Original Paper

MicroRNA-223 Suppresses Osteoblast Differentiation by Inhibiting DHRS3

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Key Words

miR-223 • DHRS3 • BMSC • Osteogenic differentiation

Abstract

Background/Aims: In this study, we aimed to use bioinformatics tools to identify the specific miRNAs and mRNAs involved in osteogenic differentiation and to further explore the way in which miRNA regulates osteogenic differentiation. **Methods:** The microarray GSE80614, which includes data from 3 human mesenchymal stromal cells (hMSCs) and 3 hMSCs after 72 hours (hr) of osteogenic differentiation, was used to screen for differentially expressed mRNAs. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of these mRNAs were conducted using Gene Set Enrichment Analysis (GSEA). Then, the miRanda website was employed to detect the binding sites of *DHRS3*. *In vitro* experiments, including RT-PCR and western blotting, were used to detect miR-223 and *DHRS3* expression levels 7 and 14 days (d) after the induction of osteogenic differentiation using human bone marrow-derived mesenchymal stem cells (hBMSCs). The target relationship between miR-223 and *DHRS3* was confirmed by a dual luciferase assay. ALP (alkaline phosphatase) staining, ARS (Alizarin Red S) staining and western blotting (*Runx2*, *OPN*, *OCN*) were used to detect the level of osteogenic differentiation after transfection with miR-223 mimics and *DHRS3* cDNA. **Results:** In this study, 127 mRNAs differentially expressed during osteogenic differentiation were identified in GSE80614. GO term and KEGG pathway enrichment analyses found that the retinol metabolism pathway was activated during osteogenic differentiation and that *DHRS3*, which is involved in the pathway, was upregulated. During osteogenic differentiation in hBMSCs, miR-223 was gradually downregulated, while *DHRS3* was upregulated. After 14 days of osteogenic differentiation, ALP and ARS staining assay results showed strong ALP activity and extracellular matrix calcification with the inhibition of miR-223 or the overexpression

of *DHRS3*. Furthermore, the expression levels of *Runx2*, *OPN*, and *OCN* were upregulated with the knockdown of miR-223 or the overexpression of *DHRS3*, while the simultaneous transfection of a miR-223 agomir and *DHRS3* cDNA resulted in no significant difference from the negative control (NC) group. **Conclusion:** The inhibition of miR-223 promotes the osteogenic differentiation of hBMSCs via the upregulation of *DHRS3*.

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Introduction

It has been widely reported that mesenchymal stem cells (MSCs) play important roles in tissue engineering and cell therapies [1, 2]. For example, exposure to a specific environmental stimulus, can induce MSCs to differentiate into varieties of adult tissues, such as bone, cartilage, fat and muscle [3, 4]. The human bone marrow-derived mesenchymal stem cells (hBMSCs), a type of pluripotent stem cells, were the first members of the mesenchymal stem cell (MSC) family to be identified [5]. As the progenitor cells of osteoblasts, hBMSCs play a critical role in bone formation and differentiation, becoming an excellent source of seed cells in bone tissue engineering [2, 6, 7].

Human quality of life may be seriously affected by bone defects caused by congenital malformations, traumas or diseases, imposing large economic and social burdens [8]. To treat or prevent these diseases, the key issue is promoting osteogenesis. A study by Wei *et al.* showed that in mouse models, the inhibition of osteoblast differentiation significantly reduced bone loss [9]. Thus, osteogenic differentiation is a key factor in bone regeneration, and clarifying the regulatory mechanism of osteoblast differentiation is very important for the improvement of the treatment of bone-related diseases.

MicroRNAs (miRNAs), a type of small noncoding RNAs, are able to bind to the 3'-untranslated regions (3'-UTRs) of specific target mRNAs, thereby regulating gene expression [10]. Studies have shown that miR-223 participates in the regulation of cardiomyocyte glucose metabolism, myoblast proliferation and differentiation, esophageal squamous cell carcinoma and human granulopoiesis [11-13]. In the osteogenic differentiation of hBMSCs, miR-223 was found to act as both a positive and a negative regulator, suggesting its potential in the treatment of bone-related diseases [14]. It was reported that the level of miR-223 is significantly lower in osteosarcoma tissues compared with non-cancerous bone tissues [15]. Moreover, the expression of miR-223 is significantly higher in the synovium of patients with osteoarthritis than in the synovium of healthy individuals [16]. In another study by Xie *et al.*, miR-223 was found to play a role during osteoblast differentiation [17]. However, the detailed mechanism of action of miR-223 during the osteogenic differentiation of hBMSCs has only been partially explored.

