1,25-Vitamin D₃ promotes cardiac differentiation through modulation of the WNT signaling pathway

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Abstract

Cardiovascular disease (CVD) remains the leading cause of death worldwide. Low levels of vitamin D are associated with high risk of myocardial infarction, even after controlling for factors associated with coronary artery disease. A growing body of evidence indicates that vitamin D plays an important role in CVD-related signaling pathways. However, little is known about the molecular mechanism by which vitamin D modulates heart development. The WNT signaling pathway plays a pivotal role in tissue development by controlling stem cell renewal, lineage selection and, even more importantly, heart development. In this study, we examined the role of 1,25-D₃ (the active form of vitamin D) on cardiomyocyte proliferation, apoptosis, cell phenotype, cell cycle progression and differentiation into cardiomyotubes. We determined that the addition of 1,25-D₃ to cardiomyocytes cells: i) inhibits cell proliferation without promoting apoptosis; ii) decreases expression of genes related to the regulation of the cell cycle; iii) promotes formation of cardiomyotubes; iv) induces the expression of casein kinase-1- α 1, a negative regulator of the canonical WNT signaling pathway; and v) increases the expression of the noncanonical *WNT11*, which it has been demonstrated to induce cardiac differentiation during embryonic development and in adult cells. In conclusion, we postulate that vitamin D promotes cardiac differentiation through a negative modulation of the canonical WNT signaling pathway and by upregulating the expression of *WNT11*. These results indicate that vitamin D repletion to prevent and/or improve cardiovascular disorders that are linked with abnormal cardiac differentiation, such as post infarction cardiac remodeling, deserve further study.

Key Words

- VDR
- Wnt11
- Csnk1 α 1
- β -catenin
- Gsk3 β
- APC

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Introduction

Hypovitaminosis D as well as other chronic medical conditions is increasingly recognized as an independent predictor for primary cardiovascular events and related

diseases (CVD; Martins *et al.* 2007, Giovannucci *et al.* 2008, Wang *et al.* 2008, van Holten *et al.* 2013). In addition, vitamin D deficiency is highly prevalent in heart failure

(HF) patients, being a significant predictor of reduced survival, whereas supplementation was associated with an improved outcome (Gotsman *et al.* 2012).

The National Health and Nutritional Examination Surveys (NHANES) (1988–1994, 2000–2004) conducted in the US have provided a means to explore the cross-sectional associations between vitamin D status and CVD. Kendrick *et al.* (2009) have reported that individuals surveyed in NHANES 1988–1994 with vitamin D deficiency (25-D <20 ng/ml) had higher prevalence of self-reported angina, myocardial infarction, and HF compared with individuals with higher levels of vitamin D. In the NHANES 2000–2004 survey, vitamin D deficiency was associated with increased prevalence of self-reported coronary heart disease, HF, and peripheral vascular disease (Kim *et al.* 2008, Melamed *et al.* 2008). Moreover, several cardiovascular risk factors have been associated with lower vitamin D status, including hypertension, diabetes, elevated BMI (>30), elevated triglyceride level, and microalbuminuria in the NHANES 1988–1994 survey report (Martins *et al.* 2007). The prevalence of peripheral arterial disease was also increased in comparisons of the lowest quartile of 25-D with the highest quartile of 25-D (Artaza *et al.* 2009). It has also been shown that in nonhypertensive individuals from the NHANES 1988–1994 survey, optimal vitamin D status (>32 ng/ml) was associated with a 20% reduction in the rate of rise in blood pressure with age (Scragg *et al.* 2007, Judd *et al.* 2008).

A few *in vitro* and *in vivo* studies have also evaluated the role of vitamin D acting directly on cardiac tissue, especially in response to injury. It has been demonstrated that matrix metalloproteinases, which contribute to aberrant cardiomyocyte remodeling in response to injury and atherosclerosis, were upregulated in vitamin D receptor (*Vdr*)-knockout mice (Rahman *et al.* 2007). It has also been shown that *Vdr*-knockout mice have impaired cardiac relaxation and contractility and develop left ventricular (LV) hypertrophy (Simpson *et al.* 2007, Tishkoff *et al.* 2008). A study in HL-1 murine cardiac myocytes showed that 1,25-D₃ significantly decreased cell proliferation; increased cell size, leading to hypoplasia, and slight hypertrophy; and altered the morphology of dividing cardiomyocytes, demonstrating that 1,25-D₃ is involved in the maintenance of heart cell structure and function at the cellular level (Nibbelink *et al.* 2007). In addition, our group demonstrated that 1,25-D₃ inhibited profibrotic markers *in vitro* in mesenchymal multipotent cells, indicating that 1,25-D₃ may also have a direct effect on the vasculature fibrotic response to injury (Artaza *et al.* 2009).

Even though most evidence indicates an adverse effect of low vitamin D levels on CVD, the role of 1,25-D₃ in cardiac cell differentiation and repair remains poorly understood. In this study, we used a cardiac myoblast cell line H9c2, derived from embryonic rat heart, which has been extensively used as an *in vitro* model for cardiac muscle function (Kimes & Brandt 1976, Artaza *et al.* 2007). The aim of this study was to test the hypothesis that 1,25-D₃ promotes myocardial cell differentiation through inhibition of the WNT signaling pathway and to determine the associated molecular mechanism(s) in a well-known and widely used heart-derived cell model.

