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Detection of 1α,25-Dihydroxyvitamin D-Regulated miRNAs in Zebrafish by Whole Transcriptome Sequencing

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Abstract

The sterol hormone, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), regulates gene expression and messenger RNA (mRNA) concentrations in zebrafish *in vivo*. Since mRNA concentrations and translation are influenced by micro-RNAs (miRNAs), we examined the influence of $1\alpha,25(OH)_2D_3$ on miRNA expression in zebrafish *in vivo* with whole transcriptome RNA sequencing, searched for miRNA binding sites in $1\alpha,25(OH)_2D_3$-sensitive genes, and performed correlation analyses between $1\alpha,25(OH)_2D_3$-sensitive miRNAs and mRNAs. In vehicle- and $1\alpha,25(OH)_2D_3$-treated, 7-day postfertilization larvae, between 282 and 295 known precursor miRNAs were expressed, and in vehicle- and $1\alpha,25(OH)_2D_3$-treated fish, between 83 and 122 novel miRNAs were detected. Following $1\alpha,25(OH)_2D_3$ treatment, 31 precursor miRNAs were differentially expressed ($p < 0.05$). The differentially expressed miRNAs are predicted to potentially alter mRNAs for metabolic enzymes, transcription factors, growth factors, and Jak-STAT signaling. We verified the role of a $1\alpha,25(OH)_2D_3$-sensitive miRNA, miR125b, by demonstrating alterations in the concentrations of the mRNA of a $1\alpha,25(OH)_2D_3$-regulated gene, *Cyp24a1*, following transfection of renal cells with a miR125b miRNA mimic. Changes in the *Cyp24a1* mRNA concentration by the miR125b miRNA mimic were associated with changes in the protein for Cyp24a1. Our data show that $1\alpha,25(OH)_2D_3$ regulates miRNA in zebrafish larvae *in vivo* and could thereby influence vitamin D-sensitive mRNA concentrations.

Introduction

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), alters gene expression in the developing zebrafish *in vivo*.[1] Initially restricted to a modest number of responding loci, by day 7 of treatment, the expression of approximately 2500 protein-coding genes is either increased or decreased in response to $1\alpha,25(OH)_2D_3$ when assessed by whole transcriptome RNA shotgun sequencing.[1] To regulate gene expression in target tissues, $1\alpha,25(OH)_2D_3$ binds to a widely expressed receptor, the vitamin D receptor (VDR), and the ligand-bound receptor, either as a heterodimer with the retinoic acid X-receptor or as a homodimer binds vitamin D response elements (VDREs) in diverse genes,[2–10] many of which encode proteins that alter calcium or phosphorus transport and bone mineralization or bone resorption.[11–16] In *Danio rerio*, there are two VDR genes (*VDRa* and *VDRb*) that encode receptors with 453 (VDRA) and 422 (VDRB) amino acids with highly conserved DNA and ligand binding domains.[17] Only the *Danio* VDRA plays a role in intestinal calcium transport.[18] As described earlier, a large number of messenger RNAs (mRNAs) encoding proteins important in lipid, xenobiotic, and cell signaling pathways were shown to be regulated by $1\alpha,25(OH)_2D_3$ in developing zebrafish.[1]

The role of post-transcriptional regulation by micro-RNAs (miRNAs) has been established as a key contributory mechanism for gene expression in several model organisms for a variety of cellular processes.[19–21] miRNAs, which represent 1%–2% of genes in worms, flies, and vertebrates,[22] post-transcriptionally regulate gene expression in plants and vertebrates by binding to the 3′ untranslated regions of mRNAs[19–21]

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and altering mRNA decay or translation.[23] miRNAs are transcribed by RNA polymerase II[24] (or sometimes RNA polymerase III)[25] to give rise to long primary RNAs that are processed by the microprocessor complex (containing Drosha and DGCR8) to generate pre-miRNAs.[26,27] Pre-miRNAs are cleaved by the cytoplasmic ribonuclease, Dicer, to generate mature miRNAs.[28,29] One of the two strands of a miRNA is loaded into the RNA-silencing (RISC) complex before interaction with a target mRNA.[30]

Steroid hormones such as estrogens, progesterones, and androgens regulate miRNAs in cell culture and *in vivo*,[31–39] and miRNAs regulate steroid hormone receptor concentrations within cells.[31] There is a paucity of information regarding the regulation of miRNAs by the calcium regulating sterol, $1\alpha,25(OH)_2D_3$. The effects of $1\alpha,25(OH)_2D_3$ on miRNA expression have been examined in cells maintained in culture.[40–51] Using microarrays, Alvarez-Diaz *et al.* recently demonstrated that $1\alpha,25(OH)_2D_3$ induces miRNA-22 expression in cultured colon cancer cells in a time-, dose- and VDR-dependent manner.[40] Gocek *et al.* studied the effects of the exogenously added $1\alpha,25(OH)_2D_3$ on miRNA expression in human myeloid leukemia cells and demonstrated the induction of miR-32 in such cells.[44] That study showed that miR-32 targets the 3′-untranslated region of the mRNA encoding the proapoptotic factor Bim, reducing its expression. Wang *et al.* examined the effects of $1\alpha,25(OH)_2D_3$ on miRNA expression in LNCap prostate cancer cells and demonstrated the regulation of a number of different miRNAs.[49] Essa *et al.* showed recently that $1\alpha,25(OH)_2D_3$ and/or epigenetic drugs modulated VDR mRNA expression in $1\alpha,25(OH)_2D_3$-responsive and -resistant melanoma cell lines.[43] Two very recent reports show that $1\alpha,25(OH)_2D_3$ regulates miRNAs in prostate tissue and in tumor xenografts *in vivo*,[52,53] but no reports have demonstrated changes in miRNA expression in normal physiological states.

