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Gene Expression Profiles in Human and Mouse Primary Cells Provide New Insights into the Differential Actions of Vitamin D3 Metabolites

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Abstract

1α,25-Dihydroxyvitamin D3 (1α,25(OH)2D3) had earlier been regarded as the only active hormone. The newly identified actions of 25-hydroxyvitamin D3 (25(OH)D3) and 24R,25-dihydroxyvitamin D3 (24R,25(OH)2D3) broadened the vitamin D3 endocrine system, however, the current data are fragmented and a systematic understanding is lacking. Here we performed the first systematic study of global gene expression to clarify their similarities and differences. Three metabolites at physiologically comparable levels were utilized to treat human and mouse fibroblasts prior to DNA microarray analyses. Human primary prostate stromal P29SN cells (hP29SN), which convert 25(OH)D3 into 1α,25(OH)2D3 by 1α-hydroxylase (encoded by the gene CYP27B1), displayed regulation of 164, 171, and 175 genes by treatment with 1α,25(OH)2D3, 25(OH)D3, and 24R,25(OH)2D3, respectively. Mouse primary Cyp27b1 knockout fibroblasts (mCyp27b1−/−), which lack 1α-hydroxylation, displayed regulation of 619, 469, and 66 genes using the same respective treatments. The number of shared genes regulated by two metabolites is much lower in hP29SN than in mCyp27b1−/−. By using DAVID Functional Annotation Bioinformatics Microarray Analysis tools and Ingenuity Pathways Analysis, we identified the agonistic regulation of calcium homeostasis and bone remodeling between 1α,25(OH)2D3 and 25(OH)D3 and unique non-classical actions of each metabolite in physiological and pathological processes, including cell cycle, keratinocyte differentiation, amyotrophic lateral sclerosis signaling, gene transcription, immunomodulation, epigenetics, cell differentiation, and membrane protein expression. In conclusion, there are three distinct vitamin D3 hormones with clearly different biological activities. This study presents a new conceptual insight into the vitamin D3 endocrine system, which may guide the strategic use of vitamin D3 in disease prevention and treatment.

Introduction

Vitamin D3 produced in the skin undergoes two sequential hydroxylation steps, first 25-hydroxylation generating 25-hydroxyvitamin D3 (25(OH)D3) and then 1α-hydroxylation generating 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3). Both of them are further metabolized by 24-hydroxylase. Among many endogenous vitamin D3 metabolites, 25(OH)D3 and 24R,25-dihydroxyvitamin D3 (24R,25(OH)2D3) are the first and second most abundant circulating metabolites of vitamin D3. 1α,25(OH)2D3 is believed to be the most active vitamin D3 metabolite. Their classic actions in calcium homeostasis and non-classical actions in cell proliferation and differentiation have been the focus of vitamin D3 research. During the past two decades, there have been some important discoveries of vitamin D3 metabolism and endocrine system. Cells can uptake 25(OH)D3 by megalin-mediated endocytosis [1,2]. 25(OH)D3 possesses an inherent hormonal activity regulating cell proliferation and gene expression, which was first evidenced in human prostate cells [3] and later in other types of human and mouse cells by in vitro and in vivo studies [4–13]. 25(OH)D3 acts synergistically with 1α,25(OH)2D3 [4]. 24R,25(OH)2D3 seems to regulate specifically chondrocytes [14] and osteogenesis [15]. 25(OH)D3 1α-hydroxylation, encoded by the gene CYP27B1 and responsible for the conversion of 25(OH)D3 to 1α,25(OH)2D3, is expressed in the kidney and in many extra-renal tissues [16–18], thus the intracellularly synthesized 1α,25(OH)2D3 was suggested to act as an intracrine factor [9]. Epidemiological studies provide
accumulating evidence showing that vitamin D₃ insufficiency is associated with infection [19], cancers [20], fractures [21], diabetes [22], and neuropsychiatric disease [23], and 25(OH)D₃ concentration gradient is related to the seasonal variation of cancer survival [24]. The optimal dose and serum concentration of vitamin D₃ is under debate. Some epidemiological studies also suggest that both low and high 25(OH)D₃ levels are harmful [9,25,26]. Based on these findings, it seems that the vitamin D₃ system is more complex than earlier thought.

To identify target genes, microarray gene expression studies have been performed in various cellular systems after treatment with 1α,25(OH)₂D₃, reviewed by C. Kriebitzsch, et al [27]. Gene expression in response to 25(OH)D₃ or 24R,25(OH)₂D₃ has not yet been studied by DNA microarray. Here we compared the effects of 1α,25(OH)₂D₃, 25(OH)D₃, and 24R,25(OH)₂D₃ on gene expression patterns to clarify similarities and differences in signal transduction. To exclude the effect of the intracellular product of 1α,25(OH)₂D₃, we also performed microarray study in mouse primary Cyp27b1 knockout fibroblasts (mCyp27b1⁻/⁻). To our knowledge, microarray gene expression studies have not been made in Cyp27b1⁻/⁻ cells. Our data show that there are three vitamin D₃ (cholecalcipherol) hormonal systems, which display partially independent gene transcriptional effects.

**Materials and Methods**

**Ethics Statement**

All the animal experiments were approved by the Ethical Committee of the University of Tampere. Animal care and experimental procedures were conducted in accordance with the European legislation and the guidelines of the European Laboratory Animal Associations.

**Mice**

*Cyp27b1* knockout (*Cyp27b1⁻/⁻*) mice were bred at the University of Tampere from the line initially generated at the Shriners Hospital, Montreal, Canada [28]. Feeding and genotyping *Cyp27b1⁻/⁻* mice were described previously [4].

