Identification of microRNAs in human circulating monocytes of postmenopausal osteoporotic Mexican-Mestizo women: A pilot study

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Abstract. MicroRNAs (miRNAs or miRs) are a class of short non-coding RNAs that serve an important regulatory role in living organisms. These molecules are associated with multiple biological processes and are potential biomarkers in multiple diseases. The present study aimed to further identify miRNAs that are differentially expressed in circulating monocytes (CMCs) from postmenopausal Mexican-Mestizo women. Microarray analyses of monocytes using Affymetrix miRNA 4.0 and Human Genome U133 Plus 2.0 arrays were performed in 6 normal and 6 osteoporotic women, followed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation. The overexpression of miR-1270, miR-548x-3p and miR-8084 were detected in the osteoporosis compared with the normal group according to the microarray analysis; miR-1270, a miRNA with several target genes associated with bone remodeling, was validated by RT-qPCR. Bioinformatics analysis identified that interferon regulatory factor 8 (*IRF8*) is the most likely target gene of miR-1270, which is associated with osteoclastogenesis. Furthermore, the findings of the present study demonstrate that an upregulation of miR-1270 may reduce the gene expression of *IRF8* in CMCs (osteoclast precursors), implicating its potential role in leading to low bone mineral density and contributing to osteoporosis development in postmenopausal women.

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Introduction

Osteoporosis (OP) is a metabolic bone disorder characterized by low bone mineral density (BMD) and decreased bone strength, which leads to an increased risk of fractures with a consequent increase in morbidity and mortality (1). In the Mexican population, OP is a major public health issue, mainly due to the increase in the life expectancy of the Mexican population (2). In OP, the balance of bone formation and resorption is impaired.

Osteoclasts are multinucleated cells that are formed from the fusion of progenitors of osteoclasts (3). In the process of osteoclast formation, there is an absolute requirement for cells of hematopoietic origin of the monocyte/macrophage linage and bone marrow stromal cells to commit the hematopoietic cell towards osteoclast development (4).

Circulating monocytes (CMCs) are an appropriate model for bone-related studies and are important cells that participate as osteoclast precursors (5,6); they also secrete osteoclastogenesis-associated factors, including interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) (5,7). In addition, previous studies have revealed associations of gene and protein expression in CMCs and OP (8-10). These studies demonstrate that CMCs are a valuable cell linage with functional relevance in the pathogenesis of OP and have been well substantiated and accepted as a suitable model for studies aiming to dissect etiological mechanisms in the bone field (6).

MicroRNAs (miRNAs or miRs) are a class of non-coding single-stranded RNAs (~22 nucleotides) that confer a crucial level of gene expression regulation, primarily by promoting mRNA degradation or inhibiting translation (11). Altered

miRNA expression is associated with bone-related diseases, including OP, and serves a key role in bone formation and resorption (12,13). Substantial evidence has demonstrated that circulating miRNAs may be used as potential non-invasive biomarkers for various human diseases (14,15). Recent reports have illustrated the importance of miRNAs in osteoclastogenesis and OP. From these studies, miR-21, miR-133a, miR-148, miR-422a and miR-194-5p have been associated with postmenopausal OP and fractures (16,17).

The present study aimed to identify potentially useful miRNAs as biomarkers for postmenopausal OP, in CMCs isolated from postmenopausal Mexican-Mestizo women. A significant increase was identified in the miR-1270 expression in women with OP compared with normal ones using combined miRNAs and transcriptomics microarray technology and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation, and a further bioinformatics analysis of miR-1270 revealed that interferon regulatory factor 8 (*IRF8*) is the most important target gene associated with osteoclastogenesis.

Materials and methods

Subjects and study design. The Ethics Committee of the Mexican Institute of Social Security and the National Institute of Genomic Medicine approved the study protocol and informed consent forms. All participants enrolled in the present study were informed of the project and provided written informed consent. The women were of Mexican-Mestizo origin and were recruited from The Mexican Health Worker Cohort Study, which is a long-term study of workers from a large health care institution in Cuernavaca, Mexico, that focuses on lifestyle and chronic illnesses. Between 2011 and 2013 (Wave 2), a total of 1,026 participants were followed-up, of which 400 were postmenopausal women (18). The detailed characteristics of the postmenopausal women are reported elsewhere (19). The clinical procedures, data recording and participant follow-up practices were standardized and validated (20).