The effects of $1\alpha,25(OH)_2D_3$, steroids, and peptide hormones can be assessed by adding hormones to the zebrafish incubation medium[54] and correlating changes in gene expression with organ, cartilage, and bone development. We previously examined $1\alpha,25(OH)_2D_3$-mediated gene expression in zebrafish embryos and larvae *in vivo* and showed that the sterol alters cartilage and bone development.[1] To determine the influence of $1\alpha,25(OH)_2D_3$ on miRNA expression *in vivo*, we treated zebrafish embryos/larvae with $1\alpha,25(OH)_2D_3$ or vehicle and assessed miRNA expression at 7 days postfertilization (dpf), following addition of $1\alpha,25(OH)_2D_3$ or vehicle with whole transcriptome shotgun sequencing (RNA-seq). We chose 7 dpf larvae in which to examine changes in the miRNA expression, to establish a model system in which changes in miRNA expression could be correlated with ossification and cartilage development, both of which are established by this time.[55,56] We now demonstrate that multiple miRNAs are altered by $1\alpha,25(OH)_2D_3$ in the developing zebrafish. These miRNAs potentially regulate multiple transcription factors, growth factors and growth factor binding proteins, peptide hormones, and various amino acid metabolic pathways (e.g., metabolic pathways for glycine, serine, threonine, and tryptophan). In addition, we demonstrate that miR125b alters *Cyp24a1* mRNA expression in cultured cells.

Materials and Methods

Treatment of zebrafish with $1\alpha,25(OH)_2D_3$

Zebrafish embryos were obtained and cultured as described from natural mating of Segrest wild-type adult zebrafish in the Mayo Clinic Zebrafish Core Facility.[57] One hundred forty zebrafish embryos were placed in a 20 mL embryo medium (pH 7.2) containing 1-phenyl-2-thiourea (PTU) (0.003% (w/v) and were maintained at 28°C–30°C. At 24 hours post-fertilization (1 dpf), 10 μL $1\alpha,25(OH)_2D_3$ in ethanol (gift of Dr. Milan Uskokovic, Hoffmann LaRoche, Nutley, NJ) was added to embryos maintained in 20 mL of fresh embryo medium (final concentration $1\alpha,25(OH)_2D_3$ 300 pM). Control zebrafish were treated with 10 μL ethanol alone (vehicle controls). The medium containing either 300 pM $1\alpha,25(OH)_2D_3$ or vehicle was changed every 24 h. At 7 dpf, larvae were removed and immediately frozen at −80°C for RNA preparation. Thirty larvae were used for preparation of RNA. Four individual cDNA libraries from $1\alpha,25(OH)_2D_3$-treated fish and four individual libraries form vehicle-treated fish were prepared at 7 days.

RNA preparation for RNA-seq and quantitative polymerase chain reaction

RNA was prepared as previously described, using RNA/protein spin columns (Clontech, Mountain View, CA).[1] A lysis solution was added to 25–30 larvae that were lysed by passage successively through 21- and 27-gauge needles. Individual lysates were applied to RNA spin columns, and RNAs were eluted into nuclease-free water. Before library construction, the RNA quality was assessed by capillary electrophoresis against a reference size standard.

miRNA library preparation

We synthesized miRNA libraries from $1\alpha,25(OH)_2D_3$- or vehicle-treated zebrafish larvae using total RNA samples and a NEBNext® Multiplex Small RNA Kit (New England BioLabs, Ipswich, MA). Adaptors were ligated to the 3′ ends of the small noncoding RNAs present in 500 ng of total RNA. A complementary primer was annealed to the 3′ adaptor sequences followed by ligation of a 5′ RNA adaptor. A cDNA library was created by reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA) of the adaptor ligated and annealed small RNA population. The library was enriched by 15 cycles of polymerase chain reaction (PCR) employing a common 5′ primer and a 3′ primer containing one of the eight index primers (3′ adaptor complement; index sequences equivalent to Illumina TruSeq Small RNA sequences). The PCR products were subsequently purified. The libraries were assessed for miRNA products by Agilent Bioanalyzer DNA 1000 (Santa Clara, CA) analysis. The 130–160 bp region was quantitated to determine equimolar amounts of vehicle- and $1\alpha,25(OH)_2D_3$-treated sample libraries to pool. The pooled small RNA libraries were fractionated to extract a miRNA-enriched sample using 3% Pippin Prep (Sage Science, Beverly, MA) gel cassettes. It is unlikely that ribosomal, transfer, and sn/sno RNAs are present in the purified fraction used for analysis. The recovered fraction was purified and reconstituted in 10 μL of nuclease-free dH_2O. Pooled miRNA fractions were assessed by a second Agilent DNA 1000 assay. A predominant peak at 140–150 bp indicated that miRNA modification and size selection steps were performed as

expected. The final concentration of each library pool was determined. Libraries were loaded onto single end flow cells at concentrations of 8–10 pM to generate cluster densities of 700,000/mm^2 following Illumina's standard protocol using the Illumina cBot cluster kit version 3. The flow cells were sequenced as two reads: read 1–51 cycles using the small RNA sequencing primer and an Index read to demultiplex the samples. Libraries were sequenced on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and SCS version 1.4.8 data collection software. Base calling was performed using Illumina's RTA version 1.12.4.2.

mRNA library preparation

mRNA-seq libraries were prepared from identical RNA samples as previously described using the mRNA v1 sample prep kit protocol (Illumina, San Diego, CA).[1] Poly-A containing mRNA was purified using poly-T oligo magnetic beads. The purified mRNA was fragmented, RNA fragments were copied into first-strand cDNA, and second strand cDNA synthesis was carried out. The cDNA was purified, ends were repaired and phosphorylated after which, an A base was added to the 3′ ends of double-stranded DNA. Paired-end DNA adaptors (Illumina) with a single T-base overhang at the 3′ end were ligated to the double-stranded DNA and separated on a 2% agarose gel. DNA fragments of approximately 250–300 bp were excised, purified, and enriched by PCR. Sequencing was carried out as previously described.[1]

Quantitative PCR

Quantitative PCR (QPCR) was carried out using a Roche LightCycler 480 QPCR apparatus in 96-well QPCR plates (Roche Diagnostics Corp., Indianapolis, IN) using SYBR® Green master mix, Universal RT (Exiqon, Woburn, MA). RNA was isolated as described earlier.[1] A Universal cDNA Synthesis Kit (Exiqon) was used to generate double-stranded DNA from 70 ng total RNA for each sample. We used predesigned locked nucleic acid LNA™-enhanced microRNA-specific QPCR primer sets from Exiqon for dre-let-7i (hsa-let-7i), dre-miR-100 (hsa-miR-100), dre-miR-96 (hsa-miR-96) (unchanging reference control), dre-miR-125b (hsa-miR-125b), U6 snRNA (additional reference control), or the custom LNA™ PCR primer set unique for zebrafish dre-miR-21. A reverse transcribed product was used to generate PCR products with each LNA primer pair. QPCR data were subjected to relative quantitation against dre-miR-96 using software supplied with the instrument.

