

# Gene Expression Profiles in Human and Mouse Primary Cells Provide New Insights into the Differential Actions of Vitamin D<sub>3</sub> Metabolites

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## Abstract

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) had earlier been regarded as the only active hormone. The newly identified actions of 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) and 24R,25-dihydroxyvitamin D<sub>3</sub> (24R,25(OH)<sub>2</sub>D<sub>3</sub>) broadened the vitamin D<sub>3</sub> 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)D<sub>3</sub> into 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> by 1 $\alpha$ -hydroxylase (encoded by the gene *CYP27B1*), displayed regulation of 164, 171, and 175 genes by treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, respectively. Mouse primary *Cyp27b1* knockout fibroblasts (m*Cyp27b1*<sup>-/-</sup>), which lack 1 $\alpha$ -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 m*Cyp27b1*<sup>-/-</sup>. 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> 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 D<sub>3</sub> hormones with clearly different biological activities. This study presents a new conceptual insight into the vitamin D<sub>3</sub> endocrine system, which may guide the strategic use of vitamin D<sub>3</sub> in disease prevention and treatment.

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## Introduction

Vitamin D<sub>3</sub> produced in the skin undergoes two sequential hydroxylation steps, first 25-hydroxylation generating 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) and then 1 $\alpha$ -hydroxylation generating 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>). Both of them are further metabolized by 24-hydroxylase. Among many endogenous vitamin D<sub>3</sub> metabolites, 25(OH)D<sub>3</sub> and 24R,25-dihydroxyvitamin D<sub>3</sub> (24R,25(OH)<sub>2</sub>D<sub>3</sub>) are the first and second most abundant circulating metabolites of vitamin D<sub>3</sub>. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is believed to be the most active vitamin D<sub>3</sub> metabolite. Their classic actions in calcium homeostasis and non-classical actions in cell proliferation and differentiation have been the focus of vitamin D<sub>3</sub> research.

During the past two decades, there have been some important discoveries of vitamin D<sub>3</sub> metabolism and endocrine system. Cells can uptake 25(OH)D<sub>3</sub> by megalin-mediated endocytosis [1,2]. 25(OH)D<sub>3</sub> 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* as well as *in vivo* studies [4–13]. 25(OH)D<sub>3</sub> acts synergistically with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> [4]. 24R,25(OH)<sub>2</sub>D<sub>3</sub> seems to regulate specifically chondrocytes [14] and osteogenesis [15]. 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase, encoded by the gene *CYP27B1* and responsible for the conversion of 25(OH)D<sub>3</sub> to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, is expressed in the kidney and in many extra-renal tissues [16–18], thus the intracellularly synthesized 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> was suggested to act as an intracrine factor [9]. Epidemiological studies provide

accumulating evidence showing that vitamin D<sub>3</sub> insufficiency is associated with infection [19], cancers [20], fractures [21], diabetes [22], and neuropsychiatric disease [23], and 25(OH)D<sub>3</sub> concentration gradient is related to the seasonal variation of cancer survival [24]. The optimal dose and serum concentration of vitamin D<sub>3</sub> is under debate. Some epidemiological studies also suggest that both low and high 25(OH)D<sub>3</sub> levels are harmful [9,25,26]. Based on these findings, it seems that the vitamin D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, reviewed by C. Kriebitzsch, et al [27]. Gene expression in response to 25(OH)D<sub>3</sub> or 24R,25(OH)<sub>2</sub>D<sub>3</sub> has not yet been studied by DNA microarray. Here we compared the effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub> on gene expression patterns to clarify similarities and differences in signal transduction. To exclude the effect of the intracellular product of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, we also performed microarray study in mouse primary *Cyp27b1* knockout fibroblasts (m*Cyp27b1*<sup>-/-</sup>). To our knowledge, microarray gene expression studies have not been made in m*Cyp27b1*<sup>-/-</sup> cells. Our data show that there are three vitamin D<sub>3</sub> (cholecalciferol) 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 Federation of European Laboratory Animal Associations.

### Mice

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

### Cell Culture, Vitamin D<sub>3</sub> Treatment, and RNA Isolation

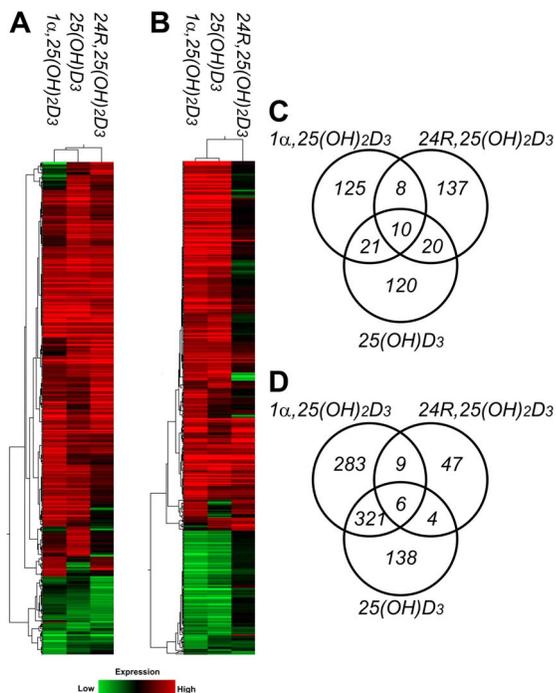
The isolation and characterization of human primary prostate stromal P29SN cells were described previously [3]. The vast majority of the primary stromal culture was shown to be fibroblasts in phenotype. Over 99% of the cells were positive for fibroblast markers vimentin and fibronectin. Less than 5% 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  $\mu$ g/ml insulin, and antibiotics (penicillin 100 units/ml, streptomycin 100  $\mu$ g/ml) (Gibco-BRL, Life Technology, Paisley, Scotland) at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 500 nM 25(OH)D<sub>3</sub>, or 25 nM 24R,25(OH)<sub>2</sub>D<sub>3</sub> (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 (m*Cyp27b1*<sup>-/-</sup> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 500 nM 25(OH)D<sub>3</sub>, or 50 nM 24R,25(OH)<sub>2</sub>D<sub>3</sub> for 24 hours. Ethanol concentration was 0.1% in all treatment conditions. Experiments were replicated four independent times for each treatment condition.

