

Vitamin D reduces the expression of collagen and key profibrotic factors by inducing an antifibrotic phenotype in mesenchymal multipotent cells

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Abstract

Hypovitaminosis D is an important public health problem. Serum 25-hydroxyvitamin D (25-OHD) is now recognized as an independent predictor for cardiovascular and related diseases (CVD) as well as other chronic medical conditions. However, the biologic pathways through which these effects are mediated remain poorly understood. We hypothesized that exposing mesenchymal multipotent cells (MMCs) to the active form of vitamin D would increase the expression of selected antifibrotic factors that in turn would ameliorate the progression of chronic diseases. MMCs were primed with 5'-azacytidine to induce a fibrotic phenotype and then treated with active vitamin D (1,25D) or ethanol <0.1% as vehicle in a time course manner (30 min, 1, 5, and 24 h, and for 4 and 7 days). The addition of 1,25D to MMCs promotes: a) increased expression and nuclear translocation of the vitamin D

receptor; b) decreased expression of TGFB1 and plasminogen activator inhibitor (SERPINE1), two well-known profibrotic factors; c) decreased expression of collagen I, III and other collagens isoforms; and d) increased expression of several antifibrotic factors such as BMP7 a TGFB1 antagonist, MMP8 a collagen breakdown inducer and follistatin, an inhibitor of the profibrotic factor myostatin. In conclusion, the addition of 1,25D to differentiated MMCs displays a decreased profibrotic signaling pathway and gene expression, leading to decrease in collagen deposition. This study highlights key mechanistic pathways through which vitamin D decreases fibrosis, and provides a rationale for studies to test vitamin D supplementation as a preventive and/or early treatment strategy for CVD and related fibrotic disorders.

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Introduction

Hypovitaminosis D, as well as other chronic medical conditions, is increasingly recognized as an independent predictor factor for cardiovascular and related diseases (CVD; Casteels *et al.* 1998, Martins *et al.* 2007, Giovannucci 2008, Kovesdy *et al.* 2008, Wang *et al.* 2008). The biologic pathways through which these effects are mediated remain poorly understood, but may be linked to vitamin D regulation of related fibrotic pathways (Repo *et al.* 2007).

Fibrosis (progressive scarring) is often defined as an exaggerated wound-healing response due to chronic sustained injury, microtrauma, oxidative stress, endogenous or exogenous insults, autoimmunity, and other factors and is attributed to an excessive deposition of extracellular matrix components, mainly collagen (Wynn 2007). Fibrosis is not restricted to CVD and may underlie numerous chronic medical conditions such as progressive kidney disease,

macular degeneration and others (Willis *et al.* 2006, Gharaee-Kermani *et al.* 2007, Henderson & Iredale 2007) and, at the present time, there are limited effective treatments for these conditions (Wynn 2007). While chronic inflammation typically precedes fibrosis, there are distinct, independent inflammatory mechanisms that regulate fibrogenesis such as the deregulation of myofibroblast progenitor cell differentiation and myofibroblast collagen synthesis and extracellular matrix repair (Iredale 2007, Wynn 2007). Contrary to previous dogma, fibrosis can now be considered as a reversible and curable process (Fallowfield *et al.* 2006, Iredale 2007) when interventions can be initiated at earlier stages of the disease (Kisseleva & Brenner 2006).

Recently, it has been clinically shown, that the administration of active vitamin D (1,25D), results in significant amelioration of chronic medical conditions such as chronic kidney disease and CVD (Kovesdy *et al.* 2008, Wolf *et al.* 2008). This effect may occur, in part, through attenuation of

profibrotic, and proinflammatory pathways (Tan *et al.* 2006, 2007), although the basic mechanism by which vitamin D may exert these effects remains poorly understood.

Vitamin D is a lipid-soluble prohormone obtained from dietary sources or from *de novo* synthesis in the skin, as a result of u.v. light-induced photolytic conversion of 7-dehydrocholesterol to previtamin D₃ followed by thermal isomerization to vitamin D₃ (Brown 1998, Dusso & Brown 1998, Feldman *et al.* 2005). The first step in the metabolic activation of vitamin D is hydroxylation of carbon 25, occurring primarily in the liver. The second and more regulated step in vitamin D bioactivation is the formation of 1,25D (1 α , 25-(OH)₂D₃) also known as calcitriol, the active form of vitamin D, which occurs mainly in the kidney (Dusso *et al.* 2005) via the 25-hydroxyvitamin D-1 α -hydroxylase enzyme. 1,25D activates the vitamin D receptor (VDR), a nuclear transcription factor of the steroid-retinoid nuclear receptor gene superfamily, ultimately promoting gene transcription (Carlberg *et al.* 2001, Zehnder *et al.* 2001). The VDR appears to be ubiquitous in the body, supporting the vitamin D endocrine system, which is involved in a wide range of physiological functions (Mathieu *et al.* 1994, Casteels *et al.* 1998, Malleuche *et al.* 2002, Dusso *et al.* 2004).

In the present study we examined, in a time-course manner, using C3H 10T1/2 cells a multipotent mesenchymal cell line of mouse origin, the effects of 1,25D on the expression and nuclear translocation of VDR, the expression of SERPINE1 and TGFB1 (both well-known profibrotic factors), the changes in the expression of collagen I, collagen III, and other collagen isoforms and the expression of antifibrotic factors such as BMP7, MMP8, and follistatin (Fst) by immunofluorescence, western blot, DNA microarrays and real-time PCR array analysis. C3H 10T1/2 cells have been shown by us and others to have the potential to differentiate into a variety of specialized cells such as osteocytes, chondrocytes, adipocytes, endothelial, and smooth muscle cells (Singh *et al.* 2003, Pittenger & Martin 2004, Artaza *et al.* 2005), and they have been used to study the effects of 1,25D on cell proliferation and cell cycle (Brackman *et al.* 1993).

Recent studies have demonstrated that mesenchymal multipotent cells (MMCs) also possess the potential to differentiate along an endothelial lineage. Additionally, other studies have revealed the contribution of MMCs to the formation of new vessels and improvement in cardiac function (Pittenger & Martin, 2004). Thus, MMCs may hold the potential to differentiate along endothelial lines and to be used for applications such as neovascularization and tissue engineering (Rabbany *et al.* 2003).

Materials and Methods

Cell culture

Mouse C3H 10T1/2 MMCs (ATCC, Manassas, VA, USA) were grown in DMEM with 10% dialyzed fetal bovine serum

at 37 °C and 5% CO₂ and then treated with 20 μ M 5'-azacytidine (AZCT) for 2 days to induce differentiation by epigenetic changes (Singh *et al.* 2003, Artaza *et al.* 2005, Schmittwolf *et al.* 2005) and to promote a fibrotic phenotype (Adams *et al.* 2005, Artaza *et al.* 2008a,b).

Cells were split in a 1:2 ratio, allowed to recover for 2 days, and replated at 60–70% confluence in T75 flasks, eight-well chamber slides or six-well plates. The next day, cells were incubated with or without 100 nM of 1,25D (Sigma–Aldrich) dissolved in less than 0.1% ethanol as vehicle in DMEM 10% dialyzed fetal bovine serum and incubated for 30 min, 1, 5 and 24 h, and for 4 and 7 days. Control groups were incubated with 0.1% ethanol in DMEM 10% dialyzed fetal bovine serum. Because of the short half-life of 1,25D, the cell culture media with and without 1,25D (100 nM) was replaced every 2 days.

Detection of VDR by immunofluorescence

After the corresponding incubation time with or without 1,25D, the cells were washed thrice with PBS and fixed by immersion in 2% *p*-formaldehyde. Cells were blocked with normal goat serum, and incubated with a rabbit polyclonal antibody against VDR at a dilution of 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The detection was followed by a 1/200 dilution of anti-rabbit biotinylated secondary antibody (Calbiochem, La Jolla, CA, USA), followed by Streptavidine-Texas Red (10 μ g/ml; Vector Labs, Burlingame, CA, USA). After several washes, cells were counterstained with DAPI (Vector Labs). The slides were detached and mounted in 'prolong anti-fade' (Molecular Probes, Eugene, OR, USA) and were examined under a Leica DMLB fluorescence microscope equipped with the appropriate filters. The merge image was obtained by fusing the red and the blue filters pictures (Artaza *et al.* 2002). Fields were photographed with a SPOT-RT digital camera and acquisition software (Diagnostic Instruments, Sterling Heights, MI, USA).