Analysis of miRNAs

The generated single-end sequence reads were analyzed by the miRDeep2 tool.[58] Known and novel miRNAs were interrogated for each sample, and their expression profiles were derived by counting the number of reads aligning to the genomic coordinates of all known and potential novel miRNAs predicted by the miRDeep tool.[59] We consider a candidate to be a novel real miRNA if its miRDeep score is larger than 0. Specifically, for genomic regions aligned against sequencing reads, the miRDeep tool (1) investigates genomic DNA bracketing the alignments and computes their secondary RNA structure; (2) identifies plausible miRNA precursor sequences and scores their likelihood to be real miRNA precursors.[60] We used a cutoff score value 0 in this study, since four samples were analyzed, and the estimated true positive rate on average is larger than 60%. In our standard bioinformatics workflow, snoRNA and other small RNAs are not assessed. For differential gene expression analysis between two conditions, we first eliminated miRNAs without any reads across all samples. Because scaling by total lane counts (e.g., reads per kilobase per million [gene counts/kilobase of exon for that gene/total counts of each biological replicate] $\times$ 1,000,000) can bias estimates of differential expression, we used quantile-based normalization on read counts to determine if genes are differentially expressed[61] using the negative binomial method[62] requiring an adjusted p-value < 0.01 controlled for multiple testing using the Benjamini and Hochberg correction.[63] miRNAs with significantly differential expression (adjusted p-value < 0.01) between two conditions were selected for downstream target prediction. Due to the lack of bioinformatics tools with statistically significant accuracy in predicting miRNA binding sites, we proposed a two-step computational approach to improve the prediction accuracy and to create an optimal framework for deciphering biological functions of miRNAs. First, we used the TargetScan algorithm[64] to predict potential mRNAs for each differentially expressed miRNA. This algorithm predicts targets of vertebrate miRNAs by identifying mRNAs with conserved complementarity to the seed sequence of the miRNA. Second, we applied Pearson correlation analysis between differentially expressed mRNAs and differentially expressed miRNAs. We assessed statistical significance by using the q value of the false discovery rate (qFDR) as described by Storey and Tibshirani[65] We defined the miRNA-mRNA correlation coefficient qFDR < 0.01 to be statistically significant. The common mRNA genes identified by both steps are treated as high-confidence targets for each miRNA. The biological effects of each miRNA, which occur through a variety of mechanisms,[21] can subsequently be experimentally validated. The hypergeometric test was performed for enrichment analysis between the mRNAs that are correlated with one differentially expressed miRNA and the predicted mRNA targets for this miRNA. The FDR value was then calculated as described by Storey.[66] Lists of predicted target genes for the differentially expressed miRNAs were used for pathway/network level analysis. We conducted pathway analysis of predicted target genes using DAVID (Database for Annotation, Visualization, and Integrated Discovery v6.7).[67] An adjusted p-value less than 0.05 was used to assign biological significance. The goal was to investigate whether the predicted target genes were enriched with genes in canonical pathways in public databases. DAVID is a web resource consisting of an integrated biological knowledgebase and analytical tools that aim to extract and understand biological themes in large gene lists. It provides the gene functional classification as well as the identification (ID) conversion of the list of gene ID accessions to any other accession of choice. It also provides pathways from other databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG),[68] Panther,[69] BioCarta (http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways), and Reactome,[70] and provides tissue expression and disease-specific information based on represented genes.

Table 1. Summary Statistics of miRNA in Each Sample

	7 dpf ET1	*7 dpf ET2*	*7 dpf ET3*	*7 dpf ET4*	*7 dpf VD1*	*7 dpf VD2*	*7 dpf VD3*	*7 dpf VD4*
Detected known miRNA	291	293	292	287	282	283	287	295
Novel miRNA	122	105	110	102	87	83	101	117
Expressed miRNA	348	349	349	346	343	346	346	346

Detected known precursor miRNA: the number of known precursor miRNAs detected by miRDeep tool.
Novel miRNA: the number of novel miRNAs detected by miRDeep tool.
Expressed miRNA: the number of known miRNAs with no less than five mapped sequencing reads.
dpf, day postfertilization; ET, ethanol; VD, $1\alpha,25(OH)_2D_3$; miRNA, micro-RNA.

VDR element analysis of promoter regions of differentially expressed miRNAs

Promoter sequences of all differentially expressed miRNAs from -5000 to $+5000$ relative to the transcription start site were retrieved from the latest available zebrafish reference genome in the ENSEMBL database (Zv9 version) using customized Perl scripts. The match program[71] provided by the TRANSFAC database in BIOBASE (www.biobase-international.com) was used to detect potential VDR binding sites in these sequences, using customized VDR profiles, in which four candidate VDR-related matrices from the MATRIX table were selected to minimize the rate of false positives. Matches exhibiting a matrix similarity scores above 0.9 were used.

VDR element analysis of promoter regions of differentially expressed mRNAs

Promoter sequences of all differentially expressed mRNAs from -5000 to $+5000$ relative to the transcription start site were retrieved from the latest available zebrafish reference genome in the ENSEMBL database (Zv9 version) using customized Perl scripts. Analysis was performed as described above to identify potential VDREs.