**Cell Culture, Vitamin D₃ Treatment, and RNA Isolation**

The isolation and characterization of human primary prostate stromal P29SN cells were described previously [3]. The vast majority of the primary stromal culture was shown to be fibroblasts in phenotype. Over 99% of the cells were positive for fibroblast markers vimentin and fibronectin. Less than 5% of the cells were positive for smooth muscle actin and less than 2% were positive for desmin [3]. The cells were maintained in phenol red-free DMEM/F12 medium (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% fetal bovine serum (FBS), 3 mM L-glutamine, 5 μg/ml insulin, and antibodies (penicillin 100 units/ml, streptomycin 100 μg/ml) (Gibco-BRL, Life Technology, Paisley, Scotland) at 37°C in a humid atmosphere with 5% CO₂. 24 hours prior to treatments, growth media containing 10% FBS-DCC (10% FBS treated with dextran coated charcoal) were added to semi-confluent hP29SN cells. Next day the cells were treated with 0.1% ethanol, 10 nM 1α,25(OH)₂D₃, 500 nM 25(OH)D₃, or 25 nM 24R,25(OH)₂D₃ (kindly provided by Leo Pharmaceuticals, Ballerup, Denmark) for 24 hours. Ethanol concentration was 0.1% in all treatment conditions. Experiments were replicated four independent times for each treatment condition.

The isolation and culture of mouse primary *Cyp27b1* knockout skin fibroblasts (mCyp27b1⁻/⁻ fibroblasts) were described previously [4]. 48 hours prior to experiments, growth media were supplemented with 10% FBS-DCC. Fibroblasts were treated with 0.1% ethanol, 10 nM 1α,25(OH)₂D₃, 500 nM 25(OH)D₃, or 25 nM 24R,25(OH)₂D₃ for 24 hours. Ethanol concentration was 0.1% in all treatment conditions. Experiments were replicated four independent times for each treatment condition.

Total cellular RNA was isolated from hP29SN stromal cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and from mCyp27b1⁻/⁻ fibroblasts by RNeasy Mini Kit (Qiagen GmbH, Hilder, Germany) following the manufacturers’ protocols. The RNA concentration and purity were verified using a GeneQuant II (Pharmacia Biotech, Piscataway, NJ, USA). The A280/A260 ratio of all RNA samples utilized for experimentation was 1.9–2.1. Randomly selected RNA samples were subjected to denaturing-gel electrophoresis. The ratio of the intensity of 28S and 18S bands was 1.5–2.0.

**Quantitative Real-time RT-PCR (qRT-PCR)**

*CYP24A1* (encoding vitamin D₃ 24-hydroxylase) gene expression was measured by using qRT-PCR to ensure that hP29SN stromal cells were successfully stimulated by vitamin D₃ metabolites. Similarly, for the validation of microarray data, the expression levels of eight differentially expressed genes in hP29SN stromal cells and two genes in mCyp27b1⁻/⁻ fibroblasts were analyzed. The qRT-PCR analysis was performed as previously described [3]. Briefly, total RNA was converted to cDNA by using High Capacity Archive Kit and the 20 ng of cDNAs were used in real time qPCR reactions by using SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). Primers were designed by using Primer Express v2.0 software (Applied Biosystems, Foster City, CA, USA).
Biosystems, Foster City, CA, USA). The primer sequences are listed in Table S1. The expression levels of target genes were normalized against the housekeeping gene acidic ribosomal phosphoprotein P0 (RPLP0) in hP29SN stromal cells and TATA box binding protein (Tbp) in Cyp27b12/2 fibroblasts.

**Affymetrix Microarray Analysis**

cRNA preparation, hybridization, and the array wash procedure were performed following the standard protocol from Affymetrix. In brief, 7 μg of total RNA from each hP29SN sample were used to synthesize double-stranded cDNA. The cDNA was used as a template to generate biotinylated cRNA by an *in vitro* transcription reaction. 20 μg of biotinylated cRNA was fragmented and added to the GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). The hybridization was carried out at 45°C for 20 hours with a rotation at 60 rpm in an Affymetrix hybridization oven, and then the arrays were washed and stained with a streptavidin-conjugated fluorescent stain followed by antibody amplification on the Affymetrix Fluidics Station 400. Scanned images were processed using Affymetrix GeneChip® Fluidics Station 450 was used to wash and stain the arrays. GeneChip® Scanner 3000 7G with AutoLoader was used to scan the arrays.

**Array Data Analysis**

Data from array scans were normalized and analyzed by GeneSpring GX 7.3.1 Expression Analysis software (Agilent Technologies, Santa Clara, CA, USA). Before normalization, expression values less than 0.01 were converted to 0.01 to enable more efficient analysis of log-transformed data. To control chip-wide variation in intensity values, each chip was normalized to the 50th percentile of all measurements (per-chip normalization). As per gene normalization, treatment samples were normalized against the median of control samples. Each measurement for each gene in the treatment samples was divided by the median of that gene’s measurements in the corresponding control samples. In all three conditions, the present call was demanded only to respective conditions. We used Pearson Correlation as similarity measure and unsupervised average linkage hierarchical clustering algorithm. The program was asked to merge similar branches with separation ratio of one and minimum distance of 0.001 and to calculate confidence levels (bootstrapping) with 100 datasets.

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**Figure 2. IPA diagrams of the top associated network generated for genes exclusively regulated by each metabolite in hP29SN stromal cells.** (A) Genes regulated by 1a,25(OH)2D3 in Cardiovascular System Development and Function, Tissue Development, Organismal Development. (B) Genes regulated by 25(OH)D3 in Cell Death and Survival, Gene Expression, Tissue Morphology. (C) Genes regulated by 25(OH)D3 in Cell Death and Survival, Cellular Growth and Proliferation, Cell Cycle. (D) Genes regulated by 24R,25(OH)2D3 in Cell Morphology, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair. Green indicates gene down-regulation and pink to red indicate gene up-regulation (the more intensive the color, the higher the expression level). An asterisk (*) indicates that multiple identifiers in the microarray set map to a single gene.

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The microarray data has been deposited to ArrayExpress with the accession numbers of E-MTAB-1773 for human data and E-MTAB-1774 for mouse data.