Negative controls were done by either omitting the first antibody or using a rabbit non-specific IgG (Singh *et al.* 2003, Artaza *et al.* 2005, 2008a,b). To ensure specific staining of VDR antibody, a negative control was done by pre-absorbing the primary VDR antibody with 1 μ M VDR specific peptide, SC1008P (Santa Cruz Biotechnology), after 1 h incubation at RT the mixture was centrifuged at full speed for 15 min and the supernatant was used as a primary antibody at the same dilution used previously for the experiment. No positive staining was observed in the cells by immunofluorescence in this negative control.

Immunocytochemical analyses of profibrotic markers and collagen

After the corresponding incubation time with or without 1,25D, the cells were washed thrice with PBS and fixed by immersion in 2% *p*-formaldehyde, quenched with H₂O₂, blocked with normal goat or horse serum and incubated with

the following antibodies: a) SERPINE1 (1:200) (Abcam Inc., Cambridge, MA, USA), b) TGFB1 (1:200) (Promega Corporation), c) collagen I (1:100) (Abcam Inc.) and d) collagen III (1:100) (Abcam Inc.).

Detection was based on a secondary biotinylated antibody (1:200), followed by the addition of the streptavidin–HRP ABC complex (1:100), Vectastain (Elite ABC System, Vector Laboratories) and 3,3'-diaminobenzidine and H₂O₂ mixture (Sigma). The cells were counterstained with Meyer's hematoxylin (Sigma). In negative controls, we either omitted the first antibody or used a rabbit non-specific IgG. (Artaza *et al.* 2002, 2005, 2008a,b, Singh *et al.* 2003).

Quantitative image analysis

In all cases, the immunoreactivity was quantified by image analysis using ImagePro-Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA). After the images were calibrated for background lighting, integrated optical density (IOD) results were proportional to the mean optical density per area. IOD determines the amount of immunoreactive antigen present in each cell. The IOD values expressed in arbitrary units were determined in at least 20 pictures per treatment and time points. The results expressed as mean \pm S.E.M. represent the average of three independent experiments (Singh *et al.* 2003, Artaza *et al.* 2005, 2008a,b).

Nuclear and cytoplasmic fraction extraction

Of 5'-azacytidine pre-treated cells grown in T75 cell culture flasks were incubated with or without 1,25D (100 nM) for 24 h, 4 and 7 days. At the end of the corresponding incubation times, cells were washed thrice with PBS and detached with trypsin, 2.5%. Cells were centrifuged and the cell number was adjusted to 2×10^6 cells. Nuclear and cytoplasmic extraction fractions were done using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions. Protein concentrations in both fractions were determined and adjusted. The cytoplasmic and nuclear fractions were subjected to western blot analysis for VDR localization/expression and to ensure extraction efficiency GAPDH was determined in each fraction.

Western blot and densitometry analysis

Cell lysates (30–40 μ g protein) were subjected to western blot analyses by 4–15% Tris–HCl PAGE (Bio-Rad) using a monoclonal antibody for glyceraldehyde–3-phosphate dehydrogenase (GAPDH) (1/10 000) (Chemicon International, Temecula, CA, USA). Polyclonal antibodies were used for VDR (1/500), (Santa Cruz Biotechnology Inc.), TGFB1 (1/1000) (Promega Corporation), and SERPINE1 (1/1000) (Abcam Inc.). The washed membranes were incubated with 1/2000 dilution (anti-rabbit) or 1/3000 dilution (anti-mouse) of secondary antibody linked to HRP

(GE Healthcare, Bucks, UK) respectively. Immunoreactive bands were visualized using the ECL plus western blotting chemiluminescence detection system (Amersham Biosciences). The densitometry analysis of the bands was done with the Scion Image software beta 4.02 (Scion Corp., Frederick, MD, USA).

Real-time quantitative PCR

After 24 h and 4 days incubation with or without 1,25D, total RNA was extracted using Trizol-Reagent (Invitrogen) and equal amounts (1 μ g) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The locations of forward/reverse PCR primers for real-time RT-PCR are as follows: VDR region 575–597/626–641 on BC074818.2 (67 bp). Mouse gene PCR primer sets (RT2) were purchased from SABiosciences (Frederick, MD, USA). The Qiagen Sybr Green PCR kit with HotStar Taq DNA polymerase (Qiagen) was used with i-Cycler PCR thermocycler and fluorescent detector lid (Bio-Rad).

The protocol included melting for 15 min at 95 °C, 40 cycles of three-step PCR including melting for 15 s at 95 °C, annealing for 30 s at 58 °C, elongation for 30 s at 72 °C with an additional detection step of 15 s at 81 °C, followed by a melting curve from 55 to 95 °C at the rate of 0.5 °C per 10 s. We confirmed that inverse derivatives of melting curves show sharp peaks for VDR at 83 °C and GAPDH at 87 °C, indicating the correct products. Samples of 25 ng cDNA were analyzed in triplicate in parallel with GAPDH controls; standard curves (threshold cycle versus log pg cDNA) were generated by log dilutions of from 0.1 pg to 100 ng standard cDNA (reverse-transcribed mRNA from C3H 10T1/2 cells in Growth Medium). Experimental mRNA starting quantities were then calculated from the standard curves and averaged using i-Cycler, iQ software as described previously. The ratios of marker experimental gene (e.g., VDR mRNA) to GAPDH mRNA were tallied and normalized to control (untreated) samples as 100%. (Singh *et al.* 2003, Artaza *et al.* 2008a,b).

DNA microarray analysis of TGFB1/BMP target genes

Total cellular RNA was isolated with Trizol-Reagent from C3H 10T1/2 cells undergoing differentiation with AZCT, and treated with or without 1,25D (100 nM) for 4 days. Isolated RNA was subjected to cDNA gene arrays (GEArray Q Series, TGFB1/BMP signaling pathway gene array (MM-023) and osteogenesis gene array (MM-026) analysis (SABiosciences Corp). This series of mice TGFB1/BMP signaling pathway gene arrays is designed to study the genes involved in TGFB1/BMP signaling pathway and in the process of osteogenic differentiation. Biotin-labeled cDNA probes were synthesized from total RNA, denatured, and hybridized overnight at 60 °C in GEHybridization solution to membranes spotted with TGFB1/BMP signaling pathway specific genes, as well as with genes involved in the regulation

of osteogenic differentiation. Membranes were washed, and chemiluminescent analysis was performed per the manufacturer's instructions. Raw data were analyzed using GEArray Expression Analysis Suite (SABiosciences Corp). Fold changes in relative gene expression were presented after background correction and normalization with a housekeeping gene. (Artaza *et al.* 2008a,b).

Confirmation of DNA microarray analysis by RT² profiler PCR array analysis of TGFB1/BMP and osteogenesis target genes

RT² profiler PCR SABiosciences analyses of TGFB1/BMP and osteogenesis target genes were applied in triplicates in order to confirm the GE Array data. Aliquots of total cellular RNA isolated with Trizol-Reagent from C3H 10T1/2 cells undergoing differentiation with AZCT were treated with or without 1,25D (100 nM) for 1 and 4 days. They were then subjected to reverse transcription, and the resulting cDNA was analyzed by: RT² profiler PCR mouse TGFB1/BMP signaling pathway (APM-035A) and by RT² profiler PCR mouse osteogenesis (AMM-026D) (SABiosciences Corp). This series of mice TGFB1/BMP and osteogenesis signaling pathway gene arrays is designed to study the genes involved in upstream and downstream of TGFB1/BMP signaling and osteogenesis differentiation respectively. Each array contains a panel of 84 primer sets related to the TGFB1/BMP signaling genes plus five housekeeping genes and two negative controls. Real time PCRs were performed as follows: melting for 10 min at 95 °C, 40 cycles of two-step PCR including melting for 15 sec at 95 °C, annealing for 1 min at 60 °C. The raw data were analyzed using the $\Delta\Delta C_t$ method following the manufacturer's instructions (SABiosciences Corp.; Artaza *et al.* 2008a,b).