Retinol (vitamin A) is a micronutrient of vital importance in cell proliferation and differentiation. In adults, retinol and metabolites such as retinoic acid play key roles in vision, tissue remodeling and metabolism, as well as immune and brain functions. For instance, in the pituitary, retinoid might be helpful for the treatment of corticotropinomas by targeting the corticotroph; in the thyroid, vitamin A deficiency aggravates iodine-deficiency-related thyroid dysfunction [18]. In addition, retinoic acid, the metabolite of retinol, can cooperate with BMP-2 to enhance osteoblast differentiation through the induction of the expression of alkaline phosphatase (*ALP*), osteocalcin (*OCN*), and osteopontin (*OPN*) [19]. It was also reported that RA promotes osteogenic differentiation in osteosarcoma cells, which is mediated in part by activating the Smad signaling pathway [20]. In all relevant studies, to the best of our knowledge, the effect of retinol metabolism during osteogenic differentiation has not yet been comprehensively identified.

The retinoic acid-inducible dehydrogenase reductase 3 (*DHRS3*) is a highly conserved member of the short-chain alcohol dehydrogenase/reductase superfamily and has been implicated in retinol metabolism [21]. Acting as a retinaldehyde reductase, *DHRS3* is thought to control the levels of retinaldehyde, the immediate precursor of bioactive retinoic acid. Reversible oxidation-reduction reactions occur between retinol and retinaldehyde that are

regulated by *RDH10/DHRS3* complex. Retinaldehyde can be further oxidized to RA [22], which may be involved in the regulation of osteogenic differentiation, as mentioned above. *DHRS3* plays a critical role in regulating the metabolism of RA. The expression of *DHRS3* is upregulated when the amount of RA is excessive and downregulated when RA synthesis or signaling is blocked [23]. In mice, mutation of *DHRS3* leads to excess RA in embryos [24]. Although there are numerous studies of the effects of *DHRS3* on retinol metabolism, information about the effects of *DHRS3* on osteogenic differentiation is still very sparse, and the exact mechanism of action of *DHRS3* remains unclear. The present study aimed to resolve this issue.

In the present study, we screened mRNAs that were differentially expressed during osteogenic differentiation and analyzed the key pathways and involved genes by using gene set enrichment analysis. Activation of the retinol metabolism pathway and overexpression of *DHRS3* were found during the differentiation of osteoblasts. MiR-223 was found to target *DHRS3* and was downregulated during osteoblast differentiation. This study was undertaken to clarify how miR-223 regulates osteogenic differentiation by affecting the retinol metabolism pathway and *DHRS3*.

Materials and Methods

Cell culture and transfection

The hBMSC cell lines and the human embryonic kidney cell line 293T were all purchased from the BeNa Culture Collection (BNCC339828, Beijing, China). The cells were all maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 4 mM L-glutamine (Gln) and sodium pyruvate and incubated in a 5% CO₂ atmosphere at 37°C. The miR-223 agomir, the miR-223 antagomir and their negative controls, generated by GenePharma (Shanghai, China), were transfected into hBMSCs according to the manufacturer's instruction. The transfection was performed with Lipofectamine 2000 reagent according to the manufacturer's instructions. Transfection efficiency was detected, and the cells were used for further experiments.

Gene expression profiles

The gene expression data matrix was obtained from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>), which is accessible through the GEO platform GPL6947 and Series Accession Number GSE80614. The matrix contained a total of 66 samples, including osteogenic and adipogenic differentiation groups at different points in time (0 hour (hr), 0.5 hr, 1 hr, 2 hr, 3 hr, 6 hr, 12 hr, 1 day (d), 2 d, 3 d and 4 d), with each time point consisting of 3 cases. In the present study, we selected 3 samples after 0 hr of osteogenic differentiation as the normal group and 3 samples after 4 days of osteogenic differentiation as the osteogenic group.

Screening differentially expressed mRNAs

R software (Version 3.3.3) and Bioconductor packages (impute and limma) were applied to analyze the differentially expressed genes (DEGs) between the osteogenic differentiation samples and the normal samples. First, quality detection on microarray data was conducted by box plot, and quantile normalization was used to eliminate parallel experimental error. A clustering analysis diagram was constructed based on the "impute" and "limma" packages. Second, "lmFit" was chosen to process the data. The empirical Bayes method was employed to search for the significant DEGs. The *P* values were adjusted by the Benjamini-Hochberg procedure, and both adjusted *P* < 0.05 and log₂(fold change (FC)) > 2 were considered statistically significant.

DAVID analysis and gene set enrichment analysis (GSEA)

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to investigate the underlying functions and some vital pathways involved in osteogenic differentiation. The DAVID Bioinformatics Tool (<https://david.ncifcrf.gov/>) was used for the GO and KEGG analyses. Three categories were exported from the GO analysis, namely, biological process (BP), cellular component (CC)

and molecular function (MF). The *P* value denotes the significance of the GO term enrichment among differentially expressed genes ($P < 0.05$). Pathway analysis was used to map the genes to KEGG pathways. The *P* value denotes the significance of the pathway correlations ($P < 0.05$).

Gene set enrichment analysis (GSEA) was conducted based on the KEGG database. The expression data of total normalized mRNAs were uploaded to the GSEA v3.0 software. The default weighted enrichment statistic was adopted to process the data 1000 times, with normalized $P < 0.05$ considered to be significantly enriched. Next, 7 of the highest up- and down-regulated results of the GSEA reports were selected to undergo graphics processing using the “ggplot2” package in R. Dotplot and ridgeplot were also used to visualize the data from the GSEA and illustrate the distribution of multiple pathways.