A total of 12 unrelated postmenopausal Mexican-Mestizo women, 6 with normal and 6 with osteoporotic hip BMD were recruited. The inclusion criteria were the following: Spine or hip T-score <-2.5 for the OP group (bottom 20% of the age-, sex- and ethnicity-matched population) and spine or hip T-score >-1.0, respectively for the normal group (top 20% of the age, sex and ethnicity-matched population). The BMD (g/cm^2) for the lumbar spine (L2-L4) and total hip were measured using a Lunar DPX NT dual energy X-ray absorptiometry instrument (Lunar Radiation Corp., Madison, WI, USA). The standard calibration of the instrument was performed daily using the settings intended for the spine and femoral neck phantom, as provided by the manufacturer. Technicians ensured that the daily variation coefficient (VC) was within the normal operational standards and that the *in vivo* VC was <1.5%. All women were aged between 63 and 85 years and had been postmenopausal for ≥12 months (their postmenopausal status was defined as the date of the last menses followed by at least 12 months of no menses). Exclusion criteria were used to minimize potential effects of any known non-genetic factors on bone metabolism and BMD determination. The present study excluded women who presented serious residuals from cerebral vascular disease, diabetes mellitus, chronic renal disease manifest by serum creatinine >1.9 mg/dl, chronic liver diseases or alcoholism, corticosteroid therapy, treatment with anticonvulsant therapy, rheumatoid arthritis or collagen disease, significant disease of any endocrine organ that may affect bone mass, hyperthyroidism and any other illness, treatment (including bisphosphonates) or condition (including hormone replacement therapy) that may be an apparent non-genetic factor underlying the variation BMD.

Monocyte isolation. A total of 80 ml whole blood was obtained from each participant. Peripheral blood mononuclear cells were obtained by density gradients using Histopaque-1077 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. $CD14^+$ monocytes were obtained by density gradient centrifugation at 400 x g for 30 min at room temperature and magnetic bead isolation. Naive monocytes were isolated using the negative isolation kit EasySep Human Monocyte Enrichment (Stemcell Technologies, Inc., Vancouver, BC, Canada) following the manufacturer's protocol. The purity and viability was ≥85% (data not shown) as determined by flow cytometry with the fluorescent-labeled antibodies CD14-phycoerythrin (anti CD14-PE) and CD45-fluorescein isothiocyanate (anti CD45-FITC; cat. no. 555574 and cat. no. 555748; BD Biosciences, Franklin Lakes, NJ, USA). Briefly, $1x10^6$ enriched monocytes were suspended in 100 μl PBS with 2% fetal bovine serum, stained with 20 μl of anti CD14-PE and anti CD45-FITC for 45 min at room temperature in the dark. The analysis was performed on a FACSAria I cytometer using FACSDiva software version 6.1.3 (both BD Biosciences; San Jose, CA USA).

miRNA profiling. Total RNA was isolated from monocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The RNA integrity and quantification was assessed using a Nanodrop (Thermo Fisher Scientific, Inc.) and 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Each RNA sample exhibited high quality with a high integrity number (>8.0). In total, 250 ng total RNA was labeled using a FlashTag Biotin HSR RNA labeling kit (Affymetrix; Santa Clara, CA USA.) and was subsequently hybridized on an GeneChip miRNA 4.0 Array (cat. no. 902411; Affimetrix), which contains 30,434 probes of mature miRNAs, of which 2,578 of the probes are human miRNAs, according to the Sanger miRBase v.21 (http://www.mirbase.org/). The array was washed two times with 1x PBS, 0.02% Tween-20 and stained with FlashTag Biotin HSR labeled RNA sample on a Fluidics Station 450 (cat. no. 901910, Affymetrix) followed by digitization in a GeneChip Scanner 3000 7G (Affimetrix), according to the manufacturer's protocol.

Gene expression microarray. An Affymetrix GeneChip Human U133 Plus 2.0 Array (Affymetrix) was used to evaluate the genome-wide gene expression levels. A total of 500 ng total RNA were used for the cDNA and biotinylated cRNA synthesis using the GeneChip expression 3' amplification reagents kits by Affymetrix (Thermo Fisher Scientific, Inc). Fragmented cDNA was applied to the hybridization, and

the scanning of the array was performed using the Affymetrix GeneChip 3000 7G scanner (Affymetrix). The expression intensity of each gene was logarithmically transformed to base 2 and was normalized using quantile normalization using R (programming language) through the Bioconductor (v3.3.3; https://www.r-project.org/).

