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# Effects of Vitamin D analogs on gene expression profiling in human coronary artery smooth muscle cells☆

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**Abstract**

Vitamin D analogs provide survival benefit for chronic kidney disease patients with cardiovascular complications. Activation of smooth muscle cells plays a role in cardiovascular diseases. It is not known how Vitamin D analogs modulate gene expression in smooth muscle cells. In this study, DNA microarray technology was used to assess the gene expression profile in human coronary artery smooth muscle cells treated with 0.1 μM 1α,25-dihydroxyvitamin $D_3$ (calcitriol) or paricalcitol (an analog of calcitriol) for 30 h. The effects of calcitriol and paricalcitol were similar. A total of 176 target genes were identified with 115 up-regulated and 61 down-regulated genes in the paricalcitol group. Target genes fall into various categories including cell differentiation/proliferation. Real-time RT-PCR analysis demonstrated that paricalcitol dose- and time-dependently regulated the expression of IGF1, WT1 and TGFβ3, three genes known to modulate cell proliferation. Paricalcitol also down-regulated the expression of natriuretic peptide precursor B and thrombospondin 1. Both drugs inhibited cell proliferation in a dose-dependent manner. This study identified genes not previously known to be regulated by VDR, providing insight into understanding the role of VDR on regulating smooth muscle cell growth, thrombogenicity, fibrinolysis and endothelial regeneration.


*Keywords:* Vitamin D analogs; Vitamin D receptor; Atherosclerosis; Paricalcitol; Human coronary artery smooth muscle cells; Gene chip array

## 1. Introduction

Vitamin $D_3$ is modified by 25-hydroxylase in the liver and 25-hydroxyvitamin D 1α-hydroxylase in the kidney to form the active metabolite, 1,25-dihydroxyvitamin $D_3$ (1α,25-$(OH)_2D_3$, calcitriol), which is then metabolized by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) [1]. The binding of 1α, 25-$(OH)_2D_3$ or its analogs to Vitamin D receptor (VDR), a nuclear receptor, activates VDR to interact with retinoid X receptor (RXR) and recruit cofactors to form the VDR/RXR/cofactor complex, which then binds to Vitamin D response elements in the promoter region of target genes to regulate gene transcription [2].

Chronic kidney disease (CKD) patients experience a high mortality rate from cardiovascular diseases [3,4]. Vitamin D analogs such as paricalcitol and calcitriol that activate VDR are commonly used to manage secondary hyperparathyroidism associated with CKD [5]. Recent clinical data show that Vitamin D analogs provide survival benefit for end-stage renal disease patients in the effectiveness order of paricalcitol > calcitriol > no Vitamin D analog therapy, independent of the PTH and calcium levels [6,7]. Moreover, the survival benefit seems to be associated with cardiovascular causes [8].

Although data from clinical studies demonstrate the positive impact of Vitamin D analogs on the cardiovascular system, the mechanism of action is largely unknown. The activation of smooth muscle cells (SMC) during vascular injury plays a role in atherosclerosis and vascular calcification, and the high prevalence of both conditions in CKD has been well documented [9–12]. Atherosclerosis, the principal cause of myocardial infarction, stroke and peripheral vascular disease, is a process that involves a complex inter-

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play among different factors and cell types including smooth muscle cells [13,14]. The pathogenesis of vascular calcification is still poorly understood. It is not known how Vitamin D analogs modulate gene expression in smooth muscle cells. In this study, we report results from gene chip microarray analysis to obtain a broad profile of the modulatory effects of paricalcitol and calcitriol on human coronary artery smooth muscle cells. The results identify categories of gene product that are selectively modulated in smooth muscle cells by the VDR system.

## 2. Materials and methods

### 2.1. Materials

1α,25-dihydroxyvitamin $D_3$ (1α,25-$(OH)_2D_3$, calcitriol) and 19-nor-1α, 25-dihydroxyvitamin $D_2$ (19-nor-1α, 25-$(OH)_2D_2$, paricalcitol) were from Abbott Laboratories. Other reagents were of analytical grade.

