-κB inhibitor, which could cause necrotic cell death [46], has never been reported to suppress the HBp17/FGFBP-1 expression. Interestingly, in our study we showed that both BAY (2μM) and $1\alpha,25(OH)_2D_3$ (40nM) down-regulate the HBp17/FGFBP-1 expression in various OSCC cell lines (Figure 1). In the end, we concluded that the suppression of HBp17/FGFBP-1 in cells by $1\alpha,25(OH)_2D_3$ occurred via the NF-κB pathway [35].

Our next hypothesis was that $1\alpha,25(OH)_2D_3$ enhanced the HBp17/FGFBP-1 inhibition in OSCC cells through the Vitamin D Receptor (VDR). This was basically due to the previously reported mechanism of action of the $1\alpha,25(OH)_2D_3$-induced transcription which is usually mediated by VDR [47]. To test the dependency of $1\alpha,25(OH)_2D_3$ on the VDR to regulate the subsequent gene expression, we examined the HBp17/FGFBP-1 expression by $1\alpha,25(OH)_2D_3$ in siVDR-transfected cells. As a result (Figure 3B), the down-regulation of HBp17/FGFBP-1 by $1\alpha,25(OH)_2D_3$ was diminished in the siVDR-transfected cells. These results suggested that the action exerted by $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1 expression was partially (if not certainly) mediated by the VDR. However, these data presented an inconsistency with the HBp17/FGFBP-1 mRNA regulation by $1\alpha,25(OH)_2D_3$ reported earlier [35], whereby the HBp17/FGFBP-1 mRNA expression in the siVDR-transfected cells was still down-regulated to a lower degree despite the treatment. The reason for this dispute could lie in the post-transcriptional mechanisms of HBp17/FGFBP-1. Nonetheless, we concluded that the down-regulation of HBp17/FGFBP-1 by $1\alpha,25(OH)_2D_3$ is a VDR-mediated event.

Furthermore, we found no difference in expression of HBp17/FGFBP-1 between siControl and siVDR-transfected cells. This was in contrast to the IκBα expression reported earlier, whereby we observed a significant suppression of IκBα in the siVDR transfected cells [35]. These data suggest that VDR has its own anti-cancer capability towards NF-κB activity, but not towards the HBp17/FGFBP-1 expression.

In squamous cell carcinomas, HBp17/FGFBP-1 has been shown to co-localize with heparan sulfate proteoglycans 2 (HSPG2) in the pericellular environment [48, 49]. As revealed by immunofluorescence staining, HBp17/FGFBP-1 was expressed in both the nucleus and cytosol region of UE cells. The treatment with $1\alpha,25(OH)_2D_3$, reduced the HBp17/FGFBP-1 expression in both regions at 12 hours (Figure 4A). At 24 hour, UE retained its nuclear HBp17/FGFBP-1 while cytosolic HBp17/FGFBP-1 was still down-regulated (Figure 4B). The reduction of the HBp17/FGFBP-1 expression in UE cells suggesting down-regulation of HBp17/FGFBP-1 mRNA expression is demonstrated in Figure 1 and 2A. Since HBp17/FGFBP-1 is known to be relevant when secreted, its roles in the intracellular space, especially in nucleus are still to be determined.

HBp17/FGFBP-1 becomes significant when it is excreted into the surrounding extracellular matrix (ECM). From tumor cells, where it was found to be unusually abundant, HBp17/FGFBP-1 is secreted and bind to FGF-2 immobilized in the ECM of the same or other cells [2]. This was followed by FGF-2 being released and bio-activated to reach its target cell receptor and exert its effects mostly in angiogenesis. Therefore, it is also important to ensure the ability of $1\alpha,25(OH)_2D_3$ to suppress the HBp17/FGFBP-1 expression which has an impact on the bio-activation of FGF-2. For that, we measured the concentration of these two molecules in the medium conditioned by $1\alpha,25(OH)_2D_3$-treated UE cells. From the ELISA result (Figure 5A), we

found a prolonged decrease in the HBp17/FGFBP-1 expression measured up to 72 hours of treatment. Interestingly, the FGF-2 protein expression was correspondingly down-regulated, although the amount was in picograms and only traceable after 72 hours (Figure 5B). These results signify the efficacy of $1\alpha,25(OH)_2D_3$ to suppress the biological role of HBp17/FGFBP-1 altogether, although an *in vivo* study is required to provide more persuasive evidence. Furthermore, our data also implied that $1\alpha,25(OH)_2D_3$ does not have a direct effect on the synthesis of FGF-2 in the cells based on the quantitative RT-PCR result. These data were verified by western blotting (Figure 2B).

This study serves as the early approach to the establishment of $1\alpha,25(OH)_2D_3$ for OSCC therapy. In this present study, we reported the role of $1\alpha,25(OH)_2D_3$ to modulate the HBp17/FGFBP-1 expression together with its acting molecule; FGF-2. In our previous report [35], we proposed the possibility that these events occurred through the NF-κB pathway. Therefore, a more comprehensive study is needed to focus on DNA binding activity of NF-κB molecules on the deduced binding site located in the HBp17/FGFBP-1 promoter region. This is pharmacologically important for establishment of $1\alpha,25(OH)_2D_3$ to be used as anti-cancer therapy specifically targeting HBp17/FGFBP-1 and FGF-2. Besides that, a careful determination of the maximum tolerated dose of $1\alpha,25(OH)_2D_3$ and optimal biological dose has to be followed *in-vivo*.

Finally, this study has successfully demonstrated the ability of $1\alpha,25(OH)_2D_3$ to down-regulate the HBp17/FGFBP-1 expression in oral squamous carcinoma cell lines. The down-regulation has significantly reduced the bio-active FGF-2 released into the conditioned medium. Therefore, $1\alpha,25(OH)_2D_3$ could possibly inhibit the FGF-2 dependent tumorigenesis and

angiogenesis. In conclusion, this study has provided a novel capacity of $1\alpha,25(OH)_2D_3$ as an anti-cancer agent in oral cancer management.

6.0 REFERENCES

1. Wu, D.Q., M.K., Kan, G.H., Sato, T. Okamoto, J.D. Sato, Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. J Biol Chem, 1991. 266(25): 16778-16685.
2. Abuharbeid, S., F. Czubayko, and A. Aigner, The fibroblast growth factor-binding protein FGF-BP. Int J Biochem Cell Biol, 2006. 38(9): 1463-1468.
3. Czubayko, F., E.D. Liaudet-Coopman, A. Aigner, A.T. Tuveson, G.J. Berchem, A. Wellstein, A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. Nat Med, 1997. 3(10): 1137-1140.
4. Czubayko, F., R.V. Smith, H.C. Chung, A. Wellstein, Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. J Biol Chem, 1994. 269(45): 28243-28248.
5. Kurtz, A., H.L. Wang, N. Darwiche, V. Harris, A. Wellstein, Expression of a binding protein for FGF is associated with epithelial development and skin carcinogenesis. Oncogene, 1997. 14(22): 2671-2681.
6. Tassi, E., A. Al-Attar, A. Aigner, M.R. Swift, K. McDonnell, A. Karavanov, A. Wellstein, Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein. J Biol Chem, 2001. 276(43): 40247-40253.
7. Takeuchi, A., N. Yoshizawa, M. Yamamoto, Y. Sawasaki, T. Oda, A. Senoo, H. Niwa, Y. Fuse, Basic fibroblast growth factor promotes proliferation of rat glomerular visceral epithelial cells in vitro. Am J Pathol, 1992. 141(1): 107-116.

8. Tassi, E., K. McDonnell, K.A. Gibby, J.U. Tilan, S.E. Kim, D.P. Kodack, M.D. Schmidt, G.M. Sharif, C.S. Wilcox, W.J. Welch, G.I. Gallicano, M.D. Johnson, A.T. Riegel, A. Wellstein, Impact of fibroblast growth factor-binding protein-1 expression on angiogenesis and wound healing. Am J Pathol. 2011. 179(5): 2220-2232.

9. Okamoto, T., Y. Tanaka, M. Kan, A. Sakamoto, K. Takada, J.D. Sato, Expression of fibroblast growth factor binding protein HBp17 in normal and tumor cells. In Vitro Cell Dev Biol Anim, 1996. 32(2): 69-71.

10. Begum, S., Y. Zhang, T. Shintani, S. Toratani, J.D. Sato, T. Okamoto, Immunohistochemical expression of heparin-binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis. Oncol Rep, 2007. 17(3): 591-596.

11. Kurtz, A., A. Aigner, R.H. Cabal-Manzano, R.E. Butler, D.R. Hood, R.B. Sessions, F. Czubayko, Differential regulation of a fibroblast growth factor-binding protein during skin carcinogenesis and wound healing. Neoplasia, 2004. 6(5): 595-602.

12. Ray, R., R. Cabal-Manzano, A.R. Moser, T. Waldman, L.M. Zipper, A. Aigner, S.W. Byers, A.T. Riegel, A. Wellstein, Up-regulation of fibroblast growth factor-binding protein, by beta-catenin during colon carcinogenesis. Cancer Res, 2003. 63(23): 8085-8089.

13. Tassi, E., R.T. Henke, E.T. Bowden, M.R. Swift, D.P. Kodack, A.H. Kuo, A. Maitra, A. Wellstein, Expression of a fibroblast growth factor-binding protein during the development of adenocarcinoma of the pancreas and colon. Cancer Res, 2006. 66(2): 1191-1198.

14. Tassi, E. and A. Wellstein, The angiogenic switch molecule, secreted FGF-binding protein, an indicator of early stages of pancreatic and colorectal adenocarcinoma. Semin Oncol, 2006. 33(6 Suppl 11): S50-56.

15. Burgess, W.H. and T. Maciag, The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem, 1989. 58: 575-606.

16. Ornitz, D.M. and N. Itoh, Fibroblast growth factors, Genome Biology. (2001). 2(3):. reviews3005.1-3005.12.

17. Powers, C.J., S.W. McLeskey, and A. Wellstein, Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer, 2000. 7(3): 165-197.

18. Moscatelli, D., Basic fibroblast growth factor (bFGF) dissociates rapidly from heparan sulfates but slowly from receptors. Implications for mechanisms of bFGF release from pericellular matrix. J Biol Chem, 1992. 267(36): 25803-25809.

19. Nugent, M.A. and R.V. Iozzo, Fibroblast growth factor-2. Int J Biochem Cell Biol, 2000. 32(2): 115-120.

20. Takahashi, J.A., M. Fukumoto, K. Igarashi, Y. Oda, H. Kikuchi, M. Hatanaka, Correlation of basic fibroblast growth factor expression levels with the degree of malignancy and vascularity in human gliomas. J Neurosurg, 1992. 76(5): 792-798.

21. Yamanaka, Y., H. Friess, M. Buchler, H.G. Beger, E. Uchida, M. Onda, M.S. Kobrin, M. Korc, Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. Cancer Res, 1993. 53(21): 5289-5296.

22. Eguchi, J., K. Nomata, S. Kanda, T. Igawa, M. Taide, S. Koga, F. Matsuya, H. Katenaka, Y. Saito, Gene expression and immunohistochemical localization of basic fibroblast

growth factor in renal cell carcinoma. Biochem Biophys Res Commun, 1992. 183(3): 937-944.

23. Yiangou, C., J.J. Gomm, R.C. Coope, M. Law, Y.A. Luqmani, S. Shousha, R.C. Coombes, C.L. Johnston, Fibroblast growth factor 2 in breast cancer: occurrence and prognostic significance. Br J Cancer, 1997. 75(1): 28-33.

24. Gorugantula, L.M., T. Rees, J. Plemons, H.S. Chen, Y.S. Cheng, Salivary basic fibroblast growth factor in patients with oral squamous cell carcinoma or oral lichen planus. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012. 114(2): 215-222.

25. Czubayko, F., A.T. Riegel, and A. Wellstein, Ribozyme-targeting elucidates a direct role of pleiotrophin in tumor growth. J Biol Chem, 1994. 269(33): 21358-21363.

26. Liaudet-Coopman, E.D. and A. Wellstein, Regulation of gene expression of a binding protein for fibroblast growth factors by retinoic acid. J Biol Chem, 1996. 271(35): 21303-21308.

27. Holick, M.F., Vitamin D and bone health. J Nutr, 1996. 126(4 Suppl): 1159S-1164S.

28. Bikle, D.D., H. Elalieh, J. Welsh, D. Oh, J. Cleaver, A. Teichert, Protective role of vitamin D signaling in skin cancer formation. J Steroid Biochem Mol Biol. 2012. 136: 271-279.

29. Liu, M., M.H. Lee, M. Cohen, M. Bommakanti, L.P. Freedman, Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev, 1996. 10(2): 142-153.

30. Plum, L.A. and H.F. DeLuca, Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov. 2010. 9(12): p. 941-955.

31. Jiang, F., J. Bao, P. Li, S.V. Nicosia, W. Bai, Induction of ovarian cancer cell apoptosis by 1,25-dihydroxyvitamin D3 through the down-regulation of telomerase. J Biol Chem, 2004. 279(51): 53213-53221.

32. Gilmore, T.D., The Rel/NF-kappaB signal transduction pathway: introduction. Oncogene, 1999. 18(49): 6842-6844.

33. Erben, R.G., D.W. Soegiarto, K. Weber, U. Zeitz, M. Lieberherr, R. Gniadecki, G. Moller, J. Adamski, R. Balling, Deletion of deoxyribonucleic acid binding domain of the vitamin D receptor abrogates genomic and nongenomic functions of vitamin D. Mol Endocrinol, 2002. 16(7): 1524-1537.

34. Krishnan, A.V., S. Swami, and D. Feldman, The potential therapeutic benefits of vitamin D in the treatment of estrogen receptor positive breast cancer. Steroids. 2012. 77(11): 1107-1112.

35. Rosli, S.N., T. Shintani, Y. Hayashido, S. Toratani, E. Usui, T. Okamoto, 1alpha,25$(OH)_2D_3$ down-regulates HBp17/FGFBP-1 expression via NF-kappaB pathway. J Steroid Biochem Mol Biol. 2012. 136: 98-101.

36. Miyauchi, S., T. Morayama, S. Kyoizumi, J. Asakawa, T. Okamoto, K. Takada, Malignant tumor cell lines produce interleukin-1-like factor. In Vitro Cell Dev Biol, 1988. 24(8): 753-758.

37. Michimukai, E., N. Kitamura, Y. Zhang, H. Wang, Y. Hiraishi, K. Sumi, Y. Hayashido, S. Toratani, T. Okamoto, Mutations in the human homologue of the Drosophila segment polarity gene patched in oral squamous cell carcinoma cell lines. In Vitro Cell Dev Biol Anim, 2001. 37(7): 459-464.

38. Fabricant, R.N., J.E. De Larco, and G.J. Todaro, Nerve growth factor receptors on human melanoma cells in culture. Proc. Natl. Acad. Sci. U S A, 1977. 74(2): 565-9.

39. Okamoto, T., R. Tani, M. Yabumoto, A. Sakamoto, K. Takada, G.H. Sato, J.D. Sato, Effects of insulin and transferrin on the generation of lymphokine-activated killer cells in serum-free medium. J Immunol Methods, 1996. 195(1-2): 7-14.

40. Sato, J.D., T. Kawamoto, and T. Okamoto, Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth in vitro. J Exp Med, 1987. 165(6): 1761-1166.

41. Sun, J., J. Kong, Y. Duan, F.L. Szeto, A. Liao, J.L. Madara, Y.C. Li, Increased NF-kappaB activity in fibroblasts lacking the vitamin D receptor. Am J Physiol Endocrinol Metab, 2006. 291(2): E315-EP322.