Materials and methods

Cell culture

H9c2 rat embryonic myocardium cells (ATCC, Manassas, VA, USA) grown in DMEM and supplemented with 10% dialyzed fetal bovine serum at 37 °C and 5% CO₂ were seeded at 60–70% confluence into T75 flasks, eight-well chamber slides, or six-well plates. The next day, the cells were incubated for 4 and 7 days with or without 100 nM of 1,25-D₃ (1 α , 25(OH)₂ vitamin D₃) also known as calcitriol (Sigma–Aldrich) dissolved in 0.1% ethanol as vehicle in DMEM–10% dialyzed fetal bovine serum. For proliferation studies, the cells were incubated with 1,25-D₃ (10–500 nM) for 4 days. The control groups were incubated in parallel with 0.1% ethanol in DMEM–10% dialyzed fetal bovine serum (Invitrogen). The 100 nM supra-physiological concentration of 1,25-D₃ applied in the experimental design was based on the present and previous dose–response studies and it is consistent with a commonly used dose in different cell lines or in primary cell culture studies (Barbosa *et al.* 2004, Cardús *et al.* 2006, Artaza & Norris 2009, Artaza *et al.* 2010, Khanna-jain *et al.* 2010, Ramirez *et al.* 2010). Because of the short half-life of 1,25-D₃, the cell culture medium was replaced every 24 h (Garcia *et al.* 2011, 2013).

Cell proliferation assay

Cell proliferation was determined in 96-well plates by the formazan dye assay (Promega Corp.). The cells were grown at an initial density of 4000 cells/well and then treated for 4 days with 1,25-D₃ in a concentration range from 10 to 500 nM. At the end of the incubation time, 100 μ l of formazan substrate buffer was added to the cultures for 3 h at 37 °C in 5% CO₂, and the absorbance was read at 490 nm. For cell counting, cells were removed by

trypsinization and the number of viable cells was counted in a hemocytometer with Trypan blue staining (Artaza *et al.* 2007).

In parallel experiments, cell proliferation was also evaluated by changes in gene expression of related cell-proliferation markers after 4 days incubation with or without 1,25-D₃. The cell proliferation markers applied were the following: i) proliferating cell nuclear antigen (PCNA), a protein essential for DNA replication, repair, and cell proliferation (Essers *et al.* 2005) and ii) gene expression, evaluated by real time PCR, of *Mki67*, a cellular marker strictly associated with cell proliferation (Gerdes *et al.* 1983).

Cell morphology/phenotype

Changes in cell morphology/phenotype were determined in living cells in culture by the PKH2 Green Fluorescent Cell Linker assay (Sigma–Aldrich) (Horan & Slezak 1989). After an initial and stable incorporation of the PKH2-GL (2 × 10^{−6} M) into the lipid regions of the cell membrane, cells were counterstained with DAPI 0.02% (Invitrogen). The cells were grown at an initial density of 8000 cells/well in eight-wells chamber slides and were treated for 7 days with or without 1,25-D₃ (10–500 nM). Serial pictures at 200× magnification were taken every 24 h with a Leica DMLB fluorescence microscope coupled to Spot RT digital camera. The areas occupied by cells per field were analyzed using ImagePro-Plus 7.1 software (Media Cybernetics, Silver Spring, MD, USA). At least ten pictures were taken per treatment group per well done in duplicate. All the experiments were repeated at least three times.

Apoptosis

The apoptotic index was determined by the TUNEL method, based on the ability of terminal TdT to catalyze the addition of digoxenin-dUTP and dATP to 3'-OH ends of cleaved DNA. The cells were seeded onto eight-well chamber slides and were incubated with or without 1,25-D₃ (100 nM) for 4 days and then fixed in 2% *p*-formaldehyde in 1× PBS. The cells were treated with 2% H₂O₂ for quenching the endogenous peroxidase activity, digoxigenin-conjugated nucleotides and TdT, and finally anti-digoxigenin-peroxidase. The slides were stained with 0.5% diaminobenzidine/0.01% H₂O₂ and counterstained with hematoxylin. As a negative control, PBS (1×) buffer was substituted for the TdT enzyme. Quantification of the apoptotic index was done at 400×. In all cases, 20 fields were randomly selected and the apoptotic index of each field was calculated as the

percentage of TUNEL-positive cells (Essers *et al.* 2005, Artaza *et al.* 2007).

Detection of VDR by immunofluorescence

The cells plated on eight-well chamber slides were incubated with or without 1,25-D₃ (100 nM; based on the results of dose–response analyses) in a time-course-dependent manner from 0 to 4 days. At the end of the corresponding incubation times, the cells were washed thrice with PBS (1×) and fixed in 2% *p*-formaldehyde. The cells were blocked with goat serum and incubated with rabbit polyclonal antibody against VDR at a dilution of 1:50 (Santa Cruz Biotechnology). 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 µg/ml) (Vector Laboratories, Burlingame, CA, USA). After several washes, the cells were counterstained with DAPI. In negative controls, we either omitted the first antibody or used a rabbit nonspecific IgG (Artaza *et al.* 2005, 2008). To ensure specific staining, an additional negative control was included by pre-absorbing the primary VDR antibody with a VDR-specific peptide (1 µM), 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 primary antibody at the same dilution applied for the experiment. No positive staining was observed in the cells by immunofluorescence in this negative control. At the end, the slides were detached and mounted in 'prolong anti-fade' (Molecular Probes, Eugene, OR, USA) and were examined under a fluorescence microscope, Leica DMLB, coupled to a Spot RT digital camera.

RT² profiler PCR array analysis of cell cycle and WNT-related target genes

Total cellular RNA was isolated using Trizol Reagent (Invitrogen) from H9c2 cells treated with or without 1,25-D₃ (100 nM) for 4 days. The isolated RNA was subjected to RT, and the resulting cDNA was analyzed by RT² profiler Rat Cell Cycle PCR array (PARN-020) and by RT² profiler Rat WNT Signaling Pathway PCR Array (PARN-043A) (SABiosciences Corp., Frederick, MD, USA). The analysis of cell cycle and WNT-related target genes were done in triplicate. The Rat Cell Cycle RT² Profiler PCR Array contains genes that regulate the cell cycle, the transitions between each of the phases, DNA replication, checkpoints, and arrest. The Rat WNT RT² Profiler PCR Array profiles the expression of genes related to

WNT-mediated signal transduction. Each array contains a panel of 84 primer sets related to the cell cycle and to WNT signaling pathway plus five housekeeping genes and two negative controls. Real-time PCRs were carried out as follows: melting for 10 min at 95 °C, 40 cycles of two-step PCR including melting for 15 s 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.) (Garcia *et al.* 2011, 2013).