Statistical analysis

All data are presented as mean \pm s.e.m. and between-group differences were analyzed using ANOVA. If the overall ANOVA revealed significant differences, then pairwise comparisons between groups were performed by Newman-Keuls multiple comparison test. All comparisons were two-tailed, and $P < 0.05$ was considered statistically significant. The *in vitro* experiments were repeated thrice, and data from representative experiments are shown. Specifically, the DNA microarrays tests were done twice and the results confirmed by RT² Profiler PCR arrays in triplicates and in some cases by qRT-PCR also in triplicate.

Results

Time course of expression and nuclear translocation of VDR in C3H 10T1/2 MMCs after incubation with 1,25 D

To determine whether the expression of VDR in our multipotent cell cultures is present and induced upon incubation with 1,25D, experiments using immunofluorescence, western blot and real-time PCR were carried out at

different time points. C3H 10T1/2 cells pre-treated with AZCT were incubated with or without 1,25D (100 nM) for 30 min, 1, 5, and 24 h, and 4, and 7 days. The dose of 1,25D used for these studies was derived after preliminary studies on the effects at 3 and 4 days exposure to 0, 10, 25, 50, 100, and 500 nM 1,25D on proliferation of C3H 10T1/2 multipotent cells. In those preliminary studies, comparison of 1,25D at 10 and 50 nM without 1,25D had no effect, while there was a dose-dependent significant decrease in cell proliferation with increasing doses that peaked at 1,25D (100 nM; not shown; Artaza *et al.* 2008a,b). The incubation with AZCT has no effect on the expression of VDR in the C3H 10T1/2 multipotent cell line (not shown).

By immunocytochemical (ICC) staining (Fig. 1) under basal conditions (no 1,25D), VDR immunofluorescence (red) was barely detected in the cell culture. After 30 min incubation with 1,25D, VDR expression was clearly increased in the cytoplasm and nuclear compartment. A similar observation was made at 1 and 5 h (Fig. 1). By contrast, after 24 h, 4 and 7 days of continuous 1,25D incubation, most of the red-fluorescence staining was observed in the nucleus (Fig. 1). The counterstaining with DAPI and the merge pictures confirm the nuclear localization of VDR in some cases, and the presence of the cells in the cell culture in others (no 1,25D incubation).

The increased expression of VDR after 1,25D incubation was further confirmed at 24 h and at 4 days by real-time PCR (qRT-PCR; Fig. 2). VDR mRNA expression increased by 3·3- and 4-fold at 1 and 4 days respectively compared with the controls without addition of 1,25D.

Furthermore, the increased expression of VDR after 1,25D incubation was also confirmed by western blot analysis using whole cell culture homogenates incubated with or without 1,25D (100 nM) at 24 h, 4 and 7 days (Fig. 3A). The densitometry analysis shows an increase of VDR expression upon incubation with 1,25D at 24 h, 4 and 7 days by 1·75-, 2·2- and twofold respectively.

In order to confirm the nuclear translocation of VDR after 1,25D incubation previously shown by immunofluorescence, nuclear and cytoplasmic fractionation were performed after 24 h, 4 and 7 days incubation with or without 1,25D (100 nM), and the resulting nuclear and cytoplasmic fractions were analyzed by western blot. Figure 3B shows that the expression of VDR in the control group (no 1,25D addition) was negligible in both cytoplasmic and nuclear fraction at all incubation time points. 1,25D addition induces a moderate expression of VDR in the cytoplasmic compartment but a much higher expression in the nuclear compartment at 24 h. Incubation with 1,25D for 4 and 7 days shows an exclusive nuclear localization in agreement with the previous observations by immunofluorescence.

To verify the purity of the subcellular fractionation, the membrane was stripped and reprobed with anti-GAPDH antibody. Expression of GAPDH (a cytoplasmic protein) was almost exclusively localized in the cytoplasmic fraction.

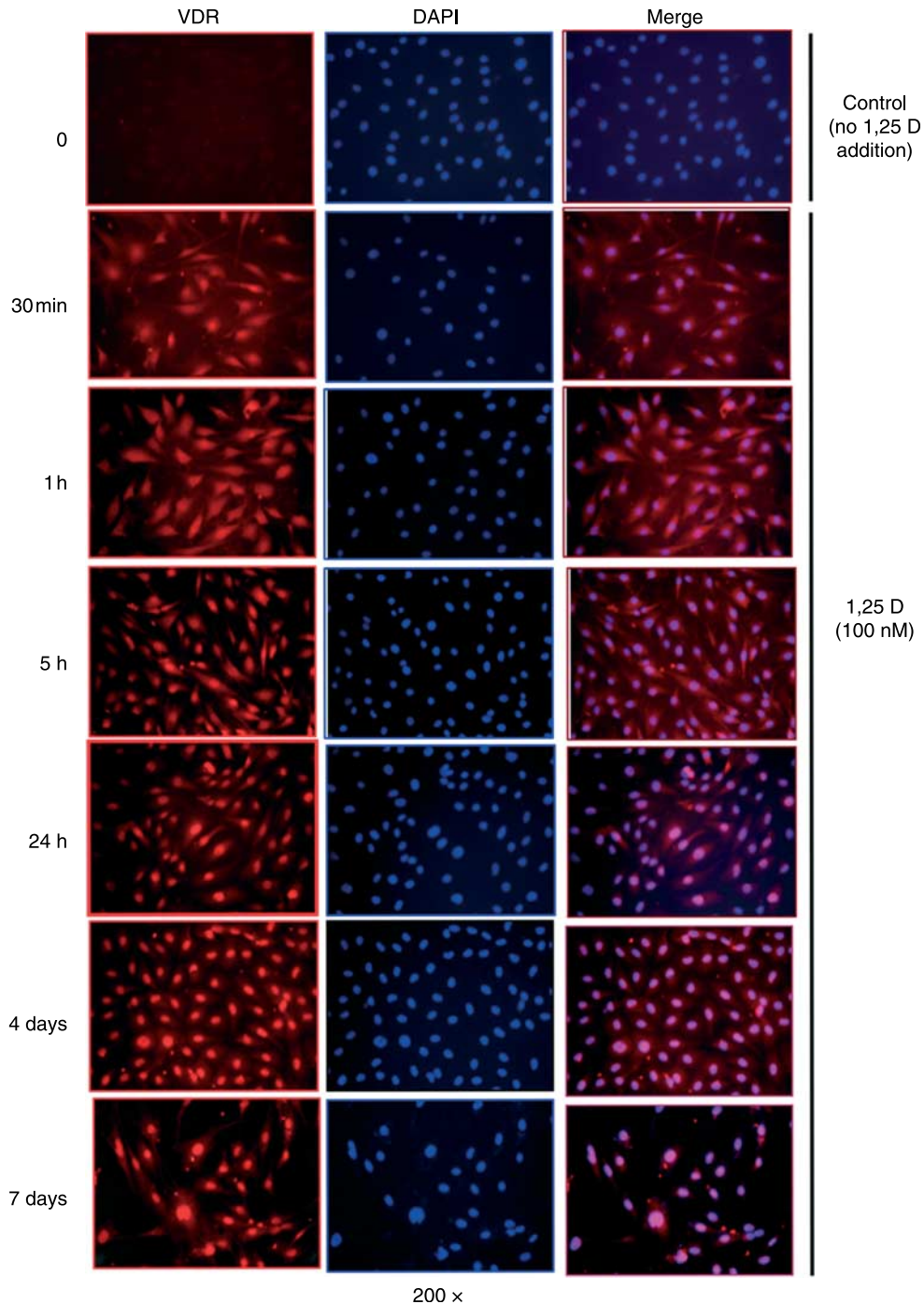


Figure 1 Time course of the expression and nuclear translocation of vitamin D receptor (VDR) upon incubation with 1,25D in mesenchymal multipotent C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated with 20 μ M azacytidine for 2 days to induce fibrosis and 2 days later were incubated with or without 1,25D (100 nM) at different time points: 30 min, 1, 5, and 24 h, and for 4 and 7 days. Cells were cultured on eight-well removable chambers slides and subjected for immunofluorescence using a polyclonal antibody for VDR followed by a Texas Red-conjugated secondary antibody (red). Cells were counterstained with DAPI (blue) to show nuclear staining. Merge pictures were done combining the red and blue pictures together (purple) and in order to show nuclear localization of VDR. Magnification=200 \times .