**Functional Annotation, Network Generation and Pathway Analyses**

The DAVID Functional Annotation Bioinformatics Microarray Analysis tools (http://david.abcc.ncifcrf.gov/) [29,30] were used to study the biological meaning of regulated genes. Functional properties of differentially expressed genes were further analyzed in the context of Gene Ontology (GO) and molecular networks using the Ingenuity Pathways Analysis (IPA; Ingenuity Systems; http://www.ingenuity.com). The number of molecules in the network was limited to default of 35, based on the number of connections between the input genes and genes in the IPA database. For each of the network, a score is calculated by the IPA according to the fit of that network to the input genes. The score (derived from a p-value) represents the likelihood of random change. For example, a score of 2 indicates that there is a 1% change that the input genes are together in a network due to random change. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random change alone. The highest scoring networks were selected.

**Results**

**Gene Expression Profiles Induced by Vitamin D₃ Metabolites**

We aimed at exploring the gene expression profiles of three vitamin D₃ metabolites using hP29SN stromal cells and mCyp27b1⁻/⁻ fibroblasts. Human P29SN stromal cells were derived from a normal area of human prostatic carcinoma [3]. Mouse Cyp27b1⁻/⁻ fibroblasts were derived from Cyp27b1⁻/⁻ mice that do not express 25(OH)D₃ 1α-hydroxylase and therefore cannot convert 25(OH)D₃ into 1α,25(OH)₂D₃. We verified the responsiveness and successful stimulation of cells by vitamin D₃ metabolites by measuring mRNA level of CYP24A1 in each sample by qRT-PCR before applying samples to microarray assays (Figure S1). RNA samples of corresponding metabolite treatments from randomly selected two sets of experiments were pooled to generate final two sets of RNA for microarray hybridizations.

The gene expression profiles in hP29SN stromal cells treated with 10 nM 1α,25(OH)₂D₃, 500 nM 25(OH)D₃, or 25 nM 24R,25(OH)₂D₃ were determined by GeneChip® Human Genome U133 Plus 2.0 Arrays that contained more than 54000 probe sets to analyze the expression level of more than 47000 transcripts and variants, including approximately 38500 well-
characterized human genes. Ethanol-treated samples served as negative control. Each vitamin D3 metabolite-treated sample was compared with ethanol-treated samples. Only those genes that exhibited at least twofold change in gene expression in parallel experiments were reported to ensure the fidelity of the data. The final result was the average of the two independent microarray experiments. For 1α,25(OH)2D3 treatment, 164 genes met the selection criteria while 171 and 175 genes were identified for 25(OH)D3 and 24R,25(OH)2D3 treatment, respectively (data not shown). All the genes that displayed at least twofold expression change in any of the treatments were clustered using hierarchical clustering method by GeneSpring software (Figure 1A). To understand the specific role of each vitamin D3 metabolite, we grouped the regulated genes into commonly and uncommonly regulated gene groups (Figure 1C). Of the genes met the selection criteria, only 10 are common in all the three experimental conditions. Interestingly, all these genes were up-regulated. The number of genes significantly regulated in two conditions are 21 for 1α,25(OH)2D3 and 25OHD3, 8 for 1α,25(OH)2D3 and 24R,25(OH)2D3, and 20 for 25OHD3 and 24R,25(OH)2D3, respectively (Figure 1C).

Mouse Cyp27b1−/− fibroblasts were treated either with 10 nM 1α,25(OH)2D3, 500 nM 25(OH)D3, or 50 nM 24R,25(OH)2D3, and GeneChip Mouse Genome 430 2.0 Arrays were used to study the gene expression profile. Using a cut-off of twofold expression change, we identified 619, 469, and 66 genes regulated by 1α,25(OH)2D3, 25(OH)D3, and 24R,25(OH)2D3, respectively (Figure 1C).

Table 1. Top five associated network functions of genes exclusively regulated by single metabolite generated by IPA.

<table>
<thead>
<tr>
<th>Human P29SN stromal cells</th>
<th>Metabolite</th>
<th>Top associated network functions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α,25(OH)2D3</td>
<td>Cardiovascular System Development and Function, Tissue Development, Organismal Development</td>
<td>16</td>
<td></td>
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<tr>
<td></td>
<td>Cellular Movement, Immune Cell Trafficking, Hematological System Development and Function</td>
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<td></td>
<td>Cardiac Proliferation, Cardiovascular System Development and Function, Cell Cycle</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Molecular Transport, Protein Synthesis, Protein Trafficking</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antimicrobial Response, Inflammatory Response, Antigen Presentation</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>Cell Death and Survival, Gene Expression, Tissue Morphology</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell Death and Survival, Cellular Growth and Proliferation, Cell Cycle</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell Cycle, Cancer, Cell Death and Survival</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell Morphology, Cellular Growth and Proliferation, Cellular Assembly and Organization</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell Cycle, DNA Replication, Recombination, and Repair, Cell Death and Survival</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>24R,25(OH)2D3</td>
<td>Cell Morphology, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gene Expression, Cellular Growth and Proliferation, Cell Cycle</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular Growth and Proliferation, Cellular Development, Cellular Movement</td>
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<tr>
<td></td>
<td>Cancer, Cell Death, Neurological Disease</td>
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<tr>
<td></td>
<td>Cell Death, Cellular Development, Digestive System Development and Function</td>
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</table>

<table>
<thead>
<tr>
<th>Mouse Cyp27b1−/− fibroblasts</th>
<th>Metabolite</th>
<th>Top associated network functions</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α,25(OH)2D3</td>
<td>Tissue Morphology, Hematological System Development and Function, Humoral Immune Response</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Cardiovascular System Development and Function, Inflammatory Response, Cellular Movement</td>
<td>28</td>
<td></td>
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<tr>
<td></td>
<td>Cancer, Respiratory Disease, Carbohydrate Metabolism</td>
<td>22</td>
<td></td>
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<tr>
<td></td>
<td>Organismal Injury and Abnormalities, Cell Morphology, Lipid Metabolism</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular Growth and Proliferation, Cell Death and Survival, Cell Cycle</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>Cellular Growth and Proliferation, Cellular Development, Hair and Skin Development and Function</td>
<td>19</td>
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<td>Cellular Function and Maintenance, Cell-mediated Immune Response, Cellular Development</td>
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<td></td>
<td>Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function</td>
<td>15</td>
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<td></td>
<td>Gene Expression, Developmental Disorder, Cancer</td>
<td>15</td>
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<tr>
<td></td>
<td>Cell Death, Cancer, Cell Cycle</td>
<td>13</td>
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</tr>
<tr>
<td>24R,25(OH)2D3</td>
<td>Cell Death and Survival, Endocrine System Development and Function, Lipid Metabolism</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Connective Tissue Development and Function, Connective Tissue Disorders, Developmental Disorder</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Developmental Disorder, Immunological Disease, Cell Cycle</td>
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<tr>
<td></td>
<td>Hereditary Disorder, Metabolic Disease, Cancer</td>
<td>2</td>
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</tbody>
</table>

