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Protection Against Cellular Stress by 25-Hydroxyvitamin D_3 in Breast Epithelial Cells

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ABSTRACT

25-Hydroxyvitamin D_3 ($25(OH)D_3$) is a prohormone and a major vitamin D metabolite. The discovery of ($25(OH)D_3$) 1α-hydroxylase in many vitamin D target organs has yielded an increased interest in defining the role(s) of $25(OH)D_3$ in these tissues. The etiology of cancer appears to be complex and multi-factorial. Cellular stress (e.g., DNA damage, hypoxia, oncogene activation) has been identified as one of the key factors responsible for initiating the carcinogenesis process. In this study, we investigated whether $25(OH)D_3$ protects breast epithelial cells from cellular stress using an established breast epithelial cell line MCF12F. To better elucidate the role of $25(OH)D_3$ in the stress response, we used multiple in vitro stress models including serum starvation, hypoxia, oxidative stress, and apoptosis induction. Under all these stress conditions, $25(OH)D_3$ (250 nmol/L) treatment significantly protected cells against cell death. Low-serum stress induced p53 expression accompanied with downregulation of PCNA, the presence of $25(OH)D_3$ consistently inhibited the alteration of p53 and PCNA, suggesting that these molecules were involved in the stress process and may be potential target genes of $25(OH)D_3$. miRNA microarray analysis demonstrated that stress induced by serum starvation caused significant alteration in the expression of multiple miRNAs including miR182, but the presence of $25(OH)D_3$ effectively reversed this alteration. These data suggest that there is a significant protective role for $25(OH)D_3$ against cellular stress in the breast epithelial cells and these effects may be mediated by altered miRNA expression. J. Cell. Biochem. 110: 1324–1333, 2010.

KEY WORDS: $25(OH)D_3$; BREAST EPITHELIAL CELLS; CELLULAR STRESS

The active metabolite of vitamin D, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) has long been recognized as one of the most potent differentiating agents. It has been reported to have both anti-proliferative and differentiating effects on various cell types [Mehta et al., 2000; Swami et al., 2005; Hussain-Hakimjee et al., 2006; Marchionatti et al., 2009; Mahajan and Stahl, 2009]. However, the use of $1,25(OH)_2D_3$ is precluded due to its toxicity (e.g., hypercalcemia) as demonstrated in several pre-clinical toxicity studies. On the other hand, the prohormone of the active metabolite, $25(OH)D_3$, is relatively non-toxic and has shown no toxicity in our pre-clinical studies (unpublished data). However, since $1,25(OH)_2D_3$ is the active vitamin D hormone and is a natural ligand for vitamin D binding receptor (VDR) not much attention has been focused on evaluating the chemopreventive efficacy of the $25(OH)D_3$ in carcinogenesis.

Epidemiological studies have demonstrated a negative correlation between breast, prostate, and colon cancer incidence and mortality rates with both sunlight exposure and serum levels of $25(OH)D_3$ [Garland et al., 1990; Gorham et al., 1990; Ainsleigh, 1993; Lipkin and Newmark, 1999; Cross et al., 2003; Friedrich et al., 2003; Grant and Garland, 2004; Lowe et al., 2005]. ($25(OH)D_3$) 1α-hydroxylase (CYP27B1), the enzyme that catalyzes hydroxylation at C1 to generate the biologically active VDR ligand—$1,25(OH)_2D_3$, has been identified in multiple vitamin D target tissues including breast [Cross et al., 2003, 2004; Friedrich et al., 2003; Zinser and Welsh, 2004; Holt et al., 2009; McCarthy et al., 2009; Zhong et al., 2009]. In addition, $25(OH)D_3$ is present at a much higher concentration in serum in comparison to that of $1,25(OH)_2D_3$ [Lou et al., 2004]. This raises the possibility that $25(OH)D_3$ could be an active hormone and be efficacious for organs where CYP27B1 is expressed. In support of

Abbreviations used: $25(OH)D_3$, 25-hydroxycholecalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; VDR, vitamin D binding receptor; DFX, deferoxamine; H_2O_2, hydrogen peroxide; CV, crystal violet.

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the concept that $25(OH)D_3$ may be an active hormone, we recently observed that $25(OH)D_3$ directly activates the VDR target gene CYP24 in mammary glands isolated from CYP27B1 knock-out mice and inhibits pre-cancerous lesion formation induced by DMBA in mouse mammary organ culture system [Peng et al., 2009]. These results indicate that $25(OH)D_3$ by itself may be active without being converted to $1,25(OH)_2D_3$. It also suggests that $25(OH)D_3$ may be the major vitamin D metabolite that modulates cell proliferation in the body. Although several vitamin D analogs have been developed in the hopes of generating potent anti-cancer agents without the toxicity exhibited by the parent compound, these data support the use of natural metabolites of vitamin D (i.e., $25(OH)D_3$) present in serum to maintain homeostasis against cell transformation at a physiological concentration. However, the mechanism of action of $25(OH)D_3$ as a chemopreventive agent is yet to be elucidated.

The genesis of cancer has been linked to several factors including cellular stress. For example, hypoxia is a well-documented tumor microenvironment factor [Kulshreshtha et al., 2007]. In recent years, many studies have shown that oxidative stress is tightly associated with carcinogenesis; moreover, many chemopreventive agents have been shown to play their role as antioxidants and improve cellular redox status [Moselhy and Al Mslmani, 2008]. Reactive oxygen species contribute to the accumulation of mutations in the genome, presumably followed by selective processes. Recent data suggest that preferred signaling pathways exist for oxidative stress-associated carcinogenesis [Toyokuni, 2006]. Thus, an ideal chemopreventive agent should protect cells from stress caused from carcinogenic stimuli. The functional significance of chemopreventive agents such as vitamin D as possible suppressors of stress-mediated damage has not been evaluated. This study aims to explore the anti-stress action of $25(OH)D_3$ in a non-malignant MCF12F breast epithelial cell line using various stress model systems in an attempt to provide evidence for its chemopreventive action from a different angle.

The miRNA represents a class of small non-coding RNAs that control gene expression by targeting mRNA and triggering translation repression or RNA degradation [Chan et al., 2005; Zhu et al., 2007]. Accumulating evidence suggests that miRNAs are subject to changes in regulation of gene expression in cancers [Iorio et al., 2005; Gaur et al., 2007; Tran et al., 2007; Tricoli and Jacobson, 2007]. Since miRNAs can contribute to cancer development and are differentially expressed in normal tissues versus cancers, they may serve as key targets for cancer chemoprevention. However, little is known about the miRNA involvement in vitamin D-mediated biological activities.