Real-time quantitative PCR

Total RNA was extracted using Trizol Reagent (Invitrogen) and equal amounts (1 µg) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems). Rat gene PCR primer sets (RT²) for *Mki67*, *Pcna*, *Casp3*, *Bcl2*, casein kinase-1- $\alpha 1$ (*Ck1 α 1*), and *Wnt11* were obtained from SABiosciences Corp. 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) (Garcia *et al.* 2011, 2013).

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. The samples of 25 ng cDNA were analyzed in quadruplicate in parallel with GAPDH controls; standard curves (threshold cycle vs log pg cDNA) were generated by log dilutions of standard cDNA (reverse-transcribed mRNA from H9c2 cells in AM) from 0.1 pg to 100 ng. Experimental mRNA starting quantities were then calculated from the standard curves and averaged using i-Cycler, iQ software as described previously (Garcia *et al.* 2011, 2013). The ratios of marker experimental gene (e.g., *Mki67*, *Pcna*, *Casp3*, *Bcl2*, *Csnk1 α 1*, and *Wnt11* mRNA) to that of *Gapdh* mRNA were computed and normalized with control (untreated) samples as 100%.

Immunocytochemical analyses of *Pcna*, cTroponin 3, Ck1 α 1 and *Wnt11* antigens

After incubating the cardiac cells for 4 days with or without 1,25-D₃ (100 nM), the cells were washed five times with PBS (1 ×) and fixed in 2% *p*-formaldehyde, quenched with H₂O₂, blocked with horse or rabbit serum, and incubated with anti-PCNA MoAb (1:400) (Millipore, Temecula, CA, USA), anti-cardiac muscle cTroponin 3 MoAb (1:500) (Santa Cruz Biotechnology), anti-CSNK1 α 1 rabbit polyclonal antibody (1/100), and anti-WNT11 rabbit polyclonal

antibody (1/100) (Abcam, Inc., Cambridge, MA, USA). The cells were detected 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 Mayer's hematoxylin solution (Sigma–Aldrich). In negative controls, we either omitted the first antibody or used a rabbit nonspecific IgG (Garcia *et al.* 2011).

Western blotting and densitometry analyses

The cell lysates (25–40 µg protein) were subjected to western blotting analyses by 4–15% Tris–HCl PAGE (Bio-Rad) in a running buffer (Tris/glycine/SDS). The proteins were transferred overnight at 4 °C onto the nitrocellulose membranes in a transfer buffer (Tris/glycine/methanol). The next day, the nonspecific binding was blocked by immersing the membranes into 5% nonfat dried milk, 0.1% (v/v) Tween 20 in PBS for 2 h at RT. After several washes with the washing buffer (PBS Tween 0.1%), the membranes were incubated with the primary antibodies for 3 h at RT or overnight at 4 °C. MoAb were as follow: i) PCNA (1/500) (Millipore), ii) GAPDH (1/10 000) (Chemicon International, Temecula, CA, USA), iii) BCL2 (1/500), iv) GSK3 β (1/500) (BD Biosciences, San Jose, CA, USA), v) cyclin D1 (1/500), vi) cyclin D3 (1/500), and vii) CDK4 (1/500) (Cell Signaling Technology, Inc., Danvers, MA, USA). The polyclonal antibodies were used for i) VDR (1/500), ii) CDK2 (1/500), iii) p21 (1/500), iv) p27 (1/500), v) APC (1/500) (Santa Cruz Biotechnology, Inc.), vi) WNT11 (1/200), and vii) CSNK1 α 1 (1/200) (Abcam, Inc.), viii) anti-cardiac muscle Troponin 3 MAB (1:500) (Santa Cruz Biotechnology). After several washes with buffer, the membranes were incubated for 1 h at RT with 1/3000 dilution (anti-mouse) or 1:2000 dilution (anti-rabbit) of secondary antibody linked with HRP (Cell Signaling Technology, Inc.). After several washes, the immunoreactive bands were visualized using the Super-Signal western blotting chemiluminescence detection system (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The densitometry analysis of the bands was done with the Scion Image software beta 4.02 (Scion Corp., Frederick, MD, USA) (Garcia *et al.* 2011, 2013).

Qualitative and quantitative immunocytochemical analyses

In all cases, the immunoreactivity was quantified by image analysis using ImagePro-Plus 7.1 software

(Media Cybernetics). Two independent observers counted fields blindly and each experiment was repeated at least three times. For PCNA and TUNEL determinations, the number of positive cells at 400 $\times$ was counted in a computerized grid and results were expressed as a ratio of the percentage of positive cells/total cells per field. In all cases, ten fields at 400 $\times$ were analyzed per well. The cell size was determined by outlining and measuring the area of each cell per field. Approximately, 100 cells were measured for each experiment and the results were plotted as cardiomyotubes area expressed in μm^2 per 1,25-D₃ concentration. For determination of cTroponin 3, CSNK1 α 1, and WNT11 immunocytochemistry (ICC), the images were first calibrated for background lighting and the integrated optical density (IOD) was determined. The IOD results are proportional to the mean optical density per area and determine the amount of immunoreactive antigen present in each cell. The IOD values expressed in arbitrary units were determined for at least 20 pictures per treatment and time point (Artaza *et al.* 2010, Garcia *et al.* 2011).

To test the precision of the quantitative immunoassay, we evaluated both inter-assay variability and intra-assay variability. s.d. was 0.38 for assays in triplicates, the coefficient of variation being 1.7% for intra-assay and 5% for inter-assay determinations. The results are expressed as mean $\pm$ s.e.m. and represent the average of three independent experiments.

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 pair-wise comparisons between groups were performed by Tukey's multiple comparison test. All comparisons were two-tailed, and a *P* value less than 0.05 was considered statistically significant. The *in vitro* experiments were repeated thrice, and data from representative experiments are shown. Specifically, the RT² Profiler PCR arrays were done in triplicate and in some cases further confirmed by qRT-PCR done in triplicate.

