

## Protective role of 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> against oxidative stress in nonmalignant human prostate epithelial cells

Bo-Ying Bao<sup>1,2,3</sup>, Huei-Ju Ting<sup>1</sup>, Jong-Wei Hsu<sup>1</sup> and Yi-Fen Lee<sup>1\*</sup>

<sup>1</sup>Department of Urology, University of Rochester Medical Center, Rochester, NY

<sup>2</sup>Department of Chemical Engineering, University of Rochester, Rochester, NY

<sup>3</sup>School of Pharmacy, China Medical University, Taichung, Taiwan

Overproduction of reactive oxygen species (ROS), through either endogenous or exogenous sources, could induce DNA damage, and accumulation of DNA damage might lead to multistep carcinogenesis. The antioxidative effects of vitamin D have been suggested by epidemiological and many *in vitro* and *in vivo* laboratory studies. While exploring the antioxidative effects of vitamin D in prostate cells, we found that the active form of vitamin D, 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> (1,25-VD), can protect nonmalignant human prostate epithelial cell lines, BPH-1 and RWPE-1, but not malignant human prostate epithelial cells, CWR22R and DU 145, from oxidative stress-induced cell death. Glucose-6-phosphate dehydrogenase (G6PD), a key antioxidant enzyme, was dose- and time-dependently induced by 1,25-VD. Mechanistic studies using chromatin immunoprecipitation (ChIP) assay revealed that a direct repeat-3 (DR3) vitamin D response element located in the first intron of the G6PD genome can be bound by liganded vitamin D receptor, thereby regulating G6PD gene expression. Increasing G6PD activity and glutathione level by 1,25-VD can scavenge cellular ROS. Moreover, the protective effects of 1,25-VD were abolished by dehydroepiandrosterone, a noncompetitive inhibitor of G6PD activity. Together, our results showed that 1,25-VD can protect nonmalignant prostate cells from oxidative stress-induced cell death by elimination of ROS-induced cellular injuries through transcriptional activation of G6PD activity. The antioxidative effect of vitamin D strengthens its roles in cancer chemoprevention and adds to a growing list of beneficial effects of vitamin D against cancer.

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**Key words:** prostate cancer; vitamin D; G6PD; oxidative stress

Reactive oxygen species (ROS) are generated after exposure to environmental stresses, such as UV-, X- and  $\gamma$ -irradiation, and carcinogens, as well as endogenous cellular stresses from leakage of electron transport chain in mitochondria, and during inflammation.<sup>1</sup> ROS are, in one way, necessary for inflammatory response to defend organisms against infection agents. In contrast, high levels of cellular ROS could damage many critical cellular components, such as protein, lipids and DNA, and eventually cause cellular injuries that might lead to a variety of clinical abnormalities, including cardiovascular disease, diabetes, neurodegenerative disorders, aging and cancer.<sup>2–6</sup>

The central defense system against ROS is the reducing equivalents, such as glutathione (GSH).<sup>7</sup> Reducing equivalents are necessary for the detoxification of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), oxidized protein sulfhydryl groups and lipid peroxides.<sup>8</sup> The maintenance of cellular reducing state requires NADPH, and glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme in the pentose phosphate pathway, is responsible for the generation of NADPH. G6PD can protect erythrocytes against ROS, and deficiency of G6PD is associated with hemolytic disorders due to the increased susceptibility of erythrocytes to oxidative stress.<sup>9</sup>

A large body of evidence suggests that ROS are directly or indirectly involved in carcinogenesis.<sup>10</sup> ROS are capable of damaging cellular components, and this destructive chain reaction can be blocked by the reducing equivalents. Therefore, compounds that can modulate the defense system against ROS could potentially hinder the process of carcinogenesis. 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> (1,25-VD) has been known to reduce the incidence of colon, breast and prostate cancers in human,<sup>11–13</sup> as well as in chemically induced animal cancer models.<sup>14–16</sup> Much research has focused on vitamin D

receptor (VDR)-mediated antiproliferative effects of 1,25-VD, the active metabolite of vitamin D. In general, VDR binds to vitamin D response elements (VDREs), and regulates target genes' expression to inhibit cancer cells proliferation.<sup>17</sup> However, little information is available on the antioxidant property of 1,25-VD. Gene expression profiling revealed that 1,25-VD and its analogs induced several genes controlling redox balance, including G6PD, glutathione peroxidase (GPx) and thioredoxin reductase (TR).<sup>18,19</sup> These studies suggest that 1,25-VD might be able to prevent cancer development through protection against oxidative stress.

Therefore, the aim of the present work was to explore the potential ability of 1,25-VD to modulate the defense system against ROS. We demonstrated that G6PD is a 1,25-VD primary target gene and G6PD is critical in the protective effects of 1,25-VD on H<sub>2</sub>O<sub>2</sub>-induced cell death in the nonmalignant human prostate epithelial cell lines, BPH-1 and RWPE-1.

### Material and methods

#### Cells and materials

1,25-VD was the generous gift of Dr. Lise Binderup from Leo Pharmaceutical Products (Ballerup, Denmark). The anti-VDR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DU 145 and RWPE-1 cells were obtained from the American Type Culture Collection. BPH-1 cells were a generous gift of Dr. William Ricke from the University of Rochester. CWR22R cells were a generous gift of Dr. Franky Chan from the University of Hong Kong. Cell culture medium (DMEM for DU 145 cells; RPMI-1640 for BPH-1 and CWR22R cells; keratinocyte serum free medium for RWPE-1) was obtained from Gibco-BRL (Carlsbad, CA).