MATERIALS AND METHODS

CELL LINE AND CULTURE CONDITIONS

MCF12F and MCF10A normal breast epithelial and T47D breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). The MCF12F and MCF10A cells were cultured in DMEM/F12 containing 5% charcoal-stripped horse serum, supplemented with 20 ng/ml EGF (Invitrogen Corp, Carlsbad, CA), 10 μg/ml insulin (Sigma–Aldrich, Milwaukee, WI), 100 ng/ml cholera toxin (Sigma–Aldrich), and 0.5 μg/ml hydrocortisone. T47D cells were cultured in MEM containing 10% FBS and supplemented with non-essential amino acids and antibiotics. $25(OH)D_3$ and $1,25(OH)_2D_3$ were purchased from Cayman Chemical (Ann Arbor, MI), dissolved in ETOH and stored in −80°C freezer. $1,25(OH)_2D_3$ served as a positive control in some of the experiments.

Induction of Experimental Cellular Stress. Four selective approaches for cellular stress induction were taken. (1) For serum starvation (low-serum stress), cells were first cultured in normal growth medium overnight and then stressed with low-serum medium (MEM containing 2% charcoal-stripped FBS without any supplements) for a period ranging from 24 h to 7 days, vitamin D metabolites $25(OH)D_3$ (250 nmol/L) or $1,25(OH)_2D_3$ (10 nmol/L) were added at the same time when normal growth medium was replaced with stress medium. Medium was replaced every other day. The end points included miRNA, protein expression, or cell number at the termination of the experiment. (2) For oxidative stress, cells were first pre-treated with ETOH (control) or 250 nmol/L $25(OH)D_3$ for 24 h, then cells were treated with 0, 100, 200, and 400 μmol/L H_2O_2 for 1 h in the absence or presence of $25(OH)D_3$, medium was then replaced with fresh normal growth medium to allow cells to recover for 24 h without any stress and cell number was evaluated using the crystal violet (CV) assay. (3) For apoptotic stress, cells were treated with the protease inhibitor MG132 (40 μmol/L) for 16 h to induce apoptosis. Three vitamin D treatment protocols were used: (a) cells were primed with $25(OH)D_3$ for 24 h first, then medium was replaced with fresh normal growth medium (without $25(OH)D_3$) and cells were exposed to MG132 in absence of $25(OH)D_3$ for 16 h (pre-treatment); (b) the cells were treated with MG132 and $25(OH)D_3$ for the entire 16 h period (co-treatment); (c) cells were primed with $25(OH)D_3$ for 24 h, then stressed with MG132 for 16 h in the presence of $25(OH)D_3$ (complete treatment). (4) For hypoxia stress, cells were treated with deferoxamine (DFX, 250 μmol/L) for 2 days. Vitamin D treatment protocols for hypoxia stress were similar for pre-treatment and co-treatment with $25(OH)D_3$ as described above for MG132, except that instead of 16 h treatment of MG132 (described above), DFX treatment was carried out for 48 h to induce significant cell death. The iron chelator and hypoxia mimetic DFX enhances both iron regulatory protein 1 expression and its endoplasmic reticulum (ER) membrane-binding activity, as occurs in hypoxia, an ER stress, in cultured cells [Yoo et al., 2008; Hiroi et al., 2009].

CELL PROLIFERATION ASSAY

Cell proliferation was evaluated using the CV assay and/or cell counting using the Z1 Coulter Particle Counter (Beckman Coulter, Brea, CA). For the CV assay, cells were cultured in 24-well plates and treated for different time points, medium was removed and cells were washed with PBS and fixed for 15 min with 0.4 ml 1% glutaraldehyde. The cells were stained with 0.4 ml 0.1% CV for 20 min at room temperature. CV was discarded; cells were washed with tap water and incubated with 0.4 ml PBS containing 0.2% Triton X-100 on a shaker for 30 min at room temperature, OD_{570} was measured using a microplate reader. Our preliminary studies showed that the absorbance (OD value) is proportional to cell number. For cell counting, cells were cultured in 6-well plates for appropriate time points then were trypsinized and cell number from each well was determined by using the Coulter Counter.

WESTERN BLOT ANALYSIS

When cells grew to 50% confluence, they were exposed to low-serum stress for 2, 4, or 6 days in the presence and absence of vitamin D metabolites, then cell lysates were prepared and 50 μg total protein was loaded for western blot analysis as previously described [Peng et al., 2006]. Rat anti-VDR monoclonal antibody was purchased from NeoMarkers, Inc. (Freemont, CA) whereas all other antibodies including monoclonal antibodies against p53 and PCNA, polyclonal antibody against β-actin and all secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

MIRNA MICROARRAY ANALYSIS

MCF12F cells were cultured in normal growth medium to 50% confluence, then the medium was replaced with low-serum medium (MEM containing 2% charcoal-stripped FBS) and cells were incubated for 24 h in the presence or absence of $25(OH)D_3$ (250 nmol/L), small RNA was isolated with mirVana™ miRNA isolation kit (Ambion, Austin, TX) as per the manufacturer's instruction. miRNA microarray was carried out using Invitrogen's NCode™ Multi-Species miRNA Microarray Kit V2 as per the manufacturer's protocol. Data were analyzed with GeneSpring GX v7.3.1 software packages. Genes from the dataset that met the fold change cut-off of 2 were selected for further analysis.

REVERSE TRANSCRIPTION AND REAL-TIME PCR

RT reaction was performed as described previously [Peng et al., 2006]. Two RT reactions for each sample were pooled and diluted with equal amount of DNase/RNase free water. Real-time PCR was performed with 2 μl diluted RT product in a MyiQ Real-time PCR Detection System (Bio-Rad, Hercules, CA) by using iQ™ SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer's guidelines. The PCR cycling conditions used were: 40 cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Fold inductions were calculated using the formula 0, where $\triangle\triangle C_t$ is $\triangle C_{t(treatment)} - \triangle C_{t(control)}$, $\triangle C_t$ is $C_{t(target\ gene)} - C_{t(5s)}$ and C_t is the cycle at which the threshold is crossed. The gene specific primers were designed based on miRNA precursor sequences (http://microrna.sanger.ac.uk/sequences/) using primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The 5S rRNA primers were forward 5′-GCCCGATCTCGTCTGATCT-3′ and reverse 5′-AGCCTACAGCACCCG-GTATT-3′ (116 bp). PCR product quality was monitored using post-PCR melt curve analysis.