Results

Time course of expression and nuclear translocation of VDR in H9c2 cardiac-derived cells incubated with 1,25-D₃

To determine whether the H9c2 cardiac cells expressed the VDR at basal level and whether its expression and nuclear translocation are induced upon incubation with 1,25-D₃,

experiments using immunofluorescence and western blotting was carried out at different time points. The cells were continuously incubated with or without 1,25-D₃ (100 nM) for 30 min, 1 h, 5 h, 24 h, and 4 days. By immunofluorescence (Fig. 1A) and under basal conditions (no 1,25-D₃ added), VDR immunofluorescence was barely detectable in the cells. After 30 min incubation with 1,25-D₃, VDR expression was clearly increased in the cytoplasm and the nuclear compartment. A similar observation was made at 1 and 5 h (Fig. 1A, upper panel). In contrast, after 24 h and 4 days of continuous 1,25-D₃ incubation, most of the red-fluorescence staining was observed in the nuclei (Fig. 1A, lower panel), indicating nuclear translocation of the VDR upon continuous 1,25-D₃ incubation for 24 h. The counterstaining with DAPI confirmed the nuclear localization of VDR after continuous incubation with 1,25-D₃.

The increased expression of VDR upon 1,25-D₃ exposure was further confirmed by western blotting analysis in whole-cell culture homogenates after 4 days of continuous incubation with 1,25-D₃ (100 nM) (Fig. 1B). The densitometry analysis shows a twofold increase in VDR expression after incubation with 1,25-D₃ at 4 days in comparison with the expression in controls.

1,25-D₃ inhibits cell proliferation of H9c2 cardiomyocytes

To assess whether 1,25-D₃ inhibits proliferation of H9c2 cardiomyocytes and stimulates cell differentiation by causing cells to exit the cell cycle, H9c2 cells were seeded at a low confluence (4000 cells/well) and incubated in triplicate with increasing concentrations of 1,25-D₃ for 4 days. At the end of the incubation time, cell proliferation was evaluated by the formazan assay (Fig. 2A). 1,25-D₃, starting at 50 nM, induced a statistically significant reduction in cell number reaching a plateau at 100 nM with no statistically significant difference at 500 nM compared with 100 nM in agreement with our data on multipotent cells published previously (Artaza *et al.* 2009). Based on these results, 100 nM was the selected dose used throughout the study to evaluate the effects of 1,25-D₃ on H9c2 cardiomyocytes.

Inhibition of cell proliferation was also demonstrated by real-time PCR by showing a decrease in the expression of *Mki67*, a well-known proliferation antigen, which is induced in G1, S, G2, and M phases of the cell cycle (Fig. 2B). Quiescent or resting cells in the G0 phase of the cell cycle do not express the *Mki67* antigen (Endl *et al.* 1997, Preusser *et al.* 2008).

To further corroborate the inhibition of cardiac embryonic cell proliferation promoted by continuous

Figure 1

Expression, nuclear translocation, and upregulation of protein levels of VDR upon incubation of H9c2 cardiac cells with 1,25-D₃. Cultures of H9c2 cardiac cells were incubated with or without 1,25-D₃ (100 nM) in a time course manner (0–4 days) on eight-well removable chamber slides and subjected to immunofluorescence using a polyclonal antibody for VDR followed by a Texas Red-conjugated secondary antibody. The cells were counterstained with DAPI to indicate nuclear localization. Magnification

incubation with 100 nM 1,25-D₃, the expression of the PCNA was studied by ICC. The upper panel of Figure 3A shows representative images at 200 $\times$ magnification of the decreased nuclear expression of PCNA after incubation of the cardiac cells with 100 nM of 1,25-D₃ for 4 days. The results from the visual inspection were confirmed by quantitative image analysis (Fig. 3A, lower panel), where PCNA expression was decreased 2.7-fold compared with expression in the controls ($P < 0.001$). The decreased expression of *Pcna* was even further confirmed by real-time PCR (*Pcna:Gapdh* ratio: -2.35) (Fig. 3B) and at the protein level by western blotting (Fig. 3C, left) with the corresponding densitometry analysis (Fig. 3C, right).

1,25-D₃ enhances cardiomyotube area and promotes the expression of cardiac troponin

In order to determine whether 1,25-D₃ promotes cardiac cell differentiation and increases cell size, the cells were pre-labeled with the cell membrane marker PKH2 Green Fluorescence Cell Linker and counterstained with DAPI to visualize cell boundaries and cell nuclei respectively. The upper panel of Figure 4A shows

representative fluorescence images of 1,25-D₃-treated and nontreated cardiac cells at different concentrations and the lower panel of Fig. 4A shows the corresponding image analysis results, indicating a statistically significant increase in cardiomyotube area upon incubating the cardiac cells with increasing doses of 1,25-D₃ starting at 50 nM for 7 days.

To further corroborate the effect of 1,25-D₃ on cardiac differentiation, the expression of cTroponin 3, a marker of cardiac tissue formation, was evaluated. Figure 4B shows an increased expression of the cardiac marker cTroponin 3 by ICC (left), with the corresponding image analysis (right) upon incubating the cells with 1,25-D₃ (100 nM) for 7 days, indicating formation of cardiac tissue.