Correlation network analysis of DEGs

The expression profiles of genes involved in the KEGG_Retinol_Metabolism were analyzed through GSE80614. The differentially expressed genes with $\log_2(\text{fold change (FC)}) > 2$ and adjusted $P < 0.05$ were selected and analyzed by hierarchical clustering. Meanwhile, TargetScan (<http://www.targetscan.org/>) and miRanda (microrna.org) were applied to predict the microRNA targeting *DHRS3*, *ADH1B*, and *ADH1A*. Then, the networks were graphed using Cytoscape software.

Osteoblast differentiation induction

To induce osteogenic differentiation, hBMSC cells were grown to 80%~90% confluence, and then the medium was changed to osteoblast-specific induction medium, α -MEM supplemented with 7.5% FBS, 10 mM glycerol-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 20 mM L-ascorbic acid (Sigma-Aldrich), and 0.1 mM dexamethasone (Sigma-Aldrich). The culture medium was replaced every 3 days for a period of 14 days. The cells were harvested and analyzed at 0, 7 and 14 days.

Quantitative Real-time PCR (qRT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the total RNA, and the iScript™ cDNA Synthesis Kit (Biorad, China) was used to synthesize cDNA following the manufacturer's protocol. iQ™ SYBR Green Supermix (Biorad, China) was used to perform qRT-PCR with a 7500 HT Fast Real-Time PCR System (Applied Biosystems). The relative gene expression levels of miR-223 and *DHRS3* were evaluated using U6 and GAPDH as the endogenous normalization controls. The primer sequences of the evaluated genes are listed in Table 1.

Western Blotting

Whole cell lysates for western blotting were extracted with lysis buffer, and the total protein concentration was determined with a protein assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Total protein (20 μ g) was boiled for 5 min in 1 $\times$ loading buffer, chilled on ice, and then separated on 10% sulfate sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The membranes were then blocked by incubation with 5% fat-free milk in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6) at 4°C for 1 h. The membranes were incubated overnight with primary antibody for *DHRS3* (ab198005, 1:1000), *Runx2* (ab23981, 1 μ g/ml), *OPN* (ab216406, 1:1000), *OCN* (ab93876, 1:500), *CRBP1* (ab154881, 1:1000), β -catenin (ab6302, 1:4000), and *GAPDH* (ab9485, 1:2500) at 4°C. Unbound antibody was removed by washing the membranes in TBST buffer three times (10 min/wash). The membranes were then incubated with secondary antibody (ab6721, 1:5000) for 1 h at room temperature, and washed with TBST buffer three times (10 min/wash). The proteins were detected with an enhanced chemiluminescence detection system (Amersham Biosciences), followed by exposure to X-ray films.

Table 1. Specific primer sequences for qRT-PCR

	Forward primers	Reverse primers
DHRS3	5'-CTCTTTTACCGTCCCAACCTT-3'	5'-GGGCATCATCATCTGTCCA-3'
miR-223	5'-CGTTGTGTCAGTTTGTCAAATAC-3'	5'-CAGTGCAGGGTCCGAGGT-3'
U6	5'-CTCGCTTCGGCAGCAC-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GTTTCGACAGTCAGCCGCATC-3'	5'-GGAATTTGCCATGGGTGGA-3'

Dual Luciferase Reporter Assay
The segments of *DHRS3*-3'UTR-WT (wild type) or *DHRS3*-3'UTR-MUT (mutant) were

constructed and then inserted into the luciferase reporter assay vector pmirGLO (Promega, Madison, USA). The vectors were transfected into 293T cells in accordance with the manufacturer's protocols. The cells were harvested 48 h after transfection with miR-223 mimics and miR-NC (GenePharma, Shanghai, China), and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

Alkaline phosphatase staining (ALP) and alizarin red staining (ARS)

Fourteen days after the induction of osteogenic differentiation, the BCIP/NBT Alkaline Phosphatase Color Development kit (Beyotime, China) was used to perform ALP staining according to the manufacturer's instructions. The Alkaline Phosphatase Assay kit (Nanjing Jiancheng Bioengineering Institute, China) was used to perform quantitative ALP measurements.

ARS was performed at 14 d to detect osteoblast calcification. Cells were washed twice with PBS, fixed in 95% ethanol for 10 min, washed with distilled water three times, and then stained by Alizarin Red S staining solution (Cyagen, China), with the pH adjusted to 8.3 by HCl, for 30 min at 37°C. After being rinsed twice with distilled water, the cells were photographed.

Statistical Analysis

All experiments were repeated at least three times. The data were analyzed using Graph Pad Prism 6.0 software. All data are presented as the mean $\pm$ standard deviation (SD). Differences between two groups were identified by unpaired *t*-tests, and one-way analysis of variance (ANOVA) was used to identify differences between three or more groups. $P < 0.05$ was considered statistically significant.

Consent for publication

Consent for publication was obtained from the participants.