Regulation of Cyp24A1 *mRNA and protein by miR125b and miR21*

The mouse *Cyp24a1* gene was examined for the presence of mir125b and miR21 binding sites using the TRANFAC program. These miRNAs were shown to be increased 1.8- and 5.1-fold by $1\alpha,25(OH)_2D_3$. A binding site for mir125b was detected in the 3′ untranslated region (3′UTR) of the *Cyp24a1* gene, but was absent in the case of miR21. Immortalized renal proximal tubular cells grown as previously described,[72] were transfected with miRNA mimics for miR125b (sequence *mmu*-miR-125b-5p: 5′ UCCCUGAGACCCUAACUUGUGA 3′) and miR21a (sequence mmu-miR21a-5p: 5′ UAGCUUAUCAGACUGAUGUUGA 3′) or a control Dy574 miRNA mimic transfection control for 10 h using the Opti-MEM1 transfection reagent (DharmaFECT1) (Thermo Scientific, Pittsburg, PA). Following transfection of cells with the miRNA mimic for 10 h, the transfecting reagent was removed, a new medium was added, and the cells were grown for an additional 38 h. Cells were then harvested, and RNA and protein were isolated. *Cyp24a1* RNA was quantitated using reverse transcription (RT)-PCR with appropriate primers (forward 5′ CCTGGGACACCATTTTCAA 3′, reverse 5′ TGCTGATAAATATCACAAAGGAAATC 3′).[72] The Cyp24A1 protein was detected using western blotting methods with an antibody produced against recombinant Cyp24A1.[73]

Generation of mouse proximal tubule cells

Proximal tubule cells were isolated from the SV40 T-antigen mutant tsA58 ImmortoMouse (Charles River, Wilmington, MA) mouse kidney.[74] The cortex from isolated

Table 2. Change in Expression of miRNAs Following Treatment of 7 dpf Zebrafish Larvae with Vehicle or $1\alpha,25(OH)_2D_3$

Mature miRNA	*Precursor miRNA*	*Fold change*	p-*Value*
dre-miR-2190	dre-miR-2190-1	6.38	0.02
dre-miR-2190	dre-miR-2190-2	6.38	0.02
dre-miR-2190	dre-miR-2190-3	6.38	0.02
dre-miR-2190	dre-miR-2190-4	6.38	0.02
dre-miR-21	dre-miR-21-1	5.07	0.9×10^{-50}
dre-miR-21	dre-miR-21-2	5.06	0.9×10^{-50}
dre-miR-735	dre-miR-735	2.69	0.02
dre-miR-150	dre-miR-150	2.50	1.92×10^{-16}
dre-miR-100	dre-miR-100-2	2.13	0.002
dre-miR-100	dre-miR-100-1	2.13	0.002
dre-miR-125b	dre-miR-125b-1	1.76	1.0×10^{-6}
dre-miR-125b	dre-miR-125b-2	1.76	1.0×10^{-6}
dre-miR-125b	dre-miR-125b3	1.75	1.0×10^{-6}
dre-miR-146a	dre-miR-146a	1.74	0.02
dre-miR-125c	dre-miR-125c	1.6	0.00069
dre-miR-17a*	dre-miR-17a-1	1.54	0.39
dre-miR-let-7e	dre-miR-let- 7e	1.52	0.021
dre-miR-146b	dre-miR-146b	1.52	0.003
dre-miR-214	dre-miR-214	1.48	0.009
dre-miR-181c	dre-miR-181c	1.44	0.032
dre-miR-126a	dre-miR-126a	1.41	0.009
dre-miR-140*	dre-miR-140	1.37	0.02
dre-miR-22a	dre-miR-22a	1.35	0.02
dre-miR-184	dre-miR-184	0.70	0.013
dre-miR-184	dre-miR-184-2	0.70	0.013
dre-miR-192	dre-miR-192	0.70	0.04
dre-miR-let-7i	dre-miR-let-7i	0.67	0.003
dre-miR-729	dre-miR-729	0.64	0.0008
dre-miR-459*	dre-miR-459	0.62	0.0007
dre-miR-122	dre-miR-122	0.52	7.64×10^{-7}
dre-miR-29a	dre-miR-29a	0.44	0.04

miRNAs, which decreased following treatment with $1\alpha,25(OH)_2D_3$, are highlighted in *gray*.
$1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3.

TABLE 3. STATISTICS OF MIRNA TARGETS DERIVED FROM TARGET SCAN AND PEARSON CORRELATION APPROACHES

Name	*Chr*	*Start*	*End*	*Strand*	*# of potential VDR binding sites*
DRE-MIR-150	chr3	32708295	32708357	+	2
DRE-MIR-21	chr10	28880713	28880774	−	3
DRE-MIR-735	chr24	39680979	39681041	−	5
DRE-MIR-100	chr5	31628647	31628704	+	3
DRE-MIR-125B	chr15	20409343	20409405	+	4
DRE-MIR-146A	chr13	11537689	11537750	+	5
DRE-MIR-125C	chr15	29150075	29150136	+	1
DRE-MIR-17A	chr1	2806111	2806172	+	5
DRE-LET-7E	chr23	5478703	5478777	−	6
DRE-MIR-146B	chr21	40390767	40390827	−	3
DRE-MIR-214	chr20	14792434	14792496	+	5
DRE-MIR-181C	chr3	34283001	34283058	+	6
DRE-MIR-126A	chr8	12065966	12066026	−	3
DRE-MIR-140	chr25	35855094	35855156	+	3
DRE-MIR-29A	chr4	10671282	10671407	−	1
DRE-MIR-459	chr1	1275358	1275421	+	1
DRE-MIR-729	chr4	12649029	12649091	+	4
DRE-LET-7I	chr25	1688209	1688287	−	0
DRE-MIR-184	chr18	25989802	25989866	−	0
DRE-MIR-192	chr10	27698862	27698925	+	0
DRE-MIR-2190	chr5	1190769	1190872	−	9

VDR, vitamin D receptor.

kidneys of a 12-week-old female ImmortoMouse was harvested, minced, and digested with 1% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C. Cell suspensions were filtered through a nylon mesh, suspended in 45% Percoll, and centrifuged at 27,000 *g* for 15 min at 4°C. Isolated proximal tubule cells were grown on Matrigel-coated tissue culture plates, in DMEM/F12, 10% FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 1.2 mg/mL sodium bicarbonate. Either 6-well tissue culture plates (seeded at 1×10^5 cells/well) or 24-well tissue culture plates (seeded at 1×10^4 cells/well) were used for miRNA mimic experiments upon cell confluence.