1,25-D₃ induces cell cycle exit of H9c2 cardiac cells without inducing apoptosis

The effect of 1,25-D₃ incubation on the expression of genes/proteins related to activation, promotion, or exiting from the cell cycle was investigated using real-time PCR arrays (Fig. 5A) and western blotting analysis (Fig. 5B, C, and D). Cell division relies on the activation of cyclins,

Figure 2

Increasing concentrations of 1,25-D₃ reduce cardiomyocyte cell proliferation. H9c2 cells were incubated for 4 days with increasing concentrations of 1,25-D₃ (0–500 nM), at the end of the incubation time, cell proliferation was evaluated by the formazan assay (A). In a parallel experiment, inhibition of cell proliferation was also demonstrated by the steady-state mRNA down-regulation of *Mki67*, a well-known proliferation antigen (B). Mean $\pm$ S.E.M. corresponds to experiments done in triplicate, ** P <0.01 and *** P <0.001. The samples and controls were normalized to the *Gapdh* housekeeping gene.

which bind to cyclin-dependent kinases (CDKs) to induce cell-cycle progression toward S phase and later to initiate mitosis. Cyclins are a group of proteins that control the progression of cells through the cell cycle by activating CDK enzymes. Figure 5 shows a general decrease in the expression of cyclins A1, D1, D3, C, and E.

CDKs are a group of protein kinases that are activated by the formation of a complex with cyclins and are involved in the regulation of the cell cycle. Figure 5C shows a decrease in the expression of *Cdk2* and *Cdk4*. 1,25-D₃ also decreases the expression of *Chek1*, a checkpoint serine/threonine kinase that is involved in the control of the cell cycle. Even more interesting is the fact that p21 and p27, which bind to cyclin–CDK complexes to inhibit their catalytic activity and induce the cell cycle, are differentially expressed in cardiac cells incubated with 1,25-D₃ (western blotting; Fig. 5D). At least, in this cell

model, the exit from the cell cycle induced by incubation with 1,25-D₃ appears to be solely due to an increased in p21 expression because the expression of p27 does not change in response to incubation with 1,25-D₃ (Fig. 5D).

Figure 6 illustrates by different approaches that the reduction in cell number observed in the cell proliferation studies (Figs 2 and 3) is not due to apoptosis. Figure 6A shows that there was not a significant effect of 1,25-D₃

Figure 3

1,25-D₃ downregulates the expression of *PcnA*. Cultures of H9c2 cells were treated as illustrated in Fig. 2 for 4 days. Immunocytochemistry (ICC) reactions, real-time PCR, and western blotting were carried out at the end of the incubation period. Arrows indicate nuclei positive for PCNA. (A) Representative ICC pictures with the corresponding image analysis to determine the percentages of positive cells (brown nuclear staining) for experiments done in triplicate. *** P <0.001. Magnification 200 $\times$. (B) Steady-state mRNA down-regulation of *PcnA*. Mean $\pm$ S.E.M. corresponds to experiments done in triplicates, *** P <0.001. (C) Protein extracts were subjected to western blotting and the corresponding image analysis. Mean $\pm$ S.E.M. corresponds to experiments done in triplicates, ** P <0.01 and *** P <0.001. Control, VD1, and VD2 are different pools of two samples each. In both cases, real-time PCR and western blotting, samples and controls were normalized to the *Gapdh* housekeeping gene. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0168>.

Figure 4

1,25-D₃ increases cardiomyotubes area and the expression of cardiac troponin. (A) Cultures of H9c2 cells pre-incubated with PKH2 Green Fluorescence Cell Linker and counterstained with DAPI were treated as illustrated in Fig. 2 for 7 days. Representative pictures are presented for control (no 1,25-D₃ addition) and for increasing the concentrations (10–500 nM) of 1,25-D₃ with the corresponding image analysis of the cardiomyotube area. ****P* < 0.001. Magnification 200×. In a parallel

experiment, cultures of H9c2 cells incubated on eight-well chamber slides were treated as described in Fig. 2 for 7 days. At the end of the incubation period, the cells were fixed and subjected to ICC. (B) Representative ICC pictures of cardiac Troponin+ cells with the corresponding image analysis expressing percentage IOD (area × intensity) for experiments done in triplicate. ***P* < 0.01 and ****P* < 0.001. Magnification, 200×. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0168>.

on the number of apoptotic cells determined by TUNEL assay followed by quantitative image analysis. There was no change in the expression of the pro-apoptotic caspase 3, evaluated by real-time PCR after 1,25-D₃ incubation (Fig. 6B), or in the expression of the anti-apoptotic *Bcl2* evaluated by real-time PCR and western blotting analysis (Fig. 6C).

1,25-D₃ modulates the expression of key components of the canonical and noncanonical WNT signaling pathway

The effects of 1,25-D₃ incubation on the expression of the key members of the canonical and noncanonical WNT signaling pathway on cardiac cells was demonstrated by real-time PCR arrays and confirmed by qPCR, western blotting analysis, and ICC analysis.

Figure 7A shows the differential expression of the key WNT signaling pathway members by determined

using real-time PCR arrays after incubation of the cardiac-derived cells with 1,25-D₃. No marked changes were noted in the expression of adenomatosis polyposis coli (*Apc*), *Axin1* and *Axin2*, *Gsk3β*, *Nkd1* and *Nkd2*, and *Nlk* after 1,25-D₃ incubation. However, the expression of *Csnk1α1*, a negative regulator of the canonical WNT signaling pathway, and *Wnt11*, a noncanonical WNT signaling member, was markedly increased. The analysis of the other components of the destruction complex responsible for degrading β-catenin and thus impairing the canonical WNT signaling pathway such as *Apc*, *Axin1*, *Axin2*, and *Gsk3β* showed no major change in their expression, as determined using real-time PCR arrays. This observation was further confirmed by western blotting and the corresponding densitometric analysis (Fig. 7B and C).