### 2.2. Cell cultures

Primary cultured human coronary artery smooth muscle cells (Cambrex) were grown to confluence in SMGM-2 containing 5.5 mM glucose, 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, 5 μg/ml insulin, 2 ng/ml human recombinant fibroblast growth factor and 0.5 ng/ml human recombinant epidermal growth factor (growth medium) at 37 °C in a humidified 5% $CO_2$–95% air atmosphere. Cells were grown to >80% confluence and used within five passages.

### 2.3. Microarray

Total RNA was extracted from primary culture of human coronary artery smooth muscle cells grown in complete media and treated with or without 100 nM of paricalcitol or calcitriol for 30 h ($n = 3$ for each condition). While the yield of total RNA was low (~2.0 μg total per sample), the RNAs were intact as judged by Agilent 2100 analysis. One microgram of total RNA from each sample was used to prepare biotin-labeled cRNA target using standard Affymetrix protocols. Prepared cRNA targets were of good quality and quantity. The Affymetrix Human chip U133Av2 was used (>22,000 probe sets) and 10 μg cRNA target was applied to each array. After hybridization and chip scanning, the quality control data report (i.e. scaling factor, glyceraldehyde-3-phosphate dehydrogenase 5′/3′ ratio, noise, background) demonstrated that every array passed all quality criteria. Scanned images were loaded into the Rosetta Resolver 4.0 database and processed using the Resolver Affymetrix error model. Within Resolver the drug treated sample replicates ($n = 3$) were informatically combined and ratios constructed relative to the combined control (no addition of drug) samples. The Resolver Affymetrix error model was used to develop each ratio and calculate a $p$-value. The Resolver error model is robust and includes reporter level, background and error values in the calculation of statistical significance. A combination of hierarchical clustering, gene ontology analysis and pathway mapping were used to assess the function of the regulated genes.

### 2.4. Primers and probes for real-time reverse transcription-PCR

For quantitative PCR (qPCR), TaqMan™ probes that were 5′ labeled with the reporter 6-carboxyfluorescein (FAM) and 3′ labeled with the quencher tetramethylrhodamine (TAMRA) were used. The primer and probe sets were obtained from the Assay-on-Demand collection (Applied Biosytsems).

### 2.5. Real-time reverse transcription-PCR

PCR was performed with a 7900HT sequence detector (Applied Biosytsems). Each sample has a final volume of 25 μl containing 50 ng of cDNA, 0.4 mM each of the forward and reverse PCR primers and 0.1 mM of the TaqMan™ probe. Temperature conditions consisted of a step of 5 min at 95 °C, followed by 40 cycles of 60 °C for 1 min and 95 °C for 15 s. Data was collected during each extension phase of the PCR reaction and analyzed with the SDS software package (Applied Biosystems). Threshold cycles were determined for each gene. The expression level of target gene of interest was given as relative expression normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### 2.6. Thymidine incorporation

Cells were plated at $1 \times 10^6$ cells/ml, 200 μl/well into 96-well plates (Costar). One day after plating, the cells were treated with test agents for 24 h and then labeled with 0.3 μCi/well of $^3$H-thymidine for another 48 h. Each well was washed with 0.3 ml/well of PBS, incubated with 0.2 ml/well of ice-cold 10% trichloroacetic acid (TCA) for 30 min at 4 °C, and then followed by another wash of 0.2 ml/well of TCA. Samples were dissolved in MICROSCINT™ 20 (Packard) before counting.

## 3. Results

### 3.1. Effects of paricalcitol versus calcitriol

Using a two-fold change in average difference in either the paricalcitol- or calcitriol-treated group as cut-off with $p < 0.05$ for significantly modulated expression, a total of 176 target genes were identified. In the paricalcitol group, 115 and 61 genes were up- and down-regulated, respectively. In the calcitriol group, 116 and 60 genes were up- and down-regulated, respectively. Fig. 1 shows the hierarchical clustering of genes regulated by either paricalcitol or