Table 1. Top five associated network functions of genes exclusively regulated by single metabolite generated by IPA.

doi:10.1371/journal.pone.0075338.t001
24R,25(OH)2D3-treated cells displays much less changes compared with ethanol control, suggesting lower bioactivity of 24R,25(OH)2D3. The Venn diagram shows six genes commonly regulated by the three metabolites, 321 by 1α,25(OH)2D3 and 25OHD3, 9 by 1α,25(OH)2D3 and 24R,25(OH)2D3, and 4 by 25OHD3 and 24R,25(OH)2D3, respectively (Figure 1D).

Functional Annotation of Genes Regulated by Vitamin D3 Metabolites

The biological relevance of the specifically regulated genes by each metabolite was further analyzed by the DAVID Functional Annotation Bioinformatics Microarray Analysis tools (http://david.abcc.ncifcrf.gov/) [29,30]. The most enriched GO categories with p-value <0.05 are listed in Tables S2, S3, and S4. The enriched GO categories show clear differences between the profiles of the three metabolites, which suggests that each of them has a unique biological role.

In hP29SN stromal cells, the highly enriched GO categories related to 1α,25(OH)2D3-treatment include those involved in nucleotide biosynthesis, ubiquitin conjugation, ion binding, and macromolecule biosynthesis (Table S2). 25(OH)D3 plays numeral roles in intracellular organelles, DNA-binding transcription factors, protein-protein interaction, transcription coregulator activity, RNA-binding motif, and organic acid biosynthesis (Table S2). 24R,25(OH)2D3 appeared to be involved in the regulation of zinc-finger motifs, methylation, transcription, epigenetics, protein kinases, and membrane targeting (Table S2).

In mCyp27b1−/− fibroblasts, 1α,25(OH)2D3 is specifically involved in cell differentiation, extracellular matrix, cell adhesion, prostate gland development, cytokine and growth factor activity.
cell motion, mesenchymal and epithelial cell proliferation, neuron development, EGF, and gland development (Table S3). On the other hand, 25(OH)D3 plays a different role, mainly in the regulation of cell apoptosis, lipid transport, glycoprotein, extracellular matrix components, blood vessel development, cell proliferation, and response to wounding (Table S3). However, 24R,25(OH)2D3 appears to have very limited biological activity based on the current detection method, only being involved in ion binding (Table S3). We were also interested to know the common biological roles of 25OHD3 and 1α,25(OH)2D3, and therefore performed functional annotation analysis of 321 commonly regulated genes. These gene products are involved in glycoproteins, extracellular matrix-related proteins and enzymes, EGF-like calcium-binding, epithelium development, and cell morphogenesis (Table S4).

Pathway Analyses and Network Generation of Genes Exclusively Regulated by Vitamin D3 Hormones

To reveal the possible biological interaction of specifically regulated genes by each metabolite, we performed network analyses using the IPA. The top five associated network functions of genes exclusively regulated by single metabolite are summarized in Table 1. 1α,25(OH)2D3-specific target genes are mostly involved in “Cardiovascular System Development and Function, Tissue Development, Organismal Development” (score 16, Figure 2A) and “Cellular Movement, Immune Cell Trafficking, Haematological System Development and Function” (score 14) in hP29SN stromal cells and “Tissue Morphology, Hematological System Development and Function, Humoral Immune Response” (score 28, Figure 3A) and “Cardiovascular System Development and Function, Inflammatory Response, Cellular Movement” (score 28, Figure 3B) in mCyp27b1−/− fibroblasts. The most populated biological networks for 25(OH)D3-specific target genes are entitled “Cell Death and Survival, Gene Expression, Tissue Morphology” (score 25, Figure 2B) and “Cell Death and Survival, Cellular Growth and Proliferation, Cell Cycle” (score 23, Figure 2C) in hP29SN stromal cells and “Cellular Growth and Proliferation, Cellular Development, Reproductive System Development and Function” (score 19, Figure 3C) in mCyp27b1−/− fibroblasts. 24R,25(OH)2D3 specific target genes are mostly involved in “Cell Morphology, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair” (score 22, Figure 2D) and “Gene Expression, Cellular Growth and Proliferation, Cell Cycle” (score 20) in hP29SN stromal cells and “Cell Death and Survival,
Endocrine System Development and Function, Lipid Metabolism” (score 18, Figure 3D) in mCyp27b1−/− fibroblasts.

Furthermore, the top five canonical pathways for genes exclusively regulated by a single metabolite are listed in Table 2. The top canonical pathway in hP29SN stromal cells affected by 1α,25(OH)2D3, 25(OH)D3, and 24R,25(OH)2D3 is “Amyotrophic Lateral Sclerosis Signaling” (p = 0.006), “Granzyme B Signaling” (p = 0.002), and “DNA Double-Strand Break Repair by Homologous Recombination” (p = 0.003), respectively. The most affected pathway in mCyp27b1−/− fibroblasts by 1α,25(OH)2D3, 25(OH)D3, and 24R,25(OH)2D3 is “Xenobiotic Metabolism Signaling” (p = 0.005), “Aryl Hydrocarbon Receptor Signaling” (p = 0.003), and “Production of Nitric Oxide and Reactive Oxygen Species in Macrophages” (p = 0.005), respectively.