42. Cohen-Lahav, M., S. Shany, D. Tobvin, C. Chaimovitz, A. Douvdevani, Vitamin D decreases NFkappaB activity by increasing IkappaBalpha levels. Nephrol Dial Transplant, 2006. 21(4): 889-897.

43. Merke, J., P. Milde, S. Lewicka, U. Hugel, G. Klaus, D.J. Mangelsdorf, M.R. Haussler, E.W. Rauterberg, E. Ritz, Identification and regulation of 1,25-dihydroxyvitamin D3 receptor activity and biosynthesis of 1,25-dihydroxyvitamin D3. Studies in cultured bovine aortic endothelial cells and human dermal capillaries. J Clin Invest, 1989. 83(6): 1903-1915.

44. Harant, H., B. Wolff, and I.J. Lindley, 1Alpha,25-dihydroxyvitamin D3 decreases DNA binding of nuclear factor-kappaB in human fibroblasts. FEBS Lett, 1998. 436(3): 329-334.

45. Harris, V.K., E.D. Liaudet-Coopman, B.J. Boyle, A. Wellstein, A.T. Riegel, Phorbol ester-induced transcription of a fibroblast growth factor-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. J Biol Chem, 1998. 273(30): 19130-19139.

46. Berger, N., H. Ben Bassat, B.Y. Klein, R. Laskov, Cytotoxicity of NF-kappaB inhibitors Bay 11-7085 and caffeic acid phenethyl ester to Ramos and other human B-lymphoma cell lines. Exp Hematol, 2007. 35(10): 1495-1509.

47. Kato, S., Molecular mechanism of transcriptional control by nuclear vitamin receptors. Br J Nutr, 2000. 84 Suppl 2: S229-233.

48. Beer, H.D., M. Bittner, G. Niklaus, C. Munding, N. Max, A. Goppelt, S. Werner, The fibroblast growth factor binding protein is a novel interaction partner of FGF-7, FGF-10 and FGF-22 and regulates FGF activity: implications for epithelial repair. Oncogene, 2005. 24(34): 5269-5277.

49. Mongiat, M., J. Otto, R. Oldershaw, F. Ferrer, J.D. Sato, R.V. Iozzo, Fibroblast growth factor-binding protein is a novel partner for perlecan protein core. J Biol Chem, 2001. 276(13): 10263-10271.

Legends and Figures

Figure 1. HBp17/FGFBP-1 expression is significantly down-regulated by 1α,25$(OH)_2D_3$ (40nM) and BAY-117085 (2μM) in various oral cancer cell lines; UE, KO and A431. (A) Growth-dose relationship curve of UE and A431 cells. (B) Growth curve for (i) UE and (ii) A431. (C) HBp17/FGFBP-1 expression in UE, KO and A431 in response to 1α,25$(OH)_2D_3$ and BAY. To determine the effect of 1α,25$(OH)_2D_3$ (40nM) and BAY-117085 (2μM) on the expression of HBp17/FGFBP-1, the cells were treated for 12 hours. Then, relative mRNA expression of HBp17/FGFBP-1 between ethanol-treated cells (control), 1α,25$(OH)_2D_3$-treated cells and BAY-treated cells were determined by quantitative RT-PCR. Each bar represents the mean $\pm$S.D., *p<0.05, n=3.

Figure 2. Down-regulation of HBp17/FGFBP-1 by 1α,25$(OH)_2D_3$ does not affect intracellular expression of FGF-2. A time dependent study on the effect of 1α,25$(OH)_2D_3$ (40nM) on the expression of (A) HBp17/FGFBP-1, (B) FGF-2, and (C) VDR in UE cells. (A) UE cells were cultured in serum-free medium with 40nM 1α,25$(OH)_2D_3$ for 0, 3, 6, 12, and 24 hours, then harvested for RNA and cell lysates. The mRNA and protein expression levels of each molecule at different time points were evaluated by quantitative RT-PCR and western blotting. Only the 12-hour data are shown in (B) FGF-2 and (C) VDR. Each bar represents the mean $\pm$S.D., *p<0.05, n.s.=not significant, n=5.

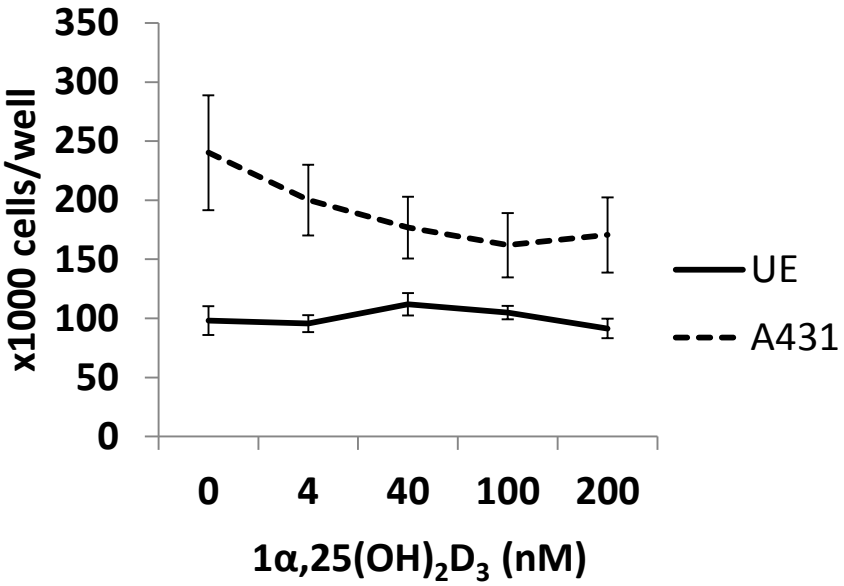
Figure 3. Inhibition of the HBp17/FGFBP-1 expression by 1α,25$(OH)_2D_3$ is mediated by VDR. To determine the significance of VDR on the regulation of HBp17/FGFBP-1 by 1α,25$(OH)_2D_3$, we constructed a siVDR-transfected UE cells (siVDR). (A) 80% suppression on VDR protein expression in siVDR. (B & C) Down regulation of HBp17/FGFBP-1 (B) and enhancement of IκBα (C) by 1α,25$(OH)_2D_3$ diminished in siVDR. The effect of 1α,25$(OH)_2D_3$ (40nM) on HBp17/FGFBP-1 expression in siVDR and siControl were subsequently examined. As a result, 1α,25$(OH)_2D_3$ failed to bring about inhibition towards HBp17/FGFBP-1 and enhancement towards IκBα protein expression in siVDR. No difference in the expression of HBp17/FGFBP-1 between siControl and siVDR in the absence of 1α,25$(OH)_2D_3$. (C) Each bar represents the mean $\pm$S.D., *p<0.05, n.s.=not significant, n=5.

Figure 4. HBp17/FGFBP-1 is expressed in the nucleus and cytosol of UE cells and down-regulated by 1α,25$(OH)_2D_3$. Immunofluorescence staining was performed to observe HBp17/FGFBP-1 expression in intracellular space of UE cells. Bar graphs represent the relative corrected total cell fluorescences (CTCF) for different sites. To validate data on immunofluorescence, HBp17/FGFBP-1 in fractionated UE cells was analyzed by western blotting. (A) HBp17/FGFBP-1 expression in UE cells after 1α,25$(OH)_2D_3$ treatment for 12 hours. The expression of HBp17/FGFBP-1 in both sites reduced by 1α,25$(OH)_2D_3$. (B) The HBp17/FGFBP-1 expression in UE cells after 24 hours exposure to 1α,25$(OH)_2D_3$. The HBp17/FGFBP-1 expression as a whole was still down-regulated although nuclear HBp17/FGFBP-1 appears to retain expression at this time. Each bar represents the mean $\pm$S.D., *p<0.05, n.s.= not significant, n=5.

Figure 5. The HBp17/FGFBP-1 and FGF-2 expression in conditioned media are reduced by $1\alpha,25(OH)_2D_3$. HBp17/FGFBP-1 and FGF-2 are released into surrounding extracellular matrix measured in conditioned media by ELISA. (A) The secreted HBp17/FGFBP-1 level has been reduced for at least 72-hour treatment with $1\alpha,25(OH)_2D_3$. (B) The reduced expression of soluble (biologically active) FGF-2 appears in measurable amounts only after 72 hours of treatment. Each bar represents the mean $\pm$S.D., *$p<0.05$, n.d.= not detected, n=3.

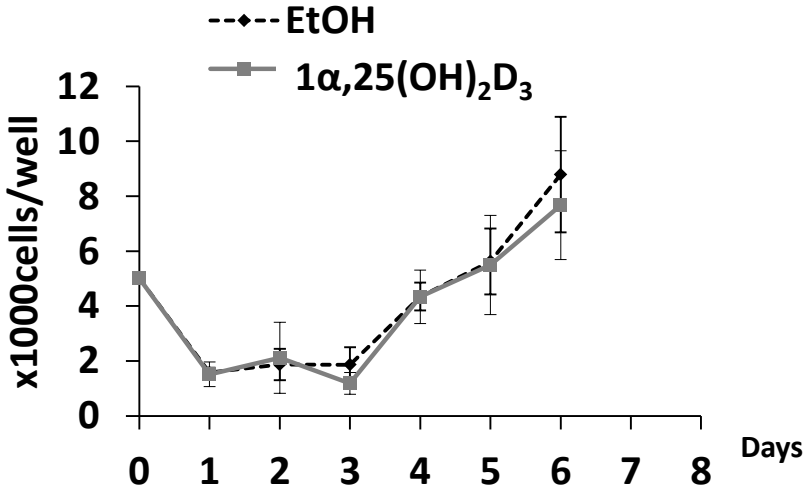
FIGURE 1.

(A)

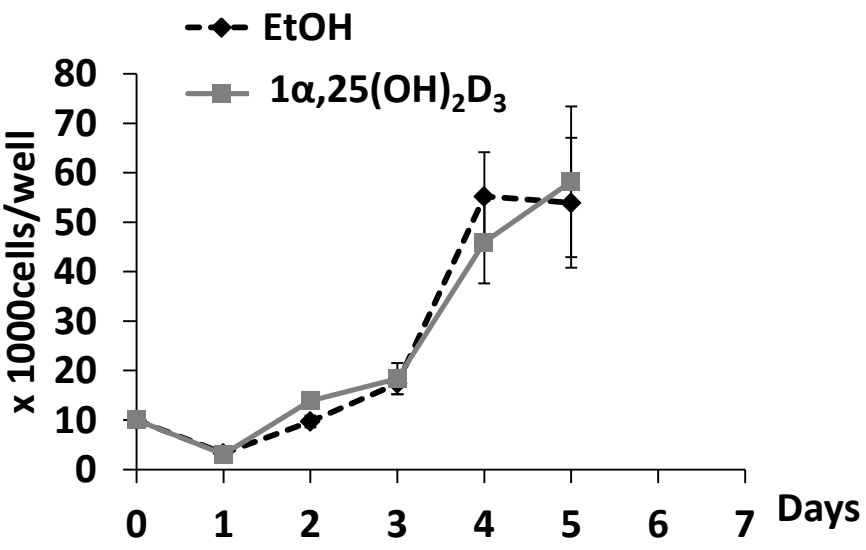


(B)

(i) UE



(ii) A431


EtOH
1α,25(OH)2D3
x 1000cells/well
80
70
60
50
40
30
20
10
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1
2
3
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7
Days

C)

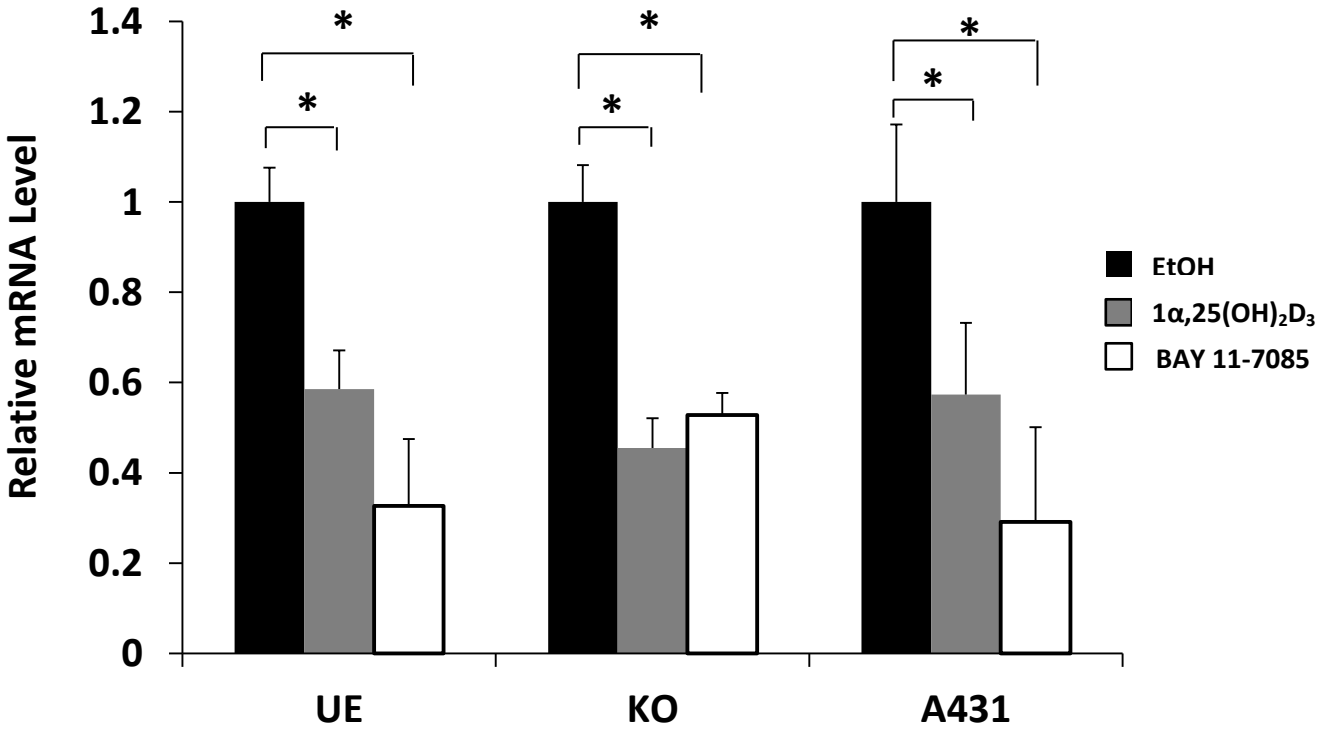
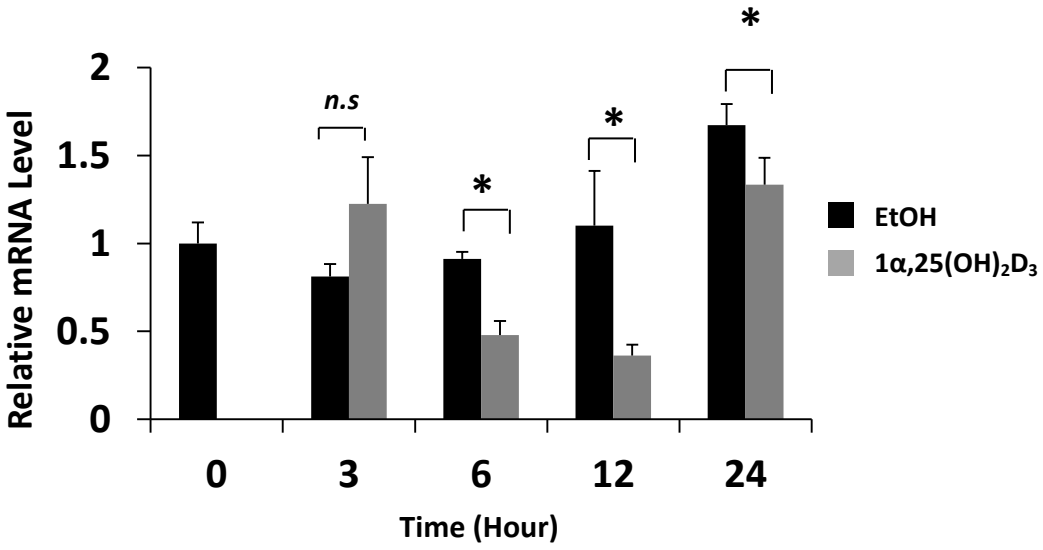

Relative mRNA Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
*
*
UE
KO
A431
EtOH
1α,25(OH)2D3
BAY 11-7085

FIGURE 2.