Results

Differentially expressed mRNAs during osteogenic differentiation and enrichment analysis

Expression levels were considered different if they were under the standard $\log_2(FC) > 2$ and adjusted $P < 0.05$. In total, 87 upregulated mRNAs and 40 downregulated mRNAs were identified (Table 2). Fig. 1A shows the top 10 mRNAs that are up- and downregulated during osteogenic differentiation. Using DAVID for the KEGG pathway and GO enrichment analyses, we identified several vital pathways involved in osteogenic differentiation. The results showed that the differentially expressed mRNAs were significantly enriched in 8 pathways ($P < 0.05$, Fig. 1B), including the AMPK signaling pathway and retinol metabolism. Fig. 1C-E shows the 8 significantly upregulated GO terms in the biological process, cellular component and molecular function categories. According to the enrichment score from the GSEA report, the top 7 scored pathways

Fig. 1. Differentially expressed mRNAs during osteogenic differentiation. (A) The top 10 up- and down-regulated mRNAs in normal and osteogenic differentiation samples were arranged by unsupervised hierarchical clustering. Data displayed as red and green represent elevated (high) and reduced expression (low), respectively. (B) Enrichment scores for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were the most significantly upregulated pathways during osteogenic differentiation. (C-E) Enrichment scores of Gene Ontology (GO) terms that were upregulated most significantly, covering the domains of biological processes (BP), cellular components (CC) and molecular functions (MF).

Table 2. Microarray GSE80614 analysis results. 87 upregulated mRNAs and 40 downregulated mRNAs were identified under the standard $\log_2(\text{FC}) > 2$ and adjusted $P < 0.05$