To have an overall view of the networks affected by each metabolite, we constructed IPA network analyses using all the regulated genes in hP29SN stromal cells (Figure 4) and mCyp27b1−/− fibroblasts (Figure 5). The top five associated network functions of all the genes regulated by single metabolite are summarized in Table 3.

Validation by qRT-PCR
To validate the microarray data by an independent method, the expression levels of eight genes in hP29SN stromal cells were measured by qRT-PCR (Figure 6A–C). A good correlation (R2 = 0.96) was detected between the two methods (Figure 6D). Similarly, the expression of two genes in mCyp27b1−/− fibroblasts was measured by qRT-PCR (Figure 6E–F).

Discussion
In order to analyze putative differential gene regulatory effects of vitamin D3 metabolites, we performed cDNA microarray analysis in hP29SN stromal cells. To rule out the effect of intracellularly 25(OH)D3-derived 1α,25(OH)2D3, we also performed cDNA microarray analysis in mCyp27b1−/− fibroblasts that lack 25(OH)D3 1α-hydroxylase enzyme activity.
The optimal serum concentrations of 25(OH)D3 for good health are still under discussion. The proposed normal range is broad and variable in different populations perhaps due to an adaptation. Several optimal concentrations have been proposed: 20–80 nM [31], 50–250 nM [32], and 40–70 nM [9] have been suggested. The international recommended optimal concentration of 25(OH)D3 is 50 nM or more. 40 nM or more is recommended in Finland, 50 nM by the Institute of Medicine, 75–185 nM (30–74 ng/ml) by MedlinePlus, and 100 nM by the Endocrine Society. On the other hand, the normal serum concentration of 1α,25(OH)2D3 is clearly defined, being 0.03–0.14 nM [31]. The concentration of 24R,25(OH)2D3 is approximately 1.44–8 nM (0.6–2.9 ng/ml) [33].

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Here we identified 1α,25(OH)2D3 regulated genes involved in immune functions, antibacterial response, and inflammation (Figure 4 and Table 1) as well as DNA repair, cell growth and death, and cell-matrix interaction (Tables S1, S3, and 3), which have been previously described in other cell systems [34–36].

### Table 3. Top five associated network functions of all the genes regulated by single metabolite generated by IPA.

#### Human P29SN stromal cells

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Top associated network functions</th>
<th>Score</th>
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<tbody>
<tr>
<td>1α,25(OH)2D3</td>
<td>Cancer, Cardiovascular System Development and Function, Cellular Movement</td>
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<tr>
<td></td>
<td>Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking</td>
<td>15</td>
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<tr>
<td></td>
<td>Cell Death, Cell Morphology, DNA Replication, Recombination, and Repair</td>
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<td></td>
<td>Hereditary Disorder, Neurological Disease, Ophthalmic Disease</td>
<td>2</td>
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<tr>
<td></td>
<td>Cardiac Proliferation, Cardiovascular System Development and Function, Cell Cycle</td>
<td>2</td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>Cell Death and Survival, Metabolic Disease, Endocrine System Disorders</td>
<td>22</td>
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<tr>
<td></td>
<td>Cancer, Hematological Disease, Cellular Development</td>
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<td></td>
<td>Cell Cycle, Cell Death and Survival, Cellular Compromise</td>
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<td></td>
<td>Gene Expression, Cell Death and Survival, Amino Acid Metabolism</td>
<td>11</td>
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<td></td>
<td>Gene Expression, RNA Post-Transcriptional Modification, Cell-To-Cell Signaling and Interaction</td>
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</tr>
<tr>
<td>24R,25(OH)2D3</td>
<td>Cell Development, Reproductive System Development and Function, Cell Morphology</td>
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<tr>
<td></td>
<td>Cell Cycle, Cell Death, Cellular Movement</td>
<td>19</td>
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<td>Gene Expression, DNA Replication, Recombination, and Repair, Cardiovascular System Development and Function</td>
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</tbody>
</table>

#### Mouse Cyp27b1−/− fibroblasts

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Top associated network functions</th>
<th>Score</th>
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</thead>
<tbody>
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<td>1α,25(OH)2D3</td>
<td>Cardiovascular System Development and Function, Organismal Development, Cancer</td>
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<td>Protein Synthesis, Cellular Development, Hematological System Development and Function</td>
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<td>Dermatological Diseases and Conditions, Hereditary Disorder, Organismal Injury and Abnormalities</td>
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<td>25(OH)D3</td>
<td>Humoral Immune Response, Inflammatory Response, Cellular Development</td>
<td>37</td>
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<td>Cancer, Cellular Growth and Proliferation, Connective Tissue Disorders</td>
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<td>Hematological System Development and Function, Humoral Immune Response, Tissue Morphology</td>
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<td>Organismal Injury and Abnormalities, Inflammatory Response, Connective Tissue Disorders</td>
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<td>24R,25(OH)2D3</td>
<td>Hematological System Development and Function, Hematopoiesis, Tissue Morphology</td>
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<td>Cellular Development, Hematological System Development and Function, Hematopoiesis</td>
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</tbody>
</table>

doi:10.1371/journal.pone.0075338.t003

### Three Distinct Vitamin D3 Hormones

Within the mouse, the concentration of 25(OH)D3 is approximately 50 nM [31]. 24R,25(OH)2D3 is approximately 8 nM (2.9 ng/ml) [33]. These results are consistent with the human studies.[31] The concentration of 1α,25(OH)2D3 is clearly defined, being 0.03–0.14 nM [31]. The concentration of 24R,25(OH)2D3 is approximately 1.44–8 nM (0.6–2.9 ng/ml) [33]. The four vitamin D metabolites are not active in cell studies. To avoid bias in comparison and ensure detectable responses, we have chosen 10-fold higher concentrations of vitamin D metabolites over their normal serum levels, which are approximately 50, 5, and 1 nM for 25(OH)D3, 24R,25(OH)2D3, and 1α,25(OH)2D3, respectively.