Microarray data analysis. The raw data from microarray platforms, microRNA 4.0 and Human U133 Plus 2.0 gene expression were pre-processed using Robust Multiarray Average (21) for background correction and the samples were normalized with quantile normalization (22) methods available in the Bioconductor oligo (23) affy packages in Bioconductor v.3.3.3 (24).

The differential expression was determined through linear models, using the limma Bioconductor package v.3.5 (25), and the miRNAs were classified as differentially expressed according to a fold-change <-0.5 or >0.5 and P<0.05.

RT-qPCR analysis. RNA was extracted from monocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Then, 100 ng of total RNA were used for first strand cDNA synthesis using a TaqMan MicroRNA Reverse Transcription kit (cat. no. 4366596. Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The expression profile for miR-1270 (Assay ID. 002807), miR-548x-3p (Assay ID. 463079_mat) and miR-8084 (Assay ID. 466802_mat) were examined using TaqMan microRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed on an Applied Biosystems QuantStudio 7 Flex system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression levels were normalized with respect to RNU44 (Assay ID. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.). For the *IRF8* and *GAPDH* gene expression analyses, cDNA was synthetized from 100 ng total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. qPCR was performed via TaqMan assay (Assay ID. Hs01128713_m1; Applied Biosystems; Thermo Fisher Scientific, Inc.) in the QuantStudio 7 Flex system (Applied Biosystems; Thermo Fisher Scientific, Inc.).

The relative quantity (RQ) of the miRNAs of each sample was determined by the $2^{-\Delta\Delta Cq}$ (26) method, where the ΔCq=[the average of the triplicate Cq of the gene target miRNA-the average of the triplicate Cq of the endogenous control (GAPDH)], and the ΔΔCq=(the ΔCq-the mean ΔCq of all samples). The RQ data were used to identify the miRNAs that were differentially expressed between the two groups in the study via Student's t-test.

Bioinformatics analysis. To predict the potential target genes of miR-1270, the algorithms from the following different databases were used: microRNA.org (http://www.microrna.org/microrna/home.do), miRDB (http://mirdb.org/miRDB/), miRWalk v2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/), PITA v5.0 (https://omictools.com/pita-tool) and TargetScan Human v7.0 (http://www.targetscan.org/vert_71/), and the target genes predicted by ≥3 databases were selected for further analysis. To validate the *in silico* results, the list of potential target genes with the expression data from the Affymetrix GeneChip Human U133 Plus 2.0 Array of monocytes from the same 12 samples included in the present study were compared, and a proof of concept was applied so that if the miRNA is upregulated, then the mRNA of the target gene must be downregulated. The target genes accomplished by the proof of concept were used to identify the binding sites for miR-1270 according to complementary sequences of the target gene in the 3'-untranslated region (UTR) using the miRDB database (www.miRDB.org) (27,28) and TargetScan (www.targetscan.org) (29). PhastCons conservation score, which measures the evolutionary conservation of sequence blocks across multiple vertebrates using a phylogenetic hidden Markov model (30), was used to filter out less conserved predicted target sites.

Functional and pathway enrichment analysis. The online tool STRING version 10.0 (www.string-db.org/) was used to search the functional enrichment analyses of potential target genes of miR-1270 by selecting the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A false discovery rate (FDR)-value <0.05 was selected as the cut-off criterion for the KEGG enrichment analysis. The PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) was used to search for literature reported as 'BMD', 'osteoclasts', 'monocytes' and 'osteoporosis' associated genes to improve the potential candidate genes of the present study and to search for the interaction association of the predicted genes contained in the significant KEGG pathways. A confidence score >0.7 was considered as the cut-off criterion for an interaction. The Ingenuity Pathways Analysis (IPA) was used to construct the protein-protein interaction networks based on the genes reported in the literature and the miRNA data. This analytical tool is based on prior knowledge of the expected effects between genes and miRNAs stored in the base Ingenuity knowledge (Qiagen, Inc., Valencia, CA, USA).

Statistical analysis. Student's t-tests were used as indicated in each case. Data plotted represent the mean ± standard deviation as indicated. For statistical analysis of microarray data of miRNAs and genes, the CEL files were imported using freely available R language. The expression was performed on log2 transformed fold-change (FC) data. The limma package in Bioconductor v.3.5 was used to compare normal and OP groups. The Robust Multiarray Analysis algorithm was applied for generation of relative signal values and normalization. For expression comparison of different groups a moderated t-statistics was used followed by calculation of FDR (31). Results were expressed as the average of three repeats of FC. All statistical analysis were performed using GraphPad Prism v. 6.0 for Mac (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the study subjects. The clinical characteristics of the study subjects are summarized in Table I. No significant differences were observed in the age of the women included in the present study; however, significant differences

Table I. Characteristics of the study subjects.