(A) HBp17/FGFBP-1

(Quantitative RT-PCR)



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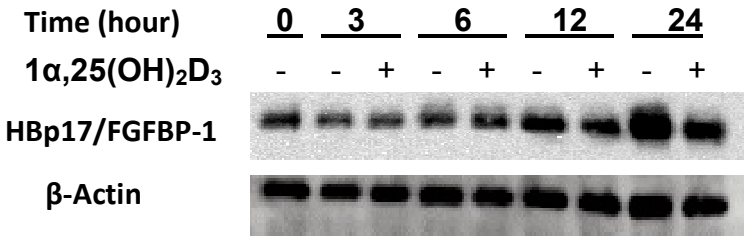
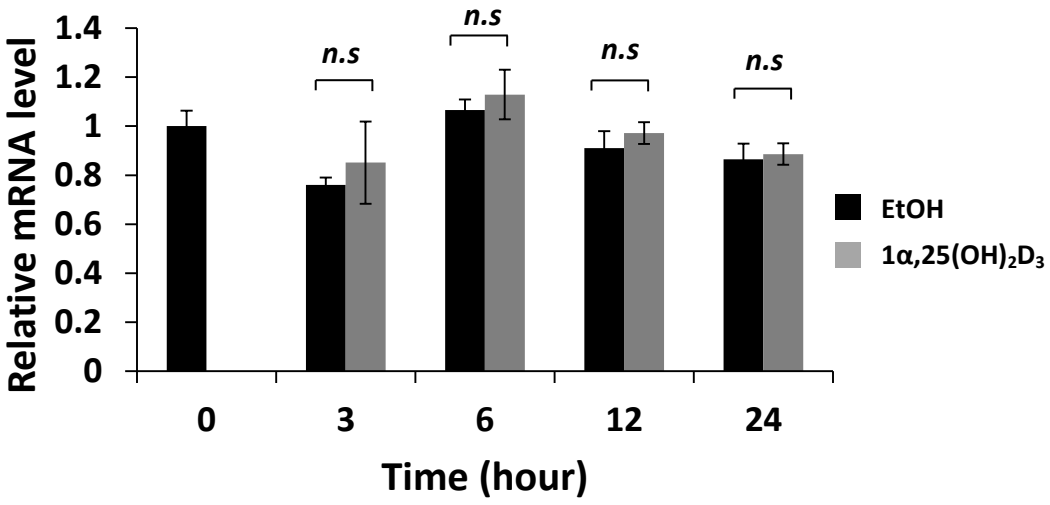


FIGURE 2.

(B) FGF-2

(Quantitative RT-PCR)



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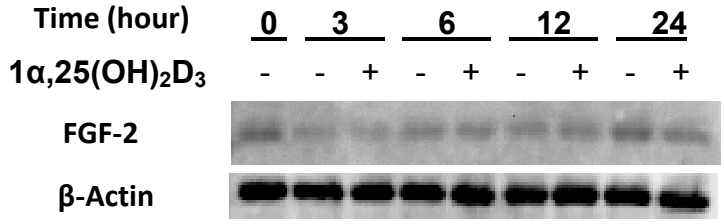
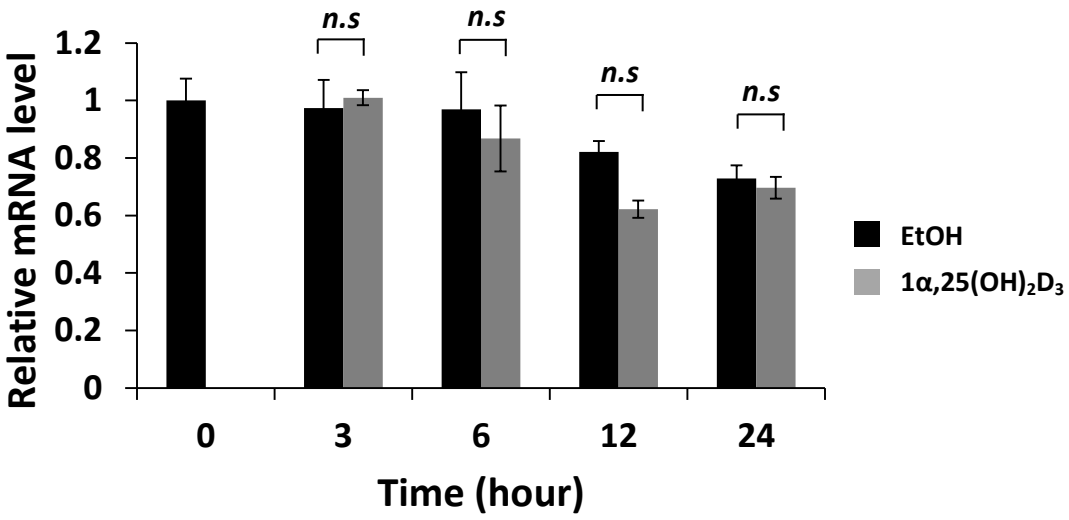


FIGURE 2.

(C) VDR

(Quantitative RT-PCR)



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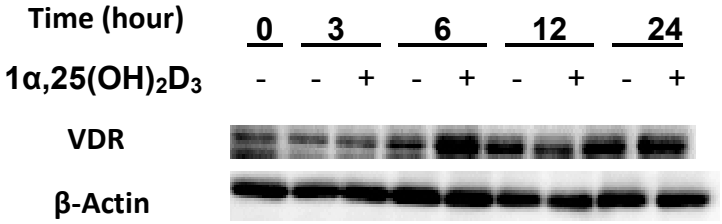
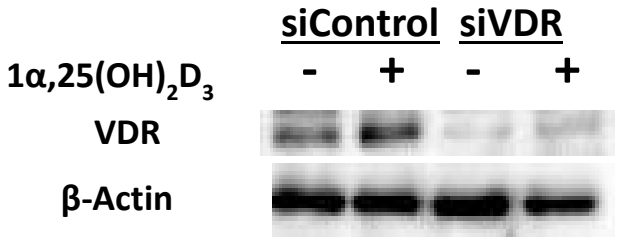


FIGURE 3.

(A) VDR



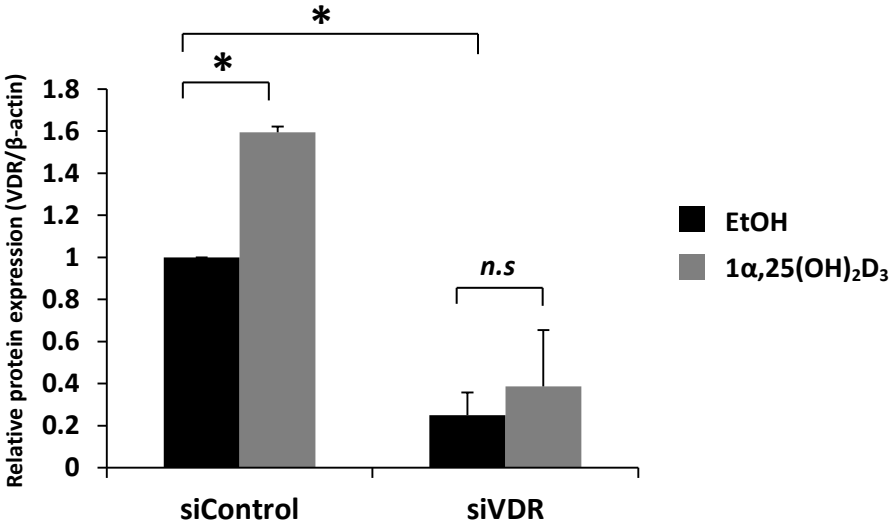
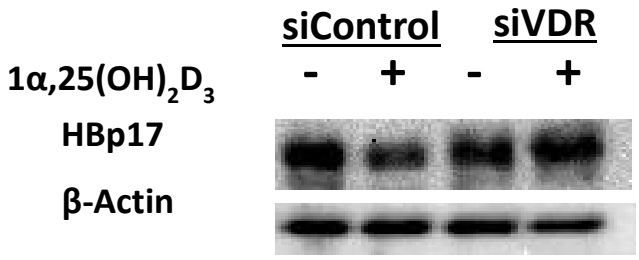


FIGURE 3.

(B) HBp17/FGFBP-1

(Western Blotting)



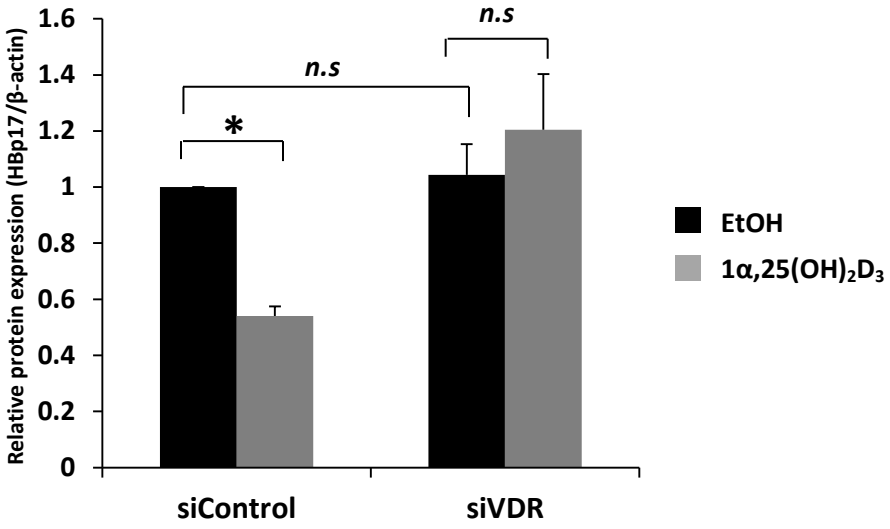


FIGURE 3.

(C) IκBα

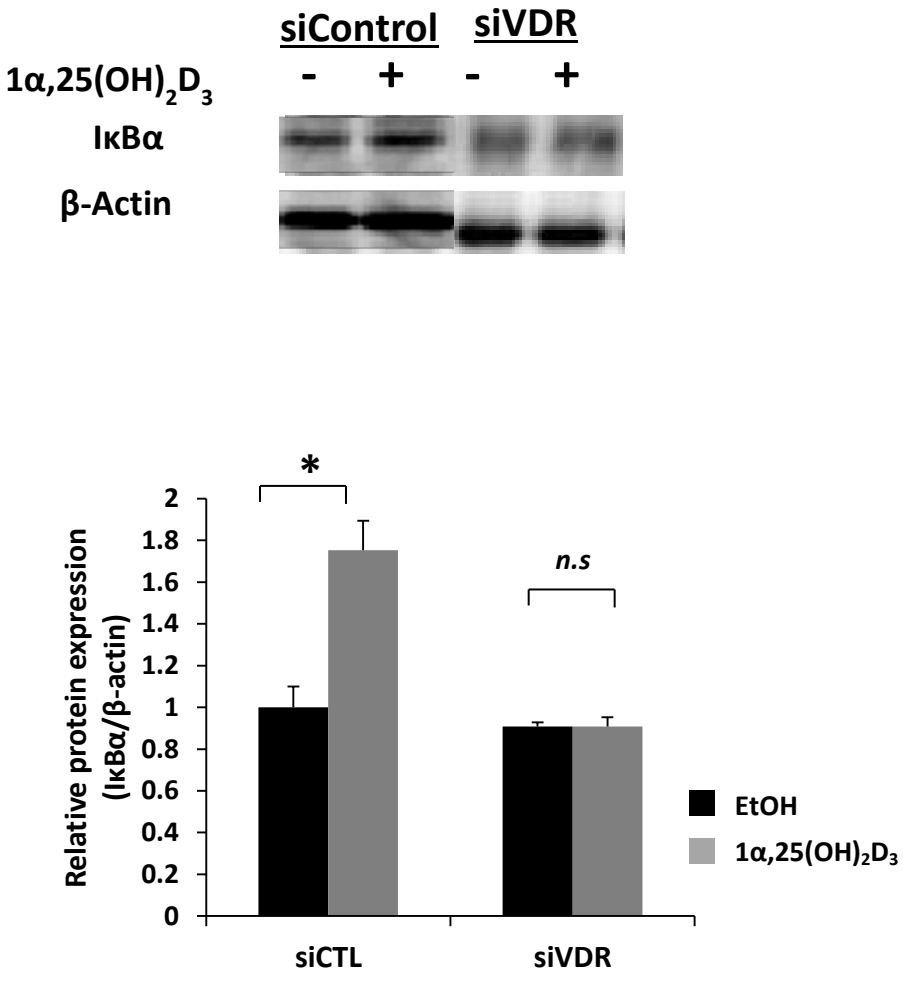
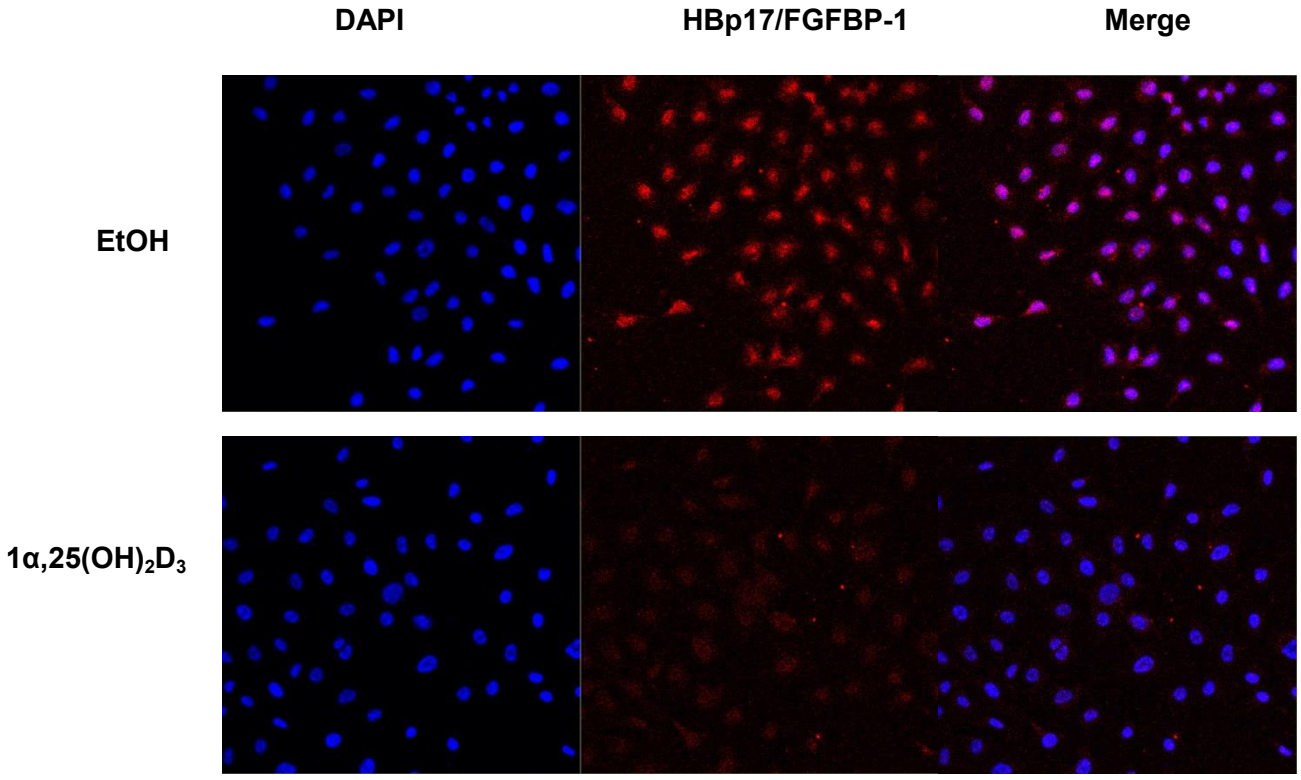
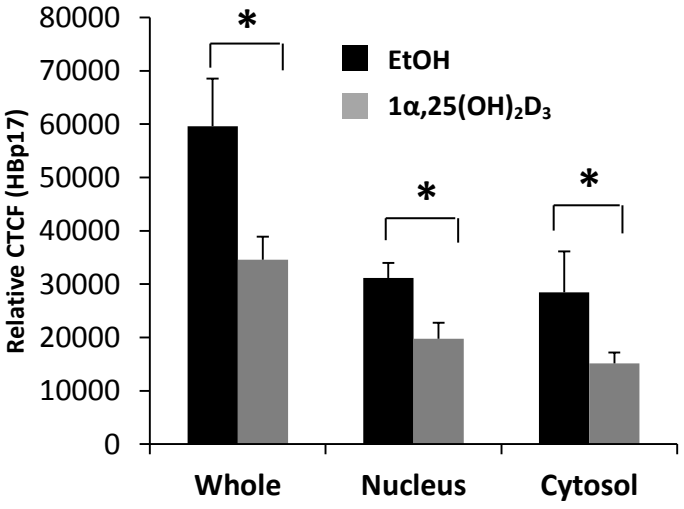