Our study in mCyp27b1−/− fibroblasts suggests that gene expression fingerprints of 1α,25(OH)2D3 and 25OHD3 are similar. Among the genes regulated by 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts are not active in cell studies. To avoid bias in comparison and ensure detectable responses, we have chosen 10-fold higher concentrations of vitamin D metabolites over their normal serum levels, which are approximately 50, 5, and 1 nM for 25(OH)D3, 24R,25(OH)2D3, and 1α,25(OH)2D3, respectively.

Here we identified 1α,25(OH)2D3 regulated genes involved in immune functions, antibacterial response, and inflammation (Figure 4 and Table 1) as well as DNA repair, cell growth and death, and cell-matrix interaction (Tables S1, S3, and 3), which have been previously described in other cell systems [34–36].

#### 25OHD3 is an Active Hormone

Our study in mCyp27b1−/− fibroblasts suggests that gene expression fingerprints of 1α,25(OH)2D3 and 25OHD3 are similar. Among the genes regulated by 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts are not active in cell studies. To avoid bias in comparison and ensure detectable responses, we have chosen 10-fold higher concentrations of vitamin D metabolites over their normal serum levels, which are approximately 50, 5, and 1 nM for 25(OH)D3, 24R,25(OH)2D3, and 1α,25(OH)2D3, respectively.
fibroblasts, 52% were also regulated by 25(OH)D₃. On the other hand, 68% of 25(OH)D₃-regulated genes in mCyp27b1²/² fibroblasts were also regulated by 1α,25(OH)₂D₃. The majority of overlapping genes are the most strongly regulated genes among the top 15 in the lists. These data once further support previous findings by us [3,4] and others [6–8,10,12] that 25(OH)D₃ is indeed an active hormone. Studies of 25(OH)D₃ analog that is hardly subjected to 1α-hydroxylation provide additional evidence that 1α-hydroxylation of 25(OH)D₃ is not required for its anti-proliferative activity [12].

Human P29SN stromal cells express 25(OH)D₃ 1α-hydroxylase that converts 25(OH)D₃ into 1α,25(OH)₂D₃ and vitamin D₃ 24-hydroxylase that converts 25(OH)D₃ into 24R,25(OH)₂D₃ [3], thus we could not rule out the effects of intracellular 1α,25(OH)₂D₃ and 24R,25(OH)₂D₃ products derived from 25OHD₃. We observed that only a few genes commonly regulated by 25(OH)D₃ and 1α,25(OH)₂D₃ in hP29SN. Of the genes regulated by one metabolite, only 12–13% were also regulated by another metabolite. CYP24A1 was the most highly up-regulated gene by 25(OH)D₃ and 1α,25(OH)₂D₃ in both mouse and human fibroblasts. It is worth mentioning that 24R,25(OH)₂D₃ did not regulate CYP24A1 gene expression. An earlier microarray study has also found the induction ratio of CYP24A1 gene expression by 1α,25(OH)₂D₃ was the highest among 3800 human genes examined and 24R,25(OH)₂D₃ at a very high concentration (1000 nM) did induce CYP24A1 gene expression [37]. The expression of cholesterol 25-hydroxylase was increased more than threefold by both 25(OH)D₃ and 1α,25(OH)₂D₃ in hP29SN, consistent with our previous study [38]. Both 25(OH)D₃ and 1α,25(OH)₂D₃ increased the expression of insulin-like growth factor 1 more than twofold in hP29SN.

These data indeed demonstrate unique roles for 25(OH)D₃ and 1α,25(OH)₂D₃. Recent molecular dynamics simulations data [4] indicate both 1α,25(OH)₂D₃ and 25(OH)D₃ bind to the vitamin D receptor (VDR) ligand-binding pocket at the same position, resulting in identical agonistic conformation [4]. Microarray data presented here further validate the biological significance of 25(OH)D₃.

A Complementary Role of 25(OH)D₃ and 1α,25(OH)₂D₃ in Calcium and Skeletal Homeostasis

1α,25(OH)₂D₃ stimulates a number of genes involved in bone formation [39,40] and osteoclast differentiation [41–43]. We discovered several new 1α,25(OH)₂D₃-target genes involved in bone physiology and pathology in hP29SN stromal cells and mCyp27b1²/² fibroblasts. Na⁺/H⁺ exchanger domain containing 2 (NHE2C2, NHA2, SLC9B2), a member of Na⁺/H⁺ exchanger...
family, was potently up-regulated by 1α,25(OH)2D3 (ca. tenfold) in hP29SN stromal cells. Thus, it is tempting to link 1α,25(OH)2D3 action to bone resorption and remodeling based on a recently-discovered role of NHEDC2 in osteoclast differentiation and fusion [44,45]. Sex determining region Y-box 6 (SOX6), an osteoporosis susceptibility gene shown by genome-wide association studies [46,47], was up-regulated by more twofold by 1α,25(OH)2D3 in hP29SN. Sex6 was shown to be essential for cartilage formation in mice [48].

Contrary to human cells, we identified a different set of bone-related genes as the targets of 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts. Secreted frizzled related protein 1 (Sfrp1) was down-regulated by 1α,25(OH)2D3. Sfrp1 was previously shown to inhibit receptor activator of nuclear factor-kB ligand-dependent osteoclast formation [49] and was strongly associated with bone mineral density and bone mineral content [50]. Loss of Sfrp1 in mice improves fracture repair [51], due to antagonism of the Wnt pathway. Bone morphogenetic protein 1 (BMP1) was increased by more than sevenfold by 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts. BMP1 has been suggested to enhance bone repair [52].