Gene symbol	log2(Fold Change)	PValue	adj.PValue	B
PPP1R14A	5.259208353	1.14E-11	4.34E-07	14.87777262
MAOA	5.003941669	2.04E-09	3.56E-06	12.22158082
FRZB	4.997395129	3.09E-09	3.95E-06	11.91247213
FKBP5	4.988723256	2.08E-09	3.56E-06	12.21002796
IGFBP2	4.692101234	3.03E-08	1.37E-05	9.979915016
EFHD1	4.570797544	1.06E-10	8.63E-07	14.01250093
DHRS3	4.394620669	2.44E-09	3.74E-06	12.08990344
SUSD2	4.375624387	9.77E-10	2.79E-06	12.73876816
SVEP1	4.267256534	3.78E-08	1.59E-05	9.775789256
ADH1A	4.237809295	5.35E-09	5.33E-06	11.48102041
LEPR	4.153297547	1.78E-11	4.34E-07	14.73576602
SAAI	4.005304937	9.16E-09	7.17E-06	11.03660251
COL11A1	3.884454718	4.76E-10	1.80E-06	13.1983427
LEP	3.781487487	1.40E-09	3.08E-06	12.49167988
TSC22D3	3.754833057	7.50E-09	6.31E-06	11.20364872
CIDEA	3.729554644	1.16E-08	7.64E-06	10.83243757
LEPR	3.685361973	1.88E-07	4.42E-05	8.219459004
IGF2	3.654617407	0.004160221	0.00444782	0.927464748
FBN2	3.562032498	1.60E-09	3.11E-06	12.40008745
COL11A1	3.556019089	5.28E-11	6.65E-07	14.3259109
CD24	3.498437578	4.43E-09	5.03E-06	11.63171209
ALPL	3.341995747	4.24E-08	1.68E-05	9.667622591
ADARB1	3.253072329	2.10E-08	1.11E-05	10.31373275
TIMP4	3.250339143	9.99E-09	7.17E-06	10.96281784
TSC22D3	3.237363067	8.63E-07	0.00011195	6.650036109
LOC388610	3.193302241	1.20E-09	2.79E-06	12.59886136
ZBTB16	3.184280012	4.89E-09	5.12E-06	11.55322636
TSPAN18	3.179930243	1.50E-06	0.00016742	6.066487301
IGF2	3.134618507	4.31E-06	0.000338548	4.92529924
OLFML3	3.108162123	2.73E-09	3.80E-06	12.00725681
C10ORF54	3.083141946	6.81E-08	2.30E-05	9.218777109
FMO3	3.04179826	5.45E-11	6.65E-07	14.31237922
LEPR	3.038544364	8.97E-06	0.000559385	4.125430786
FMO3	2.9632555	6.94E-08	2.31E-05	9.201016791
FOXO1	2.930197118	4.61E-08	1.80E-05	9.589631346
C10ORF10	2.845482359	9.73E-08	2.81E-05	8.872463074
ACTC1	2.844282686	2.74E-09	3.74E-06	12.08704912
SORT1	2.843618271	3.26E-07	5.84E-05	7.662113477
ADARB1	2.837585816	1.36E-07	3.58E-05	8.543522161
APOD	2.808071783	3.04E-09	3.95E-06	11.92359422
FBLN2	2.757607015	2.80E-07	5.30E-05	7.815756402
RABGAP1	2.755524581	1.55E-07	3.88E-05	8.413738494
RAP2A	2.70367581	7.87E-07	0.000105536	6.746231717
CRYAB	2.691607627	1.57E-06	0.000173101	6.013931687
IRS2	2.641850866	2.12E-09	3.56E-06	12.19607324
DDIT4	2.627707131	1.45E-09	3.08E-06	12.46748319
ECM2	2.607844905	1.37E-07	3.58E-05	8.538910969
FBLN2	2.599380708	3.66E-08	1.56E-05	9.806686071
HSPB7	2.573594021	7.09E-07	9.77E-05	6.856672781
JAM2	2.539107669	1.55E-10	1.08E-06	13.8262333
GCHFR	2.524081746	7.60E-08	2.44E-05	9.113117158
OLFML2A	2.511126205	2.96E-08	1.37E-05	10.00208116
COMP	2.508184177	6.96E-08	2.31E-05	9.198400556
ANGPT1	2.490210293	1.11E-06	0.000134888	6.382485317
ANGPT1	2.476817329	5.41E-06	0.000399149	5.679862201
LEPR	2.463539338	2.44E-06	0.00023022	5.54045524
TXNIP	2.45608298	1.13E-07	3.11E-05	8.722484076
PIK3R1	2.436117036	6.24E-07	8.96E-05	6.988965373
FBLN1	2.425973152	6.69E-09	6.05E-06	11.29818378
HES4	2.391289281	0.000110311	0.000110311	6.671184014
PPARG	2.387174249	1.37E-06	0.000157908	6.159992102
CLEC3B	2.384711836	1.45E-07	3.72E-05	8.479438261
IGFBP5	2.349334903	1.89E-07	4.42E-05	8.212929666
FZD4	2.34029386	2.49E-08	1.27E-05	10.1596674
TSC22D3	2.337256708	5.78E-07	5.54E-05	7.069867574
ACTA2	2.322983417	9.69E-08	2.81E-05	8.87718182
OMD	2.275793445	3.37E-07	6.00E-05	7.627781748
ADCY3	2.274867507	3.97E-08	1.64E-05	9.730479481
ZBTB16	2.216274831	6.01E-06	0.000427225	4.565199331
DCN	2.21017714	5.65E-06	0.000409095	4.631783072
FAM46B	2.207882738	2.11E-07	4.62E-05	8.103815432
CORIN	2.195436939	4.01E-07	6.78E-05	7.448775272
WASF3	2.18356917	1.93E-07	4.46E-05	8.191262434
LMCD1	2.180917064	4.68E-09	5.12E-06	11.5885619
LMOD1	2.175196335	1.15E-06	0.000139515	6.341473173
C5ORF23	2.162156061	2.89E-07	5.40E-05	7.785993457
GPD1L	2.148562302	8.88E-08	2.69E-05	8.96160013
GAS1	2.108470337	2.07E-06	0.000208541	5.717276366
IGFBP5	2.099150533	2.04E-05	0.000905353	3.220970404
GPC4	2.089484067	4.07E-10	1.80E-06	13.29314354
SPRY1	2.082671122	5.56E-09	5.43E-06	11.44945457
C10ORF198	2.07191295	1.84E-08	9.96E-06	10.43327686
C13ORF15	2.069355636	4.07E-08	1.66E-05	9.705852612
CKB	2.05497006	1.93E-07	1.46E-05	8.19273384
RAB11FIP1	2.022673678	7.00E-07	9.71E-05	6.868747533
TNS3	2.004701515	1.96E-07	4.47E-05	8.178084664
CLIC3	2.003233894	7.19E-06	0.000480923	4.36783588
BDNF	-2.011525287	9.52E-08	2.80E-05	8.894177347
CCL2	-2.035257962	3.58E-06	0.000301869	5.127481048
KCNK2	-2.046398818	1.06E-06	0.000131337	6.434715698
DCBLD2	-2.060265957	1.07E-06	0.000132586	6.419323415
PODXL	-2.080262034	8.11E-09	6.54E-06	11.13865278
ARHGAP22	-2.086957993	7.28E-09	6.23E-06	11.22882943
HAS2	-2.087383322	5.58E-06	0.000408476	4.644821988
GPR68	-2.094837815	4.93E-09	5.12E-06	11.54606355
CPA4	-2.131735371	2.63E-08	1.28E-05	10.109803
IER3	-2.168024561	1.42E-06	0.000161394	6.125298702
KIAA0101	-2.170049471	6.61E-07	9.24E-05	6.92923708
NTSE	-2.242046871	6.10E-06	0.00042979	4.547578013
PTTG1	-2.250707095	1.37E-06	0.000157908	6.158552393
LPXN	-2.305893729	1.18E-08	7.64E-06	10.82217123
KIAA1199	-2.309715313	1.73E-07	4.21E-05	8.304241094
TOM2	-2.323482523	4.25E-07	7.02E-05	7.308049219
NRG1	-2.323741377	2.01E-10	1.22E-06	13.69066124
MALL	-2.360365783	2.01E-08	1.08E-05	10.35295636
SHISA2	-2.397223478	2.89E-10	1.57E-06	13.49036625
FLG	-2.425622312	1.57E-08	1.05E-06	10.56975626
ABI3BP	-2.427150912	1.04E-07	2.94E-05	8.804249578
CLDN11	-2.492825542	1.02E-06	0.000128117	6.477252783
LOC399947	-2.58544427	2.55E-09	3.77E-06	12.05719757
SLC14A1	-2.638008629	3.41E-08	1.50E-05	9.870857276
KRTAP1-5	-2.739330401	4.78E-10	1.80E-06	13.19565963
PENK	-2.741668873	1.19E-05	0.000681288	3.815982849
APCDD1L	-2.805954068	5.85E-09	5.60E-06	11.40855012
NGF	-2.830305146	6.78E-10	2.36E-06	12.97789027
TMEM166	-2.849166435	7.21E-09	6.32E-06	11.23655005
GREM1	-2.947680066	4.17E-07	9.96E-05	7.40902673
RG54	-3.027326024	1.20E-09	2.79E-06	12.59892765
KRT34	-3.046885579	2.72E-09	3.80E-06	12.00906315
TFPI2	-3.198147849	1.28E-07	3.44E-05	8.601035575
HMGA1A	-3.258575096	1.18E-05	0.000679023	3.822544893
SERPINE1	-3.26780592	2.40E-07	4.96E-05	7.973166298
IL6	-3.35007671	4.44E-10	1.80E-06	13.2409925
KRT19	-3.412622524	1.08E-09	2.79E-06	12.66899556
PLAT	-3.529116261	2.37E-08	1.24E-05	10.20599302
HNT	-3.746359919	1.19E-09	2.79E-06	12.60593925
PHLDA1	-3.809532049	1.19E-08	7.64E-06	10.81695911