MIRNA TRANSFECTION

The miRNA transfection was conducted in MCF12F cells using lipofectomine 2000 (Invitrogen) following the manufacturer's protocol. The Pre-miR miRNA precursors for miR-182 (product ID: PM11090) and negative control (AM17110) were purchased from Ambion. The final concentration of pre-miR-182 in culture medium was 100 nmol/L. MCF12F cells were cultured overnight in normal growth medium, then transfected with pre-miR-182 or negative control in OPTI-MEM containing 3% FBS for 20 h. The transfected cells were cultured in normal growth medium for 5 days and cell proliferation was evaluated using CV assay.

STATISTICAL METHODS

Data are presented as mean values ± SD. All results were analyzed by Student's *t*-test or one-way ANOVA. The Mewman–Keuls multiple comparison test was used for all post-analyses. The statistical packgage used for these studies was Graphpad Software (San Diego, CA). Differences between means were considered significant when $P < 0.05$ was obtained.

RESULTS

$25(OH)D_3$ PROTECTS MCF12F CELLS AGAINST LOW-SERUM INDUCED STRESS IN CULTURE

The MCF12F cell line is a non-tumorigenic breast epithelial cell line, which is responsive to vitamin D treatment. The MCF12F cells are generally cultured in DMEM/F12 containing 5% charcoal-stripped horse serum with multiple supplements including insulin, EGF, hydrocortisone, and cholera toxin to maintain cell growth. In this normal culture condition, both $1,25(OH)_2D_3$ (10 nmol/L) and $25(OH)D_3$ (250 nmol/L) significantly inhibited cell growth by ~50% after 7 day treatment ($P < 0.001$; Fig. 1A). In the low-serum stressed culture condition (MEM with 2% charcoal-stripped FBS without supplements), both $25(OH)D_3$ and $1,25(OH)_2D_3$ significantly and consistently protected cells against stress (Fig. 1B). The cell proliferation studies demonstrated that the presence of $25(OH)D_3$ or $1,25(OH)_2D_3$ (positive control) either maintained cell proliferation or inhibited cell death. Results from the CV assay showed that treatment of cells with 250 nmol/L $25(OH)D_3$ increased cell number by 110% ($P < 0.001$) in comparison to the corresponding control in low-serum stressed culture condition (Fig. 1B). Dose–response experiments with MCF12F cells demonstrated that $25(OH)D_3$ at 250 nmol/L was the most effective concentration in protecting cells against low-serum induced stress (data not shown), therefore this concentration (250 nmol/L) was used for other stress models. We also tested other breast epithelial cell lines to determine whether the protective effect of $25(OH)D_3$ was common to other cell lines. Results showed that $25(OH)D_3$ at 250 nmol/L also protected T47D breast cancer cells against low-serum induced stress (data not shown); however, $25(OH)D_3$ did not show any significant protective effect for MCF10A cells which are less-responsive to vitamin D (data not shown).

$25(OH)D_3$ PROTECTS MCF12F CELLS AGAINST H_2O_2-INDUCED OXIDATIVE STRESS

Since $25(OH)D_3$ protects MCF12F cells against low-serum induced stress, we further evaluated the protective effects of $25(OH)D_3$ in MCF12F cells using various other established stress models. In order to determine the effects of $25(OH)D_3$ on oxidative stress, cells were incubated for 1 h with different concentrations of H_2O_2. As shown in Figure 2, treatment with H_2O_2 significantly induced cell death in a dose-dependent manner (left panel), however in the presence of $25(OH)D_3$, cell death induced by H_2O_2 treatment decreased (right panel) at each dose of H_2O_2 in comparison to the corresponding control (H_2O_2 only treatment without $25(OH)D_3$ protection, left panel). The CV assay demonstrated that oxidative stress reduced the normalized absorbance by 35% and 75% ($P < 0.001$, left panel) at 100 and 200 μmol/L of H_2O_2, respectively in comparison to control.

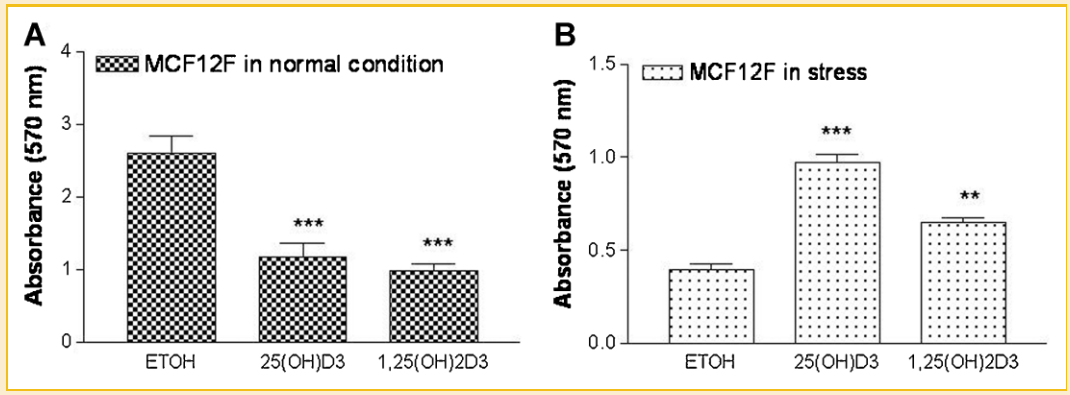

Fig. 1. Protective effect of vitamin D against low-serum-induced stress in MCF12F cells. MCF-12F cells were seeded in 24-well plates (38,000 cells/well), treated with 0.01% ETOH (control), 250 nmol/L 25(OH)D_3, and 10 nmol/L 1,25$(OH)_2D_3$ for 7 days under normal (DMEM/F12 with 5% charcoal-stripped horse serum and supplements; A) and stressed (MEM with 2% charcoal-stripped FBS; B) culture conditions. Cell proliferation was evaluated using the CV assay. Bar, mean ± SD; $^{***}P < 0.001$, $^{**}P < 0.01$ in comparison to corresponding control (ETOH), n = 8 (one-way ANOVA test). Data shown are from a representative experiment of at least three independent experiments.

However, in the presence of 25(OH)D_3, treatment with H_2O_2 at 100 and 200 μmol/L only decreased the normalized absorbance by 25% and 51% ($P < 0.001$, n = 4, right panel), respectively. Statistical analysis of the data showed that the protective effects of 25(OH)D_3 against oxidative damage were significant at the concentration of 100 ($P < 0.05$) and 200 μmol/L ($P < 0.001$) of H_2O_2, but not at the high concentration of H_2O_2 (400 μmol/L, cells might be overstressed) at this experimental setting.