Traits	Osteoporosis (n=6)	Normal (n=6)	P-value
Age (years)	73.16±7.44	66.66±2.73	0.088
Height (cm)	146.50±7.28	155.83±5.94	0.036
Weight (kg)	58.75±6.05	69.26±9.49	0.049
BMI	26.16±6.36	28.48±3.08	0.448
Children (n)	2.83±2.13	5.33±2.06	0.066
Age of menarcher (years)	12.50±1.97	13.00±0.89	0.589
Spine BMD (g/cm^2)	0.86±0.12	1.15±0.09	<0.001
Spine t-score	-3.27±0.76	-0.17±0. 68	<0.001
Hip BMD (g/cm^2)	0. 63±0.05	1.13±0.07	<0.001
Hip t-score	-2.97±0.45	1.03±0.56	<0.001

Data represent the mean ± standard deviation. BMI, body mass index; BMD, bone mineral density.

Table II. miRNAs differentially expressed between the osteoporotic and normal groups.

miRNA	Fold-change	P-value
hsa-miR-8084	1.999	0.047
hsa-miR-1270	1.483	0.003
hsa-miR-548x-3p	0.861	0.034
hsa-miR-6165	-1.098	0.022
hsa-miR-6824-5p	-1.229	0.016
hsa-miR-6124	-1.427	0.006

Fold-change (osteoporotic vs. normal) and P-values are provided. miRNA or miR, microRNA.

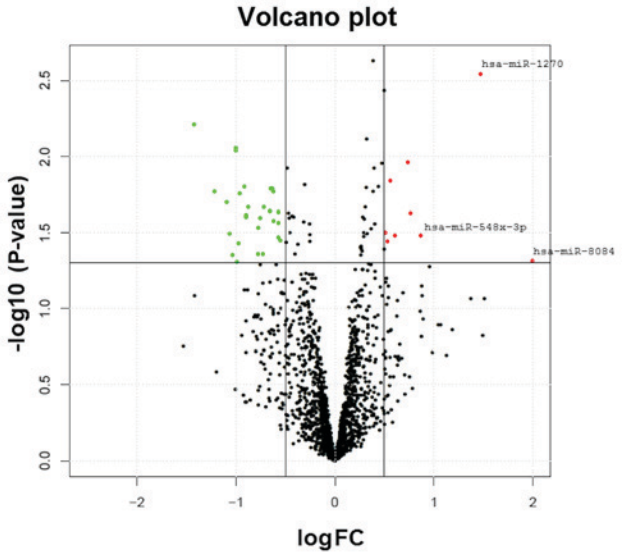

Figure 1. Volcano plot of the differentially expressed miRNAs as assessed by a microarray analysis in the circulating monocytes of osteoporotic and normal postmenopausal women. The y-axis indicates the-$\log_{10}$ of the P-values and the x-axis is the FC (measured as the log2 transformed ratio of the expression between both experimental groups). miRNA and miR, microRNA; FC, fold-change.

were identified in the weight and height between normal and OP groups (P<0.05). The mean age of the normal group was 66.66±2.73 years, whereas it was 73.16±7.44 years for the OP group, and the hip and spine BMDs were significantly different between the groups (P<0.001).

miRNAs are expressed differentially between the OP and normal groups. miRNA profiling of the OP and normal groups was performed on a microarray platform with probes for 2,578 mature human miRNAs, and several of the miRNAs were undetectable in a number of the samples. The comparison of the expression levels of the miRNAs in the OP and normal groups is depicted in Fig. 1. In total, 35 miRNAs revealed a nominally significant (P<0.05) difference in the microarray analysis between the two study groups. Table II presents the three miRNAs that were most markedly upregulated (miR-1270, miR-548x-3p and miR-8084) and downregulated (miR-6165, miR-6824-5p and miR-6124) in the OP group. In further analysis, the focus was on the upregulated miRNAs. The functions of these miRNAs are poorly understood, and none of them have previously been reported to be associated with BMD or OP.

Validation of miRNA expression by RT-qPCR. RT-qPCR was performed to validate the differential expression levels of the three-upregulated miRNAs (miR-8084, miR-1270 and miR-548x-3p). Only miR-1270 demonstrated a significant upregulation in the OP compared with the normal group (P=0.0043) (Fig. 2).