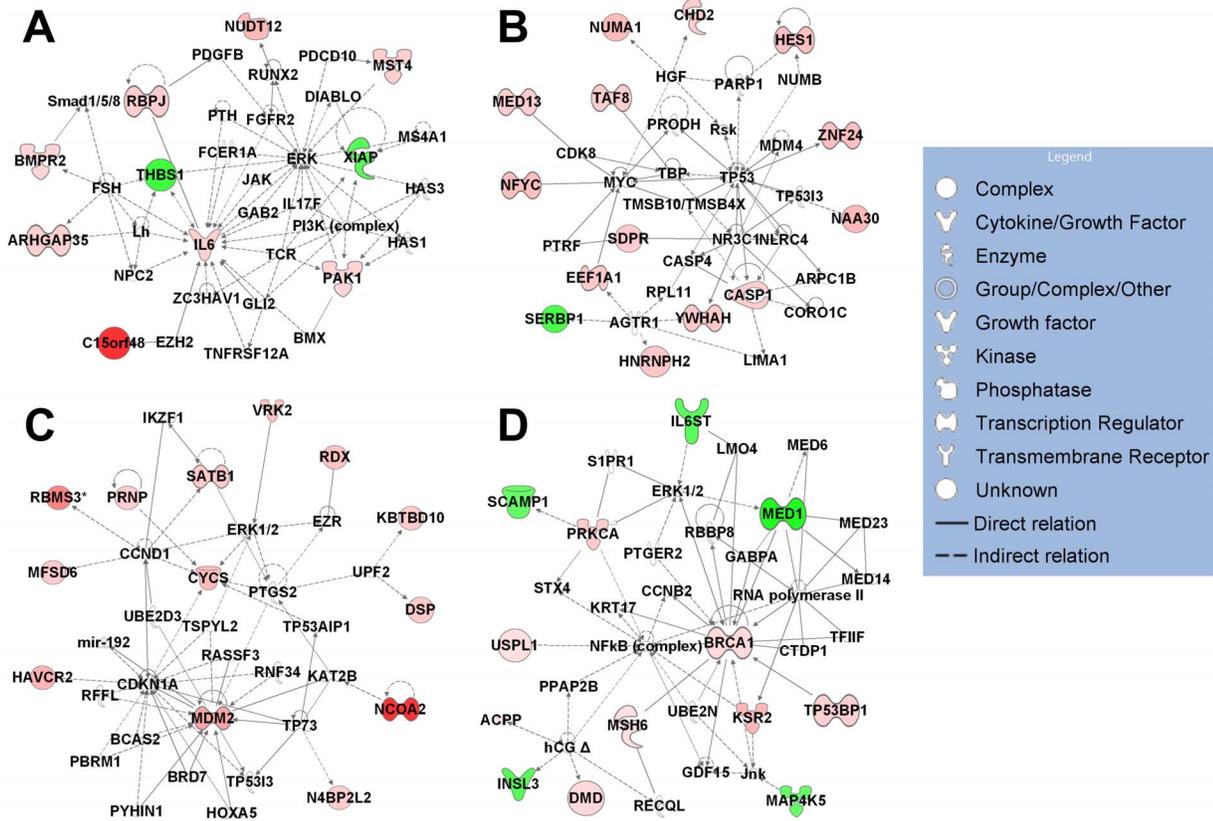
Total cellular RNA was isolated from hP29SN stromal cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and from m*Cyp27b1*<sup>-/-</sup> fibroblasts by RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturers' protocols. The RNA concentration and purity were verified using a GeneQuant 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 D<sub>3</sub> 24-hydroxylase) gene expression was measured by using qRT-PCR to ensure that hP29SN stromal cells were successfully stimulated by vitamin D<sub>3</sub> metabolites. Similarly, for the validation of microarray data, the expression levels of eight differentially expressed genes in hP29SN stromal cells and two genes in m*Cyp27b1*<sup>-/-</sup> 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



**Figure 1. Gene expression profiles.** Hierarchical clustering of the differentially expressed genes in (A) hP29SN stromal cells and (B) m*Cyp27b1*<sup>-/-</sup> fibroblasts after vitamin D<sub>3</sub> treatments. Colored-bands represent the change of the corresponding gene expression, green indicating down-regulation and red up-regulation. The key for deciphering of the color is shown below the clustering image. Venn diagrams of co-expressed and uniquely regulated genes by vitamin D<sub>3</sub> metabolites in (C) hP29SN and (D) m*Cyp27b1*<sup>-/-</sup> fibroblasts. doi:10.1371/journal.pone.0075338.g001



**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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in Cardiovascular System Development and Function, Tissue Development, Organismal Development. (B) Genes regulated by 25(OH)D<sub>3</sub> in Cell Death and Survival, Gene Expression, Tissue Morphology. (C) Genes regulated by 25(OH)D<sub>3</sub> in Cell Death and Survival, Cellular Growth and Proliferation, Cell Cycle. (D) Genes regulated by 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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. doi:10.1371/journal.pone.0075338.g002

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 *mCyp27b1*<sup>-/-</sup> fibroblasts.

**Affymetrix Microarray Analysis**

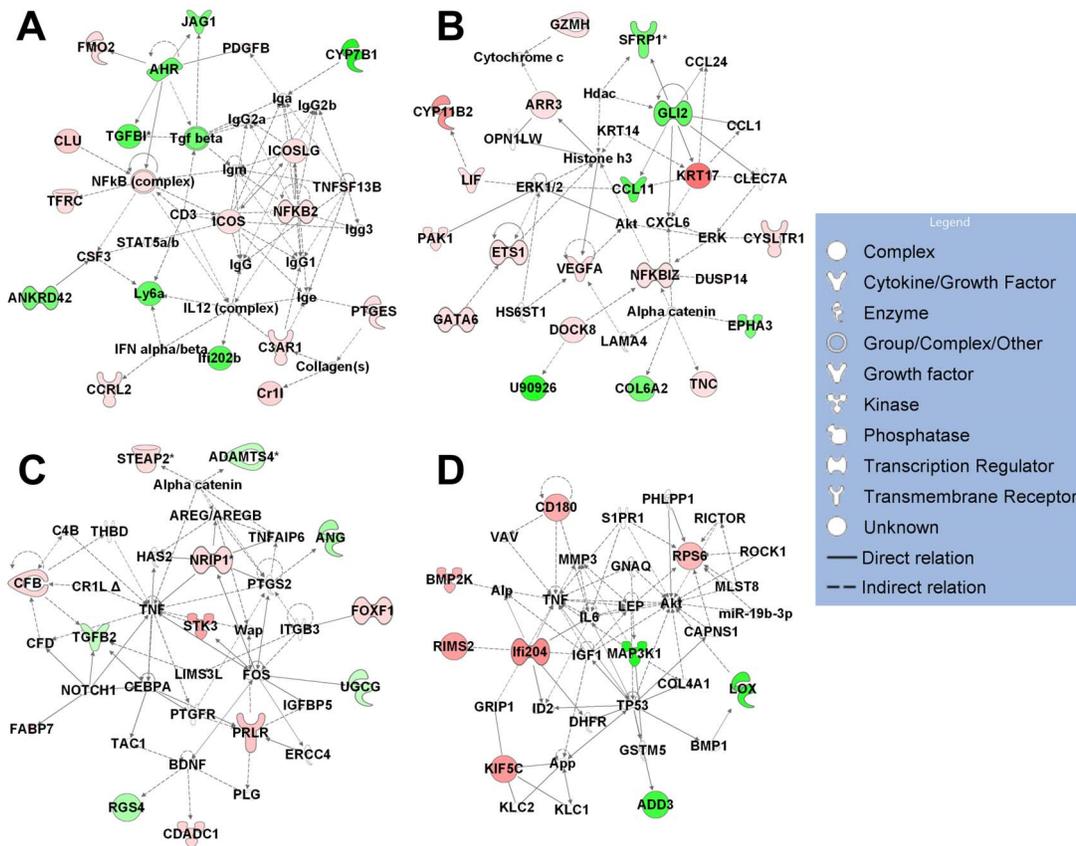
cRNA preparation, hybridization, and the array wash procedure were performed following the standard protocol from Affymetrix. In brief, 7  $\mu$ g of total RNA from each hP29SN sample were used to synthesize double-stranded cDNA. The cDNA was used as a template to generate biotinylated cRNA by an *in vitro* transcription reaction. 20  $\mu$ g of biotinylated cRNA was fragmented and added to the GeneChip<sup>®</sup> 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<sup>®</sup> Operating Software Server 1.0 (GCOS Server) (Affymetrix, Santa Clara, CA, USA).