25(OH)D3 shares some functions with 1α,25(OH)2D3 in regulating calcium and skeletal homeostasis. Three calcium homeostasis-related genes were regulated by 25(OH)D3 and 1α,25(OH)2D3 in both mouse and human fibroblasts. Voltage-dependent calcium channel γ-4 subunit (CACNG4) was up-regulated in hP29SN stromal cells (more than twofold by 25(OH)D3 and more than threefold by 1α,25(OH)2D3). S100 calcium binding protein G (S100G, CABLES1, encoding calbindin D9K) was dramatically up-regulated in mCyp27b1−/− fibroblasts (38-fold by 25(OH)D3 and 85-fold by 1α,25(OH)2D3). Prolactin receptor (PRLR) gene expression was up-regulated by more than threefold by 25(OH)D3 and more than 12-fold 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts. PRLR was responsible for the growth inhibitory effect of prolactin on prostate cells [53] and recently it was found to participate in calcium homeostasis by multiple mechanisms [54]. Two bone-related genes were regulated by both 25(OH)D3 and 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts. The expression of secreted frizzled related protein 2 (Sfrp2) was strongly down-regulated by both 25(OH)D3 (sixfold) and 1α,25(OH)2D3 (sevenfold) in mCyp27b1−/− fibroblasts. Sfrp2 is an osteoporosis susceptibility gene associated with bone mineral density [50]. In addition, both 25(OH)D3 (more than fourfold) and 1α,25(OH)2D3 (more than fivefold) also inhibited the expression of tumor necrosis factor receptor superfamily, member 11b (Tnfsf11b, OPG, osteoprotegerin) in mCyp27b1−/− fibroblasts. Osteoprotegerin functions as a negative regulator of osteoclastogenesis and bone resorption [55].

Collectively, studies in human prostate stromal cells and mouse skin fibroblasts suggest that 25(OH)D3 and 1α,25(OH)2D3 complement each other in regulating calcium homeostasis and bone remodeling. The role of 1α,25(OH)2D3 is broader than that of 25(OH)D3. Our current findings support in vivo data that show partial rescue of the phenotype of Cyp27b1−/− mice by vitamin D3 injection [10]. Although human prostate stromal cells and mouse skin fibroblasts are neither the sites of calcium absorption nor the responsible cells for bone formation, the current finding provides new information with indications that require further studies in intestine and bone cells. The action of 25(OH)D3 and 1α,25(OH)2D3 in calcium homeostasis in stromal cells and fibroblasts may have two meanings. Firstly, the second messenger Ca2+ is involved in many cellular processes, such as cell proliferation, cell differentiation, cell-cell and cell-matrix interactions, and cell movement. In fact, the role of 1α,25(OH)2D3 in calcium homeostasis has been described in a microarray study of LNCaP human prostate cancer cells [56]. Secondly, calcium homeostasis in non-bone tissues may indicate soft tissue calcification, which is also a major concern in vitamin D therapy. This is evidenced by a microarray study of human artery smooth muscle cells [57].

We did not find 24R,25(OH)2D3-target genes related to skeletal homeostasis in hP29SN stromal cells and mCyp27b1−/− fibroblasts. This should be verified by in vitro bone cell studies. It might also be possible that the previously discovered effect of 24R,25(OH)2D3 on the bone is non-genomic.

Non-classical Actions of Vitamin D3 Metabolites

To get an in-depth view of the actions of each metabolite, we further analyzed the regulated genes not involved in bone and calcium homeostasis. We discovered 1α,25(OH)2D3-mediated up-regulation of several genes that negatively regulate cell cycle and growth. Tyrosine kinase, non-receptor, 1 (Tnk1), up-regulated by more than sixfold in hP29SN, was earlier shown to facilitate tumor necrosis factor α-induced apoptosis [58]. Cdk5 and Abl enzyme substrate 1 (CABLES1), up-regulated by more than sixfold in hP29SN, is involved in regulation of the cell cycle. Reduction of CABLES1 expression was previously observed in colorectal cancers [59]. Microcephalin 1 (MCPH1) was up-regulated by more than fourfold in hP29SN. MCPH1 product acts as a DNA-damage response protein and prevents premature entry into mitosis [60].

The vitamin D endocrine system plays an important role in skin in both ligand-dependent and -independent manners [61]. Vitamin D and the VDR regulate a number of genes involved in keratinocyte differentiation and hair follicle cycle. Here, we identified three genes whose expression was specifically regulated by 1α,25(OH)2D3 in mCyp27b1−/− fibroblasts. Keratin 17, a regulatory gene in hair development [62], was strongly induced by 1α,25(OH)2D3 (more than 13-fold). Cadherin 11, which is expressed in mesenchymal aggregates during hair follicle development [63], was up-regulated by 1α,25(OH)2D3 (threefold). Transforming growth factor β2, shortening hair cycle by stimulating certain caspases [64], was down-regulated by 1α,25(OH)2D3 (twofold). These findings provide additional important mechanism by which 1α,25(OH)2D3 specifically regulates hair development in a VDR-dependent manner.

The IPA analysis shows that amyotrophic lateral sclerosis (ALS) signaling is the top canonical pathway of 1α,25(OH)2D3-mediated genes in hP29SN stromal cells (Table 2). This finding supports the recent hypothesis that vitamin D may delay ALS progression [65–68].

A number of genes involved in gene transcription were specifically modulated by 25(OH)D3 (Figures 2B and 2C). Nuclear receptor coactivator 2 (NCOA2, SRC2) was induced by 8-fold, hairless and enhancer of split 1 (HES1) by more than twofold, activating transcription factor 6 (ATF6) by more than twofold, and nuclear transcription factor Y, γ (NFYC, CBF-C) by more than twofold in hP29SN stromal cells. HES1, a Notch-target gene, is a transcription factor involved in various events during development [69–72]. ATF6 mediates RUNX2-dependent osteocalcin expression in osteoblasts [73] and apoptosis in myoblasts [74]. CBF-C represses the transactivating activity of Smad2 and Smad3 [75]. Several C2H2-like zinc-finger proteins that are involved in transcriptional regulation, for example, YY2 transcription factor and zinc finger proteins, were also induced by 25(OH)D3 in hP29SN.