FIGURE 4.

(A) 12 Hour

(Immunofluorescence)





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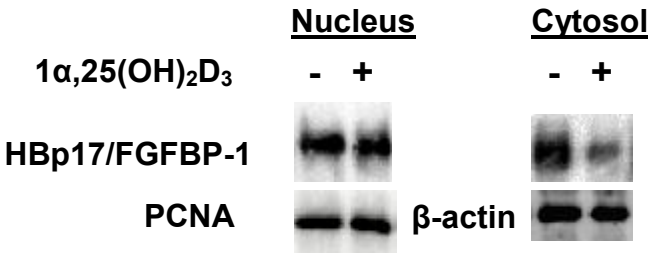


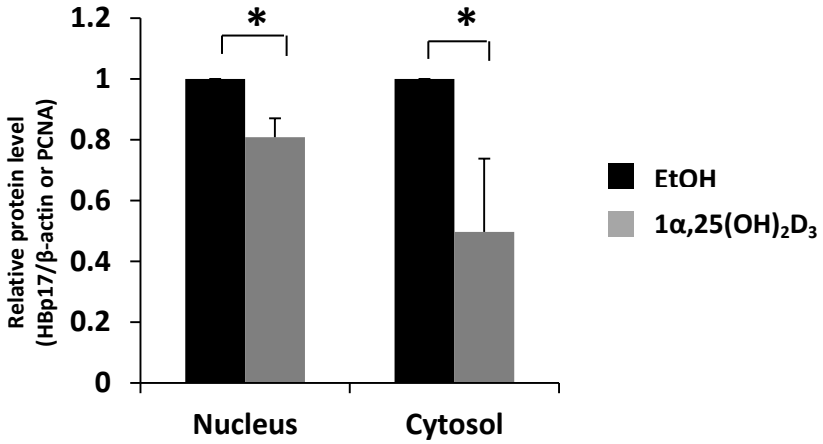
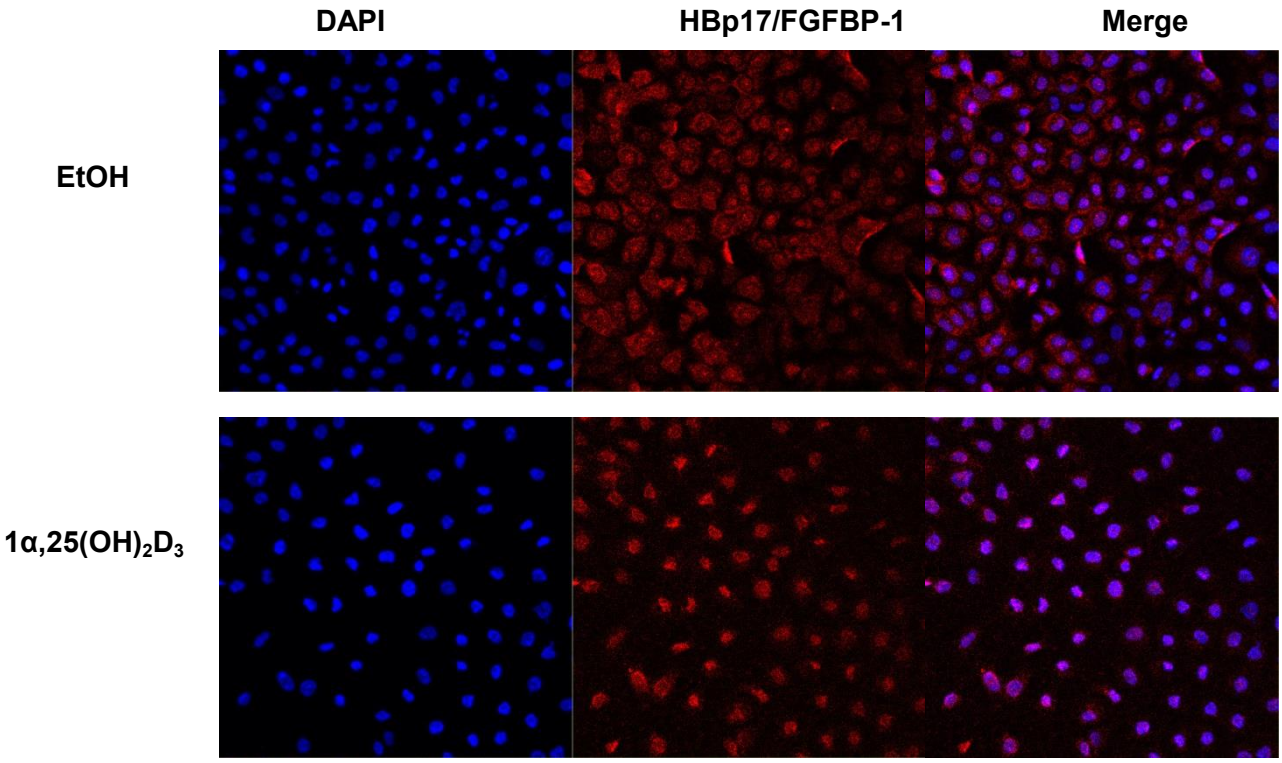
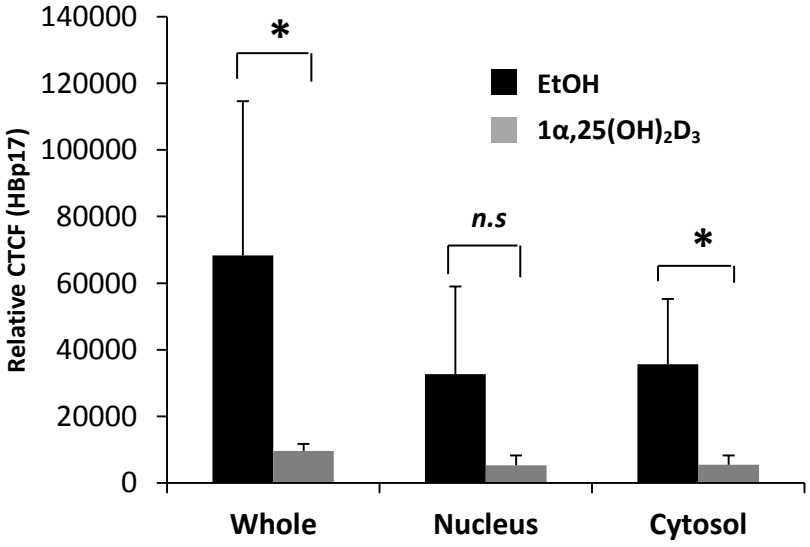


FIGURE 4.

(B) 24 Hour

(Immunofluorescence)





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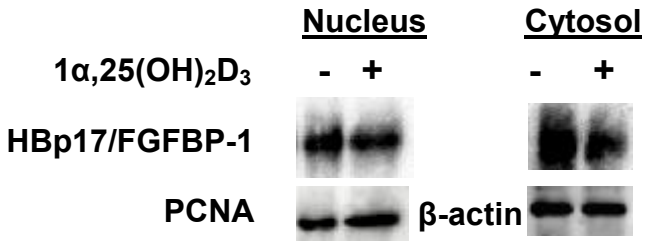


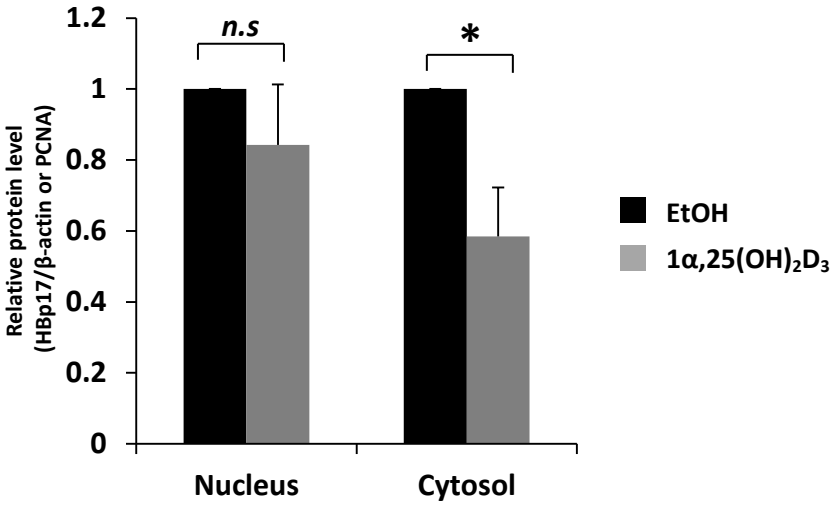
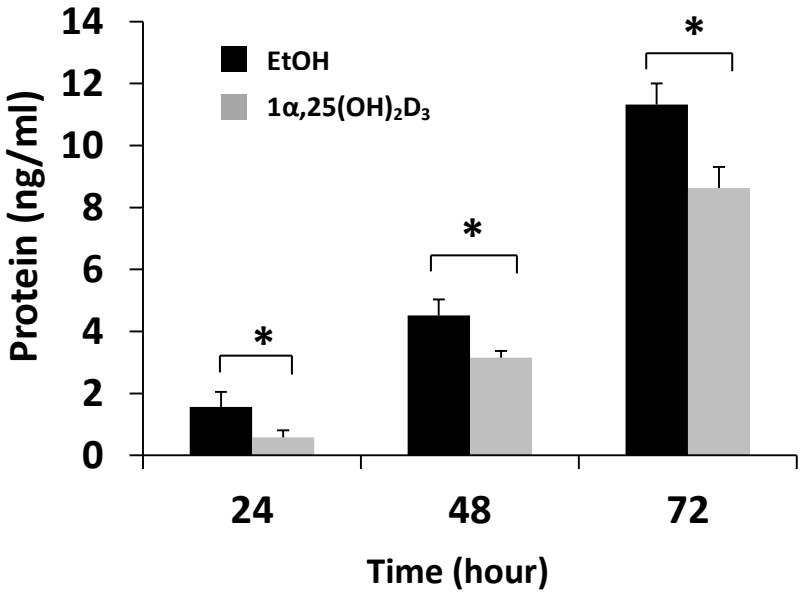
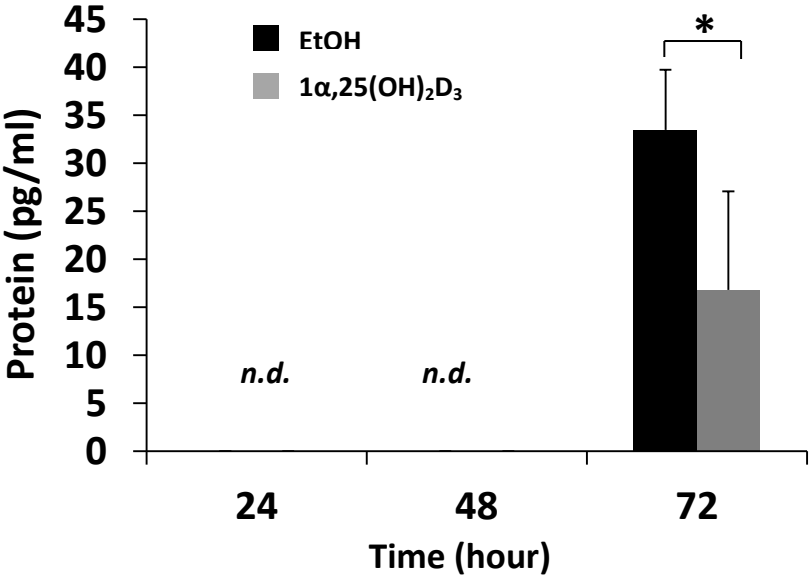


FIGURE 5.

(A) HBp17/FGFBP-1



(B) FGF-2



Chapter II

$1\alpha,25(OH)_2D_3$ Down-regulates the Heparin-Binding Protein 17/Fibroblast Growth Factor-Binding Protein-1 (HBp17/FGFBP-1) Expression through NF-κB Pathway

1.0 ABSTRACT

Heparin-binding protein 17 /fibroblast growth factor-binding protein (HBp17/FGFBP-1, GenBank accession no.NP-005121) has been reported to enhance angiogenesis as well as promotes tumor growth *in vivo*. Furthermore, this molecule was found to be highly expressed in the tissue and cell lines of oral squamous cell carcinoma (OSCC). $1\alpha,25(OH)_2D_3$ is used to study its potential to inhibit the expression of HBp17/FGFBP-1 in cancer cells. Consequently, we found that HBp17/FGFBP-1 mRNA and protein levels were significantly down-regulated. In this present study, we show that this event takes place via the NF-κB pathway since mRNA and protein levels of this pathway regulator, IκBα, were found to be significantly up-regulated. Furthermore, promoter activity of HBp17/FGFBP-1 (region between -217 and +61) measured by a luciferase reporter assay was down-regulated following treatment. Silencing of VDR with siRNA showed the effect of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1. Based on these findings, we concluded that $1\alpha,25(OH)_2D_3$ down-regulates the HBp17/FGFBP-1 expression via NF-κB pathway.