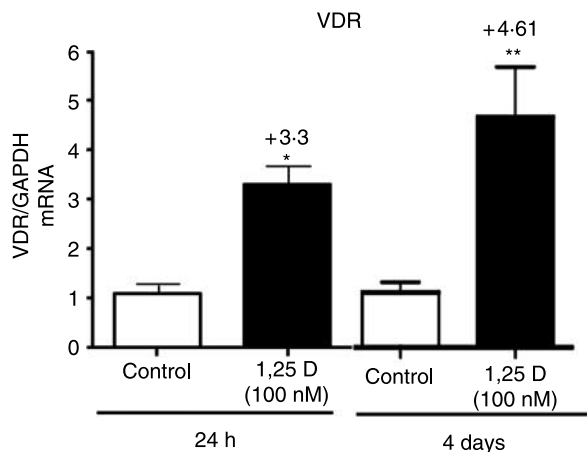


Figure 2 Transcriptional upregulation of mRNA VDR upon incubation with 1,25D in mesenchymal multipotent C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h and 4 days. Total RNA isolation followed by real-time RT-PCR was applied and normalized by GAPDH housekeeping gene. Mean \pm S.E.M. corresponds to experiments done in triplicates. * $P < 0.05$, ** $P < 0.01$.

1,25D induces an antifibrotic phenotype in C3H 10T1/2 multipotent cells due to translational downregulation of profibrotic factors

Incubations with 1,25D (100 nM) for 24 h and 4 days clearly decreased the intensity of the ICC staining of two selected profibrotic factors, TGFB1 and SERPINE1. Figure 4A and B (left panels) shows the decreased expression of TGFB1 after 24 h and 4 days incubation with 1,25D. Our visual inspection was confirmed by quantitative image analysis Fig. 4A and B (right panels) where TGFB1 expression was significantly decreased by 67% with respect to the control ($P < 0.001$) at 24 h and by 50% with respect to the control ($P < 0.01$) at 4 days. The ICC results were further confirmed by western blot at 24 h, 4 and 7 days showing that the levels of expression of TGFB1 were dramatically reduced after 7 days of continuous incubation with 1,25D Fig. 4C.

The same effect was observed on the expression of another key profibrotic factor SERPINE1 upon incubation with 1,25D. Figure 5A and B shows by ICC that SERPINE1 expression was reduced by 55% with respect to the control ($P < 0.001$) at 24 h and by 52% after 4 days incubation with 1,25D ($P < 0.001$). The ICC results for SERPINE1 were further confirmed by western blot at 24 h, 4 and 7 days of continuous incubation with 1,25D Fig. 5C.

1,25D decreases the expression of collagen I and collagen III in C3H 10T1/2 multipotent cells

C3H 10T1/2 cells pre-treated with AZCT shows high expression of collagen I as shown in Fig. 6A and B by ICC at

low and high magnification. Addition of 1,25D to the cell culture for 24 h and 4 days reduced the expression of collagen I in the cells by 65% with respect to the control ($P < 0.001$) at 24 h and by 68% with respect to the control ($P < 0.001$) at 4 days determined by quantitative image analysis.

Figure 7 shows similar results regarding the expression of collagen III under the same conditions. Addition of 1,25D to the cell culture for 24 h and 4 days reduced the expression of collagen III in the cells by 75% with respect to the control ($P < 0.001$) at 24 h and by 68% with respect to the control ($P < 0.001$) at 4 days determined by quantitative image analysis.

Effects of 1,25D on the transcriptional regulation of pro and antifibrotic factors

The antifibrotic effect of 1,25 D on the C3H 10T1/2 cells was also analyzed at the transcriptional level applying the mouse TGFB1/BMP signaling pathway gene array (MM-023) and the mouse osteogenesis gene array (MM-026). Figure 8 shows one of the two sets of membranes for each DNA microarray: TGFB1/BMP signaling pathway gene array (MM-023) (Fig. 8A) and mouse osteogenesis gene array (MM-026) (Fig. 8B) performed after 4 days incubation with or without 1,25D. Some of the genes that showed differential RNA expression between the 1,25D-treated and -untreated cells are indicated by circles (Fig. 8A and B) and were selected for the table shown in Fig. 8C, where the computer-generated ratios of spot intensities, normalized by housekeeping genes are tabulated. DNA microarray analysis shows an increase in the expression of the BMP4 as was expected due to the role of 1,25D in the osteogenic pathway. The antifibrotic effect of 1,25D was evident by the reduction of several collagen types and isoforms such as procollagen, type I α 1,2; procollagen, type III α 1; procollagen, type V α 1, and procollagen, type VI α 1,2. In addition, 1,25D increases the expression of several antifibrotic factors such as: BMP7, MMP8, and follistatin a well-known myostatin inhibitor.

This DNA microarray panel has only some of the fibrotic genes – essentially collagens, BMP members – that transduce signals triggered by the members of the TGFB1 family. For this reason, and in order to confirm and expand the DNA microarray data, we performed real-time PCR arrays using the RT2 profiler PCR SuperArray set of primers and procedures. The ratios for triplicate determinations are shown in Table 1 for 24 h and 4 days after incubation with 1,25D. The agreement between the ratios obtained by DNAmicroarrays and RT-PCR is in general adequate and provides a reasonable assessment of up- and downregulation. The results of the RT-PCR arrays confirm and provide additional data related to the antifibrotic effects of 1,25D. A substantial decrease in the mRNA expression of numerous collagen isoforms such as: collagen I α 1 and α 2, collagen II α 1, collagen III α 1, collagen V α 1, collagen VI α 1 and α 2, collagen X α 1, collagen XII α 1, and collagen XIV α 1 was found, after only 24-h incubation with 1,25D.