For mouse RNA samples, similar procedures were followed except for the following: 250 ng of total RNA from each *mCyp27b1*<sup>-/-</sup> sample was used as a starting material. Each

sample was hybridized to GeneChip<sup>®</sup> Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) at 45°C for 16 hours according to 3' IVT Express Kit User Manual (Affymetrix, Santa Clara, CA, USA). GeneChip<sup>®</sup> Fluidics Station 450 was used to wash and stain the arrays. GeneChip<sup>®</sup> 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 50<sup>th</sup> 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.



**Figure 3. IPA diagrams of the top associated network generated for genes exclusively regulated by each metabolite in *mCyp27b1*<sup>-/-</sup> fibroblasts.** (A) Genes regulated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in Tissue Morphology, Hematological System Development and Function, Humoral Immune Response. (B) Genes regulated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in Cardiovascular System Development and Function, Inflammatory Response, Cellular Movement. (C) Genes regulated by 25(OH)D<sub>3</sub> in Cellular Growth and Proliferation, Cellular Development, Hair and Skin Development and Function. (D) Genes regulated by 24R,25(OH)<sub>2</sub>D<sub>3</sub> in Cell Death and Survival, Endocrine System Development and Function, Lipid Metabolism. 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. doi:10.1371/journal.pone.0075338.g003

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 D<sub>3</sub> Metabolites**

We aimed at exploring the gene expression profiles of three vitamin D<sub>3</sub> metabolites using hP29SN stromal cells and *mCyp27b1*<sup>-/-</sup> fibroblasts. Human P29SN stromal cells were derived from a normal area of human prostatic carcinoma [3]. Mouse *Cyp27b1*<sup>-/-</sup> fibroblasts were derived from *Cyp27b1*<sup>-/-</sup> mice that do not express 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase and therefore cannot convert 25(OH)D<sub>3</sub> into 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>.

We verified the responsiveness and successful stimulation of cells by vitamin D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 500 nM 25(OH)D<sub>3</sub>, or 25 nM 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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-

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

Human P295N stromal cells		
Metabolite	Top associated network functions	Score
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Cardiovascular System Development and Function, Tissue Development, Organismal Development	16
	Cellular Movement, Immune Cell Trafficking, Hematological System Development and Function	14
	Cardiac Proliferation, Cardiovascular System Development and Function, Cell Cycle	2
	Molecular Transport, Protein Synthesis, Protein Trafficking	2
	Antimicrobial Response, Inflammatory Response, Antigen Presentation	2
25(OH)D <sub>3</sub>	Cell Death and Survival, Gene Expression, Tissue Morphology	25
	Cell Death and Survival, Cellular Growth and Proliferation, Cell Cycle	23
	Cell Cycle, Cancer, Cell Death and Survival	8
	Cell Morphology, Cellular Growth and Proliferation, Cellular Assembly and Organization	2
	Cell Cycle, DNA Replication, Recombination, and Repair, Cell Death and Survival	2
24R,25(OH) <sub>2</sub> D <sub>3</sub>	Cell Morphology, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair	22
	Gene Expression, Cellular Growth and Proliferation, Cell Cycle	20
	Cellular Growth and Proliferation, Cellular Development, Cellular Movement	18
	Cancer, Cell Death, Neurological Disease	2
	Cell Death, Cellular Development, Digestive System Development and Function	2
Mouse <i>Cyp27b1</i> <sup>-/-</sup> fibroblasts		
Metabolite	Top associated network functions	Score
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Tissue Morphology, Hematological System Development and Function, Humoral Immune Response	28
	Cardiovascular System Development and Function, Inflammatory Response, Cellular Movement	28
	Cancer, Respiratory Disease, Carbohydrate Metabolism	22
	Organismal Injury and Abnormalities, Cell Morphology, Lipid Metabolism	16
	Cellular Growth and Proliferation, Cell Death and Survival, Cell Cycle	16
25(OH)D <sub>3</sub>	Cellular Growth and Proliferation, Cellular Development, Hair and Skin Development and Function	19
	Cellular Function and Maintenance, Cell-mediated Immune Response, Cellular Development	17
	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function	15
	Gene Expression, Developmental Disorder, Cancer	15
	Cell Death, Cancer, Cell Cycle	13
24R,25(OH) <sub>2</sub> D <sub>3</sub>	Cell Death and Survival, Endocrine System Development and Function, Lipid Metabolism	18
	Connective Tissue Development and Function, Connective Tissue Disorders, Developmental Disorder	2
	Developmental Disorder, Immunological Disease, Cell Cycle	2
	Hereditary Disorder, Metabolic Disease, Cancer	2
	Cancer, Cell Death and Survival, Cellular Compromise	2

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characterized human genes. Ethanol-treated samples served as negative control. Each vitamin D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment, 164 genes met the selection criteria while 171 and 175 genes were identified for 25(OH)D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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 D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>, 8 for 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, and 20 for 25(OH)D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, respectively (Figure 1C).

Mouse *Cyp27b1*<sup>-/-</sup> fibroblasts were treated either with 10 nM 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 500 nM 25(OH)D<sub>3</sub>, or 50 nM 24R,25(OH)<sub>2</sub>D<sub>3</sub>, and GeneChip<sup>®</sup> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, respectively (data not shown). Samples were grouped using hierarchical clustering method based on the overall expression changes in the samples (Figure 1B). The gene expression profile in 50 nM

**Table 2.** Top five canonical pathways generated by IPA constructed from genes exclusively regulated by single metabolite.