Keywords: $1\alpha,25(OH)_2D_3$; Vitamin D receptor; HBp17/FGFBP-1; NF-κB; IκBα; Oral squamous cell carcinoma

2.0 INTRODUCTION

1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) is a multi-functional hormone that exerts its actions through the vitamin D receptor (VDR). Besides being well-known as an anti-inflammatory agent, this molecule has been reported to down-regulate the level of the NF-κB protein, i.e. p50, and its p105 precursor in human lymphocytes (B1) [1].

Fibroblast growth factors (FGFs) are important regulators for cell migration, proliferation and differentiation, for example, during embryogenesis, wound healing, tumor growth and angiogenesis. FGFs however bind tightly to heparan sulfate proteoglycans. They are usually immobilized in the extracellular matrix (ECM), and they are only bio-activate when freely soluble. Mobilizations of FGFs are mediated through the action of enzymes or through direct binding with secreted heparin-binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) [2]. HBp17, also known as FGFBP-1, is highly tissue specific and strictly regulated through different promoter elements. Furthermore, this molecule was found to be up-regulated in several tumors including squamous cell carcinomas [2, 3], and it was associated especially with early stages of tumor formation in which angiogenesis plays a critical role [4, 5].

This study showed that HBp17/FGFBP-1 can be manipulated by 1α,25$(OH)_2D_3$ via NF-κB pathway and therefore suggesting the potential role of this molecule as the target for anti-tumor therapy by 1α,25$(OH)_2D_3$.

3.0 MATERIALS AND METHODS

3.1. Cell Cultures

The OSCC cell line (UE) established from a patient with oral cancer in our laboratory (2.5X 10^4 cells) [6] was cultured in 6-well plates (BD Falcon™) with serum-free RD medium (RPMI1640 medium : Dulbecco's modified Eagle's medium [DMEM]; 1:1) (Sigma-Aldrich) containing 6 factors (RD6F) [6, 7]; 6F (10μg/ml bovine insulin, 4μg/ml oleic acid [oleic acid: fatty acid-free bovine serum albumin; 2:1], 10μM β-mercaptoethanol, 10μM 2-aminoethanol, 10nM sodium selenite, 5μg/ml human transferrin) (Sigma-Aldrich). Growth were allowed at 37℃ in a 5% CO_2 incubator (Thermo Fisher Scientific™) for 48 hours. Subsequently, 40nM 1α,25$(OH)_2D_3$ (Enzo) were added and further cultured for another 12 hours.

3.2. Quantitative RT- PCR (qRT-PCR) analysis

Total RNA was extracted using RNeasy total RNA spin Mini RNA Isolation kit (GE Healthcare). Reverse transcription (RT) was performed using the SuperScript first-strand synthesis system (Life Technologies). Quantitative RT-PCR analysis for HBp17/FGFBP-1, VDR and IκBα were performed using STRATAGENE MX3000PTM (Agilent Technologies) with GAPDH as the internal control. The sequences of primers and TaqMan™ fluorogenic probes for HBp17/FGFBP-1, and IκBα were designed according to Probefinder™ software of Roche Universal Probe Library system (Roche Applied Science) : 5'- cgtgtgctcagaacaaggtg-3', 5'-gagcagggtgaggctacaga-3' and #46 fluorescence probe (Roche Diagnostics) for HBp17/FGFBP-1

(NM__005130) ; 5'-cttctctggggactcctcct-3', 5'-tggacgagtccatcatgtct-3' and #15 fluorescence probe (Roche) for VDR (NM__001017536); 5'-gctgatgtcaatgctcagga-3', 5'-acaccaggtcaggattttgc-3' and #86 fluorescence probe (Roche Diagnostics) for IκBα (NM__020529) .

3.3. siRNA transfection

VDR siRNA (5'- AGAUCACUGUAUCACCUCUTTtt-3') and control scrambled siRNA (5'- AGUUCAACGACCAGUAGUCtt-3') were synthesized by the Ambion siRNA facility (Ambion). UE cells were inoculated at $15x10^4$ cells in 6-well plates for 24 hours and then, transfected with 1μM VDR or scrambled siRNA using siPORT^{TM} NeoFX^{TM} Transfection Agent (Ambion) in RD6F medium for 48 hours. Afterwards, cells were treated with 40nM 1α,25$(OH)_2D_3$ (Enzo), with ethanol as control for 12 hours. Next, mRNAs were isolated for the gene expression analysis and cell lysates were obtained for western blotting.

3.4. Plasmid Constructions

To clone DNA fragments from the HBp17/FGFBP-1 promoter region into luciferase reporter vectors, DNA fragments containing 278 base pairs (from -217 to+61) of HBp17/FGFBP-1[8] was amplified by PCR using the human genomic DNA as a template. The PCR products were cloned into the SacI-HindIII (New England Biolab, Ipswich, England) site of pGL4.10 vector (Promega, Madison, WI, USA). The fidelity of the constructed vector was confirmed by DNA sequencing (BECKMAN COULTER Genetic Analysis System).

3.5. Transient Transfection and Luciferase Reporter Assay

Cells were inoculated at $8x10^4$ cells per well in 24- well plates (BD Falcon^{TM}), and cultured in serum-free RD6F for 24 hours. Afterwards, the cells were transfected with luciferase constructs (1ng/assay) and the internal control plasmid pGL3-TK (20pg/assay) using Trans Fast^{TM} (Promega). 48 hours after transfection, cells were incubated with 1 α,25$(OH)_2D_3$ or ethanol for 6 hours and then harvested. Cell lysates were analyzed using Dual Luciferase Assay System (Promega).

3.6. Statistical Analyses

The student's *t*-test was used for group analysis and data are presented as mean $\pm$S.E.M. *p*-values <0.05 were considered significant. All experiments were performed in triplicates (n=3).

4.0 RESULTS

To investigate the effect of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1 mRNA expression in OSCC, UE cells were treated with 40nM $1\alpha,25(OH)_2D_3$ in a defined RD6F medium [6, 7] for 12 hours. As demonstrated by qRT-PCR (Figure 1A), the level of HBp17/FGFBP-1 mRNA was down-regulated by 60% subsequent to treatment. This result was validated by western blot analysis. The experiment was repeated using another OSCC cell lines; KO (Kono) and epidermoid carcinoma cell lines; A431. Results were similar to those obtained with UE (Figure 1C, chapter 1).

$1\alpha,25(OH)_2D_3$ was reported to interfere with NF-κB activity by increasing IκBα levels [42]. To investigate whether this is also true under our experimental conditions, quantitative RT-PCR was performed to measure mRNA levels of NF-κB molecules (p65, p50 and IκBα). From the data, $1\alpha,25(OH)_2D_3$ treatment caused no significant difference in mRNA levels encoding p65 and p50 (Figure 1C & D). By contrast, the mRNA for the NF-κB regulator (IκBα) was found to be significantly up-regulated by $1\alpha,25(OH)_2D_3$ (Figure 1B). This result was confirmed by western blotting.

$1\alpha,25(OH)_2D_3$ is known to exert its biological effects through regulation of the gene expression mediated by the vitamin D receptor (VDR) [9]. We hypothesized the effect of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1 and IκBα observed in UE was as well mediated by VDR. For the purpose of monitoring the vitality of VDR for the function of $1\alpha,25(OH)_2D_3$, we had UE cells transfected with VDR siRNA. This had allowed us to compare the effects of $1\alpha,25(OH)_2D_3$ in the absence (knock-down VDR; siVDR) or presence of VDR (siControl). Using siRNA, VDR

expression was suppressed as much as 75% (Figure 2A.). These cells were then treated with 1α,25$(OH)_2D_3$, followed by qRT-PCR for determination of HBp17/FGFBP-1 and IκBα mRNA levels afterwards. From the results, the HBp17/FGFBP-1 mRNA expression in both siControl and siVDR cells was significantly down-regulated with lesser efficiency observed in siVDR cells (Figure 2B). By contrast, the IκBα mRNA expression was not induced by 1α,25$(OH)_2D_3$ in siVDR cells, however significantly induced in siControl cells (Figure 2C). Taken together these results indicated the dependency of 1α,25$(OH)_2D_3$ on VDR as a mediator to trigger its effects on the regulation of the HBp17/FGFBP-1 and IκBα genes. Prior to transfection, we had VDR mRNA and protein levels measured in these cell lines following treatment with 1α,25$(OH)_2D_3$. Our results confirmed that 1α,25$(OH)_2D_3$ does not directly affect VDR mRNA as well as the protein expression in the cell lines used (Chapter 1, Figure 2C).

A previous report identified an NF-κB binding site in the HBp17/FGFBP-1 promoter from nucleotide -185 to -176 [45]. A luciferase reporter assay in which the luciferase expression was driven by the HBp17/FGFBP-1 promoter (nucleotide -217 to +61; [8]) was performed to determine whether 1α,25$(OH)_2D_3$ regulated HBp17/FGFBP-1 expression via the NF-κB binding site. We found that the HBp17/FGFBP-1 promoter activity was indeed significantly down-regulated at about 25% by 1α,25$(OH)_2D_3$ (Figure 3).

5.0 DISCUSSION

Oral squamous cell carcinoma has become more prevalent particularly among the elder generation. In our previous reports, HBp17/FGFBP-1 was found to be highly expressed in oral squamous cell carcinomas [3], and the frequency of its expression corresponds with the carcinoma malignancy state [5]. Based on the deduced NF-κB site in the HBp17/FGFBP-1 promoter region reported by Harris *et al*. [10], we decided to test the hypothesis that HBp17/FGFBP-1 was regulated by NF-κB by manipulating this particular binding site. In order to investigate the regulation mechanism involved, we treated UE, KO and A431 cells (see chapter 1, figure 1C) with the optimized concentration of $1\alpha,25(OH)_2D_3$ at 40nM. Cells were grown in a defined serum-free medium [6, 7] which therefore eliminates other possible factors that might affect the treatment condition. We found that NF-κB activity was down-regulated by $1\alpha,25(OH)_2D_3$ as a result of IκB*α* up-regulation. This series of events led to the subsequent down-regulation of HBp17/FGFBP-1 mRNA, protein expression as well as promoter activity (Figure 3.).

Previously, $1\alpha,25(OH)_2D_3$ has been shown to up-regulate IκBα by prolongation of its mRNA half-life and increase in its phosphorylation [9]. We have shown in our study of the IκBα up-regulation (Figure 1B.) and later proved that this event is dependent on VDR (Figure 2C.). Moreover, our study has verified the dependency of $1\alpha,25(OH)_2D_3$ on VDR to achieve higher degree of inhibition towards the HBp17/FGFBP-1 expression in UE cells. Our result also showed that $1\alpha,25(OH)_2D_3$ is capable of suppressing the HBp17/FGFBP-1 expression in the absence of VDR albeit in lower efficiency. Thus, we concluded that $1\alpha,25(OH)_2D_3$ is capable to exert immediate inhibition towards the HBp17/FGFBP-1 expression in UE cells, whereas VDR

benefits the reaction by make increase of IκBα, hampering subsequent NF-κB activity, leading to further down-regulation of the HBp17/FGFBP-1 expression.

Apart from that, there has been reported that VDR is capable to influence NF-κB activity by causing transcription of IκBα [11]. Another way to it is by binding to NF- κB molecule, deterring translocation of this molecule into the nucleus [11]. Therefore, even though the IκBα expression was low in siVDR, HBp17/FGFBP-1 is still significantly down-regulated by 1α,25$(OH)_2D_3$ (Figure 2B and 2C). Overall, our results suggest the indirect effect of 1α,25$(OH)_2D_3$ on IκBα, whereby VDR is much required to mediate the reaction. Meanwhile, the HBp17/FGFBP-1 regulation does not fully dependent on regulation of IκBα.

Our data successfully demonstrate the ability of 1α,25$(OH)_2D_3$ to down-regulate HBp17/FGFBP-1 by inhibiting the activity of NF-κB. These results suggest that 1α,25$(OH)_2D_3$ may be a useful agent for the oral cancer therapy by targeting NF-κB and HBp17/FGFBP-1 molecules.

6.0 REFERENCES

1. T. D. Gilmore, The Rel/NF-kappa B signal transduction pathway: introduction, Oncogene, 1999. 18: 6842-6844.
2. D. Wu, M. Kan, G.H. Sato, T. Okamoto, JD. Sato, Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors, J Biol Chem, 1991. 266: 16778-16785.
3. T. Okamoto, Y. Tanaka, M. Kan, A. Sakamoto, K. Takada, JD Sato, Expression of fibroblast growth factor binding protein HBp17 in normal and tumor cells, In Vitro Cell Dev Biol Anim, 1996. 32: 69-71
4. L. C. Center, A.Aigner, R.H.Cabal-Manzano, R.E.Bulter, D.R.Hood, R.B.Sessions, F.Czubayko, A.Wellstein, Differntial regulation of a fibroblast growth factor-binding protein during skin carcinogenesis and wound healing, Neoplasia, 2004. 6: 595-602.
5. S. Begum, Y.Zhang, T.Shintani, S.Toratani, JD.Sato, T.Okamoto, Immunohistochemical expression of heparin-binding protein17/fibroblast growth factor-binding protein (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis, Oncology Reports, 2007.17: 591-596.
6. J. D. Sato, T. Kawamoto, T. Okamoto, Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth *invitro*, J Exp Med, 1987. 165: 1761-1766.
7. T. Okamoto, R. Tani, M. Yabumoto, A. Sakamoto, K. Takada, G.H. Sato, JD. Sato, Effects of insulin and transferrin on lymphokine-activated killer cell generation in serum-free medium, J Immunol Meth, 1996. 195: 7-14

8. S. Shi, R.W. Whitney, JD. Sato, Cloning and promoter characterization of the HBp17 gene encoding an FGF-binding protein, 13th International Symposium on Cellular Endocrinology, Cell Signaling and Tumor Angiogenesis. Lake Placid, NY, September 11-14, 1997.

9. M. Cohen-Lahav, S.Shany, D.Tobvin, C.Chaimovitz, A.Douvdevani, Vitamin D decreases NF-κBactivity by increasing IκBα levels, Nephrol Dial Transplant, 2006. 21: 889-897.

10. V. K. Harris, E.D.E.Liaudet-Coopman, B.J.Boyle, A.Wellstein, A.T.Riegel, Phorbol ester-induced transcription of a fibroblast growth factor-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements, J Biol Chem, 1998. 273: 19130-19139.

11. Z. Janjetovic, et al., 20-Hydroxycholecalciferol, product of vitamin D3 hydroxylation by P450scc, decreases NF-kappaB activity by increasing IkappaB alpha levels in human keratinocytes. PLoS One, 2009. 4 (6): p. e5988.

Legends and Figures

Figure 1. 1α,25$(OH)_2D_3$ suppresses HBp17/FGFBP-1 while inducing IκBα expression in UE cells. Effect of 40nM 1α,25$(OH)_2D_3$ on (A) HBp17/FGFBP-1 (B) IκBα (C) p65 and (D) p50 mRNA expression in UE cells were analyzed by quantitative RT-PCR after 12 hours of treatment period. All data was validated by western blotting. Each bar represents the mean ± S.D. *, $P<0.05$ (n=3).