Predicted target genes of the OP-associated miRNA. Target mRNAs were predicted for miR-1270; the criterion for the target identification was consistent with the prediction by at least three databases, 3,857 target genes for miR-1270 were predicted. This list of potential target genes was linked with data derived from expression changes of the monocytes of the same samples (data not shown), assuming the association of the high expression levels of a given miRNA corresponded to the low level of target gene expression. Downregulation was defined as a minimum 0.5-FC. Using these criteria 161 putative downregulated target genes were identified for miR-1270

Table III. Putative binding sites of miR-1270 in the predicted target genes in humans.

Target gene	3'-UTR position	Sequence		PhastCons score
KRAS	2011	3'ugUGUCGA-GA-AGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'uuACUGCUGCUGUGGAUAUCUCCAu 3'	KRAS	0.5726
FOSL2	604	3'ugUGUCGA-GAAGGUA-UAGAGGUc 5'	hsa-miR-1270	
		5'ugACGCCUCCCAGUCAUCAUCUCCAg 3'	FOSL2	0.5389
MEF2C	1948	3'ugugUCGAGA-AGGUA-UAGAGGUc 5'	hsa-miR-1270	
		5'gugaAGAUCUGUCGAUUCAUCUCCAa 3'	MEF2C	0.6563
IRF8	1185	3'ugUGUCGAGAAGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'caAUAG-GCUU-GAAUCUCCAa 3'	IRF8	0.5677
SP1	2254	3'uguGUCGAGAAGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'cucCAUUUGGUCC-UUUCUCCAc 3'	SP1	0.6502
IKBKB	454	3'uguGUCGAGA-AGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'agcCUGUCCUCUCCUGCUCUCCAa 3'	IKBKB	0.5335
RASSF5	597	3'ugugUCGA-GAAGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'cugaGGCUGGCUCAGAGAUCUCCAg 3'	RASSF5	0.4772
ABL1	396	3'ugugucGAGAAGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'gccuccUUCUUCCACUUCUCCAa 3'	ABL1	0.4373
STAT2	704	3'ugugucgaGAAGGUAUAGAGGUc 5'	hsa-miR-1270	
		5'aguuuaugCUACCUAGUCUCCAc 3'	STAT2	0.5894

miR, microRNA; UTR, untranslated region; *FOSL2*, Fos-related antigen 2; *MEF2C*, myocyte enhancer factor 2C; *IRF8*, interferon regulatory factor 8; *SP1*, Sp1 transcription factor; *IKBKB*, inhibitor of κ light polypeptide gene enhancer in B-cells kinase β; *RASSF5*, Ras association domain family member 5; ABL1, Abelson murine leukemia viral oncogene homolog 1; *STAT2*, signal transducer and activator of transcription 2.

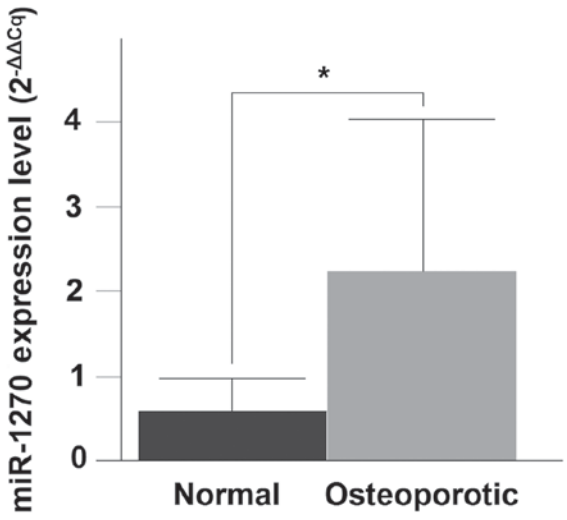

Figure 2. Expression level of miR-1270 in human circulating monocytes from osteoporotic and normal postmenopausal women as revealed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean + standard deviation of three independent experiments. *P=0.0043. miR, microRNA.

(data not shown). The interaction by combining the lists of the target genes generated by the prediction algorithms of miR-1270 and the expression dataset of the monocytes is depicted in Fig. 3A.