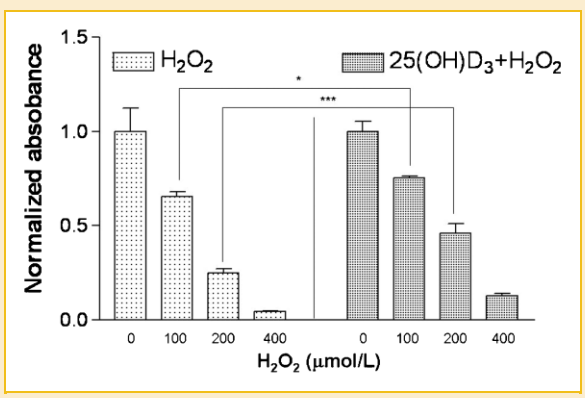

Fig. 2. Protective effect of 25(OH)D_3 against oxidative stress in MCF12F cells. MCF-12F cells were seeded in 24-well plates (38,000 cells/well) in normal culture condition and incubated overnight. MCF12F cells were first pre-treated with ETOH (control, left panel) or 250 nmol/L 25(OH)D_3 (treatment, right panel) for 24 h, then stressed with H_2O_2 (0–400 μmol/L) for 1 h in the absence (control, left panel) and presence of 25(OH)D_3 (treatment, right panel), medium was then replaced with normal growth medium to allow cells to recover for another 24 h in the absence of oxidative stress and 25(OH)D_3 protection, cell number was evaluated by CV assay. Absorbance was normalized to the control (0 μmol/L H_2O_2) in each panel. Note the cell death rate (slope) significantly decreased in the presence of 25(OH)D_3. Bar, mean ± SD; $^{*}P < 0.05$, $^{***}P < 0.001$ in comparison to its corresponding control in the left panel (25(OH)D_3 + H_2O_2 vs. H_2O_2 at 100 and 200 mmol/L of H_2O_2), n = 4 (one-way ANOVA test). Data shown are from a representative experiment of three independent experiments.

25(OH)D_3 PROTECTS MCF12F CELLS AGAINST HYPOXIA STRESS

Hypoxia-induced stress has been used as another important experimental stress model. Therefore, for the present studies DFX (250 μmol/L) was used to mimic hypoxia stress. Figure 3 shows the protective effect of 25(OH)D_3 against hypoxia stress induced by DFX in MCF12F cells. DFX treatment for 48 h caused 42% cell death ($P < 0.01$), whereas in the presence of 25(OH)D_3, DFX treatment only resulted in 25% cell death, suggesting that 25(OH)D_3 significantly inhibited DFX-induced cell death by 40% ($P < 0.05$). However, pre-treatment of MCF12F cells with 25(OH)D_3 for 24 h before exposure to DFX did not show any significant protective effect. 25(OH)D_3 treatment for 2 days in normal culture conditions only exhibited a minor inhibitory effect which was not statistically significant. Treatment with 10 nmol/L 1,25$(OH)_2D_3$ also showed a similar protective effect against hypoxia stress in the same model system (data not shown).

25(OH)D_3 PROTECTS MCF12F CELLS AGAINST APOPTOTIC STRESS INDUCED BY THE PROTEASE INHIBITOR MG132

MG132 has been used as an apoptotic stress inducer. In the present experiments, we determined whether vitamin D protects MCF12F cells against apoptotic stress induced by MG132. As shown in Figure 4, MG132 (40 μmol/L) treatment for 16 h caused 45% cell death in the absence of 25(OH)D_3, while 24 h pre-treatment of MCF12F cells with 25(OH)D_3 significantly decreased cell death (induced by MG132) by 25% in comparison to that of MG132 treatment ($P < 0.01$); full treatment (pretreatment + co-treatment with 25(OH)D_3) also decreased cell death by 19% ($P < 0.05$). Although 25(OH)D_3 co-treatment inhibited cell death induced by MG132, the inhibition was not statistically significant ($P > 0.05$). In addition, we also found that 25(OH)D_3 protected MCF12F cells against actinomycin D-induced apoptosis (data not shown). These data collectively suggest that 25(OH)D_3 protected MCF12F cells from apoptotic stress induced through different pathways and priming cells with 25(OH)D_3 appeared to be important for its effective protection against MG132-induced cell death.

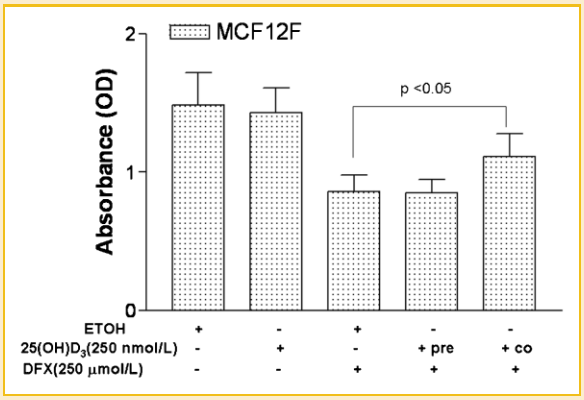

Fig. 3. Effect of 25(OH)D_3 on cell growth under hypoxia stress. MCF-12F cells were cultured in 24-well plates, treated with DFX (250 μmol/L Deferoxamine) in the presence and absence of 25(OH)D_3 (250 nmol/L) for 48 h. Then cells were subjected to the CV assay. Absorbance of each treatment is normalized to its corresponding control. Pre, cells were primed with 25(OH)D_3 for 24 h, then medium was replaced with fresh medium containing DFX (no 25(OH)D_3); co, cells were treated with 25(OH)D_3 and DFX at the same time. Bar, mean ± SD; n = 8. $^{**}P < 0.01$, $^{*}P < 0.05$ (one-way ANOVA test). Data shown are from a representative experiment of three independent experiments.