In order to corroborate the results obtained using real-time PCR arrays, the expression of *Csnk1α1* was further evaluated at the mRNA and protein levels. Figure 8A shows

Figure 5

1,25-D₃ induces cell cycle exit of H9c2 cardiac cells. (A) Differential steady-state mRNA levels of cell cyclins, cyclin-dependent kinases, DNA replication checkpoint kinases, and arrest factors between 1,25-D₃ treated and untreated H9c2 cells. Total RNA from cells treated as illustrated in Fig. 1 was subjected to RT real-time PCR using the cell-cycle PCR array, and the ratios between the 1,25-D₃-treated and 1,25-D₃-untreated cells corrected with reference to GAPDH were calculated for assays carried out in triplicate. (B) Confirmation of cyclin D1 and cyclin D3 PCR array results by western blotting. (C) Confirmation of *Cdk2* and *Cdk4* PCR array results by western blotting. (D) Changes in p21 and p27 expression after incubation with 1,25-D evaluated by western blotting analysis. VD1 and VD2 correspond to different pools of two samples each. In both cases, real-time PCR and western blotting, samples and controls were normalized to the *Gapdh* housekeeping gene.

a statistically significant increase in *Csnk1α1* determined using real-time PCR, Fig. 8B shows that *Csnk1α1* is also upregulated at the protein level as evidenced by an increased expression determined by western blotting and densitometric analysis; and Fig. 8C shows that CSNK1α1 is localized in the nucleus and cytoplasm of the cardiac cells, and after incubation with 1,25-D₃, its expression is highly up-regulated.

The increased expression of *Wnt11* was also corroborated by different approaches: Fig. 9A shows a statistically significant increase in *Wnt11* as determined by real-time PCR, Fig. 9B shows an increase in WNT11 expression as determined by western blotting and densitometric analysis; and Fig. 9C shows an increase in WNT11 expression by ICC with the corresponding quantitative image analysis.

Discussion

The discovery of adult endogenous cardiac stem cells (eCSCs; Beltrami *et al.* 2003) indicates that the heart is not a complete post-mitotic organ without any regenerative capability. The identification of eCSCs has provided an explanation for the hitherto unexplained existence of a subpopulation of immature cycling myocytes in the adult myocardium. Indeed, published evidence from a genetic fate-mapping study indicated that stem cells replenish adult mammalian cardiomyocytes lost by cardiac wear and tear and injury throughout the adult life (Hsieh *et al.* 2007). Moreover, it is now accepted that myocyte death and myocyte renewal are the two sides of the proverbial coin of cardiac homeostasis in which the eCSCs play a central role (Nadal-Ginard *et al.* 2003). These findings

Figure 6

1,25-D₃ induces no changes in apoptotic markers in H9c2 cardiac cells. (A) Cells treated as illustrated in Fig. 2 were seeded into eight-well chamber slides for TUNEL assay or in six-well plates for RNA and protein isolation. At the end of the incubation period, the samples were subjected to TUNEL assay where the apoptotic index (AI) was obtained for control and VD-incubated samples. Arrows indicate apoptotic nuclei according to the TUNEL assay. In another set of samples, total RNA isolation was followed by real-time PCR for Caspase 3 and *Bcl2* normalized to the *Gapdh* housekeeping gene (B) and (C). Mean $\pm$ S.E.M. corresponds to experiments done in triplicate. (C) Western blotting analysis was carried out for extracts also obtained at 4 days incubation time for *Bcl2* normalized to *Gapdh*. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0168>.

Figure 7

1,25-D₃ modulates the expression of key components of the canonical and noncanonical WNT signaling pathway. (A) Differential steady-state mRNA levels of WNT signaling pathway members between 1,25-D₃ treated and untreated H9c2 cells. Total RNA from cells treated as illustrated in Fig. 1 was subjected to real-time PCR using the WNT Signaling Pathway PCR Array, and the ratios between the 1,25-D₃-treated and 1,25-D-untreated cells corrected with reference to *Gapdh* were calculated for assays carried out in triplicate. (B) Confirmation of *Gsk3β* PCR array results by western blotting and densitometric analysis. (C) Confirmation of *Apc* PCR array results by western blotting and densitometric analysis. VD1 and VD2 correspond to different pools of two samples each. In both cases, real-time PCR arrays and western blotting, samples and controls were normalized with reference to the *Gapdh* housekeeping gene.

produced a paradigm shift in cardiac biology and presented new opportunities and approaches for future treatment of cardiac diseases by targeting eCSCs with growth factors and, as described in this manuscript, vitamin D. There are recently published results indicating that activation of differentiation may be critical for allowing subsequent proliferation (Naqvi *et al.* 2014).

The data presented in this manuscript demonstrate that the addition of 1,25-D₃ to H9c2 cardiac cells inhibits cell proliferation without inducing apoptosis and promotes cardiac differentiation through a mechanism mediated by increased expression and nuclear

translocation of the VDR. We cannot discard the possibility of the interaction of 1,25-D₃ with a membrane-localized receptor that binds 1,25-D₃ (Huhtakangas *et al.* 2004), especially at the supra-physiological doses used for the *in vitro* cultures. It is speculated that the VDR, normally associated with the cell nucleus and gene transcription (genomic response), can also be resident near to or associated with caveolae present in the plasma membrane to generate rapid response (RR) (Razani *et al.* 2002). The outcomes of these interactions would include opening of the voltage-gated calcium or chloride channels or generation of the indicated second messengers. Some of these second messengers, particularly RAF/MAPK, may engage in a cross talk with the nucleus to modulate gene expression (Norman 2006). It would be interesting to reproduce this experimental design *in vivo* by applying vitamin D analogs or nongenomic vitamin D activators

Figure 8

1,25-D₃ upregulates the expression of *Gsk3β*. (A) Differential steady-state mRNA levels of *Gsk3β* between 1,25-D₃ treated and untreated H9c2 cells. Total RNA from cells treated as illustrated in Fig. 1 was subjected to real-time PCR for *Gsk3β*, and the ratios between the 1,25-D₃-treated and 1,25-D₃-untreated cells corrected with reference to *Gapdh* were calculated for assays carried out in triplicate. (B) Confirmation of *CSNK1α1* PCR array results by western blotting and densitometric analysis. (C) Representative immunocytochemistry pictures of CSNK1α1+ cells with the corresponding image analysis of the percentage IOD (area × intensity) for experiments done in triplicate. Magnification 200×. Mean ± s.e.m. corresponds to experiments done in triplicate ****P* < 0.001. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0168>.