Human P29SN stromal cells			
Metabolite	Top canonical pathways	p-value	Ratio
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Amyotrophic Lateral Sclerosis Signaling	6.31E-03	3/100 (0.03)
	TNFR1 Signaling 2/51	1.43E-02	2/51 (0.039)
	Xenobiotic Metabolism Signaling	1.51E-02	4/262 (0.015)
	PXR/RXR Activation 2/64	2.35E-02	2/64 (0.031)
	RhoGDI Signaling	2.92E-02	3/188 (0.016)
25(OH)D <sub>3</sub>	Granzyme B Signaling	2.32E-03	2/16 (0.125)
	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.61E-02	2/48 (0.042)
	Aryl Hydrocarbon Receptor Signaling	2.14E-02	3/141 (0.021)
	Myc Mediated Apoptosis Signaling	2.83E-02	2/60 (0.033)
	Cell Cycle: G1/S Checkpoint Regulation	2.92E-02	2/64 (0.031)
24R,25(OH) <sub>2</sub> D <sub>3</sub>	DNA Double-Strand Break Repair by Homologous Recombination	2.58E-03	2/16 (0.125)
	ATM Signaling	3.73E-03	3/59 (0.051)
	Complement System	1.32E-02	2/33 (0.061)
	Role of BRCA1 in DNA Damage Response	4.27E-02	2/63 (0.032)
	Melatonin Signaling	5.63E-02	2/71 (0.028)
Mouse <i>Cyp27b1</i> <sup>-/-</sup> fibroblasts			
Metabolite	Top canonical pathways	p-value	Ratio
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Xenobiotic Metabolism Signaling	4.62E-03	9/263 (0.034)
	Ephrin Receptor Signaling	7.42E-03	7/184 (0.037)
	Hepatic Fibrosis/Hepatic Stellate Cell Activation	9.24E-03	6/138 (0.043)
	Renal Cell Carcinoma Signaling	1.17E-02	4/69 (0.058)
	Lipoate Biosynthesis and Incorporation II	2.66E-02	1/2 (0.5)
25(OH)D <sub>3</sub>	Aryl Hydrocarbon Receptor Signaling	2.59E-03	5/139 (0.036)
	Metabolism of Xenobiotics by Cytochrome P450	3.15E-03	4/93 (0.043)
	LPS/IL-1 Mediated Inhibition of RXR Function	1.32E-02	5/214 (0.023)
	Xenobiotic Metabolism Signaling	2.96E-02	5/264 (0.019)
	Arachidonic Acid Metabolism	4.34E-02	3/110 (-0.027)
24R,25(OH) <sub>2</sub> D <sub>3</sub>	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.67E-03	3/175 (0.017)
	Ceramide Signaling	1.01E-02	2/78 (0.026)
	Mineralocorticoid Biosynthesis	1.82E-02	1/10 (0.1)
	Glucocorticoid Biosynthesis	2.02E-02	1/11 (0.091)
	p70S6K Signaling	2.28E-02	2/120 (0.017)

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24R,25(OH)<sub>2</sub>D<sub>3</sub>-treated cells displays much less changes compared with ethanol control, suggesting lower bioactivity of 24R,25(OH)<sub>2</sub>D<sub>3</sub>. The Venn diagram shows six genes commonly regulated by the three metabolites, 321 by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25OHD<sub>3</sub>, 9 by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, and 4 by 25OHD<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub>, respectively (Figure 1D).

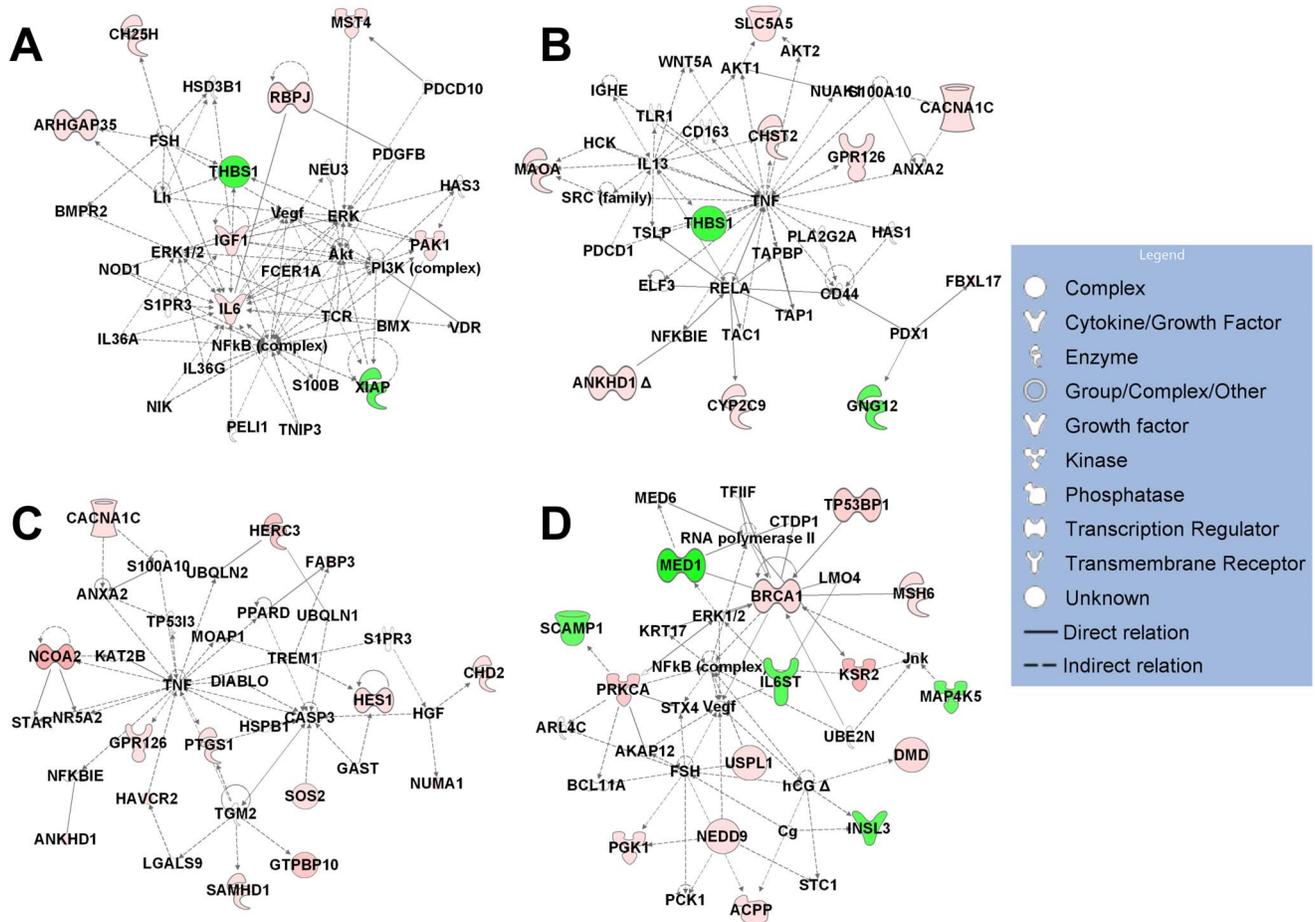
### Functional Annotation of Genes Regulated by Vitamin D<sub>3</sub> Metabolites

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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-treatment include those involved in nucleotide biosynthesis, ubiquitin conjugation, ion binding, and macromolecule biosynthesis (Table S2). 25(OH)D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> appeared to be involved in the regulation of zinc-finger motifs, methylation, transcription, epigenetics, protein kinases, and membrane targeting (Table S2).