CHARACTERIZATION OF SERUM STARVATION STRESS IN MCF12F CELLS IN THE PRESENCE OF 25(OH)D_3

Although we observed a protective effect of 25(OH)D_3 in low-serum stressed culture conditions in MCF12F cells (Fig. 1B), we did not know whether 25(OH)D_3 maintained cell growth or inhibited cell death under the stressed condition. We therefore designed an experiment by counting cells before and after cell exposure to stress to determine how 25(OH)D_3 protects cells. As shown in Figure 5A, after exposure to low-serum stress for 7 days, cell number decreased from 528,000 ± 199,600 (day 0) to 74,066 ± 28,942 (day 7, 86% decrease, $P < 0.01$, n = 3) in each well, while in the presence of 25(OH)D_3, cell number in each well was 156,717 ± 28,404 (reduction of 74% as compared to 86% in control cells) on day 7. This also indicates ~2-fold protection against cell death on day 7 ($P < 0.05$, n = 3). These data demonstrate that 25(OH)D_3 inhibited cell death caused by low-serum stress. More importantly, these results indicate that under the stressed condition, 25(OH)D_3 did not maintain or stimulate cell growth, but prevented cell death or enhanced cell survival signaling.

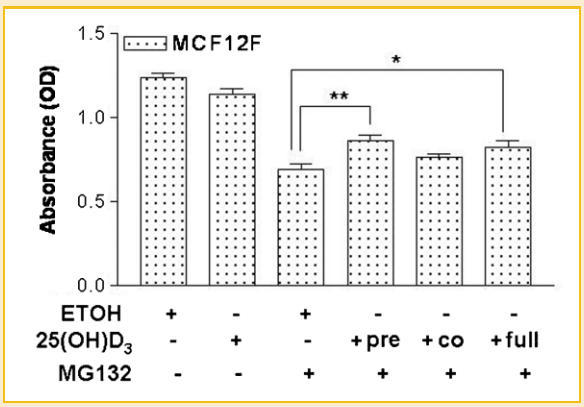

Fig. 4. Effect of 25(OH)D_3 on apoptosis induced by MG132. MCF-12F cells were seeded at 40,000 cells/well in 24-well plates and incubated overnight; cells were primed with 250 nmol/L 25(OH)D_3 (pre) or ETOH (control; co) for 24 h, then treated with 40 μmol/L MG132 for 16 h in the presence (co) or absence (pre) of 25(OH)D_3, cell number was evaluated with the CV assay. Full, pre-treatment for 24 h and co-treatment with 25(OH)D_3 for 16 h. Bar, mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to its corresponding control (MG132 treatment), n = 8 (one-way ANOVA test). Data shown are from a representative experiment of three independent experiments.

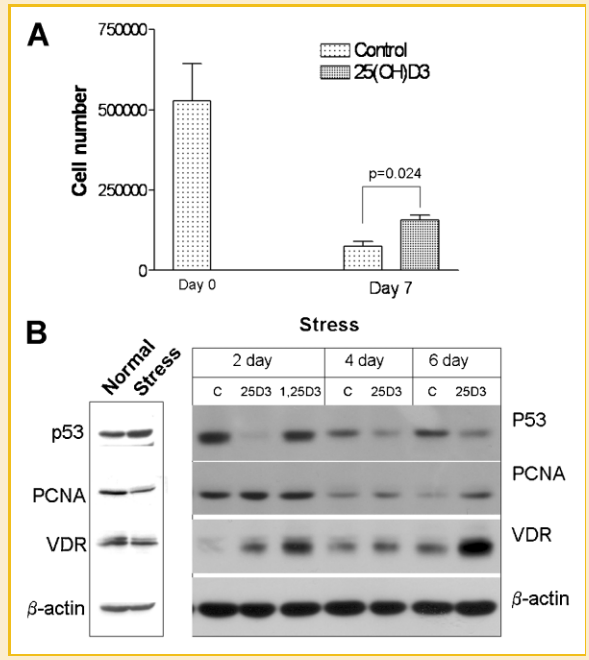

Fig. 5. Characterization of MCF12F cells in low-serum-stressed culture condition in response to 25(OH)D_3. A: Effect of 25(OH)D_3 on cell death in low-serum stressed culture condition. MCF12F cells were cultured in 6-well plates, then stressed from day 0 by culturing cells in medium containing 2% charcoal-stripped FBS in the presence/absence of 250 nmol/L 25(OH)D_3 for 7 days, cell number was counted on day 0 (right before stress) and day 7. Stress caused significant decrease of cell number, 25(OH)D_3 treatment in stressed condition significantly inhibited the decrease of cell number caused by stress, demonstrating a protective effect of 25(OH)D_3 against cell death. Bar, mean ± SD, n = 3 (student's *t*-test). B: Immunoblot analysis of stress and proliferation related molecules in MCF12F cells under stressed condition. MCF-12F cells were cultured in normal and stressed condition (MEM with 2% charcoal-stripped FBS) for 2 days in the absence of 25(OH)D_3 (left) or treated with 0.1% ETOH (C, control), 250 nmol/L 25(OH)D_3, or 10 nmol/L 1,25(OH)$_2D_3$ for 2, 4, and 6 days under stressed (MEM with 2% charcoal-stripped FBS) culture condition (right). Protein markers (p53, PCNA, and VDR) were analyzed using western blot.

Since 25(OH)D_3 significantly inhibited cell death caused by serum starvation, we next determined whether cell death, proliferation, and/or stress-related proteins were regulated under the stressed condition in the absence or presence of 25(OH)D_3. We previously found that p53 and PCNA expression levels were associated with apoptosis induced by serum starvation in other cell systems [Peng et al., 1998]. We therefore examined p53 and PCNA expression. As shown in Figure 5B, low-serum stress induced p53 protein expression and decreased PCNA expression. However, at different time points under the stressed condition, the alterations of these

molecules were significantly inhibited by both 25(OH)D_3 (250 nmol/L) and 1,25$(OH)_2D_3$ (10 nmol/L, positive control). These results are consistent with the data generated from the proliferation assay shown in Figures 1B and 5A. VDR, as expected, was upregulated by vitamin D metabolites, probably due to stabilization after ligand binding.

25(OH)D_3 DOES NOT ENHANCE TRANSFORMATION

Since 25(OH)D_3 protects MCF12F cells against multiple stress models and enhances cell survival signaling, a potential concern can be raised as to whether 25(OH)D_3 can enhance transformation under stressed conditions. We therefore designed an experiment to examine whether 25(OH)D_3 promotes transformation (Fig. 6). For this, MCF12F cells were treated with DMBA in normal culture conditions for 2 days in the absence or presence of 25(OH)D_3 using a standard transformation protocol described previously [Hussain-Hakimjee et al., 2006]. The culture medium was then replaced with MEM + 5% FBS (no supplements) and the serum level was gradually decreased to 0.1% and then gradually increased to 5%. 25(OH)D_3 was included in the medium for protection or not included for control until the experiment was terminated. Based on our experience, MCF12F cells grow poorly in MEM + 5% FBS, so as expected, almost all cells without 25(OH)D_3 protection died after a month (left panel), while cells with 25(OH)D_3 protection died slowly and were able to form colonies (right panel). However, after a subsequent month in culture with MEM + 5% FBS (transformed cells should grow in this medium), no significant cell growth was observed with 25(OH)D_3 protection, indicating that 25(OH)D_3 protected cells were not transformed. This observation suggests that 25(OH)D_3 is anti-stress protective, but not a transformation inducer or promoter. It seems 25(OH)D_3 only enhanced cell survival signaling or decreased the cell death rate. In this experiment, both DMBA and DMBA + 25(OH)D_3 did not transform MCF12F cells.