Figure 9

1,25-D₃ upregulates the expression of *Wnt11*. (A) Differential steady-state mRNA levels of *Wnt11* between 1,25-D₃ treated and untreated H9c2 cells. Total RNA from cells treated as illustrated in Fig. 1 was subjected to RT real-time PCR for *Wnt11*, and the ratios between the 1,25-D-treated and 1,25-D-untreated cells corrected with reference to *Gapdh* were calculated for assays carried out in triplicate. (B) Confirmation of *Wnt11* PCR array results by western blotting and corresponding densitometric analysis. (C) Representative immunocytochemistry pictures of WNT11+ cells with the corresponding image analysis expressing percentage IOD (area × intensity) for experiments done in triplicate. Magnification 200×. Mean ± S.E.M. corresponds to experiments done in triplicate ***P* < 0.01 and ****P* < 0.001. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0168>.

that may be able to induce the beneficial pro-differentiation and anti-proliferative effects without the adverse hypercalcemic side effects.

We also demonstrated that the effects of 1,25-D₃ in promoting cardiac differentiation involve the modulation of members of the WNT signaling pathway, including the increased expression of *Ck1α1*, a well-known inhibitor of the canonical WNT signaling pathway (Thorne *et al.* 2010). This observation is consistent with the concept that canonical WNT inhibition is required for heart development *in vivo* (Bergmann 2010). Remarkably, the inhibition of the canonical WNT signaling pathway promoted by 1,25-D₃ is accompanied by an increased expression of the noncanonical *Wnt11*, which has been reported *per se* to induce cardiac differentiation during embryonic development and in adult cells (Flaherty & Dawn 2008, Flaherty *et al.* 2008, 2012).

We showed that VDR is minimally expressed in untreated H9c2 cardiomyocytes and incubation with 1,25-D₃ stimulates *de novo* synthesis and nuclear translocation. This was expected since it is known that 1,25-D₃ auto-regulates the expression of the *Vdr* gene through intronic and upstream enhancers (Pike & Meyer 2010).

Our results indicate that the addition of 1,25-D₃ to H9c2 cardiomyocytes inhibits cell proliferation in a dose-dependent manner and down-regulates the expression of *Mki67* and *Pcna*, two well-known and broadly used cell proliferation markers/indicators (Essers *et al.* 2005, Preusser *et al.* 2008). The inhibition of cell proliferation by 1,25-D₃ is not accompanied by changes in apoptosis or the expression of the pro-apoptotic caspase 3 and/or the expression of the anti-apoptotic *Bcl2*. These results are consistent with our previous results obtained using mesenchymal multipotent cells (Artaza *et al.* 2010) and with the data presented by Assalin *et al.* (2013) where vitamin D deficiency is not associated with an increase in apoptosis in cardiac tissue.

The inhibition in cell proliferation induced by 1,25-D₃ is accompanied by an increase in cell differentiation evidenced by an increase in cardiomyotubes cell area and the expression of cardiac troponin, a major cardiac differentiation marker (Perán *et al.* 2010).

In agreement with the previous work of Eelen *et al.* (2007), we found that the addition of 1,25-D₃ to H9c2 cardiomyocytes blocks the transition of the cell cycle from G1 to S1-phase, causing cells to accumulate in G1, by decreasing the expression of cyclins such as, cyclin A1, D1, D3, C and E, as well as CDKs such as CDK2 and CDK4. Of particular interest is that in this cardiac cell model, the 1,25-D₃ G1/S-blocking effect was accompanied by an increased expression of p21, a CDK inhibitor. However, 1,25-D₃ does not increase the expression of p27, making p21 a primary candidate for the antiproliferative effect in H9c2 cardiac cells. 1,25-D₃ also decreases the expression of CHEK1, a protein required for checkpoint-mediated cell cycle arrest in response to DNA damage or the presence of unreplicated DNA.

Lastly, we demonstrated that the induction of cardiac differentiation promoted by 1,25-D₃ in H9c2 cardiomyocytes involves the modulation of the expression of key members of the WNT signaling pathway. The WNT gene family consists of secreted lipid-modified and evolutionarily conserved signaling glycoproteins (Cadigan & Nusse 1997). WNT signals are transduced to the canonical pathway for cell fate determination, and to the non-canonical pathway for control of cell movement and tissue polarity (Katoh & Katoh 2007). Canonical WNT signals

are transduced through Frizzled family receptors and LRP5/LRP6 coreceptor to the β -catenin signaling cascade. Noncanonical WNT signals are transduced through Frizzled family receptors and ROR2/RYK coreceptors to the DVL-dependent or the Ca^{2+} -dependent signaling cascades (Katoh & Katoh 2007). It has been demonstrated that canonical WNT signaling inhibition is essential for cardiogenic activity and that there is also possibility that the pathway can be targeted for the design of drug-like cardiogenic molecules (Bergmann 2010, Lanier *et al.* 2012). Also, it has been shown that inhibitors of the canonical WNT signaling pathway potently promote cardiomyocyte differentiation from human embryonic-stem-cell-derived mesoderm (Willems *et al.* 2011).

Canonical WNT signaling (or the WNT/ β -catenin pathway) causes an accumulation of β -catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of transcription factors that belong to the TCF/LEF family. β -catenin in the nucleus binds to TCF/LEF transcription factors to activate WNT/ β -catenin-responsive genes, such as *Cdk1* and cyclin D1, which are required for cell cycle progression. Inhibition of the canonical WNT signaling pathway reverts this process. In other words, there is a synergistic effect prompted by the inhibition of the canonical WNT signaling pathway and the well-known antiproliferative effect and cell cycle arrest promoted by vitamin D as demonstrated by our group and others in different cell models (Wang *et al.* 1996, Verlinden *et al.* 1998, Artaza *et al.* 2010). It is possible that modulating the WNT signaling pathway by vitamin D replenishment and/or supplementation will promote better outcomes in heart remodeling by inducing cardiac differentiation. This is a very promising and possible translation to the clinic. Also, results from several studies indicate that canonical WNT signals play distinct roles during discrete developmental windows, first positively regulating pre-cardiac mesoderm commitment and then playing a negative role in the initial induction of cardiac progenitors (Marvin *et al.* 2001, Kwon *et al.* 2007). The results of experiments performed by Naito *et al.* (2006) on ES cells indicate that WNT/ β -catenin signaling, when activated after cells are committed to the cardiac lineage, negatively regulates cardiomyocyte differentiation, and that inhibition of WNT signaling at this stage decreases cell proliferation and even more importantly enhances cardiomyocyte differentiation. Those results are in agreement with the data presented in this manuscript where the experiments were carried out on a cell model, the H9c2 cardiomyocytes, already committed to the cardiac lineage. Without WNT signaling, the