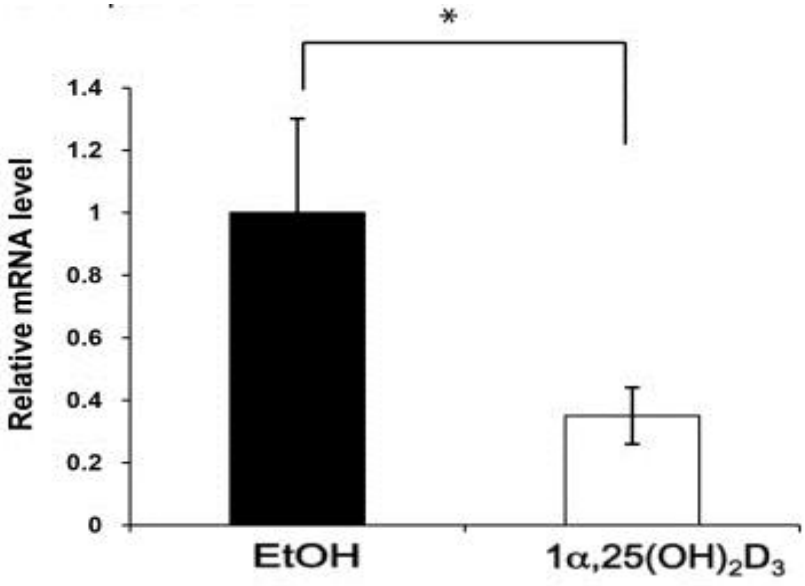
Figure 2. 1α,25$(OH)_2D_3$ requires VDR to achieve higher degree of inhibition on HBp17/FGFBP-1 expression in cancer cells. (A) Construction of siVDR with 80% knock-down of the VDR mRNA expression compared to control (siControl) in UE cells. (B) The HBp17/FGFBP-1 and (C) The IκBα mRNA expression in siControl and siVDR treated with 40nM 1α,25$(OH)_2D_3$ for 12 hours. Transcripts levels of these three molecules were evaluated by quantitative RT-PCR. Each bar represents the mean ± S.D. *, $P<0.05$ (n=3).

Figure 3. 1α, 25$(OH)_2D_3$ suppresses HBp17/FGFBP-1 promoter activity in UE cells. Luciferase activity of PGL-HBp-Luc treansfected UE cells were measured in the presence or absence of 40nM 1α,25$(OH)_2D_3$ for 12 hours . Data are shown as percentage of luciferase activity enhanced by respective activator plasmid. Each bar represents the mean ± S.D. *, $P<0.05$ (n=3).

FIGURE 1.

(A) HBp17/FGFBP-1

(Quantitative RT-PCR)



(Western Blotting)

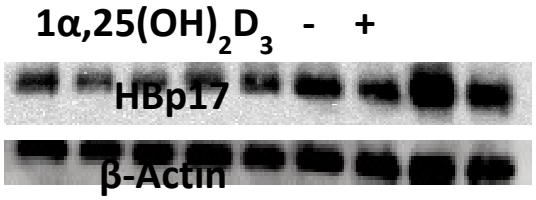
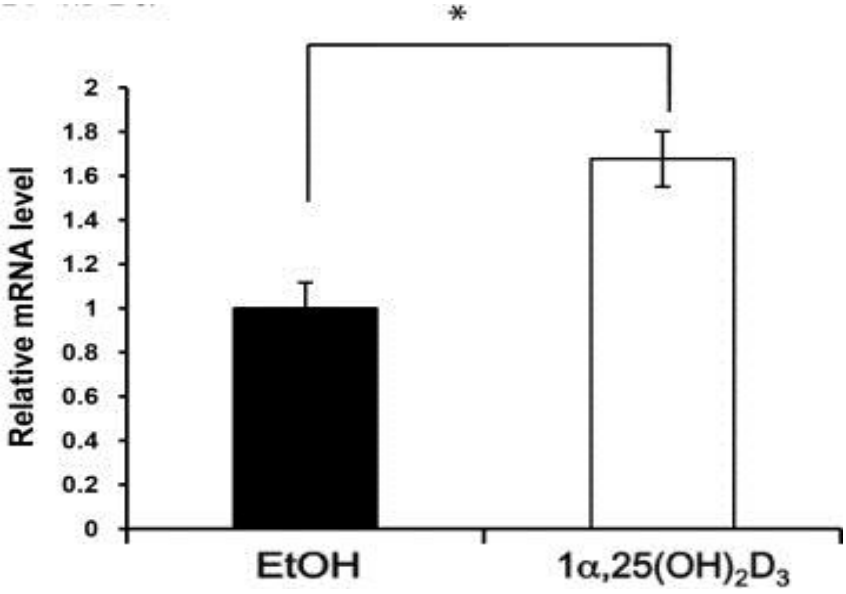


FIGURE 1.

(B) IκBα

(Quantitative RT-PCR)



(Western Blotting)

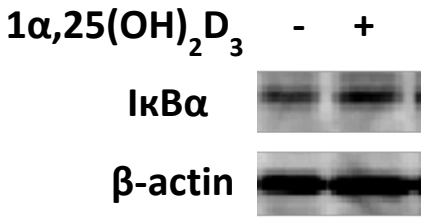
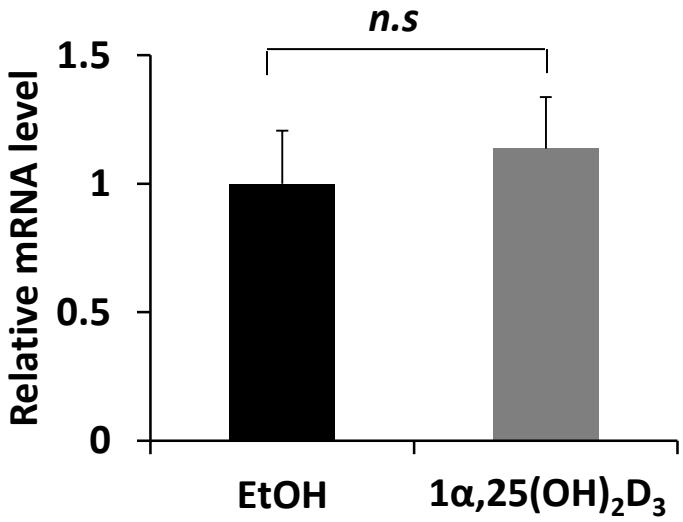


FIGURE 1.

(C) p65

(Quantitative RT-PCR)



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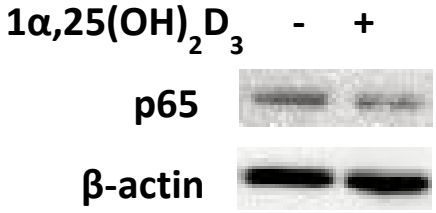
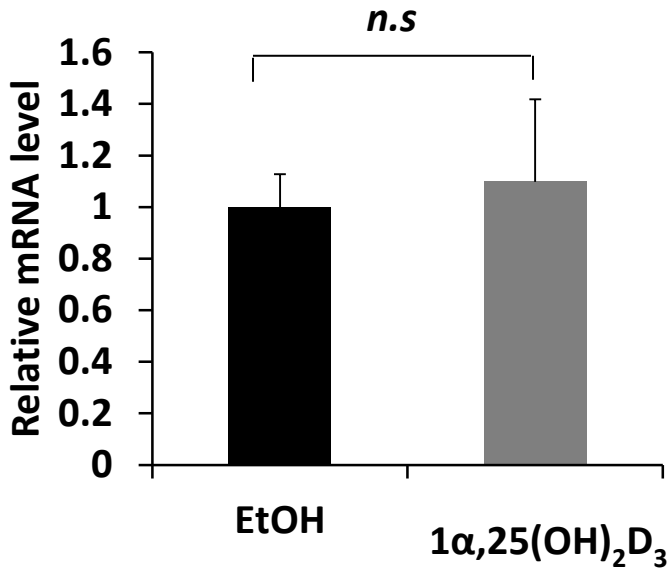


FIGURE 1.

(D) p50

(Quantitative RT-PCR)



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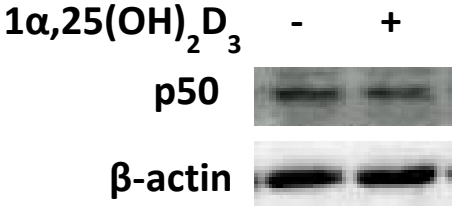
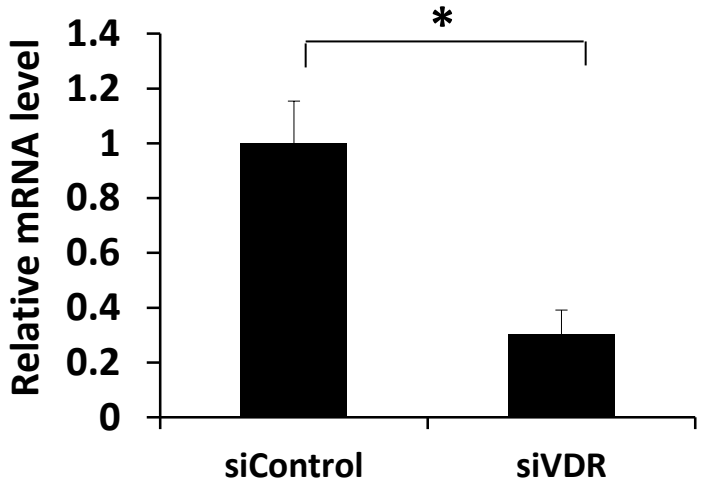


FIGURE 2.

(A) VDR

(Quantitative RT-PCR)



(B) HBp17/FGFBP-1

(Quantitative RT-PCR)

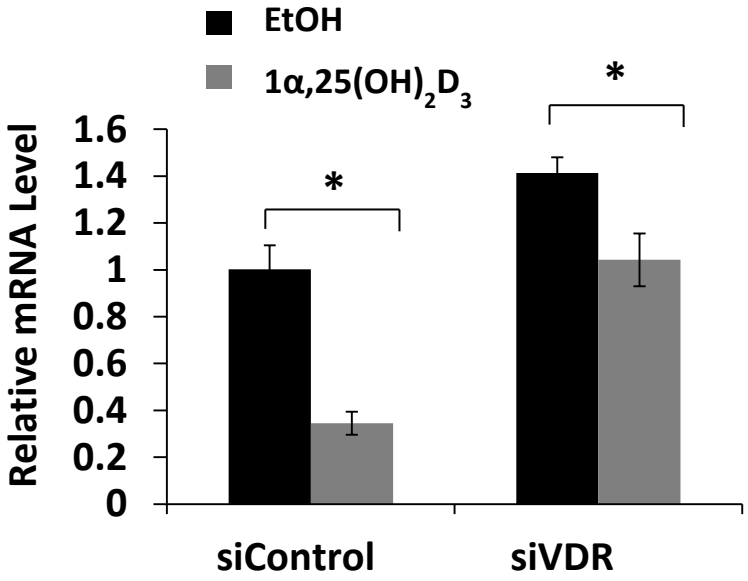


FIGURE 2.

(C) IκBα

(Quantitative RT-PCR)

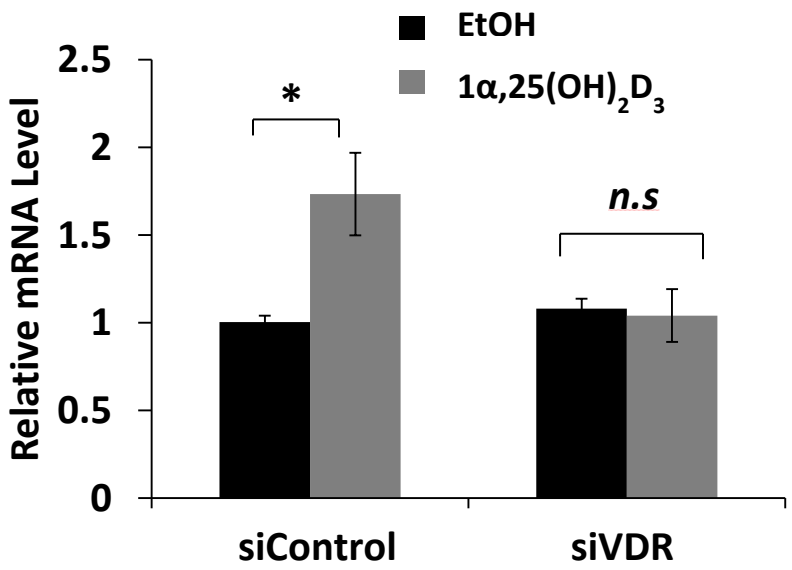
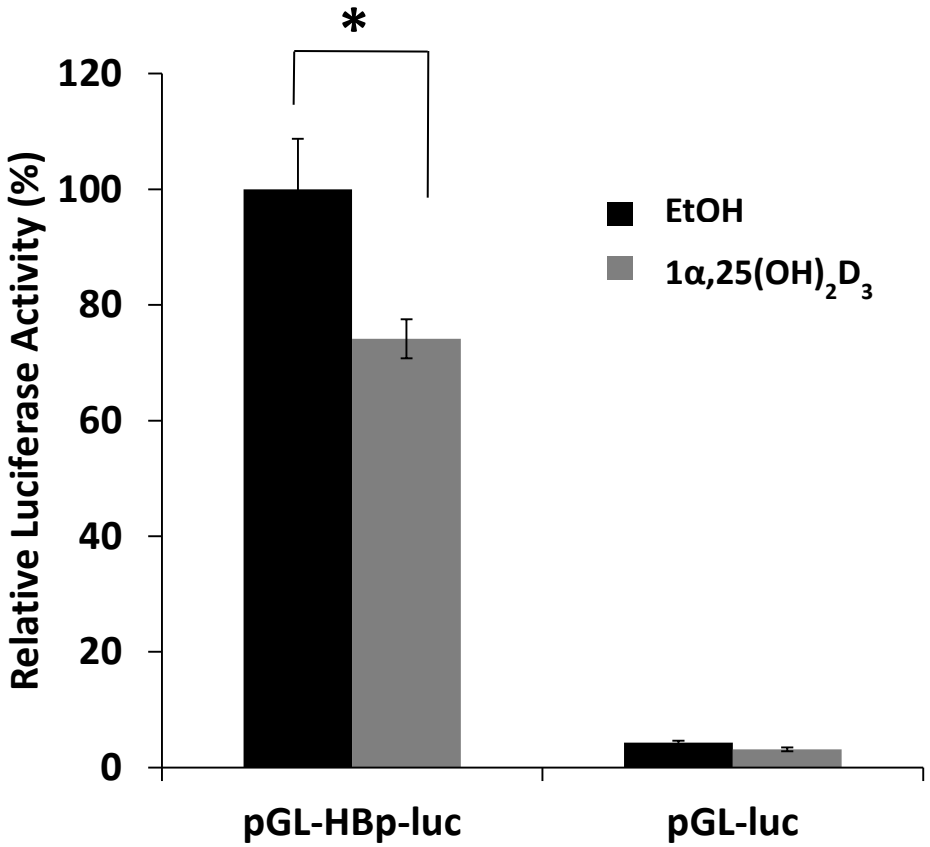


FIGURE 3.