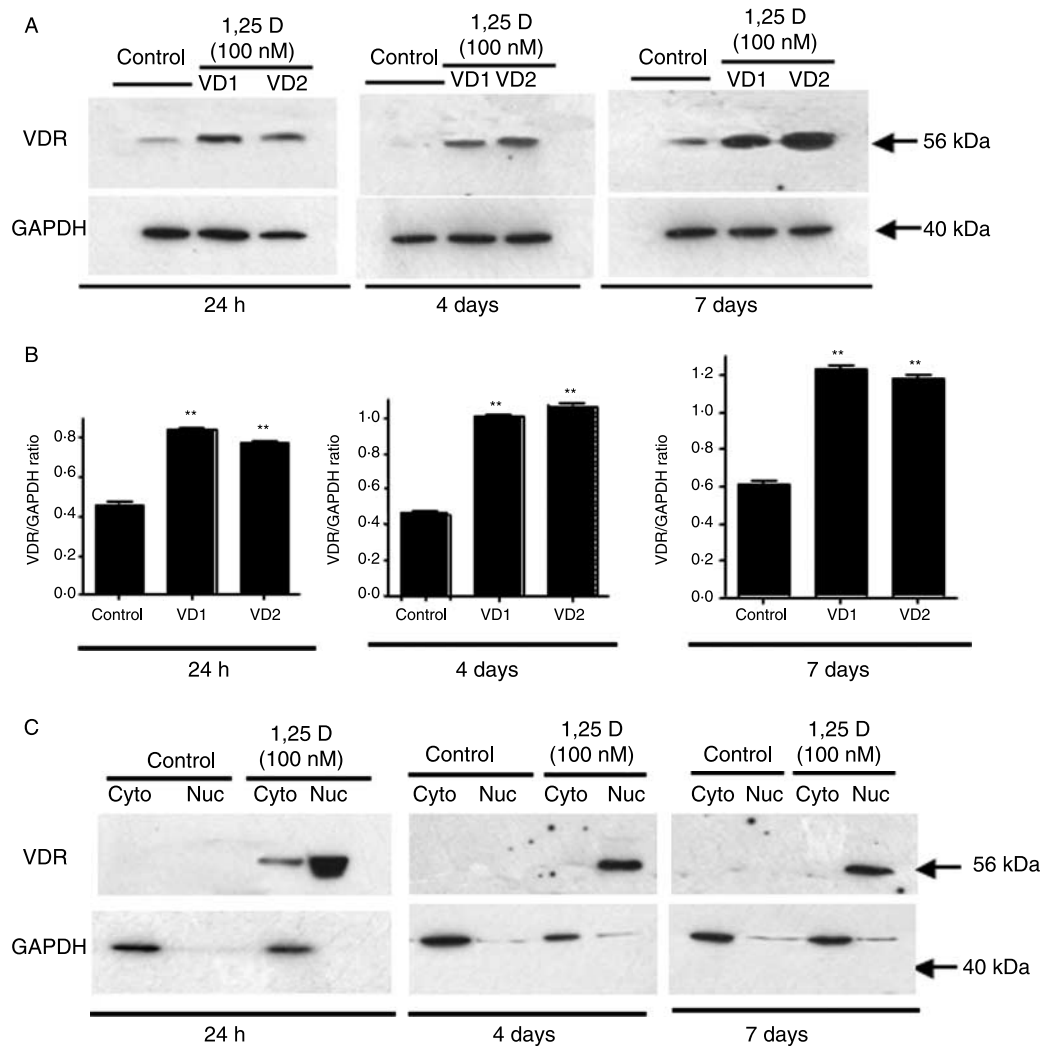


Figure 3 Translational upregulation of VDR expression and nuclear translocation upon incubation with 1,25D in mesenchymal multipotent C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h, 4 and 7 days. Western blot immunoblot analysis was performed in the whole extracts for each time point (A, top panel) and the corresponding quantitative densitometry analysis, $**P < 0.01$ (A, bottom panel). Distribution of VDR protein in subcellular fractions (B) The nuclear and cytoplasmic fractions were isolated and subjected to western immunoblot analysis for VDR as described in the text. The same membrane in each case was stripped and reprobed with anti-GAPDH antibody to verify the purity of the subcellular fractionation.

In addition, activation of the BMP signaling pathway was found as it is shown by the increase mRNA expression of *Bmp4* and *BMP6* (Table 1).

Table 1 also confirmed that incubation with 1,25D at 24 h and 4 days induced gene expression of selected antifibrotic factors such as: *Mmp8* (metalloproteinase) a collagen breakdown inducer; *Bmp7* an antagonist of transforming growth factor- β -mediated renal fibrosis; and *folistatin*, an inhibitor of the profibrotic factor myostatin. The difference in ratios between DNA microarrays and real-time PCR arrays reflects the fact that the real-time PCR arrays are much more sensitive than the DNA microarrays.

Discussion

The data presented in this manuscript to the best of our knowledge constitute the first demonstration that 1,25D also known as calcitriol, the physiologically active form of vitamin D, promotes an antifibrotic phenotype in C3H 10T1/2 cells through the increased expression and nuclear translocation of VDR. In our MMC line model, incubation with 1,25D stimulates *de novo* synthesis of VDR within 30 min to 1 h upon incubation, reaching maximal induction between 5 and 24 h. There were parallel changes in VDR specific mRNA levels indicating that the upregulation

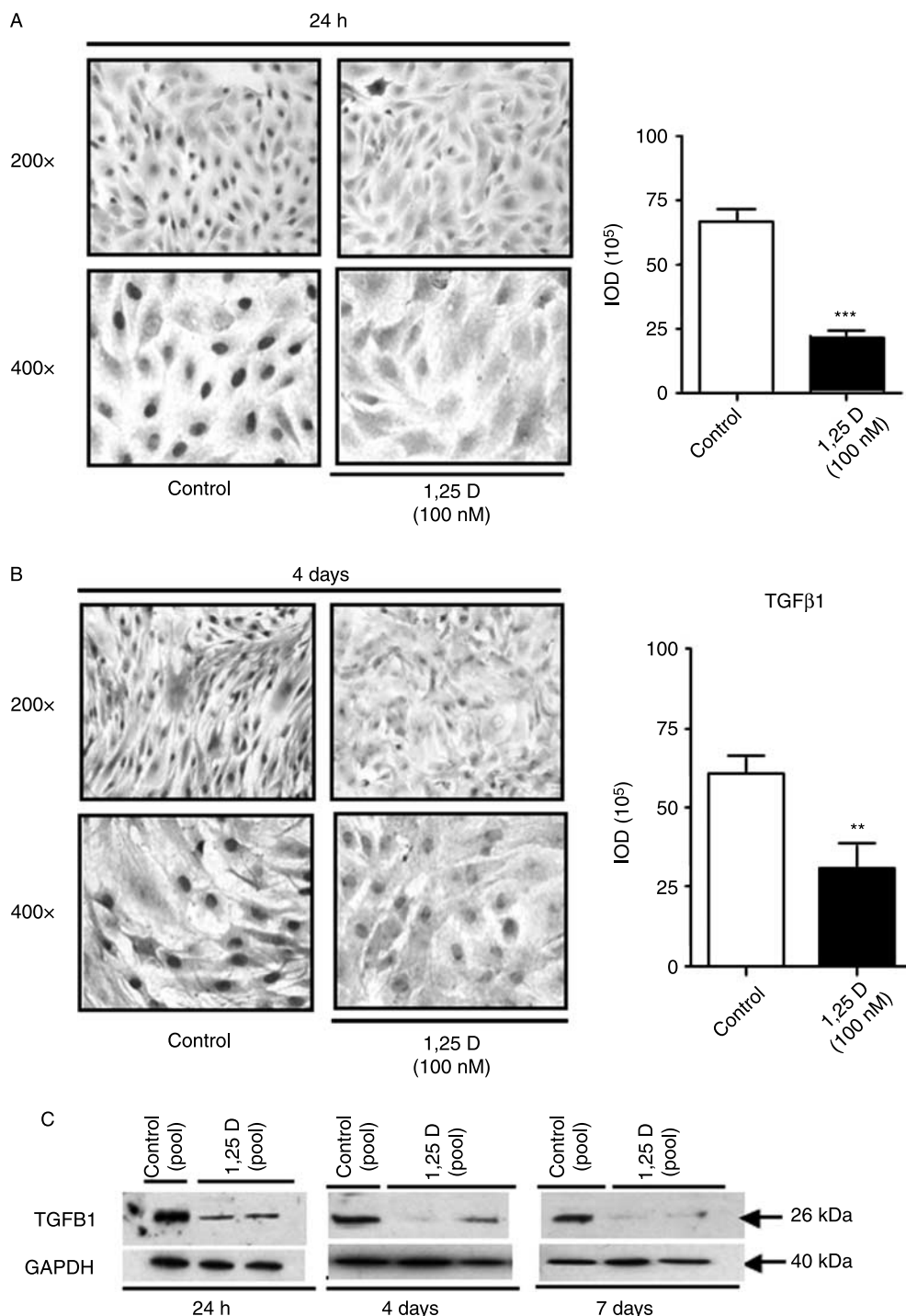


Figure 4 Effects of 1,25D on the expression of the profibrotic marker TGFβ1 in C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h, 4 and 7 days. Immunocytochemistry for TGFβ1 were performed at 24 h (A) and 4 days (B) on eight-well removable chambers slides. Mean \pm S.E.M. corresponds to experiments done in triplicates of the integrated optical density (IOD) by quantitative image analysis. ** $P < 0.01$, *** $P < 0.001$. (C) Western blot analysis were performed with an anti-TGFβ1 antibody to confirm the results obtained at 24 h, 4 and 7 days using a pool of cells done in triplicates from two different experiments. The same membrane was stripped and reprobbed with anti-GAPDH antibody (housekeeping gene).