#### Cell viability assay

Cells were seeded in 96-well plates at a density of 3,000 cells/well. After overnight incubation, cells were treated with either vehicle (ethanol (0.1% v/v)) or 100 nM 1,25-VD for 24 hr, and then challenged with indicated concentrations of H<sub>2</sub>O<sub>2</sub> for another 24 hr. Cell viability was determined by MTT assay as described previously.<sup>20</sup>

#### Chromatin immunoprecipitation (ChIP) assay

RWPE-1 cells were treated with either vehicle (ethanol (0.1% v/v)) or 100 nM 1,25-VD for 2 hr. ChIP was performed by ChIP assay kit according to the manufacturer's suggested procedures

**Abbreviations:** 25-dihydroxyvitamin D<sub>3</sub>; ChIP, chromatin immunoprecipitation; DHEA, dehydroepiandrosterone; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, glutathione; ROS, reactive oxygen species; TR, thioredoxin reductase; VDR, vitamin D receptor; VDRE, vitamin D response element.

Grant sponsor: Department of Defense; Grant number: PC040630. Grant sponsor: National Science Council; Grant number: NSC-96-2321-B-039-005-MY2. Grant sponsor: China Medical School; Grant number: CMU96-096, Taiwan.

The first two authors contributed equally to this work.

\*Correspondence to: Department of Urology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 656, Rochester, NY 14642, USA. Fax: +585-756-4133. E-mail: yifen\_lee@urmc.rochester.edu

Received 24 April 2007; Accepted after revision 10 January 2008

DOI 10.1002/ijc.23460

Published online 17 March 2008 in Wiley InterScience (www.interscience.wiley.com).

(Upstate, Lake Placid, NY). DNA fragments were purified using QIAGEN QIAquick Spin kit (Valencia, CA) and then subjected to PCR using primers designed to amplify either the proximal human CYP24A1 promoter region or various regions of the human G6PD genome. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide. CYP24A1 VDRE sense primer 5'-CCTGGCAGACGCGGCA-3' and antisense primer 5'-GGCCA GACTCCGAGGGA-3'. G6PD VDRE-A sense primer 5'-CAAACC TGGGAGATGGAG-3' and antisense primer 5'-CCTGGATTCAA GCGATTC-3'. G6PD VDRE-B sense primer 5'-AATTAGTCAG AAGTTGATACC-3' and antisense primer 5'-CACTCTAACCT CCTCCTC-3'. G6PD VDRE-C sense primer 5'-CTGTTTGTGTT GTGCTTGAG-3' and antisense primer 5'-ATGGTGTATTCC GACTAC-3'. G6PD VDRE-D sense primer 5'-TGTATTGTTAG TAGAGACG-3' and antisense primer 5'-GAAGTGTAGTGAA GAGG-3'.

#### G6PD enzyme activity assay

The assay was performed as described previously.<sup>21</sup> Briefly, after treating with vehicle (ethanol (0.1% v/v)) or indicated concentrations of 1,25-VD for 24 hr, cells were lysed in assay buffer (50 mM Tris/pH 8.1, 1 mM MgCl) by freeze-thaw cycles, and then cell lysates were incubated with reaction mixtures (assay buffer containing 100  $\mu$ M NADP<sup>+</sup> and either 200  $\mu$ M glucose-6-phosphate for total dehydrogenase activity or 200  $\mu$ M 6-phosphogluconate for 6-phosphogluconate dehydrogenase (6-PGD) activity). Enzyme activities were determined by measuring the rate of increase in absorbance at 340 nm due to the conversion of NADP<sup>+</sup> to NADPH by either G6PD or 6-PGD, and G6PD activity was then determined by subtracting 6-PGD activity from total dehydrogenase activity.

#### GSH assay

The assay was performed as described previously.<sup>22</sup> Briefly, after treating with vehicle (ethanol (0.1% v/v)) or indicated concentrations of 1,25-VD for 24 hr, cells were lysed in assay buffer (0.1 M sodium phosphate–0.005 M EDTA buffer/pH 8.0) by freeze-thaw cycles, and then cell lysates were deproteinized by adding equal volumes of 100 mg/ml metaphosphoric acid for 5 min. After centrifugation, the supernatants were incubated with reaction mixture (assay buffer containing 0.05 mg/ml *o*-phthalaldehyde) for 15 min at room temperature. Fluorescence was measured in a SPECTRAMax GEMINI spectrofluorometer by excitation at 350 nm and emission at 420 nm.

#### Measurement of intracellular ROS

The intracellular ROS levels were determined using the oxidation-sensitive fluorescent probe (2',7'-dichlorofluorescein diacetate (DCFH-DA)). Intracellular ROS oxidize this probe to a highly fluorescent compound, DCF. After treating with vehicle (ethanol (0.1% v/v)) or 100 nM 1,25-VD for 24 hr, cells were incubated with 10  $\mu$ M DCFH-DA and challenged with or without 1 mM H<sub>2</sub>O<sub>2</sub> for 20 min. Fluorescence was measured in a SPECTRAMax GEMINI spectrofluorometer by excitation at 485 nm and emission at 535 nm.