Data sharing

All sequence data that were analyzed in this report have been deposited in the Gene Expression Omnibus.

Results

We show that known and novel miRNAs are expressed in 7 dpf zebrafish embryos in both vehicle- and $1\alpha,25(OH)_2D_3$-treated fish. Multiple miRNAs are repressed or induced by $1\alpha,25(OH)_2D_3$. Table 1 lists the detailed information of different categories of miRNAs for each sample treated with vehicle or $1\alpha,25(OH)_2D_3$. Between 287 and 293 known precursor miRNAs were expressed in 7 dpf zebrafish in the vehicle-treated group, and between 282 and 295 known precursor miRNAs were expressed in the $1\alpha,25(OH)_2D_3$-treated group. The detailed information of known precursor miRNAs in each sample is listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/zeb). Besides known miRNAs in the miRBase, novel miRNAs in each sample were detected using the miRDeep2 tool. In the vehicle-treated group, 266 unique novel miRNAs were detected in 7 dpf zebrafish larvae in at least one out of four replicates, while in the $1\alpha,25(OH)_2D_3$-treated group, 246 unique novel miRNAs were detected in at least one out of four replicates. Supplementary Tables S2a and b list the sequence of these novel miRNAs in each sample in either the vehicle- or the $1\alpha,25(OH)_2D_3$-treated group.

In Table 2 are listed miRNAs that are increased or decreased following 6 days of treatment with $1\alpha,25(OH)_2D_3$.

TABLE 4. ENRICHED PATHWAYS IN PREDICTED MESSENGER RNA TARGETS OF DIFFERENTIALLY EXPRESSED MICRO-RNAS

miRNA	*Number of potential targets by TargetScan*	*Number of mRNAs correlated with each miRNA*	*Number of targets detected by both approaches*
DRE-MIR-2190	468	895	10
DRE-MIR-21	3590	1235	113
DRE-MIR-735	2638	1331	77
DRE-MIR-150	2240	1474	70
DRE-MIR-100	163	45	0
DRE-MIR-125B	539	261	2
DRE-MIR-146A	2395	1200	88
DRE-MIR-125C	98	920	4
DRE-MIR-17A*	4345	1654	205
DRE-LET-7E	880	942	27
DRE-MIR-146B	469	154	2
DRE-MIR-214	4559	1653	195
DRE-MIR-181C	0	460	0
DRE-MIR-126A	384	1210	6
DRE-MIR-140*	3327	1255	108
DRE-MIR-29A	2208	1555	104
DRE-MIR-459*	4699	1645	225
DRE-MIR-729	2157	1485	82
DRE-LET-7I	123	1566	8
DRE-MIR-184	801	1637	31
DRE-MIR-192	2809	1655	123

Analysis was performed using the (DAVID) Database for Annotation, Visualization, and Integrated Discovery v6.7 program.
miRNA, micro-RNA; mRNAs, messenger RNAs.

We confirmed the changes in concentrations of these miRNAs by performing quantitative PCR specific for miRNAs. We observed changes in dre-*let-7i* (0.85-fold change by QPCR vs. 0.68 change by RNA-seq), dre-miR-21 (6.5-fold change by QPCR vs. 5.1-fold change by RNA-seq), dre-miR-100 (2.4-fold change by QPCR vs. 2.1-fold change by RNA-seq),), and dre-miR-125b (1.7-fold change by QPCR vs. 1.8 change by RNA-seq). Dre-miR-96 was used to normalize the data as this was found to be an invariant miRNA on initial sequencing. We further investigated whether these differentially expressed miRNAs are directly regulated by the $1\alpha,25(OH)_2D_3$ treatment by performing transcription factor binding site analysis for all differentially expressed miRNAs. We searched for four candidate VDR elements from the TRANSFAC database both upstream and downstream of the transcription start sites of these differentially expressed miRNAs. Eighteen out of 21 differentially expressed mature miRNAs have at least one potential VDR element site within -5000 to $+5000$ bps of their transcription start sites (Table 3).

We identified genes that might potentially be altered by changes in miRNA amounts by performing TargetScan analysis, which identifies sequences in genes, to which a miRNA might bind, based on sequence specificity. The results of such analyses are shown in Supplementary Table S3. Supplementary Table S4 annotates the genes identified in Supplementary Table S3. Next, we analyzed the correlation between changes in miRNAs ascertained in the current study, and changes in expressed mRNAs measured by whole transcriptome analysis published by us earlier and deposited in the Gene Expression Omnibus (Accession #GSE38575). The results of such an analysis are shown in Table 4. The number of miRNAs common to both lists is considerably smaller than the number present in each list individually. Figure 1 shows the distribution plots of Pearson correlation coefficient values in different miRNA-mRNA groups. The correlations between differentially expressed miRNAs and their predicted mRNA targets are more enriched in larger absolute correlation coefficient values (i.e., either positively correlated or negatively correlated). To evaluate whether the mRNA lists generated from both correlation analysis and TargetScan prediction have a significant overlap, a hypergeometric test was performed for the results for each miRNA. A FDR value less than 0.1 indicated that our two-step computational approach identified potential mRNA targets with statistical significance.