involved in osteogenic differentiation were ranked and shown in Fig. 2A. The enrichment of these pathways is shown in Fig. 2B using Cytoscape software. Based on the adjusted *P*-value of each pathway, the distributions of several KEGG pathways were drawn in dotplot (Fig. 3A) and ridgeplot (Fig. 3B). Both dotplot and ridgeplot showed that retinol metabolism was one of the most significantly activated pathways during osteogenic differentiation. Combining these bioinformatics results, we speculated that retinol metabolism could influence gene functions, thereby affecting osteogenic differentiation.

Retinol metabolism during osteogenic differentiation

The GSEA enrichment plot showed that most genes involved in retinol metabolism were found in the region where genes were upregulated during

Fig. 2. Enrichment maps of KEGG pathways during osteogenic differentiation. (A) The 7 most distinctively activated pathways in the normal and osteogenic differentiation samples. The retinol metabolism pathway ranked the sixth in the most highly upregulated pathways during osteogenic differentiation. (B) Two pathways were identified as upregulated pathways, and 5 pathways were downregulated.

Fig. 3. Distributions of several KEGG pathways as shown by dotplot and ridgeplot. (A) The distributions of some KEGG pathways gene sets during osteogenic differentiation. The retinol metabolism pathway was upregulated. In the dotplot, the enrichment significance (the adjusted *P* value) is reflected by the color intensity of the nodes. The node size represents the gene count in the analyzed gene set. The gene ratio on the horizontal axis represents the proportion of differentially expressed genes in the gene set. (B) The distributions of some KEGG pathways gene sets during osteogenic differentiation. The retinol metabolism pathway gene sets were mainly located in the up-regulated region. In the ridgeplot, the enrichment significance (the adjusted *P* value) is reflected by the color intensity of the peaks. The value on the horizontal axis is the logarithm of the fold change. Peaks to the left of 0 indicate downregulation, while those to the right of 0 indicate upregulation.

osteogenic differentiation (Fig. 4A). Therefore, retinol metabolism was activated in osteogenic differentiation. Among the genes that are involved in retinol metabolism, the 3 genes with the most significantly upregulated expression were selected (Fig. 4B), namely, *DHRS3*, *ADH1A*, and *ADH1B*. Then, miRanda and TargetScan were used to screen for the microRNAs targeting those three genes (Fig. 4C). According to a previous study, miR-223 plays an important role during osteoblast differentiation [17]. Thus, in the present study, we aimed to further investigate the mechanism by which miR-223 affects osteogenic differentiation. Moreover, because *ADH1A* and *ADH1B* are two dehydrogenases, we further investigated the roles of miR-223 and *DHRS3*. The binding sites of miR-223 and *DHRS3* are shown in Fig. 4D.

Fig. 4. Most of genes involved in retinol metabolism were upregulated during osteogenic differentiation. (A) GSEA enrichment plots of genes involved in KEGG_Retinol_Metabolism in osteogenic differentiation. Most genes involved in retinol metabolism were in the upregulated region. (B) Hierarchical cluster analysis of 3 genes differentially expressed during osteogenic differentiation. In the heat map, the differentially expressed genes were all upregulated. (C) The interaction networks for differentially expressed mRNAs/miRNAs concerned with retinol metabolism. (D) The interaction of miR-223 and *DHRS3* was predicted based on TargetScan and miRanda.