#### Lipid peroxidation assay

Cells were treated with vehicle (ethanol (0.1% v/v)) or 100 nM 1,25-VD for 24 hr, and then challenged with or without 1 mM H<sub>2</sub>O<sub>2</sub> for another 1 hr. Lipid peroxidation assay was performed as described previously.<sup>23</sup> Briefly, cells were lysed in PBS by freeze-thaw cycles, and then cell lysates were mixed with reaction mixture (15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 N hydrochloric acid and 0.5% SDS) for 30 min in a boiling water bath. After cooling, the mixtures were extracted with *n*-butanol, and the absorbance of the organic layer was measured at 532 nm.

#### Real-time polymerase chain reaction (PCR) analysis

Real-time PCR analysis was performed as described previously.<sup>24</sup> The G6PD sense primer was 5'-CTTACCACGC

CACCCTTC-3' and antisense primer was 5'-CAAGACAA CATCTGCCTATCG-3'. Primers for 1,25-VD 24-hydroxylase (CYP24A1) and  $\beta$ -actin were described previously.<sup>20</sup>

#### Statistical analysis

The results are the mean  $\pm$  SEM of values obtained from at least 3 experiments. ANOVA and Student's *t*-test were used to assess the statistical significance, and the statistically significant difference was considered to be present at  $p < 0.05$ .

## Results

### 1,25-VD protects nonmalignant human prostate epithelial cell lines against H<sub>2</sub>O<sub>2</sub>-induced cell death

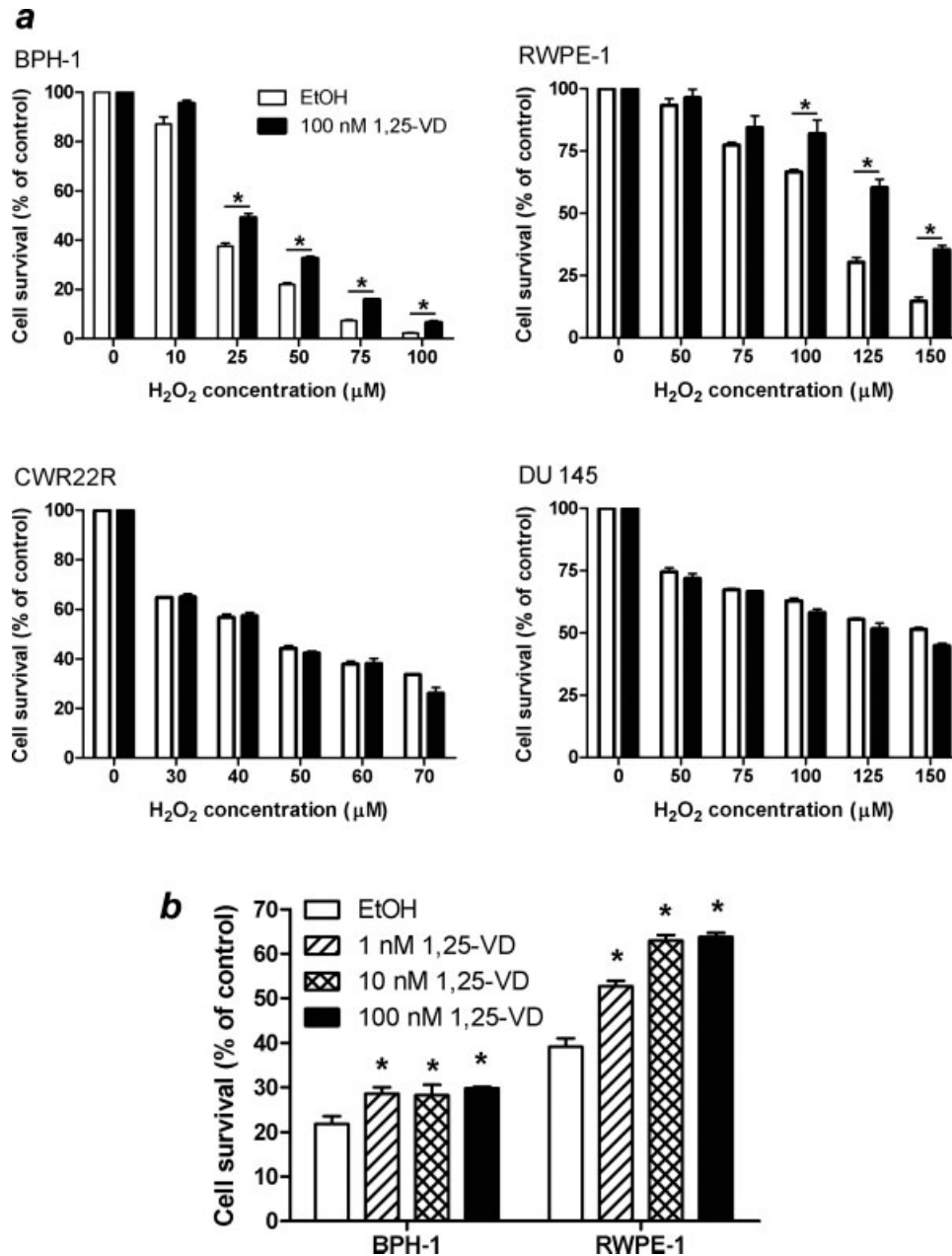
To examine if 1,25-VD can protect cells from oxidative stress-induced cell death, 2 nonmalignant human prostate epithelial cell lines, BPH-1 and RWPE-1, were used to compare with 2 malignant human prostate epithelial cancer cell lines, CWR22R and DU 145. Cells were treated with vehicle control or 100 nM 1,25-VD for 24 hr, and then challenged with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for another 24 hr. Cell viability was determined by MTT assay. As shown in Figures 1a and 1b, 1,25-VD protects BPH-1 and RWPE-1 cells against H<sub>2</sub>O<sub>2</sub>-induced cell death in a dose-dependent manner (increased cell survival from 22 to 30% in BPH-1 and from 39 to 64% in RWPE-1), but not CWR22R and DU 145 cells. The major circulating form of vitamin D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>, can be converted by 1 $\alpha$ -hydroxylase to 1,25-VD and the 1 $\alpha$ -hydroxylase is found to be expressed in higher levels in normal prostate cells compared with prostate cancer cells.<sup>25</sup> Therefore, we expect that treatment with 25-hydroxyvitamin D<sub>3</sub> will result in similar protective effects as treatment with 1,25-VD. These differential protective effects of 1,25-VD in response to H<sub>2</sub>O<sub>2</sub> between nonmalignant and malignant human prostate epithelial cells also imply that 1,25-VD specifically regulates genes involved in ROS elimination pathways in nonmalignant cells, but not in malignant cells. This finding might provide a rationale for using 1,25-VD as a chemoprevention reagent to protect against prostate carcinogenesis.