Previous studies have linked vitamin D deficiency to various inflammatory diseases and suggest a role for vitamin D in immunity [76]. Our current study provides an alternative mechanism of the immunomodulatory property of 25(OH)D3, 25(OH)D3 induced the expression of membrane-associated ring
finger C3HC4) 1 (MARCHI) more than sixfold in hP29SN stromal cells. MARCHI is an E3 ubiquitin ligase that down-regulates major histocompatibility complex class I membrane expression [77]. The immunosuppressive effect of interleukin-10 is mediated by MARCHI [78]. Furthermore, 25(OH)D3 increased SAM domain and HD domain 1 (SAMHD1) expression more than twofold in hP29SN. SAMHD1 plays a role in the innate immune response and may regulate tumor necrosis factor α proinflammatory responses [79]. Hepatitis A virus cellular receptor 2 (HAVCR2, TIM3, T cell immunoglobulin-3) gene expression was increased threefold by 25(OH)D3 in hP29SN. TIM3 fails to be up-regulated in T cells in human autoimmune diseases, such as multiple sclerosis [80]. TIM3 locus and specific gene polymorphisms are associated with various immune-mediated diseases, such as rheumatoid arthritis [81] and atopic disease [82]. These findings are primary but important. Confirmation utilizing monocyes, dendritic cells, and T cells for in vitro studies, together with functional analyses, will bolster the importance of our conclusions.

24R,25(OH)2D3 is produced by CYP24A1 when there is enough active vitamin D3 available. We identified two 24R,25(OH)2D3-target genes involved in methylation and epigenetics in hP29SN stromal cells. MLL5 (a trithorax homolog, myeloid/lymphoid or mixed-lineage leukemia 5) was up-regulated more than threefold by 24R,25(OH)2D3. MLL5 is a known chromatin regulator in H3K4 methylation [83]. Methyltransferase like 15 (METTL15, METT5D1) was increased more than 15-fold by 24R,25(OH)2D3.

Human microarray data show that 24R,25(OH)2D3 modulate cell differentiation and proliferation, in particular by strongly influencing breast cancer 1, early onset (BRCA1) signaling pathway (Figure 2D). BRCA1 expression was increased more than twofold by 24R,25(OH)2D3. Induction of BRCA1 was previously found to be partially responsible for the anti-proliferative effects of 1α,25(OH)2D3 [84]. Interestingly, cofactor of BRCA1 (COBRA1) was reduced threefold by 24R,25(OH)2D3. BRCA1 and COBRA1 seem to function in coordinate gene regulatory pathways [85]. Tumor protein p53 binding protein 1 (TP53BP1) was up-regulated by more than threefold by 24R,25(OH)2D3. TP53BP1 up-regulates the promoter of BRCA1 [86]. Kinase suppressor of ras 2 (KSR2), a target of 1α,25(OH)2D3 [87], was increased by more than fivefold by 24R,25(OH)2D3 in hP29SN. 24R,25(OH)2D3 regulates two hormone receptor coactivators. It decreased the expression of mediator complex subunit 1 (MED1, TRAP220, DRIP205) threefold. MED1 is a VDR coactivator [88]. It also decreased thyroid hormone receptor associated protein 3 (twofold).

24R,25(OH)2D3 influences the expression of several membrane proteins. Erythrocyte membrane protein band 4.1 like 5 (EPB4.1L5), which contributes to the correct positioning of tight junction during the establishment of cell polarity [89], was increased ninefold by 24R,25(OH)2D3 in hP29SNstromal cells. Synaptotagmin XIV (SYT14) was up-regulated more than sixfold in hP29SN. Regulating synaptic membrane exocytosis 2 (Rim2) was up-regulated more than twofold in mCyp27b1−/− fibroblasts. Both the gene products are involved in exocytosis.

Conclusions

The present study is the first systematic comparison of global gene expression in hP29SN stromal cells and mCyp27b1−/− fibroblasts after treatment with 1α,25(OH)2D3, 25(OH)D3, and 25R,25(OH)2D3. It seems that there are three partially different vitamin D3 (cholecalciferol) hormonal systems. 25(OH)D3 and 1α,25(OH)2D3 complement each other in regulating calcium homeostasis and bone remodeling. Each vitamin D3 metabolite has unique non-classical actions in various physiological and pathological processes. Our study presents a novel perspective for the function of vitamin D3 within the endocrine system, and furthers our understanding of the roles and relationships between vitamin D3 metabolites. The limitation of our study is that some regulated genes need to be clarified in relevant cells and tissues, e.g. immune responsive genes and bone-related genes.

Supporting Information

Figure S1 Quantitative real-time RT-PCR (qRT-PCR) analysis of CYP24A1 gene expression. Human P29SN stromal cells were treated with either 0.1% ethanol (vehicle), 10 nM 1α,25(OH)2D3, 500 nM 25(OH)D3, or 25 nM 24R,25(OH)2D3 for 24 h. Relative mRNA expression was normalized to the control gene RPLP0, and fold inductions were calculated in reference to vehicle. Results are expressed as means ± SD (n = 4). The same samples were then used in microarray assays.

Table S1 Sequences of the primers used in qPCR. (PDF)

Table S2 Enriched gene ontology (GO) categories for exclusively regulated genes in human P29SN stromal cells generated by DAVID. (PDF)

Table S3 Enriched gene ontology (GO) categories for exclusively regulated genes in mouse Cyp27b1−/− fibroblasts generated by DAVID. (PDF)

Table S4 Enriched gene ontology (GO) categories for commonly regulated genes by both 1α,25(OH)2D3 and 25(OH)D3 in mouse Cyp27b1−/− fibroblasts generated by DAVID. (PDF)

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We thank Dr. Merja Matilainen (University of Kuopio) for help in human Affymetrix microarrays, Mrs. Arja Ahola for her reagent orders, Leo Pharmaceuticals (Ballerup, Denmark) for the generous gift of vitamin D3 metabolites.

Author Contributions

Conceived and designed the experiments: YRL PT. Performed the experiments: YRL JHW MK TM. Analyzed the data: YRL PT JHW SK. Contributed reagents/materials/analysis tools: YRL PT SK AB MV. Performed the experiments: YRL JHW MK TM. Wrote the paper: YRL PT SK MV.

References


Three Distinct Vitamin D$_3$ Hormones