HBp17/FGFBP-1



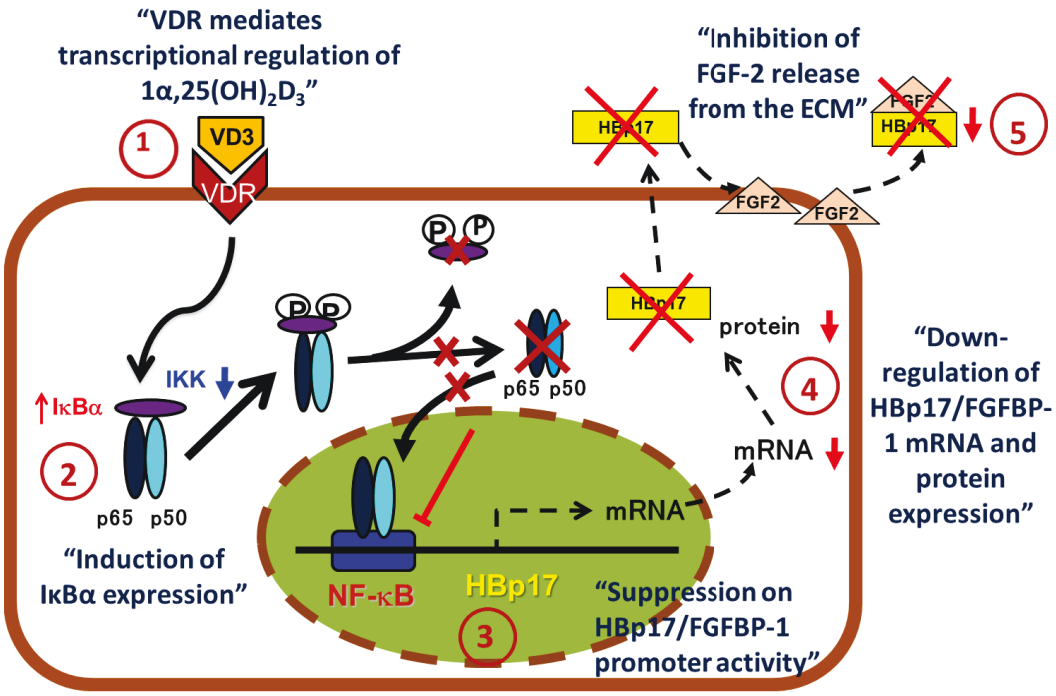
CONCLUSION

This present study represents a novel pathway for $1\alpha,25(OH)_2D_3$ anti-cancer capacity in oral cancer; i.e. targeting HBp17/FGFBP-1 expression.

The current study has demonstrated:

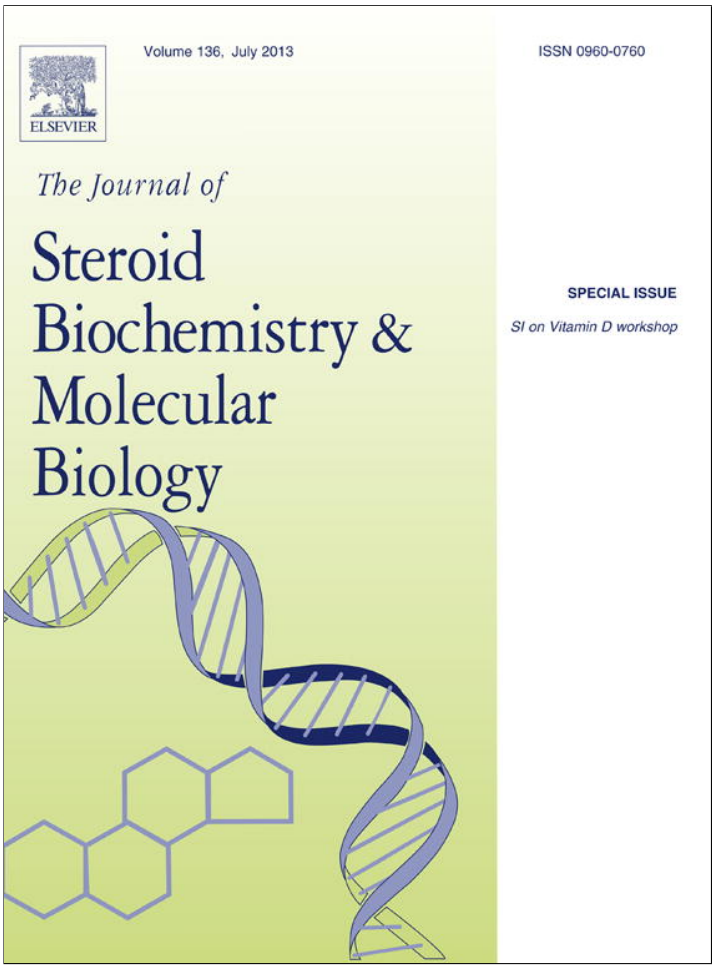
1. HBp17/FGFBP-1 expression is down-regulated by $1\alpha,25(OH)_2D_3$ (40nM) in oral cancer cell lines.
2. $1\alpha,25(OH)_2D_3$ (40nM) exhibited comparable effect with Bay 11-7085 (2μM) on HBp17/FGFBP-1 expression.
3. HBp17/FGFBP-1 expression (mRNA, protein) down-regulated by $1\alpha,25(OH)_2D_3$ in both cytosol and nuclear.
4. $1\alpha,25(OH)_2D_3$ does not have effect on the synthesis of the FGF-2.
5. Down-regulation of HBp17/FGFBP-1 by $1\alpha,25(OH)_2D_3$ resulted in the inhibition of FGF-2 released into conditioned medium.
6. HBp17/FGFBP-1 down-regulation by $1\alpha,25(OH)_2D_3$ takes place through NF-κB binding site located at the promoter region of HBp17/FGFBP-1.
7. Existence of functional NF-κB binding site at the promoter region of HBp17/FGFBP-1 (region between -185 to -176) has been discovered.
8. Inhibition of HBp17/FGFBP-1 and NF-κB activity by $1\alpha,25(OH)_2D_3$ are mediated by VDR.

Taking together, we summarized our findings as follows.



1. VDR mediates transcriptional regulation of $1\alpha,25(OH)_2D_3$.
2. Liganded VDR will trigger many gene transcriptions including the up-regulation of IκBα levels. This event will lead to inhibition of NF-κB activity. Therefore, p50 and p65 dimer could not translocate to bind to its binding site on the promoter region of HBp17/FGFBP-1.
3. This inhibition was well reflected by suppression on HBp17/FGFBP-1 activity (revealed in luciferase reporter assay).
4. Suppression on the promoter activity reasoning down-regulation of HBp17/FGFBP-1 mRNA as well as protein expression.
5. Due to low levels of secreted HBp17/FGFBP-1, FGF-2 will remain bound to the extracellular matrix, in latent state.

SUPPLEMENTARY



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Review

1α,25(OH)2D3 down-regulates HBp17/FGFBP-1 expression via NF-κB pathway

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ABSTRACT

The heparin binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1, GenBank accession no. NP-005121) has been reported to enhance angiogenesis as well as promotes tumor growth in vivo. Furthermore, this molecule was found to be highly expressed in the tissue and cell lines of oral squamous cell carcinoma (OSCC).

$1\alpha,25(OH)_2D_3$ is used to study its potential to curb the expression of HBp17/FGFBP-1 in cancer cells. Consequently, we found that HBp17/FGFBP-1 mRNA and protein levels were significantly down-regulated. In this present study, we show that this event takes place via the NF-κB pathway since mRNA and protein levels of this pathway regulator, IκBα, were found to be significantly up-regulated. Furthermore, the promoter activity of HBp17/FGFBP-1 (region between −217 and +61) measured by a luciferase reporter assay was down-regulated following treatment. Silencing of VDR with siRNA showed the effect of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1. Based on these findings, we concluded that $1\alpha,25(OH)_2D_3$ down-regulated HBp17/FGFBP-1 expression via NF-κB.

This article is part of a Special Issue entitled 'Vitamin D Workshop'.

Contents

1. Introduction

$1\alpha,25$ dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) is a multi-functional hormone that exerts its actions through the vitamin D receptor (VDR). Besides being well-known as an anti-inflammatory agent, this molecule has been reported to down-regulate the level of the NF-κB protein, i.e. p50, and its p105 precursor in human lymphocytes (B1) [1].

Fibroblast growth factors (FGFs) are important regulators for cell migration, proliferation and differentiation, for example, during embryogenesis, wound healing, tumor growth and angiogenesis. FGFs however bind tightly to heparan sulfate proteoglycans. They are usually immobilized in the extracellular matrix (ECM), and they are only bio-activate when freely soluble. Mobilizations of FGFs are mediated through the action of enzymes or through direct binding

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with secreted heparin binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) [2]. HBp17, also known as FGFBP-1, is highly tissue specific and strictly regulated through different promoter elements. Furthermore, this molecule was found to be up-regulated in several tumors including squamous cell carcinomas [2,3], and it was associated especially with early stages of tumor formation in which angiogenesis plays a critical role [4,5]. This study showed that HBp17/FGFBP-1 can be manipulated by $1\alpha,25(OH)_2D_3$ via NF-κB pathway and therefore suggesting the potential role of this molecule as the target for anti-tumor therapy by $1\alpha,25(OH)_2D_3$.

2. Materials and methods

2.1. Cell cultures

The OSCC cell line (UE) [6] established from a patient with oral cancer in our laboratory was cultured at 2.5×10^4 cells/well in 6-well plates with serum free RD medium (RPMI1640 medium: Dulbecco's modified Eagle's medium [DMEM]; 1:1) containing six factors (RD6F) [7,8]; 6F (10 μg/ml bovine insulin, 4 μg/ml oleic acid [oleic acid:fatty acid-free bovine serum albumin; 2:1], 10 μM β-mercaptoethanol, 10 μM 2-aminoethanol, 10 nM sodium selenite, 5 μg/ml human transferrin). Growth was allowed at 37 °C in a 5% CO_2 incubator for 48 h. Subsequently, 40 nM $1\alpha,25(OH)_2D_3$ (BML-DM200 Enzo) were added and further cultured for another 12 h.

2.2. Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted using RNeasy total RNA spin Mini RNA Isolation kit (GE Healthcare UK Ltd., England). Reverse transcription (RT) was performed using SuperScript first-strand synthesis system. qRT-PCR analysis for HBp17/FGFBP-1, VDR and IκBα were performed using STRATAGENE MX3000P™ system (Stratagene, CA, USA) with GAPDH as the internal control. The sequences of primers and TaqMan™ fluorogenic probes for HBp17/FGFBP-1, and IκBα were designed according to Probefinder™ software of Roche Universal Probe Library system (Roche Applied Science): 5′-cgtgtgctcagaacaaggtg-3′, 5′-gagcagggtgaggctacaga-3′ and #46 fluorescence probe (Roche) for HBp17/FGFBP-1 (NM_005130); 5′-cttctctggggactcctcct-3′, 5′-tggacgagtccatcatgtct-3′ and #15 fluorescence probe (Roche) for VDR (NM_001017536); 5′-gctgatgtcaatgctcagga-3′, 5′-acaccaggtcaggattttgc-3′ and #86 fluorescence probe (Roche) for IκBα (NM_020529).

2.3. siRNA transfection

VDR siRNA (5′-AGAUCACUGUAUCACCUCUTTtt-3′) and control scrambled siRNA (5′-AGUUCAACGACCAGUAGUCtt-3′) were synthesized by the Ambion siRNA facility (Austin, TX). UE cells were inoculated at 15×10^4 cells in six-well plates for 24 h and then, transfected with 1 μM VDR or scrambled siRNA using siPORT™ NeoFX™ Transfection Agent (Ambion) in RD6F medium for 48 h. Afterwards, cells were treated with 40 nM $1\alpha,25(OH)_2D_3$, or ethanol as control for 12 h and mRNAs were isolated for gene expression analysis.

2.4. Plasmid constructions

To clone DNA fragments from the HBp17/FGFBP-1 promoter region into luciferase reporter vectors, DNA fragments containing 278 bp (from −217 to +61) of HBp17/FGFBP-1 [9] was amplified by PCR using the human genomic DNA as a template. The PCR products were cloned into the SacI-HindIII site of pGL4.10 vector (Promega, Madison, WI, USA). The fidelity of the constructed vector was confirmed by DNA sequencing (BECKMAN COULTER Genetic Analysis System).

2.5. Transient transfection and luciferase reporter assay

Cells were inoculated at 8×10^4 cells/well in 24-well plates, and cultured in serum free RD6F for 24 h. Afterwards, the cells were transfected with the luciferase constructs (1 μg/assay) and the internal control plasmid pRL3-TK (20 ng/assay) using Trans Fast™ (Promega). Two days after transfection, cells were incubated with 40 nM $1\alpha,25(OH)_2D_3$ or with ethanol for 12 h and then harvested. Cell lysate was analyzed using Dual Luciferase Assay System (Promega). The luciferase activities were normalized by the Remilla luciferase activities of the internal control pRL-TK, and data were plotted as mean values ±S.D. in triplicate assay as a ratio to the control assay in the absence of $1\alpha,25(OH)_2D_3$.

2.6. Statistical analyses

Data are presented as mean values ± standard deviation (S.D.). The Student's *t*-test was used for group analysis and *P*-values < 0.05 were considered significant. All the experiments were independently repeated at least three times and performed in triplicate assay ($n = 3$).

3. Results

To investigate the effects of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1 mRNA expression in OSCC, UE cells were treated with 40 nM $1\alpha,25(OH)_2D_3$ in well-defined RD6F medium [6,7] for 12 h. As demonstrated by qRT-PCR, the level of HBp17/FGFBP-1 mRNA was down-regulated by 60% subsequent to treatment (Fig. 1A). This result was validated by Western blot analysis (data not shown). The experiment was repeated using another OSCC cell lines; KO (Kono: established from a patient with oral cancer in our laboratory) [10] and dermoid cancer cell lines; A431 [11]. Results were similar to those obtained with UE.

$1\alpha,25(OH)_2D_3$ was reported to interfere with NF-κB activity by increasing IκBα levels [12]. To investigate whether this is also true under our experimental conditions, qRT-PCR was performed to measure mRNA levels of NF-κB molecules (p65, p50 and IκBα). From the data, $1\alpha,25(OH)_2D_3$ treatment caused no significant difference in mRNA levels encoding p65 and p50 (data not shown). By contrast, the mRNA for the NF-κB regulator (IκBα) was found to be significantly up-regulated by $1\alpha,25(OH)_2D_3$ (Fig. 1B). This result was confirmed by Western blot analysis for IκBα protein (data not shown).