### An antioxidant enzyme, G6PD, is upregulated by 1,25-VD and is a primary target of 1,25-VD

Large-scale profiling of gene expression revealed that 1,25-VD upregulates several genes controlling redox balance, such as G6PD, GPx and TR. The effects of 1,25-VD on the expression of these genes were examined in human prostate epithelial cell lines by real-time PCR. Among those genes we tested, G6PD, a key enzyme to generate reducing equivalents, NADPH, was found to be consistently induced by 1,25-VD. Therefore, G6PD could be the candidate gene responsible for the antioxidative effect mediated by 1,25-VD.

Furthermore, as shown in Figure 2a, we found that 1,25-VD upregulates G6PD mRNA expression in a time-dependent manner, and reaches to the highest induction at 24 hr, which is similar to CYP24A1, a well-known 1,25-VD target gene. Interestingly, the induction of G6PD by 1,25-VD can be detected in nonmalignant cell lines, but not in malignant cell lines, which correlates with the protective effects of 1,25-VD on H<sub>2</sub>O<sub>2</sub>-induced cell death. The induction of G6PD by 1,25-VD in prostate cells is also dose-dependent (Fig. 2b), suggesting that G6PD might be a primary target gene for 1,25-VD.

To examine if 1,25-VD directly regulates G6PD expression, we analyzed  $\pm 2$  kb genomic sequence of G6PD for putative VDREs. From *in silico* analysis for sequence similarity with consensus VDRE (<http://bio.chip.org/mapper>),<sup>26</sup> 4 putative VDREs, marked as VDRE-A, -B, -C and -D, were found and shown in Figure 2c. Functional analyses of these 4 putative VDREs in the natural chromatin context by ChIP assays found that only the DNA fragment containing VDRE-C can be amplified by PCR from 1,25-VD/VDR-associated immuno-complexes (Fig. 2d). To validate the VDR-DNA association, we used the same chromatin template to