Interaction network of the target genes and the miRNAs. To explore a potential functional association of the deregulated miRNAs identified in the present study, the 161 target gene list was submitted to the online bioinformatics tool STRING software to identify canonical pathways. The analysis revealed 16 KEGG pathways with overrepresented monocytes, osteoclasts and OP-related genes. The pathways predicted as most markedly enriched by the miR-1270 were the chemokine, Ras, estrogen, adenosine monophosphate-activated protein kinase, insulin, phosphatidylinositol-3-kinase-protein kinase B, mitogen-activated protein kinase, forkhead box O, mechanistic target of rapamycin and TNF signaling pathways, focal adhesion and osteoclast differentiation. Other pathways were identified including: ErbB, Wnt, Janus kinase/signal transducers and activators of transcription and nuclear factor-κB (NF-κB) signaling pathways (Fig. 3B).

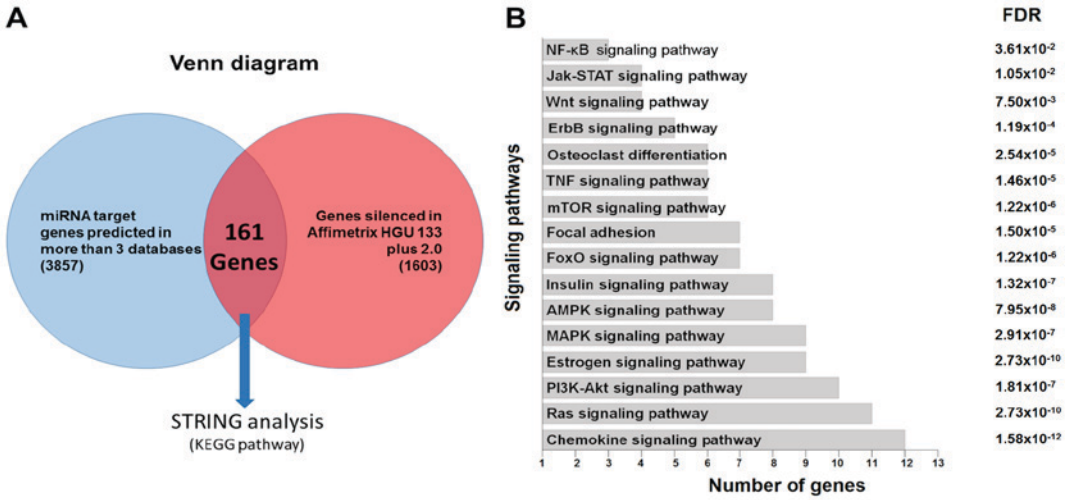

Figure 3. (A) Venn diagram demonstrating the basis for selecting the potential target genes of hsa-miR-1270, by linking the list obtained from the prediction algorithms with those generated from the expression data of the monocytes of the samples included in the present study (1,603 genes with fold-change <-0.5 or >0.5 and P<0.05). (B) KEGG pathway enrichment analysis of the putative miRNA targets from the previous analysis. miRNA or miR, microRNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; NF-κB, nuclear factor-κB; Jak-STAT, Janus kinase/signal transducers and activators of transcription; TNF, tumor necrosis factor; mTOR, mechanistic target of rapamycin; FoxO, forkhead box O; AMPK, adenosine monophosphate-activated protein kinase; MAPK, mitogen-activated protein kinase; PI3 K, phosphatidylinositol-3-kinase; FDR, false discovery rate.