$1\alpha,25(OH)_2D_3$ is known to exert its biological effects through regulation of gene expression mediated by the vitamin D receptor (VDR) [12]. We hypothesized the effect of $1\alpha,25(OH)_2D_3$ on HBp17/FGFBP-1 and IκBα observed in UE was as well mediated by VDR. For the purpose of monitoring the vitality of VDR for the function of $1\alpha,25(OH)_2D_3$, we had UE cells transfected with VDR siRNA. This had allowed us to compare the effects of $1\alpha,25(OH)_2D_3$ in the absence (knock-out VDR; VDR/KO) or presence (wild-type VDR; VDR/WT) of VDR. Using siRNA, VDR expression was suppressed as much as 75% (Fig. 2A). These cells were then treated with $1\alpha,25(OH)_2D_3$, followed by qRT-PCR for determination of HBp17/FGFBP-1 and IκBα mRNA levels afterwards. From the results, HBp17/FGFBP-1 mRNA expression in both VDR/WT and VDR/KO cells was significantly down-regulated with lesser efficiency observed in VDR/KO cells (Fig. 2B). By contrast, IκBα mRNA expression was not induced by $1\alpha,25(OH)_2D_3$ in VDR/KO cells, however significantly induced in VDR/WT cells (Fig. 2C). Taken together these results indicated the dependency of $1\alpha,25(OH)_2D_3$

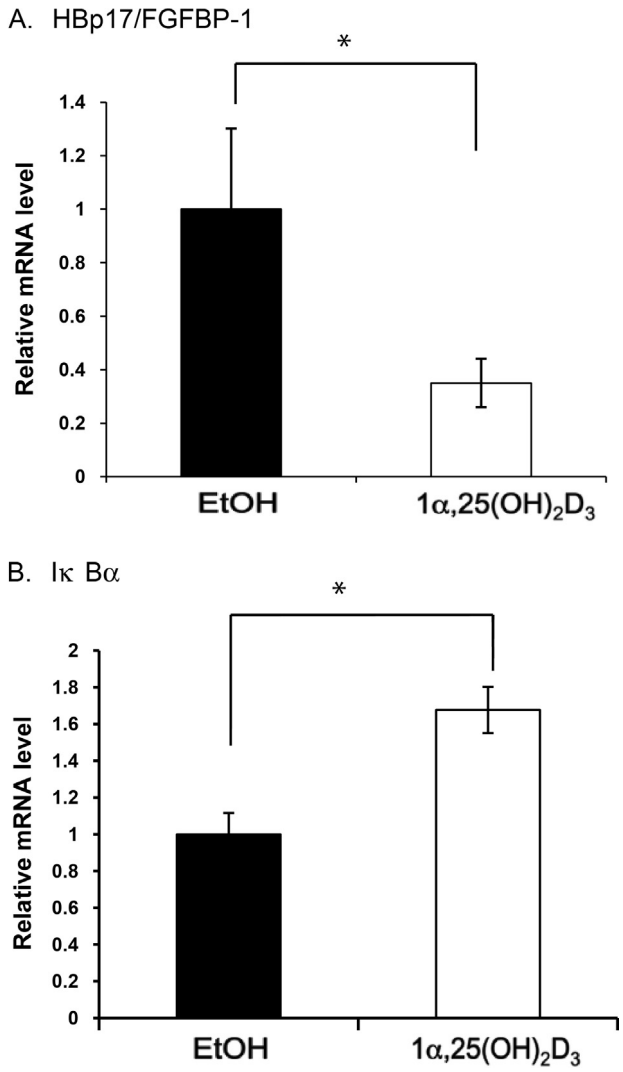

Fig. 1. "1α,25(OH)$_2$D$_3$ suppresses HBp17/FGFBP-1 while inducing IκBα expression in UE cells." Effect of 40 nM 1α,25(OH)$_2$D$_3$ on (A) HBp17/FGFBP-1 and (B) IκBα mRNA expression in UE cells were analyzed by quantitative real-time RT-PCR after 12 h of treatment period. Each bar represents the mean ± S.D., $^*P < 0.05$ ($n = 3$).

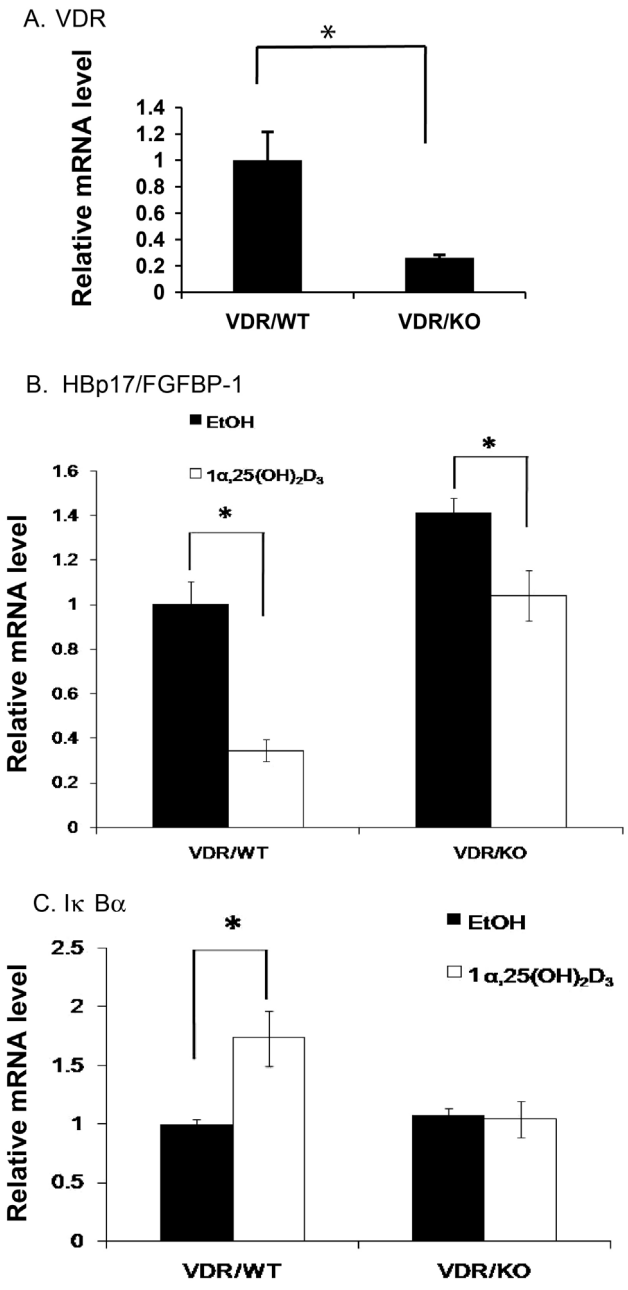

Fig. 2. "1α,25(OH)$_2$D$_3$ requires VDR to achieve higher degree of inhibition on HBp17/FGFBP-1 expression in UE cells." (A) Construction of VDR/KO with 80% knock-down of VDR mRNA expression compared to control (VDR/WT) in UE cells. (B) HBp17/FGFBP-1 and (C) IκBα mRNA expression in VDR/WT and VDR/KO treated with 40 nM 1α,25(OH)$_2$D$_3$ for 12 h. Transcript levels of these three molecules were evaluated by quantitative real-time RT-PCR. Each bar represents the mean ± S.D., $^*P < 0.05$ ($n = 3$).

on VDR as a mediator to trigger its effects on the regulation of the HBp17/FGFBP-1 and IκBα genes. Prior to transfection, we had VDR mRNA and protein levels measured in these cell lines following treatment with 1α,25(OH)$_2$D$_3$. Our results confirmed that 1α,25(OH)$_2$D$_3$ does not directly affect VDR mRNA level as well as protein expression in the cell lines used (data not shown).

A previous report by Harris et al. [13] identified an NF-κB binding site in the HBp17/FGFBP-1 promoter from nucleotide −185 to −176 [10]. A luciferase reporter assay in which the luciferase expression was driven by the HBp17/FGFBP-1 promoter (nucleotide −217–+61 [9]) was performed to determine whether 1α,25(OH)$_2$D$_3$ regulated HBp17/FGFBP-1 expression via the NF-κB binding site. We found that HBp17/FGFBP-1 promoter activity was indeed significantly down-regulated at about 25% by 1α,25(OH)$_2$D$_3$ (Fig. 3).

4. Discussion

Oral carcinoma has become more prevalent particularly among the elder generation. In our previous reports, HBp17/FGFBP-1 was found to be highly expressed in oral squamous cell carcinomas [3], and the frequency of its expression corresponds with the carcinoma malignancy state [5]. Based on the deduced NF-κB site in the HBp17/FGFBP-1 promoter region reported by Harris et al. [13], we decided to test the hypothesis that HBp17/FGFBP-1 was regulated by NF-κB by manipulating this particular binding site. In order to investigate the regulation mechanism involved, we treated UE, KO and A431 cells (KO and A431; data not shown) with the optimized concentration of 40 nM 1α,25(OH)$_2$D$_3$. Cells were grown in a well-defined serum-free medium [7,8] which therefore eliminates other possible factors that might affect the treatment condition. We found that NF-κB activity was down-regulated by 1α,25(OH)$_2$D$_3$ as a result of IκBα up-regulation. This series of events led to the subsequent down-regulation of HBp17/FGFBP-1 mRNA, protein expression as well as promoter activity (Fig. 3).

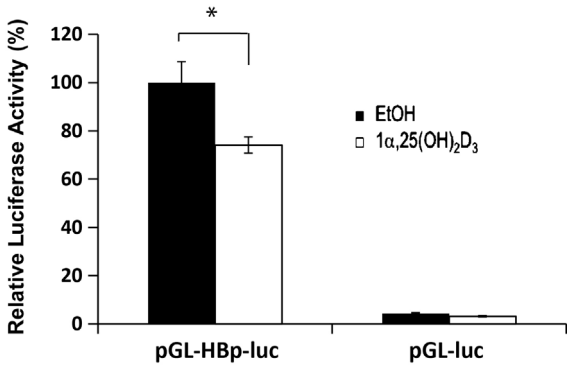

Fig. 3. "1α,25(OH)$_2$D$_3$ suppresses HBp17/FGFBP-1 promoter activity in UE cells." Luciferase activity of pGL-HBp-Luc transfected UE cells were measured in the presence or absence of 40 nM 1α,25(OH)$_2$D$_3$ for 12 h. Data are shown as percentages of luciferase activity enhanced by the respective activator plasmid. Each bar represents the mean ± S.D., $^*P < 0.05$ ($n = 3$).

Previously, 1α,25(OH)$_2$D$_3$ has been shown to up-regulate IκBα by prolongation of its mRNA half-life and increase in its phosphorylation [12]. We have shown in earlier phase of our study of this phenomenon (Fig. 1B) and later proved that this event is dependent on VDR (Fig. 2C). Moreover, our study has verified the dependency of 1α,25(OH)$_2$D$_3$ on VDR to achieve higher degree of inhibition toward HBp17/FGFBP-1 expression in UE cells. Absolute reliant of 1α,25(OH)$_2$D$_3$ on VDR to cause up-regulation of IκBα may have assisted inhibition of HBp17/FGFBP-1 observed as higher inhibition in VDR/KO. Our result also showed that 1α,25(OH)$_2$D$_3$ is capable of suppressing HBp17/FGFBP-1 expression in the absence of VDR albeit in lower efficiency. Thus, we concluded that 1α,25(OH)$_2$D$_3$ capable to exert immediate inhibition toward HBp17/FGFBP-1 expression in UE cells, whereas VDR benefits the reaction by make increase of IκBα, hampering subsequent NF-κB activity, leading to further down-expression of HBp17/FGFBP-1.

Apart from that, there was also evidence showing VDR is capable to influence NF-κB activity by causing transcription of IκBα [14]. Another way to it is by binding straight to NF- κB molecule, deterring translocation of this molecule into the nucleus [14]. Therefore, even though IκBα expression was low in VDR/KO, HBp17/FGFBP-1 is still significantly down-regulated by 1α,25(OH)$_2$D$_3$ (Fig. 2B and C). Overall, our results suggest the indirect effect of 1α,25(OH)$_2$D$_3$ on IκBα, whereby VDR is much required to mediate the reaction. Meanwhile, HBp17/FGFBP-1 regulation does not fully dependent with regulation of IκBα.

We have shown the effectually of 1α,25(OH)$_2$D$_3$ to cause suppression on HBp17/FGFBP-1 mRNA expression as much as 60% in UE cells. In order to comprehend this phenomenon, we extended our research to luciferase reporter assay. Subsequent to treatment with 40 nM 1α,25(OH)$_2$D$_3$, HBp17/FGFBP-1 promoter activity within the region of -217 and +61 (deduced region of NF-κB binding site [13]) was found to be indeed suppressed, however, weaker (30% reduction compared to that of non-treated). Thus far, our research demonstrated a modest discrepancy between the mRNA expression and promoter activity despite of similar tendency in general. In addition, this data may have illustrated the possibility of more than one NF-κB binding sites are exist and responsible for the down-regulation of HBp17/FGFBP-1 mRNA. Until we could prove this arising hypothesis, the mechanisms of this phenomenon are yet to be explained in detail.

Our data successfully demonstrate the ability of 1α,25(OH)$_2$D$_3$ to down-regulate HBp17/FGFBP-1 by inhibiting the activity of NF-κB. These results suggest that 1α,25(OH)$_2$D$_3$ may be a useful agent for the oral cancer therapy by targeting NF-κB and HBp17/FGFBP-1 molecules.

References

[1] T.D. Gilmore, The Rel/NF-kappa B signal transduction pathway: introduction, Oncogene 18 (1999) 6842–6844.
[2] D. Wu, M. Kan, G.H. Sato, T. Okamoto, J.D. Sato, Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors, Journal of Biological Chemistry 266 (1991) 16778–16785.
[3] T. Okamoto, Y. Tanaka, M. Kan, A. Sakamoto, K. Takada, J.D. Sato, Expression of fibroblast growth factor binding protein HBp17 in normal and tumor cells, In Vitro Cellular and Developmental Biology. Animal 32 (1996) 69–71.
[4] L.C. Center, A. Aigner, R.H. Cabal-Manzano, R.E. Bulter, D.R. Hood, R.B. Sessions, F. Czubayko, A. Wellstein, Differential regulation of a fibroblast growth factor-binding protein during skin carcinogenesis and wound healing, Neoplasia 6 (2004) 595–602.
[5] S. Begum, Y. Zhang, T. Shintani, S. Toratani, J.D. Sato, T. Okamoto, Immunohistochemical expression of heparin-binding protein17/fibroblast growth factor-binding protein (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis, Oncology Reports 17 (2007) 591–596.
[6] S. Miyauchi, T. Moroyama, S. Koizumi, et al., Malignant tumor cell lines produce interleukin-1-like factor, In Vitro Cellular and Developmental Biology 24 (1988) 753–758.
[7] J.D. Sato, T. Kawamoto, T. Okamoto, Cholesterol requirement of P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth in vitro, Journal of Experimental Medicine 165 (1987) 1761–1766.
[8] T. Okamoto, R. Tani, M. Yabumoto, A. Sakamoto, K. Takada, G.H. Sato, J.D. Sato, Effects of insulin and transferrin on lymphokine-activated killer cell generation in serum-free medium, Journal of Immunological Methods 195 (1996) 7–14.
[9] S. Shi, R.W. Whitney, J.D. Sato, Cloning and promoter characterization of the HBp17 gene encoding an FGF-binding protein, in: 13th International Symposium on Cellular Endocrinology, Cell Signaling and Tumor Angiogenesis, Lake Placid, NY, September 11–14, 1997.
[10] E. Michigami, N. Kitamura, Y. Zhang, H. Wang, Y. Hiraishi, K. Sumi, Y. Hayashido, S. Toratani, T. Okamoto, Mutations in the human homologue of the *Drosophila* segment polarity gene patched in oral squamous cell carcinoma cell lines, In Vitro Cellular and Developmental Biology. Animal 37 (2001) 459–464.
[11] R.N. Fabricant, J.E. DeLarco, G.J. Todaro, Nerve growth factor receptors on human melanoma cells in culture, Proceedings of the National Academy of Sciences of the United States of America 74 (1977) 565–569.
[12] M. Cohen-Lahav, S. Shany, D. Tobvin, C. Chaimovitz, A. Douvdevani, Vitamin D decreases NFκB activity by increasing IκBα levels, Nephrology, Dialysis, Transplantation 21 (2006) 889–897.
[13] V.K. Harris, E.D.E. Liaudet-Coopman, B.J. Boyle, A. Wellstein, A.T. Riegel, Phorbol ester-induced transcription of a fibroblast growth factor-binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements, Journal of Biological Chemistry 273 (1998) 19130–19139.
[14] Z. Janjetovic, M.A. Zmijewski, R.C. Tuckey, D.A. Deleon, M.N. Nguyen, L.M. Pfeffer, A.T. Slominski, 20-Hydroxycholecalciferol, product of vitamin D3 hydroxylation by P450scc, decreases NF-kappaB activity by increasing IkappaB alpha levels in human keratinocytes, PLoS One 4 (6) (2009) e5988.