INVOLVEMENT OF MIRNAS IN 25(OH)D_3 ACTION AGAINST STRESS INDUCED BY SERUM STARVATION

In order to evaluate if miRNAs are involved in stress and the action of 25(OH)D_3 against stress, we performed a miRNA microarray using Invitrogen's NCode^{TM} Multi-species miRNA Microarray Kit V2 per the manufacturer's instruction. Samples were prepared from MCF12F cells that were stressed for 24 h with low-serum medium without any supplements in the presence or absence of 25(OH)D_3 (250 nmol/L). To simplify the analysis, only human miRNAs with twofold change were selected for analysis. The alteration of miRNAs (stress vs. normal and 25(OH)D_3 treatment vs. control in stressed condition) in MCF12F cells, qRT-PCR confirmation of the corresponding miRNA precursors, and the putative p53 and VDR binding sites in the 1 kb 5′-flanking sequences of the miRNA precursors are summarized in Table I. The expressions of multiple miRNAs were altered after exposure to low-serum stress for 24 h in comparison to the control in normal culture conditions. The miRNA array demonstrated that low-serum stress upregulated multiple miRNAs including miR-26b, miR-182, and let-7a; and also downregulated several miRNAs including miR-18a, miR-106, and miR-30c. We were able to confirm most of the miRNA alterations by qRT-PCR at corresponding miRNA precursor levels (Table I). We also found that the alteration of miRNA expression induced by stress was either inhibited or reversed in the presence of 25(OH)D_3 (Table I). As p53 is regulated by vitamin D metabolites (Fig. 5B), we analyzed the potential p53 and VDR/RXR binding sites in the 1 kb 5′-flanking sequence of the miRNA precursors using MatInspector (http://www.genomatix.de). In silico analysis identified multiple putative p53 and/or VDR/RXR binding sites in some of the 5′-flanking regions of the altered miRNA precursors including miR-26b, miR-182, and let-7a (Table I).

On the basis of miRNA microarray data analysis, qRT-PCR confirmation and analysis of 1 kb 5′-flanking sequences, five miRNAs (miR-26b, miR-182, miR-200c, miR-200b, and let-7b) appear to be significantly involved in the cellular response to stress and the protective action 25(OH)D_3 (Table II). Target gene prediction of these miRNAs using two public miRNA databases (http://pictar.bio.nyu.edu/; http://microrna://microRNA.sanger.ac.uk) suggests that cell proliferation, cell cycle, and apoptosis relevant genes appear to be the common target genes of these miRNAs, which is in line with our previous observations (Figs. 1 and 5).

Fig. 6. Transformation assay of 25(OH)D_3. For detailed protocol, please refer to reference (Hussain-Hakimjee et al., 2006). MCF12F cells were initially cultured in 40 tissue culture dishes and subjected to the transformation assay in the absence (20 dishes) or presence (20 dishes) of 25(OH)D_3 (250 nmol/L) for 1 month. 25(OH)D_3 protected cells against stress, but failed to induce transformation, demonstrating that 25(OH)D_3 is not a pro-carcinogen or transformation promoter. After an additional month's culture in MEM + 5% FBS, no further cell growth was observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Microarray Analysis of miRNAs in MCF12F Cells Stressed With Low-Serum Medium in the Presence/Absence of 25(OH)D_3, qRT-PCR Confirmation and Putative p53 and VDR-Binding Sites in the 1 kb 5′-Flanking Sequences of the Corresponding miRNA Precursors

	miRNA array		qRT-PCR confirmation		No of binding sites for	
miRNA	S/N	25(OH)D3/S	S/N	25(OH)D3/S	p53	VDR/RXR
hsa_miR_26b	5.25	−6.16	8.28	−2.82	5	2
hsa_miR_182	5.07	−4.00	32.00	−4.44	2	1
hsa_let_7a	4.5	−2.64	2.07	1.27	1	2
hsa_miR_203	3.62	−2.63	–	–	0	1
hsa_miR_98	2.99	1.12	5.77	−1.39	–	–
hsa_miR_191	2.84	−2.76	–	–	0	2
hsa_let-7f	2.78	−1.39	3.14	−1.15	–	–
hsa_miR_200c	2.73	−6.81	6.06	−1.82	0	1
hsa_miR_16	2.68	−3.17	–	–	0	0
hsa_miR_21	2.5	1.42	1.87	2.63	1	0
hsa_let_7e	2.5	−3.93	–	–	1	2
hsa_mir_205	2.46	−1.4	–	–	5	1
hsa_let_7b	2.28	−3.28	3.36	−2.76	2	1
hsa_miR_200b	2.26	−4.47	7.73	−1.75	0	6
hsa_miR-92	2.17	−4.24	–	–	0	2
hsa_let_7d	2.08	−2.58	3.24	−1.11	0	1
hsa_miR_93	−2.01	1.03	–	–	–	–
hsa_20b	−2.43	2.19	–	–	1	0
hsa_miR422b	−2.83	2.8	–	–	–	–
hsa_miR_30c	−4.53	1.21	–	–	–	–
hsa_miR_106a	−4.78	2.17	–	–	1	0
hsa_miR18a	−7.42	1.28	–	–	0	1

miRNA miroarray: NCode™ multi-species miRNA microarray kit, S/N, stress versus normal, only genes with twofold change cut-off were shown. 25(OH)D_3/S: 25(OH)D_3 treatment in stressed condition versus stress control. qRT-PCR confirmation was performed at miRNA precursor level. –, not tested. 1 kb 5′-flanking sequence of miRNA precursor was analyzed for the putative binding sites for p53 and VDR/RXR using MatInspecotor (http://www.genomatix.de). These are putative binding sites, but the presence of these binding sites provides a clue that these genes could be regulated by p53 or VDR/RXR.