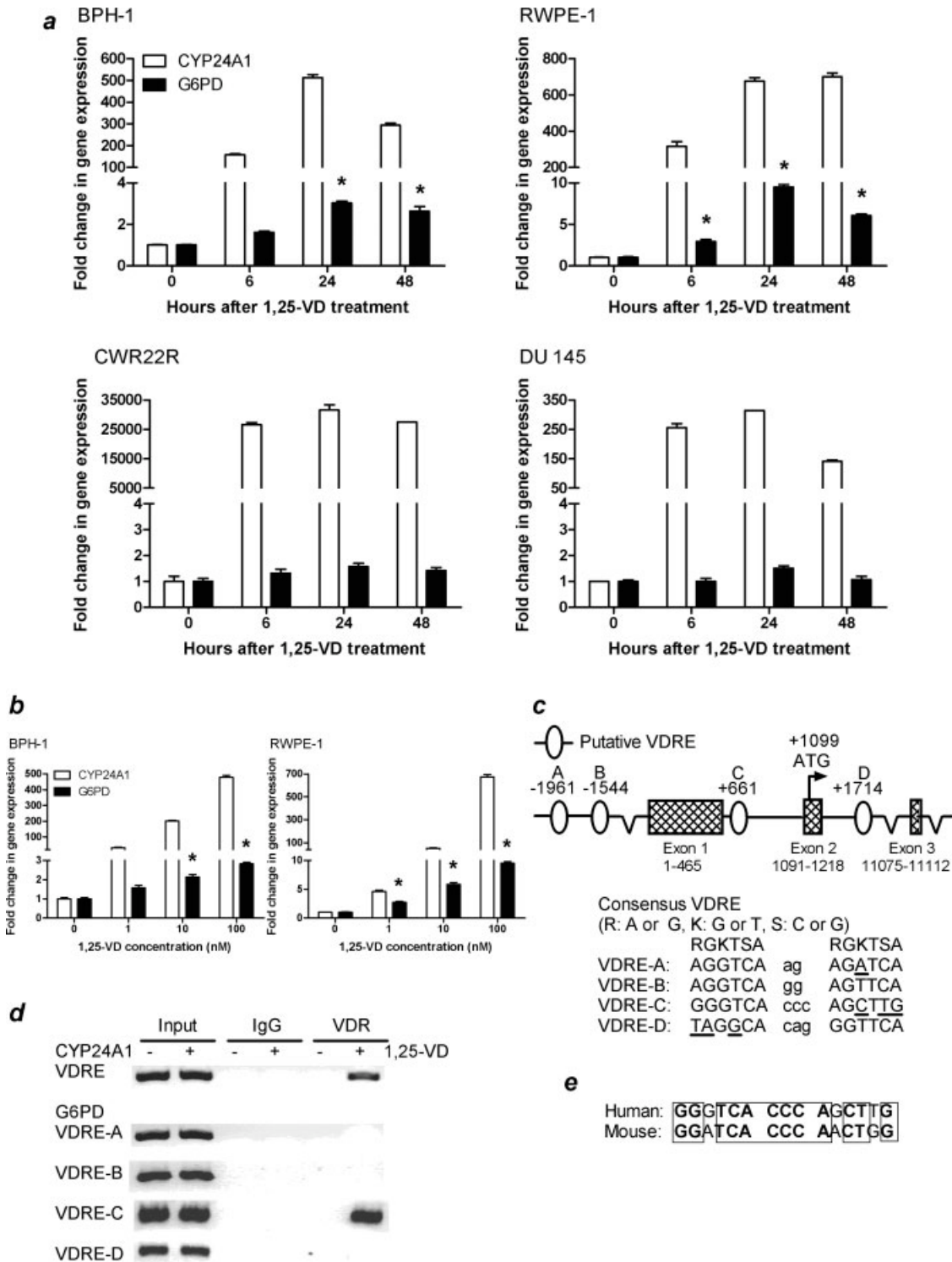


**FIGURE 1** – The effects of 1,25-VD on H<sub>2</sub>O<sub>2</sub>-induced cell death in human prostate epithelial cell lines. (a) 1,25-VD protects nonmalignant human prostate epithelial cell lines against H<sub>2</sub>O<sub>2</sub>-induced cell death. Nonmalignant human prostate epithelial cell lines, BPH-1 and RWPE-1, and malignant cell lines, CWR22R and DU145, were treated with vehicle or 100 nM 1,25-VD for 24 hr, and then challenged with increasing concentrations of H<sub>2</sub>O<sub>2</sub> for another 24 hr. Cell viability was determined by MTT assay. BPH-1 and RWPE-1 cells were treated with vehicle or indicated concentrations of 1,25-VD for 24 hr, and then treated with or without H<sub>2</sub>O<sub>2</sub> (50 μM for BPH-1 and 125 μM for RWPE-1 cells) for another 24 hr. Cell viability was determined by MTT assay. Results are the means ± SEM of at least 3 experiments. \**p* < 0.05, with vs. without 1,25-VD treatment.

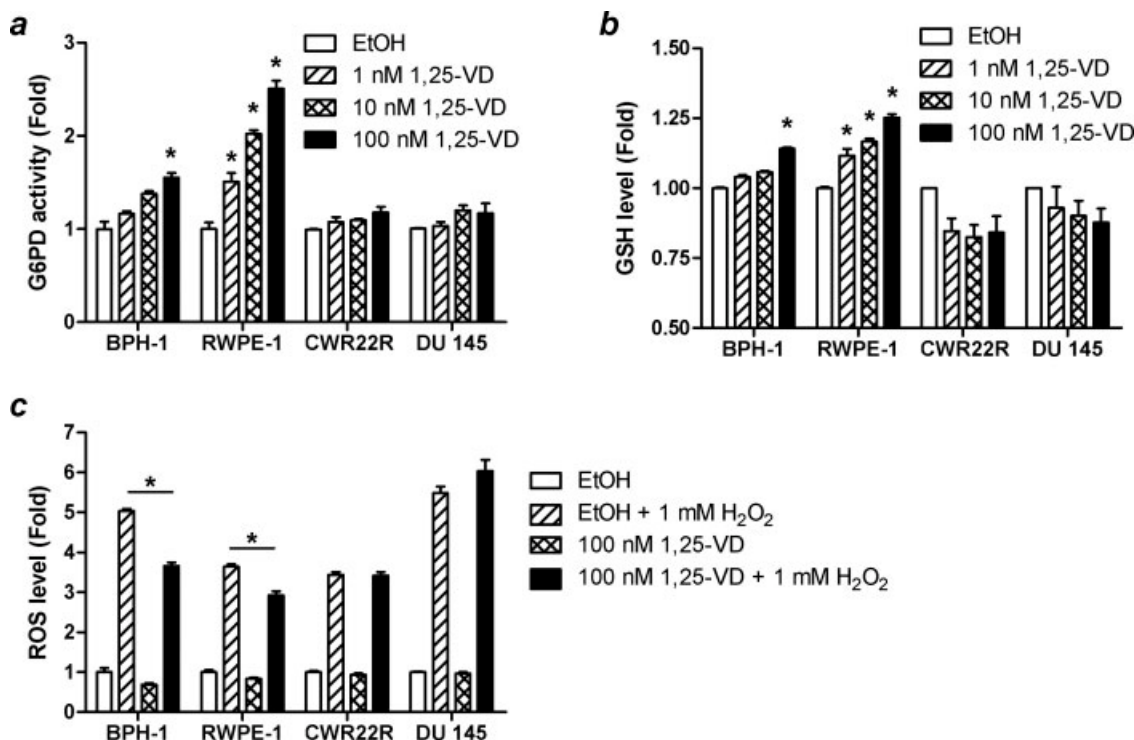
amplify human CYP24A1 proximal VDRE, as a positive control. As shown in Figure 2d, the 1,25-VD/VDR can associate with human CYP24A1 proximal VDRE as well. This novel identified VDRE-C is located in the first intron of the G6PD genome with a DR3 motif, a characteristic of classical VDRE, and is highly conserved between human and mouse (<http://genome.ucsc.edu>) (Fig. 2e). These data showed that VDR is recruited to the VDRE-C upon 1,25-VD treatment *in vivo*, and then consequently induces G6PD expression. Thus, this induction of G6PD might account for the antioxidative potential mediated by 1,25-VD.