The expression of the *Cyp24a1* gene is dramatically upregulated by $1\alpha,25(OH)_2D_3$. We determined whether the *Cyp24a1* gene had binding sites for miRNAs that were upregulated by $1\alpha,25(OH)_2D_3$. An examination of the mouse *Cyp24a1* gene revealed the presence of a mir125b-binding site in the 3′UTR of the gene. miR125b is increased 1.8-fold by $1\alpha,25(OH)_2D_3$. Immortalized renal proximal tubular cells transfected with the miRNA mimic for miR125b showed a

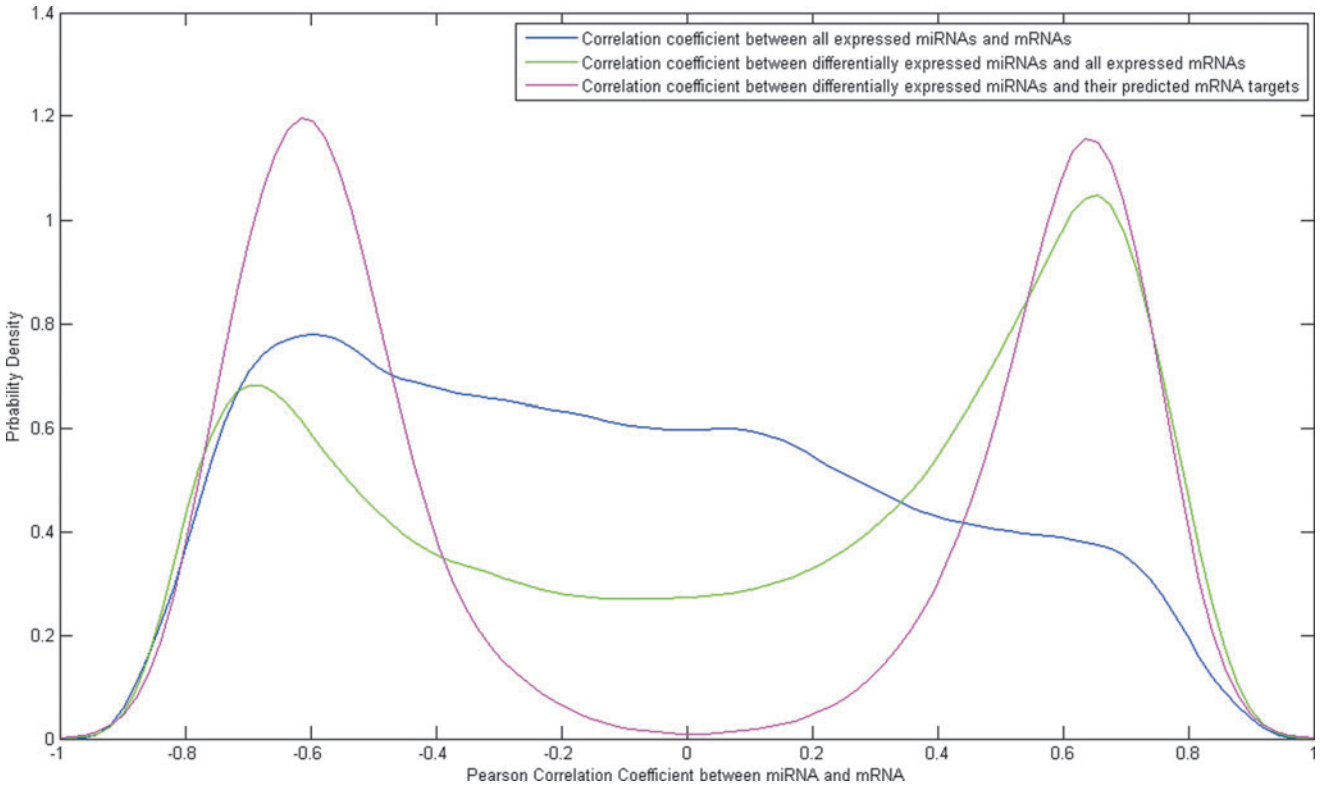

FIG. 1. The probability distribution of different zebrafish miRNA-mRNA groups whose expression is altered by exposure to $1\alpha,25(OH)_2D_3$ *in vivo*. The distribution of all miRNA-mRNA PCC shows a skewed normal distribution. The distribution of differentially expressed miRNAs and all expressed mRNAs demonstrate a two-peak distribution, and the distribution of differentially expressed miRNAs and their predicted mRNA targets as determined by a two-step computational approach has even higher peaks around -0.85 and 0.85, respectively. Results were confirmed by the hypergeometric test. $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; miRNA, micro-RNA; mRNA, messenger RNA; PCC, Pearson correlation coefficient.

2.02-fold increase in *Cyp24a1* mRNA assessed by RT-PCR (control 0.74 ± 0.17 RU vs. 1.5 ± 0.38 RU, $p < 0.011$). Identical experiments performed with a miR21a mimic, which lacks a binding site in the *Cyp24a1* gene, showed no changes in *Cyp24a1* mRNA assessed by RT-PCR (control 0.88 ± 0.34 RU vs. 0.91 ± 0.41 RU, $p = 0.91$). Assessment of transfection efficiency with Dy574 miRNA showed greater than 85% transfection efficiency. The Cyp24A1 protein, detected using western blotting methods, was increased following transfection of cells with the miRNA mimic for miR125b (control 31466.73 ± 1139.52 [standard error of mean] vs. miR125b mimic treated 39943.47 ± 2532.04, $p = 0.038$), but not following transfection with the miRNA mimic for miR21a.

Analysis of the promoters of genes encoding mRNAs regulated by $1\alpha,25(OH)_2D_3$ demonstrated that 93% (3406 out of a total of 3665) of these genes had at least one VDRE in the region 5 kb before or after the transcription start site. Two hundred fifty-nine genes did not have a VDRE in this region. Of these genes, 50 had a miRNA binding site in the 3′ region of the cognate gene (Supplementary Table S5). The data suggest that these genes might be exclusively regulated through miRNA-dependent mechanisms.