In m*Cyp27b1*<sup>-/-</sup> fibroblasts, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is specifically involved in cell differentiation, extracellular matrix, cell adhesion, prostate gland development, cytokine and growth factor activity,



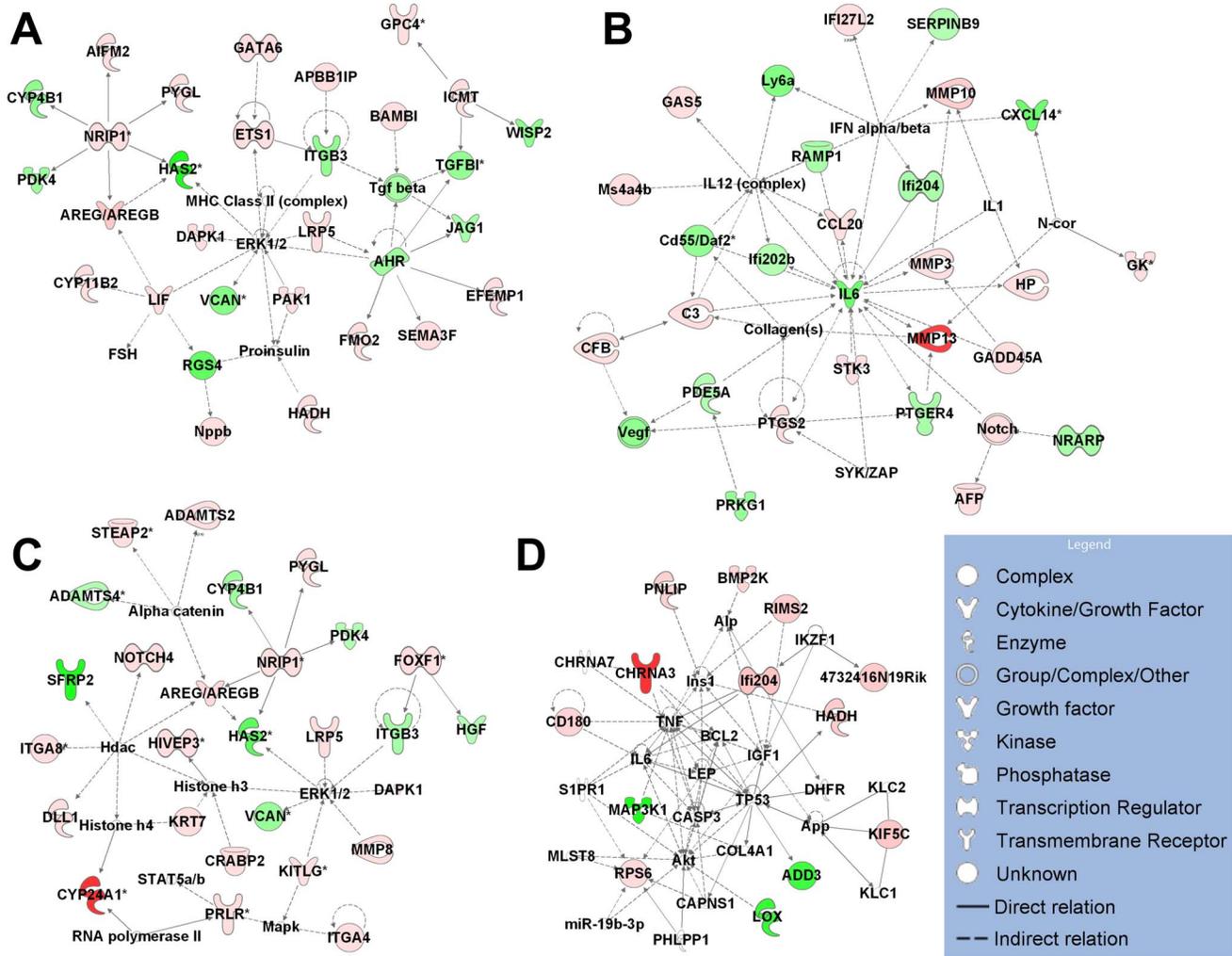
**Figure 4. IPA diagrams of the top associated network generated for all the regulated genes in hP29SN stromal cells.** (A) Genes regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$  in Cancer, Cardiovascular System Development and Function, Cellular Movement. (B) Genes regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$  in Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking. (C) Genes regulated by  $25(\text{OH})\text{D}_3$  in Cell Death and Survival, Metabolic Disease, Endocrine System Disorders. (D) Genes regulated by  $24\text{R},25(\text{OH})_2\text{D}_3$  in Cellular Development, Reproductive System Development and Function, Cell Morphology. Green indicates gene down-regulation and pink to red indicate gene up-regulation (the more intensive the color, the higher the expression level). doi:10.1371/journal.pone.0075338.g004

cell motion, mesenchymal and epithelial cell proliferation, neuron development, EGF, and gland development (Table S3). On the other hand,  $25(\text{OH})\text{D}_3$  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,  $24\text{R},25(\text{OH})_2\text{D}_3$  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  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$ , 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 D<sub>3</sub> Metabolites

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.  $1\alpha,25(\text{OH})_2\text{D}_3$ -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*<sup>-/-</sup> fibroblasts. The most populated biological networks for  $25(\text{OH})\text{D}_3$ -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, Hair and Skin Development and Function” (score 19, Figure 3C) in *mCyp27b1*<sup>-/-</sup> fibroblasts.  $24\text{R},25(\text{OH})_2\text{D}_3$  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,



**Figure 5. IPA diagrams of the top associated network generated for all the regulated genes in *mCyp27b1*<sup>-/-</sup> fibroblasts.** (A) Genes regulated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in Cardiovascular System Development and Function, Organismal Development, Cancer. (B) Genes regulated by 25(OH)D<sub>3</sub> in Humoral Immune Response, Inflammatory Response, Cellular Development. (C) Genes regulated by 25(OH)D<sub>3</sub> in Cancer, Cellular Growth and Proliferation, Connective Tissue Disorders. (D) Genes regulated by 24R,25(OH)<sub>2</sub>D<sub>3</sub> in Hematological System Development and Function, Hematopoiesis, Tissue Morphology. 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. doi:10.1371/journal.pone.0075338.g005

Endocrine System Development and Function, Lipid Metabolism” (score 18, Figure 3D) in *mCyp27b1*<sup>-/-</sup> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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*<sup>-/-</sup> fibroblasts by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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*<sup>-/-</sup> 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 ( $R^2 = 0.96$ ) was detected between the two methods (Figure 6D). Similarly, the expression of two genes in *mCyp27b1*<sup>-/-</sup> fibroblasts was measured by qRT-PCR (Figure 6E–F).