Among the five selected miRNAs involved in the stress response, miR-182, confirmed by qRT-PCR, was found to be the most upregulated miRNA in response to stress. In addition, miR-182 has not been characterized in breast epithelial cells. Therefore, miR-182 was selected to examine its functional role in MCF12F cells. MCF12F cells were transiently transfected with miR-182 precursor. Results show that miR-182 precursor over-expression suppressed cell proliferation by 24% ($P < 0.05$, n = 6) after a 5 day culture in normal growth medium following transfection (Fig. 7), which is consistent with the observation that cell death was enhanced and accompanied by increased miR-182 expression under stressed condition (Fig. 5A; Table I).

DISCUSSION

One of the goals of breast cancer prevention research is to understand what molecular alterations occur early during carcinogenesis and to determine how chemopreventive agents prevent normal

TABLE II. Target Gene Prediction of Selected miRNAs

miRNA	Target gene prediction[a]
miR_26b	RPS6KA6, PCNA, HMGA1, GSK3β
hsa_miR_182	Cyclin D2, BCL2, Caspase2, myb, EGFR
hsa_miR_200c	myc
hsa_miR_200b	cyclin D2, myc
has_let_7b	cyclin D1, D2, D3, Rb1, myc, Cdk4

[a]A selection of predicted target molecules of microRNA is shown, data derived from two public microRNA databases (http://pictar.bio.nyu.edu/; http://micorna.sanger.ac.uk).

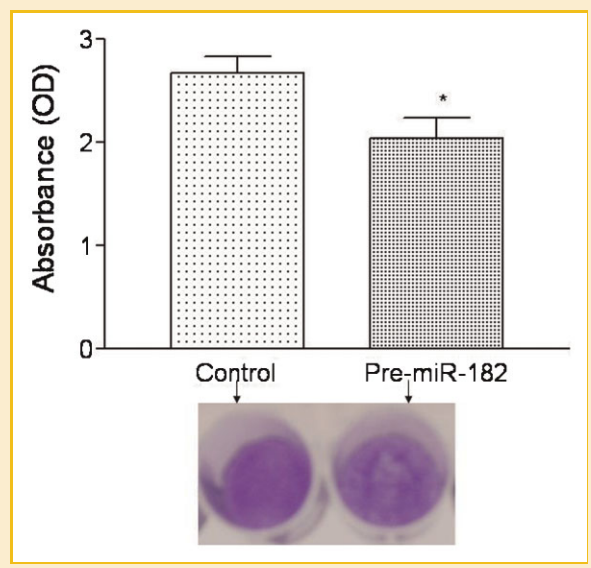

Fig. 7. Effect of miR182 over-expression on the growth of MCF12F cells. Upper panel, MCF12F cells were seeded in 24-well plates with 25,000 cells/well-transfected with negative control and miR182 precursor, then cells were maintained in normal growth medium for 5 days. Cell proliferation was evaluated using the CV assay. miR-182 transfection significantly inhibited cell proliferation. Bar, mean ± SD; *$P < 0.05$ in comparison to control, n = 6 (one-way ANOVA test). Lower panel, image of CV staining after 5 days of culture following miR-182 transfection in normal growth medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

breast epithelial cells from transformation. As transformation is generally accompanied by stress, which in itself could also be a causative factor of carcinogenesis, an ideal chemopreventive agent should also protect normal cells against stress. This study was initiated in an attempt to better elucidate how $25(OH)D_3$, the vitamin D metabolite present in the serum, can serve as a possible natural chemopreventive agent against stress mediated cell death.

$25(OH)D_3$ is the major metabolite of vitamin D in the circulation system. It was widely considered as an inactive vitamin D metabolite displaying low affinity to VDR; however, recent evidence suggests that $25(OH)D_3$ could directly bind to VDR and activate VDR target genes [Lou et al., 2004; Peng et al., 2009] and therefore it is an agonistic VDR ligand [Lou et al., 2010]. Moreover, in recent years, it has been shown that it can also be locally converted to $1,25(OH)_2D_3$ by 25-hydroxyvitamin D 1α-hydroxylase to play its functional role. Since epidemiological studies have established an inverse correlation of reduced serum level of $25(OH)D_3$ with increased cancer risk [Garland et al., 1990; Gorham et al., 1990; Ainsleigh, 1993; Lipkin and Newmark, 1999; Cross et al., 2003; Friedrich et al., 2003; Grant and Garland, 2004; Lowe et al., 2005], more laboratory work is needed to investigate the functional role of $25(OH)D_3$. On the basis of the literature and our previous work [Peng and Mehta, 2007; Peng et al., 2009], we focused on evaluating the role of $25(OH)D_3$ in stress-mediated alterations. This is the first report on the anti-stress function of $25(OH)D_3$ in breast epithelial cells. We first showed that $25(OH)D_3$ protected MCF12F cells using multiple stress models, including serum starvation, oxidative stress, hypoxia, and apoptotic stress. These studies suggested that $25(OH)D_3$ had broad protective effects in many stress model systems. Consistent with our data, the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, has been previously reported to protect cells from cell death induction through various pathways including stress [Riachy et al., 2005; Zhang et al., 2005; Diker-Cohen et al., 2006]. In HaCaT keratinocytes, $1,25(OH)_2D_3$ protected the cells from all the examined stress mediated pathways leading to cell death, including TNFα, oxidative stress, hyperosmotic, and heat shock-induced cell death [Diker-Cohen et al., 2006].