We analyzed the role of the altered miRNAs in different metabolic pathways. Shown in Figure 2 is the regulatory network containing miRNAs and their predicted candidate mRNA targets. The edge color denotes their correlation relationship. We observed a comparable number of both positive and negative correlation relationships between miRNAs and their putative mRNA targets. Although the canonical miRNA regulation model suggests that miRNAs repress the expression of their downstream target genes,[22] it has been demonstrated in several studies that the miRNA regulatory network will generate complex expression patterns, causing either positive or negative correlations between miRNAs and

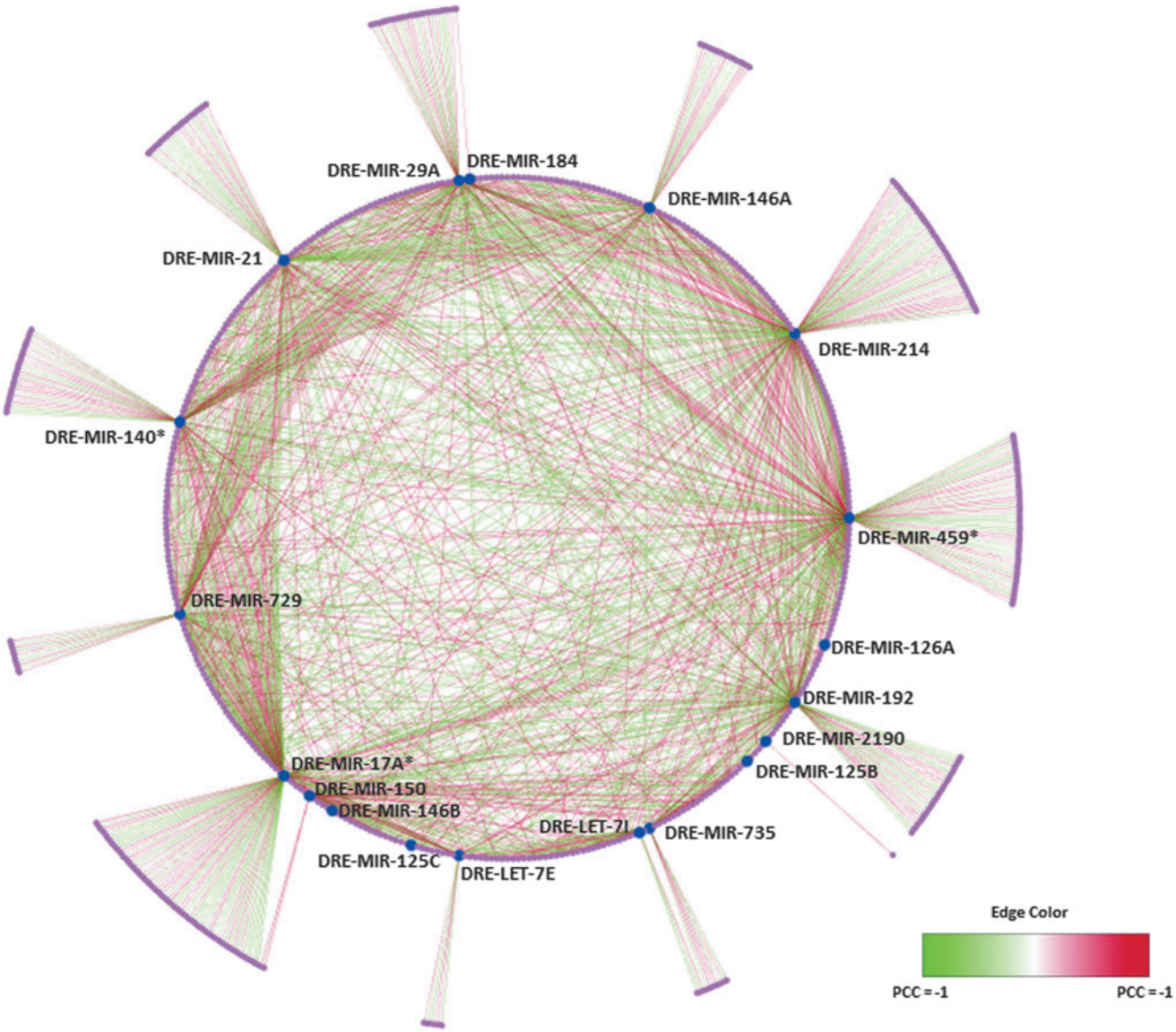

FIG. 2. Overview of the regulatory networks containing differentially expressed miRNAs and their candidate mRNA targets in response to $1\alpha,25(OH)_2D_3$ *in vivo*. *Blue nodes* denote differentially expressed miRNAs, and *red nodes* denote their predicted mRNA targets. Edges denote the regulatory relationships between miRNAs and their predicted mRNA targets.

Table 5. Enriched KEGG Pathways in the Putative mRNA Targets

KEGG pathway term	*Gene count*	p-*Value*
Alanine, aspartate, and glutamate metabolism	8	0.00075
Jak-STAT signaling pathway	14	0.00096
Aminoacyl-tRNA biosynthesis	8	0.0013
p53 signaling pathway	10	0.003
Tryptophan metabolism	7	0.0073
Porphyrin metabolism	5	0.043
Glycine, serine, and threonine metabolism	5	0.047

KEGG, Kyoto Encyclopedia of Genes and Genomes.

their target genes.[75–78] Competing endogenous RNAs, which sequester miRNAs, act as miRNA decoys and are likely to have additional effects on the miRNA function.[79–82] Our results demonstrate that miRNAs and their target genes have not only negative correlation expression patterns but much more complex regulation mechanisms. The pathway and gene ontology enrichment analyses for the putative mRNA target list were performed by the DAVID program. Table 5 presents the enriched KEGG pathways in the putative mRNA targets. The pathways altered by miRNAs include those for glycine, serine, threonine, and tryptophan metabolism, and the Jak-STAT signaling pathway. We further investigated which KEGG pathways are enriched in putative mRNA targets for each differentially expressed miRNA. Supplementary Table S6 shows the KEGG pathways enriched in the putative mRNA targets (adjusted *p*-value less than 0.01) for each differentially expressed miRNA.

Discussion

Steroid hormones, and sterols such as vitamin D and its metabolites, alter biochemical processes and development by regulating gene expression and by altering mRNAs for a number of protein coding genes.[1,5,7,11–16,83–97] In addition to regulating mRNA expression, steroids such as estrogen, progesterone, and androgen also regulate noncoding RNA, specifically, miRNA expression in cell culture and *in vivo*.[31–39] By altering mRNA stability, miRNAs change the expression of coding mRNAs.