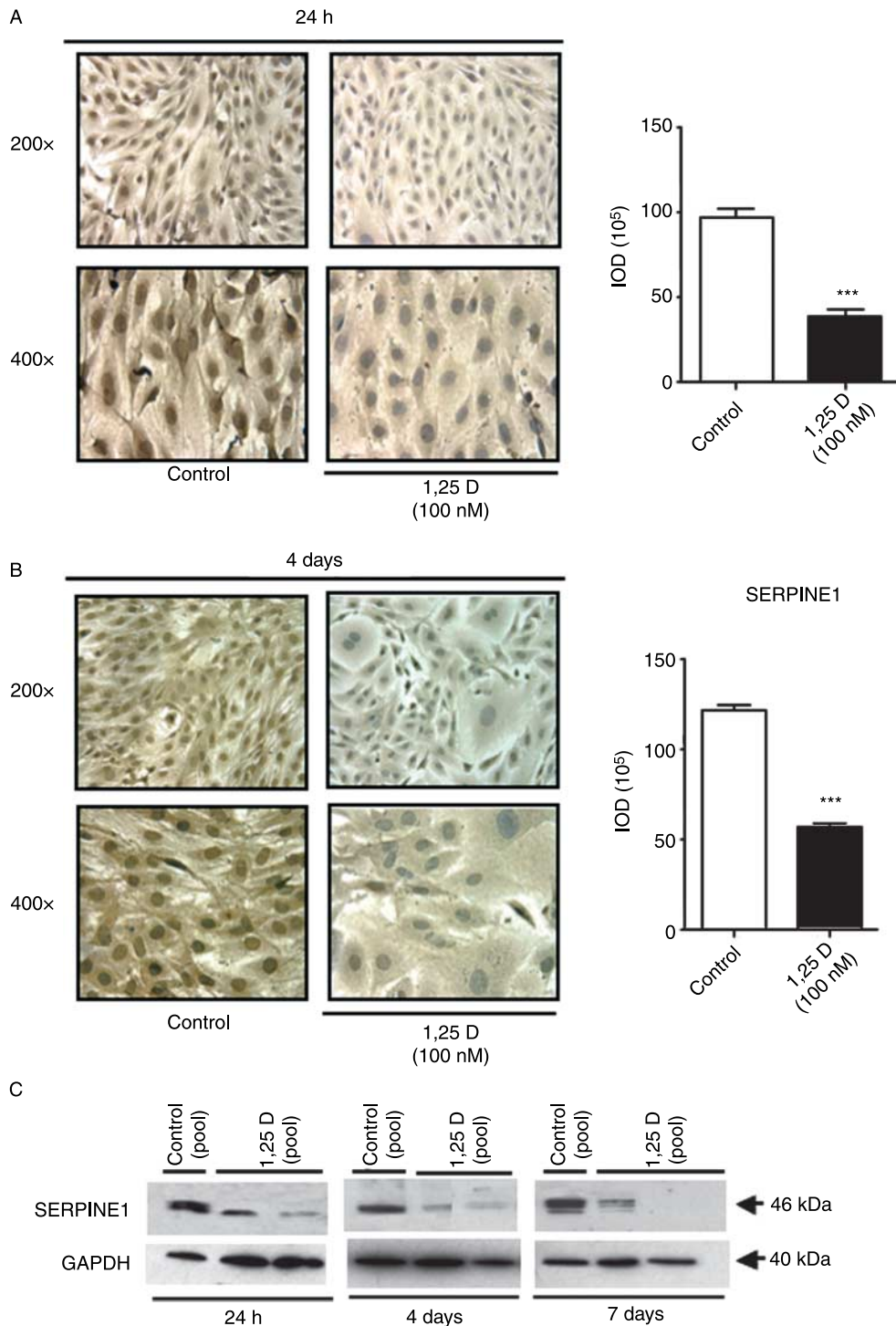


Figure 5 Effects of 1,25D on the expression of the profibrotic marker SERPINE1 in C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h, 4 and 7 days. Immunocytochemistry for SERPINE1 were performed at 24 h (A) and 4 days (B) on eight-well removable chambers slides. Mean \pm S.E.M. corresponds to experiments done in triplicates of the integrated optical density (IOD) by quantitative image analysis *** $P < 0.001$. (C) Western blot analysis were performed with an anti-SERPINE1 antibody to confirm the results obtained at 24 h, 4 and 7 days using a pool of cells done in triplicates from two different experiments. The same membrane was stripped and reprobred with anti-GAPDH antibody (housekeeping gene).

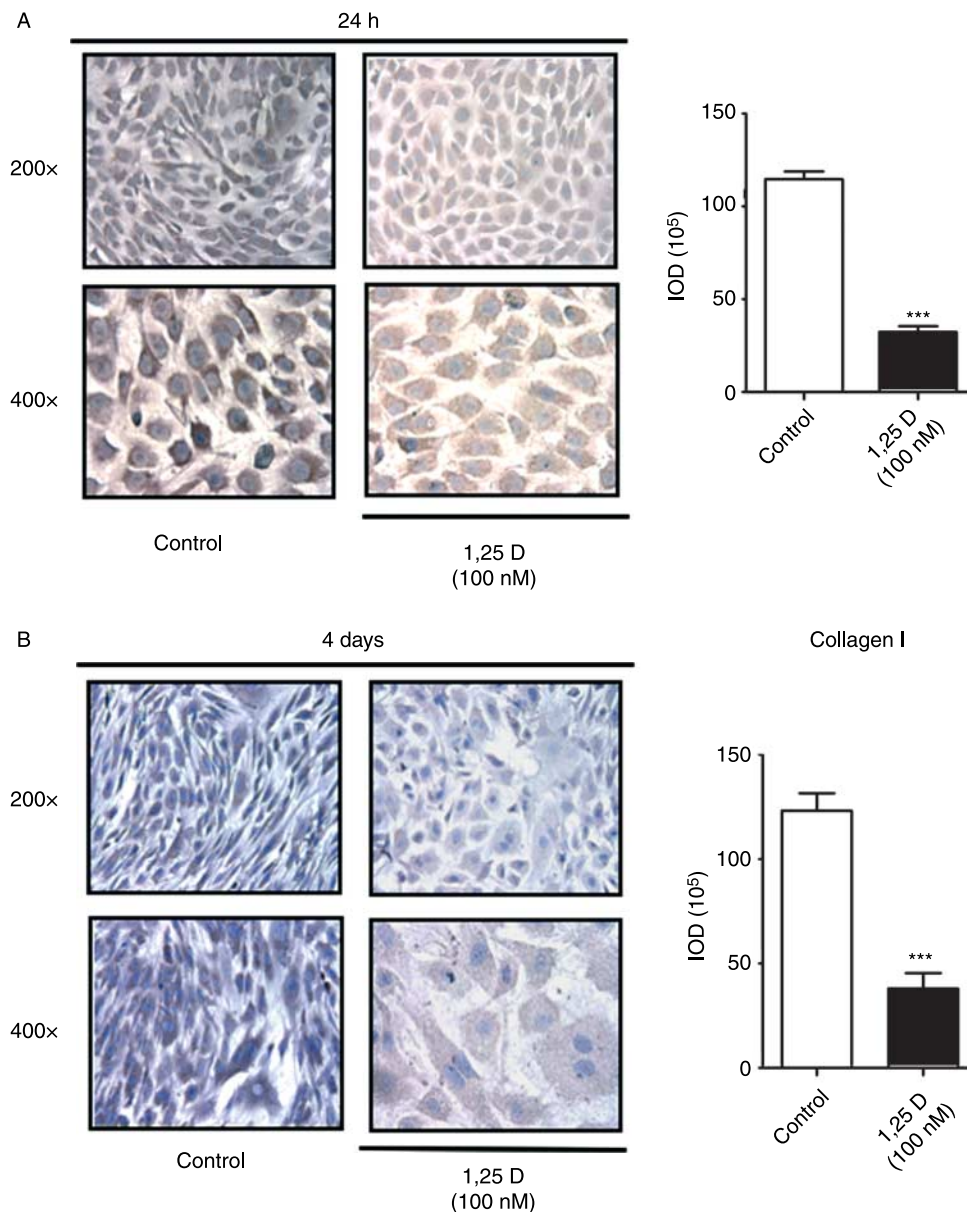


Figure 6 Effects of 1,25D on the expression of collagen I in C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h and 4 days. Immunocytochemistry for collagen I were performed at 24 h (A) and 4 days (B) on eight-well removable chambers slides. Mean \pm s.e.m. corresponds to experiments done in triplicates of the integrated optical density (IOD) by quantitative image analysis *** $P < 0.001$.

of VDR is dependent on *de novo* mRNA synthesis. Our finding that 1,25D reduces the expression of TGFB1 and SERPINE1 (two well-known profibrotic factors; Li *et al.* 2004, Mauviel 2005, Eddy & Fogo 2006) is consistent with that of Wu-Wong *et al.* (2006) who demonstrated that vitamin D analogs downregulated SERPINE1 in coronary smooth cells, and with that of Tan *et al.* (2006), who showed that paricalcitol reduced renal TGFB1 in unilateral ureteral obstructed rats, although in these studies

it was postulated that this reduction was mediated by inhibition of the renin-angiotensin system and not a direct effect of vitamin D (Li *et al.* 2002, Zhang *et al.* 2008). In addition, Aschenbrenner *et al.* (2001) reported that 1,25D treated rats had a significant reduction of TGFB1 *in vivo*, in agreement with our *in vitro* experiments, although RT-PCR demonstrated no difference in TGFB1 mRNA expression suggesting their findings were mediated through non-genomic pathways.