MiR-223 had a low expression level, while DHRS3 was highly expressed during the osteogenic differentiation of hBMSCs

The expression levels of miR-223 and *DHRS3* after the osteogenic differentiation of hBMSCs were detected by qRT-PCR and western blotting. As shown in Fig. 5A-B, the expression of miR-224 gradually decreased while the expression of *DHRS3* gradually increased during osteogenic differentiation (from 0 d to 14 d). The western blotting results showed the same results; the expression of *DHRS3* was increased 7 d and 14 d after the induction of osteogenic differentiation (Fig. 5C, $P < 0.01$), which was consistent with the microarray analysis results.

MiR-223 targeted the 3'UTR region of DHRS3

As mentioned above, the prediction by TargetScan and miRanda suggested that *DHRS3* might be the target of miR-223. The luciferase reporter gene assay was performed to verify their binding relationship. The result revealed that the luciferase activity of cells co-transfected with miR-223 mimics and 3'UTR-WT was significantly lower than that of cells co-transfected with NC and 3'UTR-WT (Fig. 6A, $P < 0.01$), whereas miR-223 mimics had no effect on the fluorescence of the MUT recombinant plasmid (Fig. 6A, $P > 0.05$). Moreover, we found that miR-223 could not target Runx2, OCN, or OPN (data not shown). Taken together, these results revealed that miR-223 directly targeted the 3'UTR of *DHRS3*.

MiR-223 prevented the osteogenic differentiation of hBMSCs by co-regulating retinol metabolism via suppressing DHRS3 expression

The results of qRT-PCR showed that miR-223 expression was upregulated in the miR-223 agomir group, while it was downregulated in the miR-223 antagomir group, indicating

Fig. 5. Expression levels of miR-223 and DHRS3 changed during osteogenic differentiation. (A-B) QRT-PCR results detected that miR-223 expression was downregulated while DHRS3 mRNA expression was up-regulated during osteogenic differentiation. ** $P < 0.01$, compared with the control group. (C) Western blotting results suggested that DHRS3 expression increased after induction of osteogenic differentiation. ** $P < 0.01$, compared with control group.

successful plasmid transfection (Fig. 6B, $P < 0.01$). The results of both qRT-PCR and western blotting showed that the expression of *DHRS3* was significantly downregulated with miR-223 overexpression, whereas the inhibition of miR-223 markedly elevated the expression of *DHRS3* (Fig. 6C-D, $P < 0.01$), indicating that miR-223 negatively regulates the expression of *DHRS3*. Meanwhile, the expression of *DHRS3* was upregulated in the cDNA group, suggesting that the overexpression of *DHRS3* (*DHRS3* cDNA) was successfully obtained. The protein expression level of the cells was detected on day 14 after the induction of osteogenic differentiation. The ALP staining and ARS staining results showed that on day 14, ALP activity and calcification were enhanced in both the cDNA and antagomir groups, while the group simultaneously co-transfected with miR-223 agomir and *DHRS3* cDNA showed no significant difference in ALP activity and calcification from the NC group (Fig. 7A-B, $P < 0.01$). Consistent with the results above, the western blotting results showed that the expression levels of Runx2, OPN and OCN were upregulated in both the cDNA and antagomir groups, but there was no significant difference in protein expression in the group simultaneously co-transfected with miR-223 agomir and *DHRS3* cDNA (Fig. 7C, $P < 0.01$). The above results demonstrated that miR-223 targeted *DHRS3* and inhibited osteogenic differentiation.

Fig. 6. MiR-223 targeted the 3'UTR region of *DHRS3*. (A) miR-223 targeted the *DHRS3* 3'UTR. Dual luciferase reporter gene assay results demonstrated that the luciferase activity in the miR-223 mimics+WT group was significantly weaker than in the NC+WT group or the MUT recombinant plasmid groups. *** $P < 0.001$, compared with the NC group. (A) QRT-PCR results showed that the expression of miR-223 was increased in the agomir group, while it was decreased in the antagomir group. ** $P < 0.01$, compared with the NC group. (B-C) QRT-PCR and western blotting results detected that *DHRS3* expression was significantly upregulated in both the *DHRS3* cDNA and miR-223 antagomir groups, whereas it was downregulated in the miR-223 agomir group. ** $P < 0.01$, compared with the NC group.

Discussion

Through microarray and enrichment analyses, *DHRS3* was found to be differentially expressed and retinol metabolism was discovered to be activated during osteogenic differentiation. Through MiRanda and TargetScan, we found that *DHRS3* may be targeted by miR-223. Our further studies showed that the expression of miR-223 decreased, while that of *DHRS3* increased during osteogenic differentiation. In addition, osteogenic differentiation was upregulated after knocking down miR-223, while there were no obvious changes when miR-223 and *DHRS3* were simultaneously overexpressed. The above results revealed that miR-223 suppressed retinol metabolism by directly targeting *DHRS3*, thereby inhibiting the osteogenic differentiation of hBMSCs.