#### 1,25-VD upregulates G6PD activity and GSH level to protect cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress

Since we found that 1,25-VD transcriptionally upregulated G6PD expression, we next examined if 1,25-VD can regulate its enzyme activity. Different doses of 1,25-VD were used to treat BPH-1 and RWPE-1 cells, and G6PD activities were measured. As shown in Figure 3a, 1,25-VD dose-dependently upregulated G6PD activity in these 2 nonmalignant prostate cells, but not in malignant prostate cells. To further confirm the antioxidative potential of 1,25-VD, we determined whether the change of GSH,



**FIGURE 2** – The effects of 1,25-Vitamin D<sub>3</sub> on G6PD expression in human prostate epithelial cell lines. (a) 1,25-Vitamin D<sub>3</sub> time-dependently upregulates G6PD mRNA expression in BPH-1 and RWPE-1 cells, but not in CWR22R and DU 145 cells. (b) 1,25-Vitamin D<sub>3</sub> dose-dependently upregulates G6PD mRNA expression in BPH-1 and RWPE-1 cells. Human prostate epithelial cells were treated with 100 nM 1,25-Vitamin D<sub>3</sub> for indicated time periods (a), or indicated concentrations of 1,25-Vitamin D<sub>3</sub> for 24 hr (b). CYP24A1 and G6PD mRNA expressions were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to control. Results are the means ± SEM of at least 3 experiments. \**p* < 0.05 vs. ethanol vehicle control. (c) *In silico* analysis indicated 4 putative VDREs (VDRE-A to VDRE-D) found within ±2 kb of the human G6PD genome. The 2 hexameric half sites of each VDRE core sequence are shown in capitals. Deviations from the consensus sequence are underlined. (d) Identification of functional VDREs in G6PD genome that are transcriptionally activated by 1,25-Vitamin D<sub>3</sub> in RWPE-1 cells. RWPE-1 cells were treated with vehicle or 100 nM 1,25-Vitamin D<sub>3</sub> for 2 hr, and then subjected to ChIP analysis using antibodies against VDR or IgG. Immunoprecipitated DNA was subjected to PCR amplification, and an amplification of the DNA using primers to CYP24A1 was also examined as a control. Representative agarose gels are shown. (e) The putative VDRE-C sequence is highly conserved between human and mouse.



**FIGURE 3** – 1,25-VD upregulates G6PD activity and GSH levels to protect BPH-1 and RWPE-1 cells from  $H_2O_2$ -induced oxidative stress. 1,25-VD upregulates G6PD activity (a) and GSH level (b). Human prostate epithelial cells were treated with vehicle or indicated concentrations of 1,25-VD for 24 hr. G6PD activities and GSH levels were determined, and data represent the fold differences relative to control. Results are the means  $\pm$  SEM of at least 3 experiments. \* $p < 0.05$ , 1,25-VD treatment vs. ethanol vehicle control. (c) 1,25-VD decreases  $H_2O_2$ -induced ROS accumulation. Human prostate epithelial cells were treated with vehicle or 100 nM 1,25-VD for 24 hr and then treated with or without 1 mM  $H_2O_2$  for another 20 min. ROS accumulation was measured by DCF fluorescence. Data represent the fold differences relative to control. Results are the means  $\pm$  SEM of at least 3 experiments. \* $p < 0.05$ , with vs. without 1,25-VD treatment.

downstream of G6PD in the ROS elimination pathway and one of the most abundant intracellular antioxidants, provides an alternative way of monitoring oxidative stress within cells. As shown in Figure 3b, increased G6PD activity upon 1,25-VD treatment results in increasing GSH levels, which were also observed in non-malignant cells only, suggesting that 1,25-VD might reduce ROS through increasing GSH level. Intracellular ROS levels upon 1,25-VD treatment were also examined using the nonfluorescent compound, DCFH-DA, that turns fluorescent upon oxidation by ROS. Figure 3c showed that ROS levels increased when cells were treated with  $H_2O_2$ , and 1,25-VD pretreatment reduced  $H_2O_2$ -induced ROS levels in nonmalignant prostate cells. However, 1,25-VD didn't show any significant effects in malignant cells, which might be due to the lack of 1,25-VD-induced responses on G6PD expression, activity and GSH level. Together, these data proved that 1,25-VD upregulates G6PD activity and the level of its downstream reducing equivalent, GSH, to scavenge ROS.