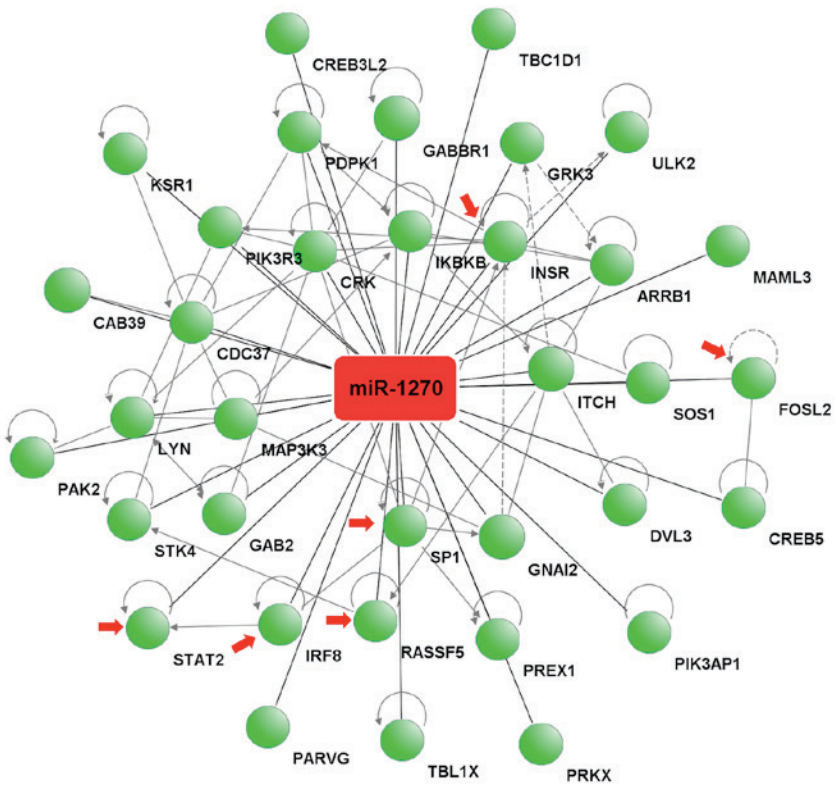

Figure 4. Interaction pathway of miR-1270 and target genes. These data reveal the interaction of 35 target genes for hsa-miR-1270. Red arrows indicate the genes related to bone remodeling. STAT2, signal transducer and activator of transcription 2; IRF8, interferon regulatory factor 8; RASSF5, Ras association domain family member 5; SP1, Sp1 transcription factor; FOSL2, FOS like 2, AP-1 transcription factor subunit and IKBKB, inhibitor of nuclear factor κ B kinase subunit β. miR, microRNA.

The target genes associated with these pathways were analyzed by IPA software to construct an interaction network between miR-1270 and the target genes. The analysis revealed that miR-1270 binds to the 3'-UTR of 35 potential target genes (Fig. 4).

Target gene prediction and verification. By performing the bioinformatics analyses, potential target genes associated with monocytes, osteoclasts and OP were identified and predicted by at least three databases. By searching the relevant references for all 35 target genes of miR-1270, it was revealed that

9 are potential targets genes for miR-1270 and are associated with osteoclastogenesis, including myocyte enhancer factor 2C (*MEF2C*) (32,33), Sp1 transcription factor (*SP1*) (4), signal transducer and activator of transcription 2 (*STAT2*) (34), *KRAS* (KRAS proto-oncogene, GTPase) (35) and FOS like 2 (*FOSL2*) (36), Inhibitor of κ light polypeptide gene enhancer in B-cells kinase β (*IKBKB*) (37), Ras association domain family member 5 (*RASSF5*) (38), Abelson murine leukemia viral oncogene homolog 1 (*ABL1*; non-receptor tyrosine kinase) (39) and *IRF8* (40). The predicted specific binding sites of miR-1270 to the 3'-UTR of these target genes are presented in Table III. The RT-qPCR analysis for the *IRF8* gene demonstrated a significant decrease in mRNA expression between the OP and normal samples (P=0.0288) (Fig. 5).

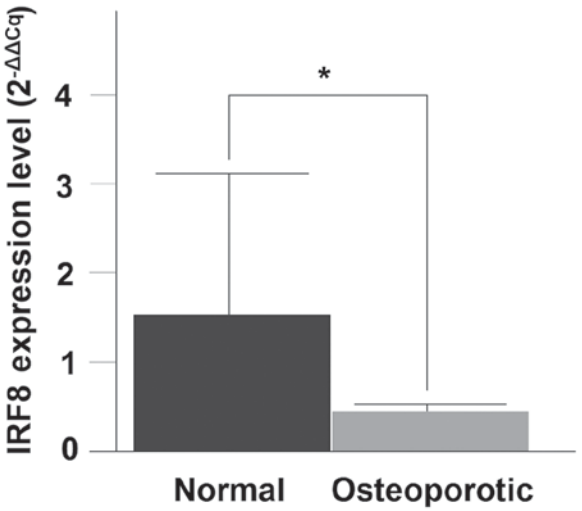

Figure 5. Expression level of the *IRF8* gene in human circulating monocytes from osteoporotic postmenopausal and normal women as revealed by reverse transcription quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation of three independent experiments. *P=0.0288. *IRF8*, interferon regulatory factor 8.

Discussion

In the present study, a microarray-based approach was performed followed by RT-qPCR validation and pathway analysis to identify important circulating miRNA for postmenopausal OP. The main observation of the present study is the evidence for miR-1270 as a potential novel biomarker for postmenopausal OP in a Mexican-Mestizo population, in addition to four previously recognized miRNAs, miR-21, miR-133a, miR-422a and miR-194-5p in Asian and Caucasian populations (16,17).