### Discussion

In order to analyze putative differential gene regulatory effects of vitamin D<sub>3</sub> metabolites, we performed cDNA microarray analysis in hP29SN stromal cells. To rule out the effect of intracellularly 25(OH)D<sub>3</sub>-derived 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, we also performed cDNA microarray analysis in *mCyp27b1*<sup>-/-</sup> fibroblasts that lack 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase enzyme activity.

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

Human P295N stromal cells		
Metabolite	Top associated network functions	Score
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Cancer, Cardiovascular System Development and Function, Cellular Movement	15
	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	15
	Cell Death, Cell Morphology, DNA Replication, Recombination, and Repair	13
	Hereditary Disorder, Neurological Disease, Ophthalmic Disease	2
	Cardiac Proliferation, Cardiovascular System Development and Function, Cell Cycle	2
25(OH)D <sub>3</sub>	Cell Death and Survival, Metabolic Disease, Endocrine System Disorders	22
	Cancer, Hematological Disease, Cellular Development	20
	Cell Cycle, Cell Death and Survival, Cellular Compromise	18
	Gene Expression, Cell Death and Survival, Amino Acid Metabolism	11
	Gene Expression, RNA Post-Transcriptional Modification, Cell-To-Cell Signaling and Interaction	2
24R,25(OH) <sub>2</sub> D <sub>3</sub>	Cellular Development, Reproductive System Development and Function, Cell Morphology	25
	Cell Cycle, Cell Death, Cellular Movement	19
	Gene Expression, DNA Replication, Recombination, and Repair, Cardiovascular System Development and Function	17
	Cell Cycle, Cellular Growth and Proliferation, DNA Replication, Recombination, and Repair	3
	Hereditary Disorder, Neurological Disease, Ophthalmic Disease	2
Mouse <i>Cyp27b1</i> <sup>-/-</sup> fibroblasts		
Metabolite	Top associated network functions	Score
1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	Cardiovascular System Development and Function, Organismal Development, Cancer	40
	Inflammatory Response, Hematological System Development and Function, Tissue Morphology	38
	Inflammatory Response, Cellular Movement, Immune Cell Trafficking	26
	Protein Synthesis, Cellular Development, Hematological System Development and Function	21
	Dermatological Diseases and Conditions, Hereditary Disorder, Organismal Injury and Abnormalities	19
25(OH)D <sub>3</sub>	Humoral Immune Response, Inflammatory Response, Cellular Development	37
	Cancer, Cellular Growth and Proliferation, Connective Tissue Disorders	37
	Hematological System Development and Function, Humoral Immune Response, Tissue Morphology	25
	Organismal Injury and Abnormalities, Inflammatory Response, Connective Tissue Disorders	18
	Cellular Development, Connective Tissue Development and Function, Tissue Morphology	15
24R,25(OH) <sub>2</sub> D <sub>3</sub>	Hematological System Development and Function, Hematopoiesis, Tissue Morphology	26
	Connective Tissue Development and Function, Connective Tissue Disorders, Developmental Disorder	2
	Developmental Disorder, Immunological Disease, Cell Cycle	2
	Cellular Development, Lipid Metabolism, Molecular Transport	2
	Cellular Development, Hematological System Development and Function, Hematopoiesis	2

doi:10.1371/journal.pone.0075338.t003

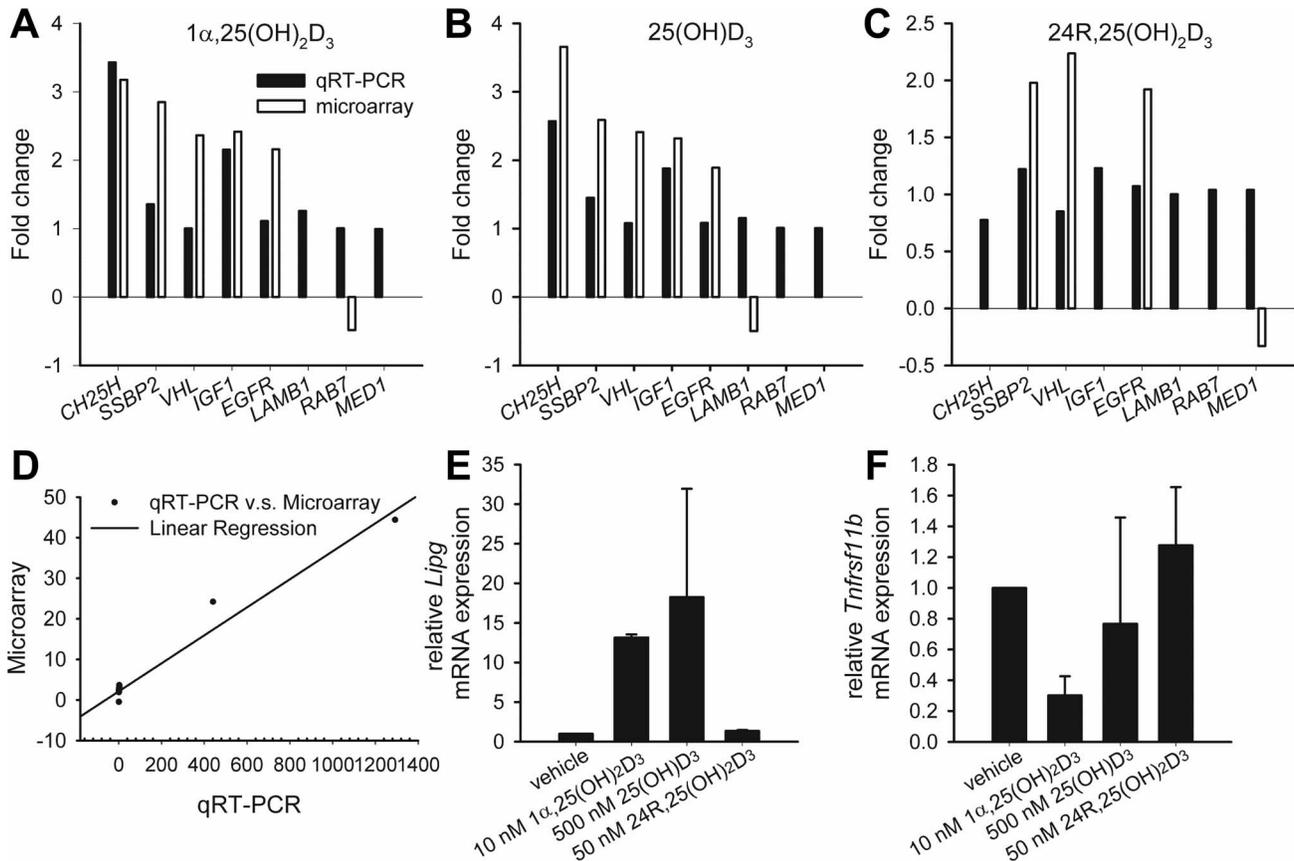
The optimal serum concentrations of 25(OH)D<sub>3</sub> for good health are still under discussion. The proposed normal range is broad and variable in different populations perhaps due to an adaptation. Several optimal concentrations have been proposed: 20–80 nM [31], 50–250 nM [32], and 40–70 nM [9] have been suggested. The international recommended optimal concentration of 25(OH)D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is clearly defined, being 0.03–0.14 nM [31]. The concentration of 24R,25(OH)<sub>2</sub>D<sub>3</sub> is approximately 1.44–8 nM (0.6–2.9 ng/ml) [33]. The perfect experimental setup would be using physiological concentrations of metabolites; however, it has been shown that the metabolites at physiological concentrations