#### *Inhibition of G6PD activity by DHEA diminishes the protective effects of 1,25-VD*

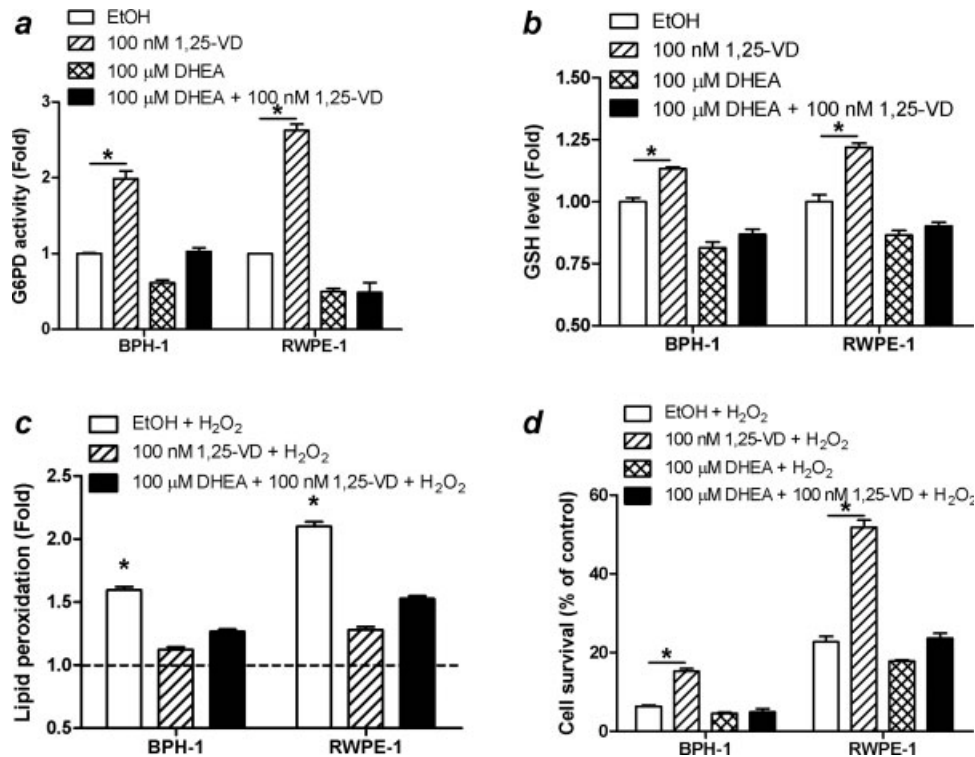
To assess the involvement of G6PD in the protective effects of 1,25-VD against  $H_2O_2$ -induced cell death, the G6PD activity was blocked by treating cells with the known noncompetitive G6PD inhibitor, dehydroepiandrosterone (DHEA),<sup>21</sup> and then we measured the GSH levels, lipid peroxidation and cell viability. As expected, 1,25-VD induced around 2- to 3-fold G6PD activities, while DHEA can inhibit ~40–50% of basal G6PD activities. The induction of G6PD activity by 1,25-VD was also abolished by DHEA treatment (Fig. 4a). Furthermore, the inhibition of G6PD with DHEA also decreased 1,25-VD-induced GSH levels (Fig. 4b). The cellular damages induced by oxidative stresses can be also determined by the lipid peroxidation status. As shown in Figure 4c, the levels of lipid peroxidation were significantly

increased after  $H_2O_2$  challenge. Administration of 1,25-VD markedly prevented  $H_2O_2$ -induced lipid peroxidation, however, with the inhibition of G6PD by DHEA, the preventive effects of 1,25-VD on lipid peroxidation were reduced. Finally, we examined whether the protective effects of 1,25-VD against  $H_2O_2$ -induced cell death could also be attenuated by adding DHEA to block G6PD. As shown in Figure 4d, 1,25-VD alone can protect cells from  $H_2O_2$ -induced cell death, and its protective effects were abolished by DHEA.

In summary, our data, for the first time, provides a molecular mechanism to illustrate that 1,25-VD can protect nonmalignant human prostate epithelial cells from  $H_2O_2$ -induced cell death by promoting G6PD activity, and GSH levels to reduce oxidative cellular injuries. This protective effect of 1,25-VD is indeed distinct from its antitumor effects, where 1,25-VD inhibits cancer cells growth. These findings would certainly add to our knowledge about the versatility of desirable activities mediated by 1,25-VD, and demonstrate that the cellular microenvironment affects 1,25-VD activities.

#### **Discussion**

The generation of ROS is a consequence of aerobic life and is unavoidable. Overproduction of ROS and accumulation of oxidative damages during the life span have been proposed to play a key role in the development of age-dependent diseases, such as cancer.<sup>27</sup> In this study, we have demonstrated that 1,25-VD can protect nonmalignant human prostate epithelial cells against  $H_2O_2$ -induced cell death through modulating the ROS defense systems, suggesting a possible role of 1,25-VD in prostate cancer prevention.



**FIGURE 4** – Inhibition of G6PD activity by DHEA diminishes the protective effects of 1,25-Vit D. DHEA decreases 1,25-Vit D-induced G6PD activity (a) and GSH level (b). BPH-1 and RWPE-1 cells were treated with vehicle, 100 nM 1,25-VD, 100  $\mu$ M DHEA, or in combination for 24 hr and then G6PD activities (a) and GSH levels (b) were determined. Data represent the fold differences relative to control. DHEA diminishes the protective effect of 1,25-VD on  $H_2O_2$ -induced lipid peroxidation (c) and cell death (d). (c) BPH-1 and RWPE-1 cells were treated with vehicle, 100 nM 1,25-VD, 100  $\mu$ M DHEA, or in combination for 24 hr, and then treated with or without 1 mM  $H_2O_2$  for another 1 hr. Lipid peroxidation was measured by lipid peroxidation assay. Data represent the fold differences relative to control. (d) BPH-1 and RWPE-1 cells were treated with vehicle, 100 nM 1,25-VD, 100  $\mu$ M DHEA, or in combination for 24 hr, and then treated with or without  $H_2O_2$  (75  $\mu$ M for BPH-1 and 125  $\mu$ M for RWPE-1 cells) for another 24 hr. Cell viability was determined by MTT assay. Results are the means  $\pm$  SEM of at least 3 experiments. \* $p$  < 0.05, with vs. without 1,25-VD treatment.