The concentration of $25(OH)D_3$ (250 nmol/L) used in this study was selected based on several criteria: (1) a dose-course study suggested that this concentration is effective in both normal culture conditions and stressed culture conditions (data not shown); (2) the concentration selected can also be a maximally achievable physiological dose [Lou et al., 2004]; (3) this concentration has been shown by us to significantly inhibit pre-cancerous lesion formation induced by a carcinogen in mouse mammary organ culture [Peng et al., 2009]. Both $25(OH)D_3$ and $1,25(OH)_2D_3$ at the selected concentration significantly inhibited cell proliferation of breast epithelial cells (MCF12F) under normal culture conditions, while in a stressed (serum-starved) setting, the vitamin D metabolites at the same concentration inhibited stress-induced cell death. Serum starvation might represent a lack of nutrients model, which could promote cell transformation. It has been suggested that genomic vitamin D action is necessary to protect against nutrition-linked hyperproliferation and oxidative stress [Kallay et al., 2002] and maintain genomic stability [Chatterjee, 2001]. The protective anti-stress effect exhibited by vitamin D in the serum starvation model was accompanied by the downregulation of p53 (a stress responsive molecule) [Levine, 2006] as well as the upregulation of PCNA, a marker for proliferating cells. These results are also consistent with our previous observation showing that p53 and PCNA are associated with serum starvation in primary granulosa cells [Peng et al., 1998]. The importance of stress-induced p53 expression on cell fate (cell cycle arrest or apoptosis) has been well established. Levine [2006] has demonstrated that p53 is a key molecule in stress response and tumorigenesis. Given that $25(OH)D_3$ regulates p53 expression in stress conditions, either directly or indirectly, we can infer that VDR could also be involved in both processes and that $25(OH)D_3$ might protect cells against transformation through its anti-stress function. Our data also demonstrate that $25(OH)D_3$ attenuates common pathways leading to cell death induced by a wide spectrum of stresses or enhances cell survival signaling through multiple pathways, which makes $25(OH)D_3$ a general protector for breast tissue. In support of our results, $1,25(OH)2D_3$ has been shown to decrease the formation of apoptotic cells in epidermis exposed to UV [Hanada et al., 1995] and to reduce cell death in growing hair follicles following treatment with chemotherapeutic drugs [Schilli et al., 1998]. The hormone also protected keratinocytes in vitro from UVB- and TNFα-induced cell death [Diker-Cohen et al., 2006]. As for the protective effect of $25(OH)D_3$ in breast epithelial cells, this is the first report to document its anti-stress action.

Oxidative damage to cellular macromolecules can arise through overproduction of ROS and faulty antioxidant and/or DNA repair mechanisms. In addition, ROS can stimulate signal transduction pathways and lead to activation of key transcription factors such as Nrf2 and NF-kappaB. The altered gene expression patterns evoked by ROS contribute to the carcinogenesis process [Klaunig et al., 2010]. Some chemopreventive agents such as epigallocatechin gallate (EGCG), may assert its chemopreventive actions by serving as potent antioxidants [Jagtap et al., 2009]. In human HaCat keratinocytes, $1,25(OH)_2D_3$ has been previously shown to protect cells against H_2O_2-induced cell death through inhibiting H_2O_2-induced caspase activity via inhibition of multiple signaling pathways including p38 MAPK and JNK activation [Diker-Cohen et al., 2006]. In line with this, our results demonstrated that $25(OH)D_3$ also protected MCF12F breast epithelial cells against H_2O_2-induced cell death. However, attenuation of p38 MAPK and JNK activation only partially accounts for the protective effect of $1,25(OH)_2D_3$ in keratinocytes [Diker-Cohen et al., 2006]. Therefore, the mechanism of action of $25(OH)D_3$ in the stress response in breast epithelial cells still needs further investigation. Since $25(OH)D_3$ protects MCF12F cells in various stress models, we expect that $25(OH)D_3$ may affect a site common to all stress models. While some may argue that protecting cells under stress conditions might lead to cell transformation, however our studies have provided evidence for only the chemopreventive effect of $25(OH)D_3$ [Murillo et al., 2007; Peng et al., 2009] and there is no evidence showing that $25(OH)D_3$ is carcinogenic or promotes cell transformation.

Recent evidence suggests that dietary components as diverse as folate, retinoids, and curcumin exert cancer-protective effects by modulating miRNA expression [Davis and Ross, 2008]. miRNAs may be useful as biomarkers of cancer prevention or nutritional status, and furthermore may serve as potential molecular targets that are

influenced by dietary interventions. Although the regulation of these miRNA genes involved in carcinogenesis is not yet very clear, they have the potential to serve as novel targets for both cancer prevention and treatment. However, the mechanism for how miRNAs are involved in vitamin D action is still not known. Our miRNA array data show that multiple miRNAs are involved in the stress response of MCF12F cells and more importantly that $25(OH)D_3$ can regulate several of them under stressed culture conditions. Our observation that alteration of miRNA expression induced by stress was either inhibited or reversed in the presence of $25(OH)D_3$ suggests that this vitamin D metabolite can function as a protective hormone against stress to maintain normal expression of the miRNAs involved in stress. 5′-flanking sequence analysis of altered miRNA precursors (Table I) suggest that these miRNAs could be potential targets of $25(OH)D_3$. It should be pointed out that the 1 kb 5′-flanking sequences of the corresponding miRNA precursors do not necessarily represent the proximal promoter region of the miRNA genes, since the premature miRNAs are generally larger than the miRNA precursors and so far it is still difficult to locate the promoter region of miRNA genes; however, the in silico analysis indeed provides a clue that these miRNAs could potentially be regulated by vitamin D metabolites either directly or indirectly (i.e., through regulating p53). It should also be pointed out that the initial signaling [triggered by stress and $25(OH)D_3$] is important for the biological effect even after several days and the initial signaling is generally more consistent and reproducible, this is the reason we only analyzed the miRNA alteration at an early time point.

Limited information regarding the functional role of these miRNAs in relation to stress and vitamin D action is available in the literature. Our transfection experiment, for the first time, demonstrated that miR-182 over-expression inhibited cell growth in breast epithelial cells under normal culture conditions, which supports our observation associated with low-serum stress: low-serum stress increased miR-182 expression in MCF12F cells, accompanied with significant cell death. Interestingly, miR-182 has previously been linked to oncogenic transformation in colon, prostate, and breast cancer [Sarver et al., 2009; Schaefer et al., 2010; Guttilla and White, 2009]. However, it was also reported to be upregulated in primary cultures of human diploid fibroblast and human trabecular meshwork cells associated with stress-induced premature senescence [Li et al., 2009], which is consistent with our observation. The apparent differential functional role of miR-182 could be due to the differential target gene expression in different cells. Further evaluation of other vitamin D targeted miRNAs and their involvement in stress and vitamin D action are yet to be elucidated. These data provide novel information for vitamin D action in breast cancer prevention.

In summary, both $25(OH)D_3$ and $1,25(OH)_2D_3$ could protect cells against cellular stress, which is a potential causative factor for carcinogenesis. The major metabolite $25(OH)D_3$ at 250 nmol/L is as efficacious as $1,25(OH)_2D_3$ at 10 nmol/L in its anti-stress activity in MCF12F cells. The concept that $25(OH)D_3$ could be an anti-stress hormone is supported by experimental evidence, miRNA microarray and in silico analysis of the selected miRNAs. The anti-stress function of $25(OH)D_3$ could be part of the mechanism underlying the chemopreventive action of this vitamin D metabolite against breast carcinogenesis. It can be speculated that the circulating form of vitamin D metabolite ($25(OH)D_3$) may serve as an anti-stress natural chemopreventive hormone to prevent cell transformation.

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