Currently, Gene Set Enrichment Analysis (GSEA) has been widely used to analyze gene expression profiles. The goal of GSEA is to identify pathways where the constituent genes show coordinated changes in expression during the experimental conditions. GSEA is able to highlight genes connected to the phenotype through pathway analysis, even if the connection is weak [25].

Therefore, to clarify some potential genes and pathways involved in osteogenic differentiation, GSEA is likely to be more powerful than the conventional single-gene method. Through GSEA, we found that retinol metabolism was activated during osteogenic differentiation. Among the genes involved in retinol metabolism, *DHRS3* was significantly upregulated.

DHRS3 is involved in the control of retinol metabolism and is codependent with retinol dehydrogenase 10 (*RDH10*). The first step of retinol metabolism is a reversible step, in which retinol associates with the *DHRS3*/*RDH10* complex and is oxidized to retinaldehyde by *RDH10*, which process is enabled by *DHRS3*. Retinaldehyde can then be reduced back to retinol by the *RDH10*-activated *DHRS3*, or it can be further irreversibly oxidized to retinoic acid (RA) by the *ALDH* genes [22, 26]. By functionally activating each other, *DHRS3* and *RDH10* work together to maintain the appropriate level of RA [23]. Many studies have demonstrated the significant role of RA in osteogenic differentiation. It was reported that RA promoted the osteogenic

Fig. 7. MiR-223 inhibited osteogenic differentiation via targeting *DHRS3*. (A-B) ALP and ARS staining results demonstrated that inhibited miR-223 or overexpressed *DHRS3* significantly increased the osteogenic differentiation of hBMSCs. The group simultaneously co-transfected with *DHRS3* cDNA and miR-223 agomir showed no significant differences from the NC group. ***P<0.001, compared with the NC group. (C) The expression levels of Runx2, OPN and OCN were higher in the miR-223 antagomir group and the cDNA group than in the NC group. There was no significant difference between the NC group and the agomir+cDNA group. ***P<0.001, compared with the NC group. Scale bar: 50 μ m.

differentiation of mouse adipose-derived adult stromal cells (ADAS), suggesting that the osteogenic commitment of mouse ADAS requires RA [27]. In addition, other study found that BMP9-induced ALP activity, one of the early osteogenic markers, was significantly enhanced by RA in a dose-dependent manner. The results strongly suggested that RA may function synergistically with BMP9 activity to promote the osteogenic differentiation of MPCs, which could potentially be useful in bone regeneration therapy [28]. As has been mentioned above, in association with *RDH10*, *DHRS3* plays a critical role in maintaining appropriate levels of RA, which promotes in a dose-dependent manner the osteogenic differentiation of MPCs. Consequently, there are strongly supported reasons to believe that *DHRS3* plays a significant role during osteogenic differentiation. In the present study, we further found that *DHRS3* can promote osteogenic differentiation *in vitro*.

Recent reports have demonstrated regulatory roles for miR-223 in bone metabolism, including in mediating the repression of the key proteins required for osteoblast differentiation [29]. For instance, miR-223 downregulates osteoblast differentiation through a C/EBP α /miR-223/FGFR2 regulatory feedback loop [29]. To maintain physiological bone metabolism, osteoblasts and osteoclasts coordinate with each other and maintain the balance between bone formation and resorption, which is always determined by the differentiation of osteoblasts and osteoclasts [30]. Once osteoblast differentiation decreases or osteoclast differentiation is enhanced, abnormal bone metabolism leads to a number of pathological bone destruction diseases [31]. It was reported that regulatory networks involving miR-223 play multiple roles in regulating bone metabolism. MiR-223 is a critical regulator in diseases with bone disorders, and changes in its expression are associated with the development stages and the control of clinical signs [17]. In the present study, we found that the expression of miR-223 gradually decreased during osteogenic differentiation, while the expression of *DHRS3* gradually increased. By directly targeting *DHRS3*, rather than *Runx2*, *OCN*, or *OPN*, miR-223 could inhibit osteogenic differentiation.

There were some limitations in this study. First, through GSEA, several pathways were screened out, which might also be involved in osteogenic differentiation. However, because of limited time, we did not study the pathways and genes other than retinol metabolism and *DHRS3*. Second, only *in vitro* experiments were performed due to limited funding, and it is necessary to perform *in vivo* experiments to confirm the effect of retinol metabolites on osteogenic differentiation. Third, there are studies indicating that excess retinol stimulates bone resorption and inhibits bone formation, which might contribute to bone loss and further lead to osteoporosis [32]. Therefore, more experiments are needed to clarify the effect of different doses of retinol on bone formation and absorption. Finally, in the present study, we demonstrated that the overexpression of miR-223 inhibited the osteogenic differentiation of hBMSCs, but the effect of miR-223 on osteoblastic proliferation after 14 d requires further investigation.

Conclusion

In conclusion, we found that miR-223 and *DHRS3* play significant roles during osteogenic differentiation. MiR-223 inhibited osteogenic differentiation via targeting *DHRS3*, providing the basis for future research.

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Disclosure Statement

No conflict of interests exists.

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