A miRNA microarray platform combined with the transcript profile of the same samples determined by the Human Genome U133 plus 2.0 microarray for the high-throughput detection of hundreds of miRNAs were used. The strength of this method resides in identifying potential miRNA biomarkers associated with bone metabolism. However, a major pitfall of this approach is a high false positive rate (41,42). Therefore, RT-qPCR is generally performed to validate significant miRNAs identified by microarray. This experimental approach aims to reveal novel potential associations between miRNA expression and the BMD status, assuming an inverse correlation between the levels of a given miRNA and the expression levels of its targets (43). The results of the present study identified an upregulation of miR-1270 in the OP group. However, only miR-1270 expression was validated by RT-qPCR. This may be due to the lower sensitivity of the qPCR method compared to the microarray, or an incorrect microarray hybridization, which leads to erroneous signals.

Using a bioinformatics analysis, target genes for miR-1270 were identified and classified by a KEGG pathway analysis. In total 16 KEGG pathways were identified that were significantly associated with categories associated with bone metabolism. As CMCs are osteoclast precursors, the focus was on miR-1270 as it was the most promising signal and its expression was validated by RT-qPCR. The bioinformatics sequence analyses and literature searches of the present study identified several potential target genes of miR-1270 associated with monocytes, osteoclastogenesis and OP: *KRAS, FOSL2, MEF2C, IRF8, SP1, IKBKB, RASSF5, ABL1* and *STAT2*.

Of these genes, *IRF8* serves an important role in bone osteoclast differentiation (44). It is a gene that encodes a transcription factor that is expressed in immune cells, including monocytes (45). Mice deficient in *IRF8* are susceptible to severe OP, accompanied by higher numbers of osteoclasts (44). Furthermore, *IRF8* has been revealed to suppress osteoclastogenesis and *in vivo* bone remodeling, in part, by inhibiting the function of nuclear factor of activated T cells c1 (NFATc1) (40). A recent study provided evidence and further emphasized the importance of *IRF8* as a negative regulator of osteoclastogenesis (46). The observations of the present study demonstrate that miR-1270 may serve an important role in monocyte differentiation, regulating the expression of *IRF8*. In the same context, a recent report demonstrated that interferon-α1 (*IFN-α1*) mRNA is a target gene for miR-1270, revealing that *IFN-α1* antisense (AS) enhances the stability of *IFN-α1* mRNA. The data indicated that *IFN-α1* AS functions as a competing endogenous RNA to prevent miR-1270 from acting on *IFN-α1* mRNA (47).

This complex regulatory mechanism indicates a key role for the innate immune system in maintaining specific physiological type I *IFN* levels via post-transcriptional regulatory mechanisms, including miRNAs and implies the involvement of the interferon pathway in bone metabolism. Previously, it was reported that *IFN-α* and *IL-1* induce the activation of signaling molecules, including NF-κB and p38, which are key molecules in the process of the differentiation of monocytes-osteoclasts (48). It highlights the role of *type I IFN* in osteoclastogenesis, revealing that an uncontrolled activation of *IFN* signaling in Usp18-knockout mice increases osteoclast differentiation (49). However, additional studies are required to understand the role of *IFN-α1* in CMCs as osteoclast precursors.

BMD is the single best predictor and is currently the gold standard, for the diagnosis of OP (50). Several studies indicate that miR-21, miR-133a, miR-422a and miR-194-5p identified in CMCs could be considered as biomarkers for postmenopausal OP (16,17,51-53), however, the present study did not replicate those results. Furthermore, the discrepancies in the observed results may be associated with the study design, for example, the astringents methods used to select the miRNAs, the differences in the study populations, the

different experimental methods, the arrays and number of participants.

Finally, there were several limitations in the present study, including the sample size being relatively small (n=12), although this was similar to previously published studies (6,43,51-53). Furthermore, raw, rather than adjusted, P-values were used for the multiple comparisons which was in agreement with other studies and was mainly for avoiding further loss of power. In this context, the significant RT-qPCR P-value confirmed the differential expression of miR-1270 in the microarray analysis.

In conclusion, the results of the present study in combination with the functional role of *IRF8* in CMCs indicate that miRNA-1270 may be a viable biomarker for postmenopausal OP in a Mexican population. Nevertheless, further studies are required to validate these observations.

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