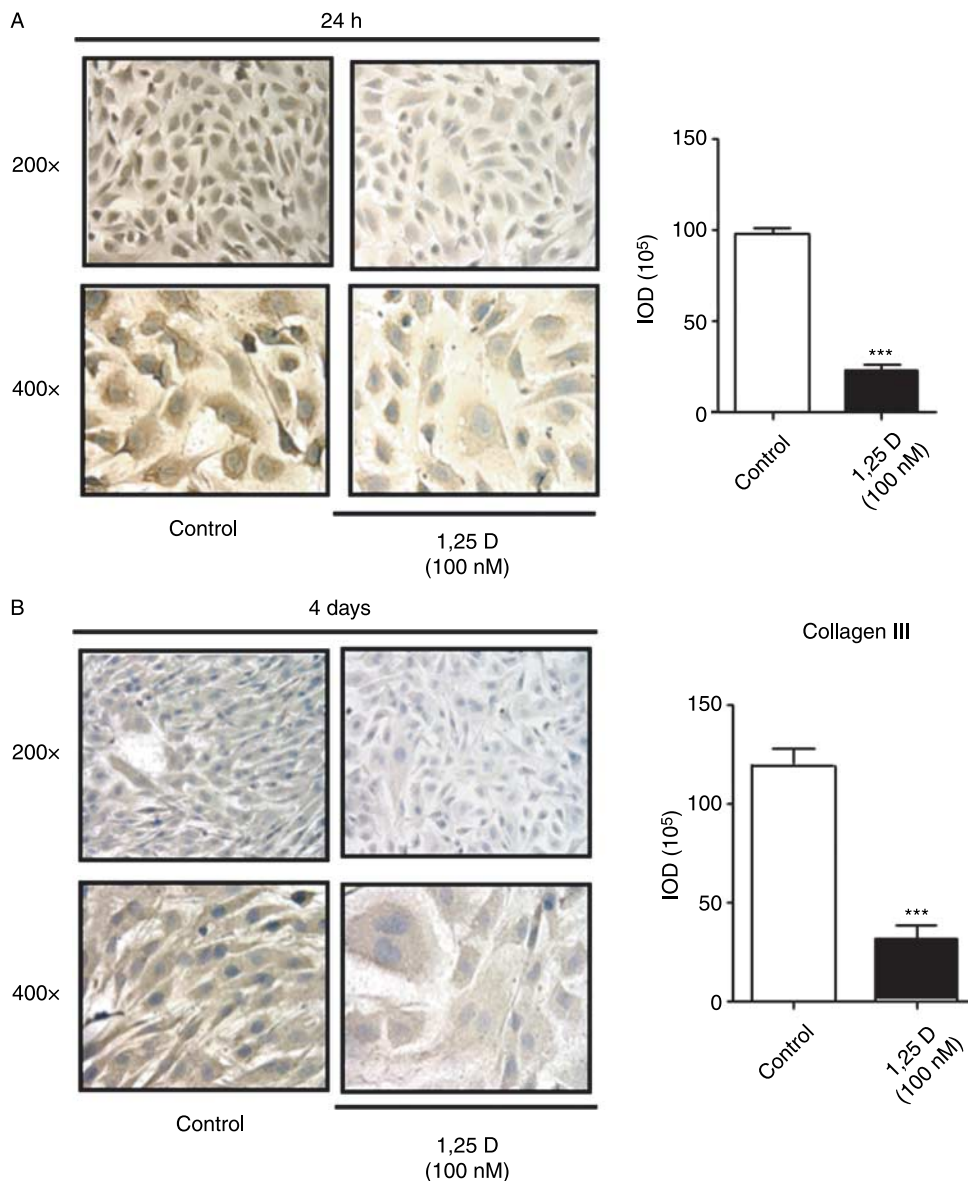
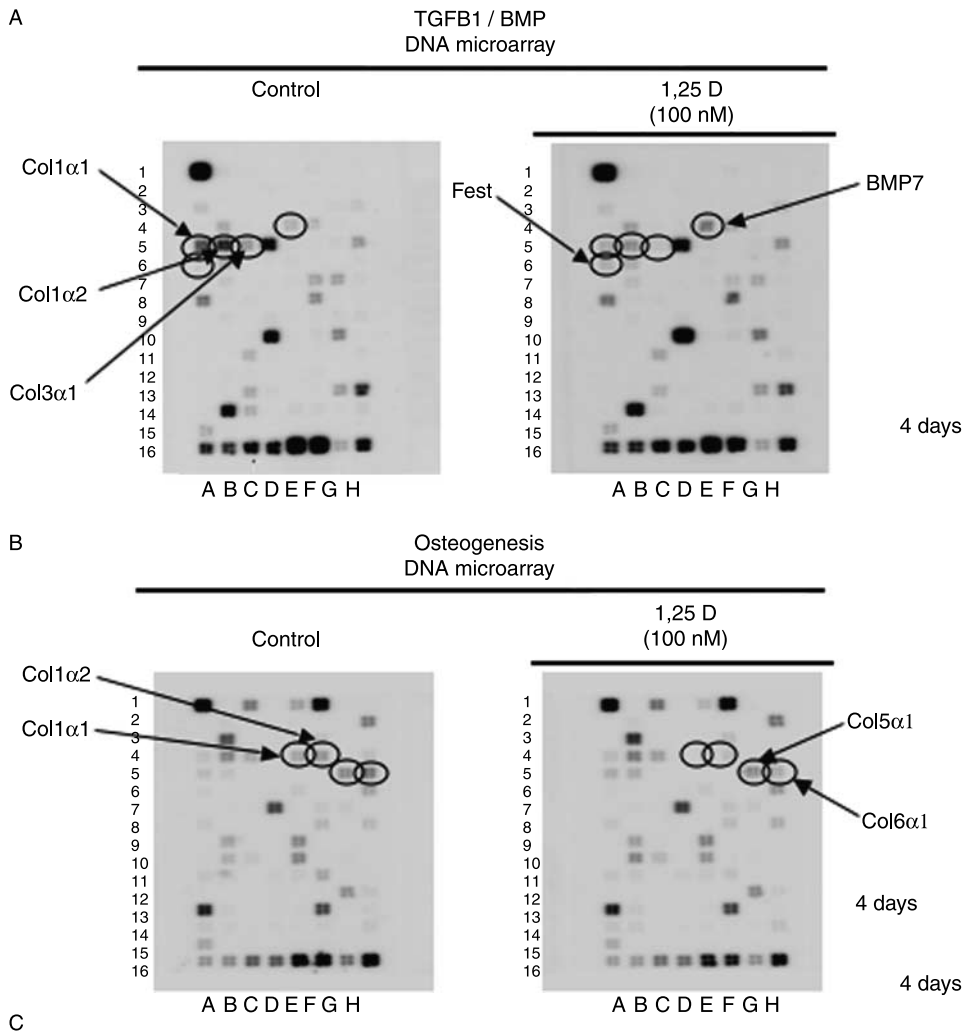


Figure 7 Effects of 1,25D on the expression of collagen III in C3H 10T1/2 cells. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h and 4 days. Immunocytochemistry for collagen III were performed at 24 h (A) and 4 days (B) on eight-well removable chambers slides. Mean \pm S.E.M. corresponds to experiments done in triplicates of the integrated optical density (IOD) by quantitative image analysis *** $P < 0.001$.