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)D<sub>3</sub>, 24R,25(OH)<sub>2</sub>D<sub>3</sub>, and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, respectively.

Here we identified 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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].

### 25OHD<sub>3</sub> is an Active Hormone

Our study in *mCyp27b1*<sup>-/-</sup> fibroblasts suggests that gene expression fingerprints of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25OHD<sub>3</sub> are similar. Among the genes regulated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in *mCyp27b1*<sup>-/-</sup>



**Figure 6. Validation of gene expression by qRT-PCR.** (A–C) Gene expression in hP29SN stromal cells. *CH25H*: cholesterol 25-hydroxylase; *SSBP2*: single-stranded DNA binding protein 2; *VHL*: von Hippel-Lindau tumor suppressor; *IGF1*: insulin-like growth factor; *EGFR*: epidermal growth factor receptor; *LAMB1*: laminin subunit  $\beta$ 1; *RAB7*: RAB7A, member RAS oncogene family; *MED1*: mediator complex subunit 1. (D) Fold changes of gene expression in hP29SN detected by either qRT-PCR or microarray are plotted and linear regression is shown by a solid line. (E–F) The expression of endothelial lipase (*Lipg*) and tumor necrosis factor receptor superfamily, member 11b (*Tnfrsf11b*) in *mCyp27b1*<sup>-/-</sup> fibroblasts was measured by qRT-PCR. Randomly selected two sets of experiments were pooled to generate final two sets of RNA samples for both microarray and qRT-PCR. Results are expressed as means  $\pm$  SD. doi:10.1371/journal.pone.0075338.g006

fibroblasts, 52% were also regulated by 25(OH)D<sub>3</sub>. On the other hand, 68% of 25(OH)D<sub>3</sub>-regulated genes in *mCyp27b1*<sup>-/-</sup> fibroblasts were also regulated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. 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)D<sub>3</sub> is indeed an active hormone. Studies of 25(OH)D<sub>3</sub> analog that is hardly subjected to 1 $\alpha$ -hydroxylation provide additional evidence that 1 $\alpha$ -hydroxylation of 25(OH)D<sub>3</sub> is not required for its anti-proliferative activity [12].

Human P29SN stromal cells express 25(OH)D<sub>3</sub> 1 $\alpha$ -hydroxylase that converts 25(OH)D<sub>3</sub> into 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and vitamin D<sub>3</sub> 24-hydroxylase that converts 25(OH)D<sub>3</sub> into 24R,25(OH)<sub>2</sub>D<sub>3</sub> [3], thus we could not rule out the effects of intracellular 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 24R,25(OH)<sub>2</sub>D<sub>3</sub> products derived from 25(OH)D<sub>3</sub>. We observed that only a few genes commonly regulated by 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in both mouse and human fibroblasts. It is worth mentioning that 24R,25(OH)<sub>2</sub>D<sub>3</sub> did not regulate *CYP24A1* gene expression. An earlier microarray study has also found the induction ratio of *CYP24A1* gene expression by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> was the highest among 3800 human genes

examined and 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in hP29SN, consistent with our previous study [38]. Both 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression of insulin-like growth factor 1 more than twofold in hP29SN.

These data indeed demonstrate unique roles for 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Recent molecular dynamics simulations data [4] indicate both 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> 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)D<sub>3</sub>.

#### A Complementary Role of 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in Calcium and Skeletal Homeostasis

1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> stimulates a number of genes involved in bone formation [39,40] and osteoclast differentiation [41–43]. We discovered several new 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-target genes involved in bone physiology and pathology in hP29SN stromal cells and *mCyp27b1*<sup>-/-</sup> fibroblasts. Na<sup>+</sup>/H<sup>+</sup> exchanger domain containing 2 (*NHEDC2*, *NHA2*, *SLC9B2*), a member of Na<sup>+</sup>/H<sup>+</sup> exchanger

family, was potently up-regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$  (ca. tenfold) in hP29SN stromal cells. Thus, it is tempting to link  $1\alpha,25(\text{OH})_2\text{D}_3$  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 than twofold by  $1\alpha,25(\text{OH})_2\text{D}_3$  in hP29SN. *Sox6* 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  $1\alpha,25(\text{OH})_2\text{D}_3$  in mCyp27b1<sup>-/-</sup> fibroblasts. Secreted frizzled related protein 1 (*Sfrp1*) was down-regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$ . *Sfrp1* was previously shown to inhibit receptor activator of nuclear factor- $\kappa\text{B}$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  in mCyp27b1<sup>-/-</sup> fibroblasts. *BMP1* has been suggested to enhance bone repair [52].