β -catenin would not accumulate in the cytoplasm because a 'destruction complex' would normally degrade it. This destruction complex includes the following proteins: Axin, APC, protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3), and casein kinase 1 α (CSNK1 α). Our results indicate that the only member of the destruction complex that increases upon 1,25-D₃ incubation is CSNK1 α 1, probably making CSNK1 α 1 solely responsible for canonical WNT inhibition in this system, thus promoting cardiac differentiation.

In this study, we have shown that 1,25-D₃ incubation induces the increased expression of *Wnt11*, a noncanonical WNT signaling member that has been shown to induce cardiomyogenesis both during embryonic development and in adult cells (Flaherty & Dawn 2008, Flaherty *et al.* 2012). It has also been reported that *Wnt11* induces robust cardiomyogenic differentiation in bone marrow mononuclear cells (Flaherty *et al.* 2008, Zhang *et al.* 2012). The general consensus in the literature regarding cardiac differentiation indicates that an initial cardiac specification requires balanced expression of both canonical and noncanonical WNT signaling, but once cells are committed, as in the case of our cardiomyocytes, the balance must be tipped in the direction of canonical inhibition for cardiac specification to occur (Eisenberg & Eisenberg 2006, 2007, Ueno *et al.* 2007).

There is increasing evidence that the WNT signaling pathway is important in the regulation of cardiac function (Dawson *et al.* 2013). It has been documented that the WNT signaling pathway plays a pivotal role in heart development but also during adult cardiac hypertrophy and remodeling (Bergmann 2010). As Bergmann pointed out, inhibition of nuclear β -catenin signaling downstream of the canonical WNT pathway significantly reduced post-infarct mortality and functional decline of LV following chronic left anterior descending coronary artery ligation. Cardiac hypertrophy is characterized by an increase in cell size, and is accompanied by protein synthesis, fibrosis, and upregulation of a fetal gene expression pattern (including atrial natriuretic peptide (*Anp* (*Nppa*)), brain natriuretic peptide (*Bnp*), and beta myosin heavy chain (β -*Mhc*) (Rohini *et al.* 2010). Initially an adaptive response, in later stages cardiac hypertrophy can lead to maladaptive remodeling and HF, especially if proliferation occurs before appropriate cardiomyocyte differentiation. In normal adult cardiomyocytes, WNT/FZD signaling is quiescent (Cingolani 2007). However, the pathway becomes reactivated in disease states, including hypertrophy. Inhibiting WNT signaling with 1,25-D₃ by activating *CSNK1 α 1* would attenuate the hypertrophic

response. Also, cardiac fibrosis can result from cardiac diseases such as congestive HF or acute myocardial infarction (MI), but also from cardiac senescence, genetic predisposition, and intense exercise (Benito *et al.* 2011, Rohr 2012). A fibrotic process can impair cardiac relaxation, causing diastolic dysfunction and potentially HF. It also impedes electrical wave propagation, potentially causing arrhythmias. The WNT pathway is well established to be involved in the fibrosis of several organ systems (lung, kidney, and liver) (He *et al.* 2009, Henderson *et al.* 2010, Akhmetshina *et al.* 2012). As canonical WNT signaling is required for transforming growth factor- β (TGF β)-mediated fibrosis (Akhmetshina *et al.* 2012). The inhibition of the canonical WNT signaling pathway promoted by 1,25-D₃ could ameliorate the fibrotic process. Moreover, our group has previously shown that 1,25-D₃ is a potent anti-fibrotic factor promoting the decreased expression of TGF- β , PAI1, and several collagen isoforms in mesenchymal multipotent cells (Artaza & Norris 2009). There is an *in vivo* study the results of which indicate that vitamin D deficiency results in maladaptive cardiac remodeling attributable to progressive myocyte hypertrophy and interstitial fibrosis (Meems *et al.* 2012). Results from a recent study by our group have indicated that repletion of hypovitaminosis D in humans led to the downregulation of protein expression in abdomen fat biopsies for tumor necrosis factor alpha, IFN gamma, interleukin 6, and soluble intercellular adhesion molecule 1, a biomarker of inflammatory processes associated with endothelial damage and platelet activation, supporting an *in vivo* role of vitamin D in related signaling pathways (Martins *et al.* 2014).

The natural conclusion is that targeting or modulating the WNT signaling pathway with vitamin D may be a suitable natural and cost-effective therapeutic intervention that deserves further investigation.

In conclusion, the inhibition of the canonical WNT signaling pathway through increased expression of *CSNK1 α 1* and at the same time increased expression of *Wnt11*, promoted in both cases by 1,25-D₃ supplementation, enhanced cardiomyocyte differentiation and may prove useful for clinical strategies such as preprogramming stem cells before myocardial transplantation.

These results indicate that vitamin D repletion might enhance the outcomes of cardiac cell therapy or even cardiac cell remodeling, contributing to the prevention/treatment of CVD and other related cardiac conditions by promoting cardiac differentiation.

The data presented in this manuscript establish a novel role of vitamin D in the modulation of WNT

signaling in cardiac-derived cells, promoting cardiac differentiation, which may ultimately be useful in directing differentiation of multipotent cells into cardiomyocytes for cardiac repair applications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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