Our findings indicate that an early (24 h) and late (4 and 7 days) VDR mediated mechanism induces the suppression of TGF β 1 and SERPINE1. Nevertheless, continuous incubation (4 and 7 days) with 1,25D to our cell cultures decreased the expression of the products that ultimately define a fibrotic process, which is collagen expression (Bhagal *et al.* 2005, Attallah *et al.* 2007). We observed a substantial decrease in collagen I, collagen III expression, and many other collagen isoforms at early (24 h) and late time points (4 and 7 days) after continuous incubation with vitamin D.

In addition to the inhibitory effects on the expression of profibrotic factors and collagen expression, 1,25D also induces upregulation of antifibrotic factors. Of particular interest is the increased expression of BMP7, a bone morphogenic protein factor that has been shown to protect against diabetic nephropathy in humans (Wang *et al.* 2006), and, in *in vitro* studies to be a potent antagonist of TGF β 1 mediated fibrotic effects on renal cells (Gould *et al.* 2002, Patel & Dressler 2005, Zhang *et al.* 2005), including TGF β 1 induced epithelial-to-mesenchymal transition



Symbol	Description	DNA microarray Ratios (4 days)
BMP4	Bone morphogenetic protein 4	+1.75
Col1 α 1	Procollagen, type I, α 1	-2.1
Col1 α 2	Procollagen, type I, α 2	-2.03
Col3 α 1	Procollagen, type III, α 1	-1.96
Col5 α 1	Procollagen, type V, α 1	-2.2
Col6 α 1	Procollagen, type VI, α 1	-2.1
Col6 α 2	Procollagen, type VI, α 2	-2.5
Fst	Follistatin	+3.36
MMP8	Matrix metalloproteinase 8	+2.35
BMP7	Bone morphogenetic protein 7	+2.01

Table 1 Effects of 1,25D on the transcriptional expression of different collagen isoforms and other and antifibrotic genes in C3H 10T1/2 cells by real-time PCR arrays

Symbol	Description	Real-time PCR array ratios (24 h)	Real-time PCR array ratios (4 days)
BMP4	Bone morphogenetic protein 4	+2.30	+3.17
BMP6	Bone morphogenetic protein 6	+1.63	+2.41
Col1 α 1	Procollagen, type I, α 1	-1.74	-4.3
Col1 α 2	Procollagen, type I, α 2	-2.38	-5.4
Col2 α 1	Procollagen, type II, α 1	-2.65	-5.61
Col3 α 1	Procollagen, type III, α 1	-2.64	-4.7
Col5 α 1	Procollagen, type IV, α 1	-2.0	-4.2
Col6 α 1	Procollagen, type VI, α 1	-3.25	-4.39
Col6 α 2	Procollagen, type VI, α 2	-3.48	-7.64
Col10 α 1	Procollagen, type X, α 1	-2.26	-3.32
Col12 α 1	Procollagen, type XI I, α 1	-1.87	-2.89
Col14 α 1	Procollagen, type XIV, α 1	-1.56	-2.5
Fst	Follistatin	+6.56	+7.82
BMP7	Bone morphogenetic protein 7	+4.21	+6.35
MMP8	Matrix metalloproteinase 8	+3.46	+5.35

Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 24 h and 4 days. Total RNA was subjected to RT-real-time PCR by the RT²-PCR profiler TGF β 1/BMP and osteogenesis array and the ratios between the 1,25D treated and 1,25D-untreated cells corrected by GAPDH were calculated for the assays performed in triplicates.

(EMT) of tubular epithelial cells (Zeisberg *et al.* 2004, 2005).

In addition, 1,25D induced upregulation of other key antifibrotic factors providing additional insights into the possible mechanism by which 1,25D promotes an antifibrotic phenotype. Incubation with 1,25D increased the expression of matrix metalloproteinase 8 (MMP-8), a potent collagen breakdown inducer, which cleaves collagen at a single site and renders it susceptible to degradation by other MMPs and proteases leading to a reversion of early fibrosis (Siller-Lopez *et al.* 2004, Newby 2007) and follistatin (Hill *et al.* 2002, Amthor *et al.* 2004, Kocamis *et al.* 2004), an important myostatin inhibitor. Myostatin is well known as a negative regulator of muscle mass (McPherron *et al.* 1997, Lee & McPherron 2001, Lee 2004), and more recently described by us and others to be an important profibrotic factor (McCroskery *et al.* 2005, Zhu *et al.* 2007, Artaza *et al.* 2008a,b).

Thus, the antifibrotic effect observed in the current study in MMCs by the active form of vitamin D suggests a potential therapeutic or preventive capacity of vitamin D, consistent with similar suggestions for the antifibrotic potential of other proven antifibrotic and profibrotic genes such as decorin (Li *et al.* 2004), Smad7 cDNA (Forbes *et al.* 2006), follistatin (Aoki *et al.* 2005, Patella *et al.* 2006), and deacetylase inhibitors which induce follistatin expression (Iezzi *et al.* 2004).

Besides, the antifibrotic effect found in our study, incubation with 1,25D induces the expression of bone morphogenetic proteins, Bmp4 and Bmp6, which is in agreement with prior studies showing that 1,25D activates the BMP signaling pathway in MCF10 breast epithelial cells (Lee *et al.* 2006a,b) indicating a biological activity of 1,25D through VDR.

We believe that this study has clinical relevance, because it provides a clear and direct mechanistic explanation for the antifibrotic effect of 1,25D that is consistent with clinical observations that low levels of vitamin D are associated with increased cardiovascular risk factors (Martins *et al.* 2007, Wang *et al.* 2008) and increased mortality (Wolf *et al.* 2007, Melamed *et al.* 2008, Ravani *et al.* 2008), and the clinical observation that continuous administration of 1,25D attenuates the progression of glomerular and tubular interstitial fibrosis in patients with chronic kidney disease (Tian *et al.* 2007) and is associated with reduced mortality (Kovesdy *et al.* 2008, Wolf *et al.* 2008). Further studies are needed to establish a direct transcriptional control of these pro and antifibrotic genes by the 1,25D-VDR complexes, even though we demonstrated a parallel correlation between the expression of these genes and VDR at early time points suggesting a likely direct effect of the 1,25D-VDR complexes on transcriptional regulation of antifibrotic genes.

Figure 8 Effects of 1,25D on the transcriptional expression of different collagen isoforms and other fibrotic genes in C3H 10T1/2 cells by DNA microarrays. Cultures of C3H 10T1/2 cells were treated as in Fig. 1 and incubated with or without 1,25D (100 nM) for 4 days on 75 cm² flasks. Total RNA was isolated and subjected to DNA microarray analysis for genes related to the TGF β 1/BMP signaling pathway (A) and for osteogenesis gene array (B). Representative membranes for each array are displayed. The circles show changes in the expression of some antifibrotic and profibrotic genes that were upregulated or downregulated by 1,25D treatment. (C) Ratios between the 1,25D treated and 1,25D-untreated cells at 4 days, corrected by GAPDH were calculated for the assays performed in duplicates.

We acknowledge that our current findings in a model of multipotent mesenchymal cells primed with azacytidine, which have been shown in humans (Adams *et al.* 2005) and in cell culture (Artaza *et al.* 2008a,b) to promote a fibrotic phenotype, may not accurately reflect what occurs in a clinical state. However, they have important implications for understanding the mechanistic pathways that are independent of calcium and parathyroid hormone, and may be useful in assessing potential future interventions with vitamin D analogs with a lesser calcemic effect than the parent compound that may have enhanced antifibrotic effects.

In summary, 1,25D (the active form of vitamin D) induced a VDR-mediated antifibrotic signaling phenotype in multipotent mesenchymal cells characterized by a decreased expression of profibrotic markers and an increased expression of antifibrotic markers leading to an effective reduction in collagen expression. This study demonstrates direct pathways through which vitamin D modulates fibrosis, and supports the emerging clinical findings linking vitamin D deficiency to adverse cardiovascular health and other chronic medical conditions. The multipotent mesenchymal cell system may be a viable model for exploring parathyroid and calcemic independent non-classic effects of vitamin D and vitamin D analogs as potential therapeutic agents.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this scientific work.

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