$25(\text{OH})\text{D}_3$  shares some functions with  $1\alpha,25(\text{OH})_2\text{D}_3$  in regulating calcium and skeletal homeostasis. Three calcium homeostasis-related genes were regulated by  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  in both mouse and human fibroblasts. Voltage-dependent calcium channel  $\gamma$ -4 subunit (*CACNG4*) was up-regulated in hP29SN stromal cells (more than twofold by  $25(\text{OH})\text{D}_3$  and more than threefold by  $1\alpha,25(\text{OH})_2\text{D}_3$ ). S100 calcium binding protein G (*S100G*, *CABP9K*, encoding calbindin D9K) was dramatically up-regulated in mCyp27b1<sup>-/-</sup> fibroblasts (88-fold by  $25(\text{OH})\text{D}_3$  and 85-fold by  $1\alpha,25(\text{OH})_2\text{D}_3$ ). Prolactin receptor (*PRLR*) gene expression was up-regulated by more than threefold by  $25(\text{OH})\text{D}_3$  and more than 12-fold  $1\alpha,25(\text{OH})_2\text{D}_3$  in mCyp27b1<sup>-/-</sup> fibroblasts. *PRLP* 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  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  in mCyp27b1<sup>-/-</sup> fibroblasts. The expression of secreted frizzled related protein 2 (*Sfrp2*) was strongly down-regulated by both  $25(\text{OH})\text{D}_3$  (sixfold) and  $1\alpha,25(\text{OH})_2\text{D}_3$  (sevenfold) in mCyp27b1<sup>-/-</sup> fibroblasts. *Sfrp2* is an osteoporosis susceptibility gene associated with bone mineral density [50]. In addition, both  $25(\text{OH})\text{D}_3$  (more than fourfold) and  $1\alpha,25(\text{OH})_2\text{D}_3$  (more than fivefold) also inhibited the expression of tumor necrosis factor receptor superfamily, member 11b (*Tnfrsf11b*, *OPG*, osteoprotegerin) in mCyp27b1<sup>-/-</sup> 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  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  complement each other in regulating calcium homeostasis and bone remodeling. The role of  $1\alpha,25(\text{OH})_2\text{D}_3$  is broader than that of  $25(\text{OH})\text{D}_3$ . Our current findings support *in vivo* data that show partial rescue of the phenotype of Cyp27b1<sup>-/-</sup> mice by vitamin D<sub>3</sub> 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  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  in calcium homeostasis in stromal cells and fibroblasts may have two meanings. Firstly, the second messenger  $\text{Ca}^{2+}$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $24\text{R},25(\text{OH})_2\text{D}_3$ -target genes related to skeletal homeostasis in hP29SN stromal cells and mCyp27b1<sup>-/-</sup> fibroblasts. This should be verified by *in vitro* bone cell studies. It might also be possible that the previously discovered effect of  $24\text{R},25(\text{OH})_2\text{D}_3$  on the bone is non-genomic.

### Non-classical Actions of Vitamin D<sub>3</sub> 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  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated up-regulation of several genes that negatively regulate cell cycle and growth. Tyrosine kinase, non-receptor, 1 (*TNKI*), up-regulated by more than sixfold in hP29SN, was earlier shown to facilitate tumor necrosis factor  $\alpha$ -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 (*MCPHI*) was up-regulated by more than fourfold in hP29SN. *MCPHI* 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  $1\alpha,25(\text{OH})_2\text{D}_3$  in mCyp27b1<sup>-/-</sup> fibroblasts. Keratin 17, a key regulatory gene in hair development [62], was strongly induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  (more than 13-fold). Cadherin 11, which is expressed in mesenchymal aggregates during hair follicle development [63], was up-regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$  (threefold). Transforming growth factor  $\beta$ 2, shortening hair cycle by stimulating certain caspases [64], was down-regulated by  $1\alpha,25(\text{OH})_2\text{D}_3$  (twofold). These findings provide additional important mechanism by which  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $1\alpha,25(\text{OH})_2\text{D}_3$ -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  $25(\text{OH})\text{D}_3$  (Figures 2B and 2C). Nuclear receptor coactivator 2 (*NCOA2*, *SRC2*) was induced by 8-fold, hairy and enhancer of split 1 (*HES1*) by more than twofold, activating transcription factor 6 (*ATF6*) by more than twofold, and nuclear transcription factor Y,  $\gamma$  (*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  $25(\text{OH})\text{D}_3$  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  $25(\text{OH})\text{D}_3$ .  $25(\text{OH})\text{D}_3$  induced the expression of membrane-associated ring

finger (C3HC4) 1 (*MARCH1*) more than sixfold in hP29SN stromal cells. *MARCH1* 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 *MARCH1* [78]. Furthermore, 25(OH)D<sub>3</sub> 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  $\alpha$  proinflammatory responses [79]. Hepatitis A virus cellular receptor 2 (*HAVCR2*, *TIM3*, T cell immunoglobulin-3) gene expression was increased threefold by 25(OH)D<sub>3</sub> 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 monocytes, dendritic cells, and T cells for *in vitro* studies, together with functional analyses, will bolster the importance of our conclusions.

24R,25(OH)<sub>2</sub>D<sub>3</sub> is produced by *CYP24A1* when there is enough active vitamin D<sub>3</sub> available. We identified two 24R,25(OH)<sub>2</sub>D<sub>3</sub>-target genes involved in methylation and epigenetics in hP29SN stromal cells. *MLL5* (a trithorax homolog, myeloid/lymphoid or mixed-lineage leukaemia 5) was up-regulated more than threefold by 24R,25(OH)<sub>2</sub>D<sub>3</sub>. *MLL5* is a known chromatin regulator in H3K4 methylation [83]. Methyltransferase like 15 (*METTL15*, *METT5DI*) was increased more than 15-fold by 24R,25(OH)<sub>2</sub>D<sub>3</sub>.

Human microarray data show that 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub>. Induction of *BRCA1* was previously found to be partially responsible for the anti-proliferative effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> [84]. Interestingly, cofactor of *BRCA1* (*COBRA1*) was reduced threefold by 24R,25(OH)<sub>2</sub>D<sub>3</sub>. *BRCA1* and *COBRA1* seem to function in coordinate gene regulatory pathways [85]. Tumor protein p53 binding protein 1 (*TP53BPI*) was up-regulated by more than threefold by 24R,25(OH)<sub>2</sub>D<sub>3</sub>. *TP53BPI* up-regulates the promoter of *BRCA1* [86]. Kinase suppressor of ras 2 (*KSR2*), a target of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> [87], was increased by more than fivefold by 24R,25(OH)<sub>2</sub>D<sub>3</sub> in hP29SN. 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> in hP29SN stromal cells. Synaptotagmin XIV (*SYT14*) was up-regulated more than sixfold in hP29SN. Regulating synaptic membrane exocytosis 2 (*Rims2*) was up-regulated more than twofold in mCyp27b1<sup>-/-</sup> 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<sup>-/-</sup>

fibroblasts after treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub>. It seems that there are three partially different vitamin D<sub>3</sub> (cholecalciferol) hormonal systems. 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> complement each other in regulating calcium homeostasis and bone remodeling. Each vitamin D<sub>3</sub> metabolite has unique non-classical actions in various physiological and pathological processes. Our study presents a novel perspective for the function of vitamin D<sub>3</sub> within the endocrine system, and furthers our understanding of the roles and relationships between vitamin D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 500 nM 25(OH)D<sub>3</sub>, or 25 nM 24R,25(OH)<sub>2</sub>D<sub>3</sub> 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  $\pm$  SD (n = 4). The same samples were then used in microarray assays.

(TIF)

**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*<sup>-/-</sup> fibroblasts generated by DAVID.**

(PDF)

**Table S4 Enriched gene ontology (GO) categories for commonly regulated genes by both 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> in mouse *Cyp27b1*<sup>-/-</sup> fibroblasts generated by DAVID.**

(PDF)

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## Author Contributions

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

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