G6PD has been considered to have important housekeeping functions on producing reducing equivalents and maintaining the cellular redox state in most tissues. Oxidative stress, generated from dietary or pharmaceutical sources, has been demonstrated to cause hemolysis of erythrocytes in G6PD-deficient individuals. The G6PD gene is X-linked, and thus only a single copy presents in human and other lower life forms. Subsequent studies have shown that G6PD gene mutations are one of the most common human genetic abnormalities and affect around 400 million people world-wide.<sup>28,29</sup> In the promoter studies, the proximal 400 bp of the human G6PD promoter is sufficient to drive the basal gene expression, and the distal promoter contains *cis*-elements responsible for hormone (insulin), nutrition (glucose) and oxidative stress.<sup>30</sup> For example, insulin has been clearly shown to upregulate G6PD expression through an insulin-response sequence,<sup>31</sup> and, not surprisingly, agents that increase oxidative stress are also known to induce G6PD through the oxidative stress-response element.<sup>32</sup> However, the level of upregulation of promoter activity is not compatible with the increase in endogenous G6PD mRNA induced by insulin and oxidative stress.<sup>33</sup> Therefore, it is possible that additional regulatory elements are located in the first intron, as G6PD sequence is highly conserved between human and mouse.<sup>34</sup> These observations support our finding that 1,25-VD/VDR could upregulate G6PD expression through binding to VDRE identified in the first intron.

Induction of G6PD is consistent with the protective effect of 1,25-VD against oxidative stress. However, this finding is controversial in the vitamin D field in determining whether 1,25-VD acts as a pro-oxidant or antioxidant. Cotreatment of breast cancer

MCF-7 cells with 1,25-VD markedly increases the susceptibility to the cytotoxic cytokines (TNF, IL-1 and IL-6), anticancer drugs (doxorubicin) and ionizing radiation.<sup>35–37</sup> It was found that 1,25-VD exacerbated TNF-induced depletion of the GSH pool, and the thiol antioxidant, *N*-acetylcysteine, partially abolished the enhancement of cytotoxicity by 1,25-VD.<sup>35,38</sup> 1,25-VD also reduced Cu/Zn superoxide dismutase activity, mRNA and protein levels in MCF-7 cells.<sup>36</sup> These data suggested that 1,25-VD increased ROS and decreased antioxidant capacity, therefore, potentiated the effects of ROS-generating drugs.

On the other hand, there are also several lines of evidence indicating that 1,25-VD exerts antioxidative activities. Vitamin D can act as a membrane antioxidant to inhibit iron-induced lipid peroxidation of brain liposomes.<sup>39</sup> Administration of vitamin D suppressed the elevated lipid peroxidation in vitamin D-deficient rats.<sup>40</sup> 1,25-VD has been found to inhibit endotoxin shock through regulation of free radical formation in mice.<sup>41</sup> 1,25-VD enhanced intracellular GSH pools, and thus decreased free radical formation in rat primary astrocytes.<sup>42</sup> Furthermore, vitamin D has also been reported to reduce oxidative stress by upregulating antioxidant systems, including GSH, GPx and superoxide dismutase in rats.<sup>43</sup> The results from these studies suggested that vitamin D participated in antioxidant machinery and protected cells from oxidative challenge.

The conclusion from these studies, as to the roles of 1,25-VD in regulating ROS systems, seems to be dependent on the cellular environment. The pro-oxidative effects of 1,25-VD are usually found in cancer cells, and the antioxidant effects of 1,25-VD usually occur in animal and primary cultured cells. This idea was fur-

ther supported in our studies, as we found that the protective effects of 1,25-VD against ROS occurred in nonmalignant human prostate epithelial cells, but was lost in malignant cells (Fig. 1a). It suggested that 1,25-VD acts as a pro-oxidant in malignant cells, but as an antioxidant in nonmalignant cells. Our data further indicated that the low induction of G6PD activity and expression by 1,25-VD in malignant prostate cells contribute to these differential effects (Fig. 2a). In addition, vitamin D signals are frequently found to be downregulated during the late stages of cancer progression through overexpression of SNAIL or other unknown mechanisms.<sup>44,45</sup> These data suggest that vitamin D might exert a subtle oxidative stress, which could stimulate the detoxification mechanisms to protect cells from the subsequent stress challenges; yet cancer cells lose the 1,25-VD-induced detoxification responses, therefore, 1,25-VD acts as

a pro-oxidant in cancer cells. Together, the cell microenvironment might contribute to the differential 1,25-VD actions, but detailed mechanisms need to be further investigated. Our results provide one mechanism to explain how 1,25-VD protects nonmalignant human prostate epithelial cells from oxidative stress to attenuate the accumulation of oxidative damages during the life. Therefore, vitamin D might be beneficial for preventing the development of age-dependent diseases.

### Acknowledgements

The authors are grateful to Dr. Lise Binderup from Leo Pharmaceutical Products for providing the 1,25-VD. They also thank Ms. Karen Wolf for manuscript preparation.

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