There is only limited information about how the active metabolite of vitamin D_3, $1\alpha,25(OH)_2D_3$, regulates the expression of miRNAs *in vivo*. In previously published work, the effects of $1\alpha,25(OH)_2D_3$ on miRNA expression have been examined in cells maintained in culture.[40–51] Two very recent reports show that $1\alpha,25(OH)_2D_3$ regulates miRNAs in prostate tissue and in tumor xenografts *in vivo*,[52,53] but no reports have demonstrated changes in miRNA expression in normal physiological states. Furthermore, how the changes in miRNAs are associated with the changes in mRNAs *in vivo* is also unknown. We used a zebrafish model to assess the effects of $1\alpha,25(OH)_2D_3$ on miRNA expression for the following reasons: (1) miRNAs are expressed in developing zebrafish embryos and larvae and regulate several processes, including neural, cardiac, and fin development[98–107]; (2) zebrafish are readily treated with hormones and drugs[1,54]; (3) the effects of $1\alpha,25(OH)_2D_3$ on mRNA expression as a function of the developmental stage has been examined by our laboratory in the past and allow correlation between changes in miRNA expression and mRNA expression; and (4) the consequences of altered miRNA expression can be assessed morphologically because of the transparency of developing zebrafish.

We made several novel observations regarding miRNA expression in zebrafish larvae. First, in 7 dpf zebrafish larvae, many more miRNAs are expressed than in zebrafish at the earlier stages of development. Chen *et al.* showed that 154 distinct miRNAs were expressed in zebrafish embryos up to 2 dpf.[98] Kloosterman *et al.* identified 139 known and 66 new miRNAs in 5 dpf zebrafish larvae.[101] We show that in 7 dpf zebrafish, approximately 295 known miRNAs and 120 new, previously undescribed miRNAs, are expressed in vehicle-treated fish, with approximately an equal number being expressed in $1\alpha,25(OH)_2D_3$-treated zebrafish. We believe that the higher number of miRNAs expressed in 7 dpf zebrafish larvae compared with the number expressed at earlier stages is likely to be due to the increased complexity of organs and the need for fine tuning of mRNA expression rather than any methodological differences in library preparation or sequencing. The sequences and identities of these miRNAs are presented in the data and serve as a reference for expressed miRNAs at this stage of zebrafish larval development.

Second, we demonstrate that $1\alpha,25(OH)_2D_3$ regulates 31 separate precursor miRNAs following 6 days of treatment with hormone. Of these, eight are decreased, whereas 23 are increased. We noted that 18 of the 21 $1\alpha,25(OH)_2D_3$-regulated mature miRNAs had VDREs in regions of DNA 5000 bp upstream or downstream of the transcription start site. We recognize that additional VDREs may be present in regions of DNA adjacent to the miRNA gene beyond those analyzed in this report, as Pike and colleagues have shown that VDREs are often located >10–100 kb from the transcriptional start sites of vitamin D-sensitive genes.[85,108] Whether such VDREs are present at more distant locations is unknown. In addition, the impact of such VDREs on miRNA gene expression is unknown. However, our data do demonstrate the presence of authentic VDREs that are likely to be relevant to the regulation of miRNA gene expression in $\sim 85\%$ of the $1\alpha,25(OH)_2D_3$-sensitive miRNAs. Of note, two *vdr* genes, *vdra* and *vdrb*, are present in the zebrafish genome.[17] The VDRa is the longer of the two receptors with 37 additional NH_2-terminal amino acids. Both VDRa and VDRb have highly similar DNA-binding domains that are similar to the DNA-binding domains of VDRs of mammalian origin. It is therefore likely that the receptors bind to similar DNA elements. The analysis in our report was carried out assuming that VDRa and VDRb bound similar DNA nucleotide elements. Further analyses to detect long-range VDREs and an examination of VDREs for binding affinities to the two VDRs are required in the future.

Third, we show that many genes are potentially regulated by these miRNAs when assessed by the Target Scan program. However, because we previously described changes in mRNA concentrations in response to $1\alpha,25(OH)_2D_3$, we can correlate changes in miRNAs with the changes in mRNAs following treatment with hormones. The changes can be statistically associated and by using a combined approach, genes regulated by changes in miRNA concentrations can be identified. Of great interest, many of the miRNAs shown to

be altered, regulate $1\alpha,25(OH)_2D_3$-sensitive mRNAs. For example, miRNAs shown to be altered potentially regulate mRNAs for transcription factors (e.g., *vdrb*, *runx2b*, *klf11*, *mycch*, *myccl1b*), growth factors and growth factor binding proteins (*igf2a*, *igf2b*, *igfbp2a*, *igfbp2b*, *tgfβ1a*), leptin receptor (*lepr*), RNA encoding proteins involved in glycine, serine, threonine, alanine, aspartate, glutamate, and tryptophan metabolism, and JAK-STAT and p53 signaling. These results were further validated by analysis using DAVID.

We showed that the expression of the mRNA and protein of a highly $1\alpha,25(OH)_2D_3$-regulated gene, the *Cyp24A1*, is increased by the $1\alpha,25(OH)_2D_3$-regulated miRNA miR125b, in renal proximal tubular cells, thus demonstrating the importance of this miRNA in vitamin D-regulated gene expression. Whereas these results are not generalizable to the mRNAs of other genes that contain miRNA-binding sites, the data are consistent with the notion that $1\alpha,25(OH)_2D_3$-regulated miRNAs alter $1\alpha,25(OH)_2D_3$-sensitive mRNA expression. Although the canonical miRNA regulation model suggests that miRNAs repress the expression of their downstream target genes,[22] it has been demonstrated in several studies that the miRNA regulatory network will generate complex expression patterns, causing either positive or negative correlations between miRNAs and their target genes.[75–78] Several reports have suggested that miRNAs can positively regulate mRNA expression through binding to 3′ UTRs.[109–113]

In conclusion, we demonstrate that $1\alpha,25(OH)_2D_3$ alters miRNA expression in zebrafish *in vivo*. The changes in miRNAs correlate with the changes in mRNA expression. The data show complex regulation of gene expression by $1\alpha,25(OH)_2D_3$.

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Disclosure Statement

No competing financial interests exist.

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