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Alteration of the microRNA expression profile in human osteosarcoma cells transfected with APE1 siRNA

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Apurinic/apyrimidinic endonuclease1 (APE1), which has the dual functions of DNA repair and redox regulation, is considered to be a promising potential target in cancer treatment. Microarray and qRT-PCR were used to confirm the change of miRNA followed by analysis with comprehensive bioinformatics-based analysis. Both microarray and qRT-PCR demonstrated that 13 microRNAs (miRNAs) were significantly changed (>2-fold) in APE1 knockdown HOS cells; seven of them (hsa-miR-451, hsa-miR-1290, hsa-miR-765, hsa-miR-483-5p, hsa-miR-513a-5p, hsa-miR-129-5p and hsa-miR-31) were up-regulated and the other six (hsa-miR-29b, hsa-miR-197, has-let-7b, hsa-miR-324-5p, hsa-let-7i and hsa-miR-484) were down-regulated. Furthermore, pathway analysis showed that these miRNAs and their target genes affected by the expression of APE1 were involved in pathways relating to developmental processes, regulation of cellular processes, cell signaling (such as TGF-β, Wnt, MAPK and the p53 signaling pathway) and cancers. There are putative binding sites of NF-κB, p53, HIF-1α, AP-1, PEBP2, ATF, NF-Y, Pax-2,CREB and c-Myb in the promoters of several down regulated miRNAs, indicating that APE1 may regulate miRNAs via transcription factors. Our data suggest that our understanding of the biological functions of APE1 will inevitably expand due to the novel pathways that APE1 uses to regulate gene expression through miRNAs.

Key words: APE1, microRNA, bioinformatics, microarray, osteosarcoma

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents and leads to the lowest survival probabilities in pediatric cancer patients [1]. Although survival rates of osteosarcoma have improved dramatically through the application of neoadjuvant chemotherapy followed by surgical resection during the late 20th century and by approximately 68% in recent years [2], the resistance to chemotherapy agents remains an important element interfering with therapeutic effect and prognosis [3,4]. Current standard chemotherapy of osteosarcoma is a combination of high dose methotrexate with cisplatin, doxorubicine and ifosfamide. Most of these agents are DNA damaging agents and therefore the DNA repair pathway acts as an essential mechanism for intrinsic drug resistance.

As a key gene in the base excise repair (BER) pathway, the multifunctional, apurinic/apyrimidinic endonuclease (APE1) plays a pivotal role in DNA repair and influences the sensitivity of tumor cells to chemotherapeutic agents [5]. It is not only responsible for the repair of DNA AP sites caused by oxidative and alkylation damage, but also functions as a reduction-oxidation (redox) factor maintaining the DNA binding activity of transcription factors such as p53, NF-κB, HIF-1, Myb, PAX, Egr-1 and AP-1 [6]. Previous studies have shown that aberrantly high expression of APE1, which is commonly found in a number of cancers including osteosarcoma, ovarian, cervical, prostate, lung cancer, rhabdomyosarcomas, and germ cell tumors, has been closely linked to poor prognosis and poor survival as well as resistance to DNA damaging agents like cisplatin and methyl methanesulfonate [6,7,8]. Knocking down APE1 expression in human osteosarcoma and colorectal cells by APE1 siRNA significantly enhanced cell sensitivity to DNA damaging agents and radiation [8,9,10]. However, although the essential function of APE1 has been confirmed, knowledge of its regulation or interactome is still scanty. To date, we have found several different regulatory mechanisms of the APE1 interactome including acetylation, phosphorylation, ubiquitination, and redox [11]. But all of these results do not yet fully explain the mechanism of how APE1 regulates other genes in apoptosis, angiogenesis and other various biological processes.

Ever since microRNAs (miRNAs) entered the gene network world, it provided a new basis for interpreting the interaction and regulation of genes. miRNAs are a class of endogenous single-stranded non-coding RNAs composed of 18-25 nucleotides [12]. Through suppressing gene expression by binding to the 3'-untranslated region (3'-UTR) of target genes, they perform a large range of biological functions in a variety of organisms and are involved in development, proliferation, differentiation, apoptosis and human diseases such as cancer [13,14]. The potential influence of miRNAs may be involved in almost every genetic pathway. Recent studies have shown that miRNAs can interact with important cancer-related genes. For example, hsa-miR-125a/b, hsa-miR-25 and hsa-miR-30d are known to target p53 and play an important role in the regulation of oncogenes [15-17]. Moreover, Lin et al reported that the hsa-miR-34s family are direct transcriptional targets of p53, reinforcing the growing awareness which places miRNAs in a central role in a well-known tumor-suppressor network [18]. However, until today, little reports concerning the interaction between APE1 and miRNAs are available in the current scientific literature. Recently, Kim et al reported that APE1 was able to directly cleave hsa-miR-21 and hsa-miR-10b in vitro, suggesting a novel connection between APE1 and miRNA [19]. We propose that APE1 is able to indirectly mediate miRNA expression via its downstream transcription factors or via miRNA-target interactions with itself. To test this issue, we used APE1 siRNA to suppress the expression of the APE1 gene in human HOS cells and observed changes in the miRNA expression profile by miRNA microarray and qRT-PCR. We used a bioinformatics approach to analyze and predict the role of APE1 in regulating miRNAs expression. Pathway analysis showed that miRNAs and their target genes that were influenced by APE1 expression were involved in the alteration of pathways including developmental processes, regulation of cellular processes, cell signaling pathways, regulation of gene expression, and cancer pathways. Our study demonstrated that the expression of miRNA was directly affected by APE1 siRNA in osteosarcoma cells, and that these processes might also provide a novel pathway that APE1 uses to regulate gene expression through miRNAs and a possible functional role for the APE1 interactome.

Materials and methods

Cell culture. Human osteogenic sarcoma (HOS) cells were purchased from Cell Culture Center, Institute of Basic Medical Sciences, CAMS and PUMC, Beijing, China. HOS cells were maintained in DMEM, which was supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

APE1 knockdown. Recombinant adenovirus vector Ad5/F35-APE1 siRNA and Ad5/F35-EGFP were constructed as described previously [10]. HOS cells were plated in six-well plates, transfected with Ad5/F35-APE1 siRNA or Ad5/F35-EGFP at 20 MOI for 4h and then washed to remove the adenoviruses. After 48h of culture, cells were harvested for further analysis.

Western blot. Western blot was used to confirm siRNA-mediated down-regulation of APE1 protein in HOS cells. Total protein was prepared by standard procedures and quantified by the Bradford method. Protein extracts (10 μg per sample) were run by 10% SDS-PAGE. After transferring and blocking, the membranes were incubated with mouse anti-APE1 (1:10,000, SantaCruz) and β-actin monoclonal antibody (1:2,000, Sigma). After washing, membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000, Pierce).

miRNA microarray assay. miRNA expression profiling was performed using Exiqon chip technology (Denmark). Ad5/F35-EGFP cells and Ad5/F35-APE1-siRNA cells were washed with pre-cooled PBS and total RNA was harvested using TRIzol (Invitrogen) and RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. After having passed RNA measurement on the Nanodrop instrument, the samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.11.0). The samples were hybridized on a hybridization station following the scheme outlined in the sample submission. Scanning was performed with the Axon GenePix 4000B microarray scanner. GenePix pro V6.0 was used to read the raw intensity of the image. The intensity of green signal was calculated after background subtraction and the median value of four replicated spots of each probe on the same slide were calculated. We used the Median Normalization Method to obtain "Normalized Data"; Normalized Data = (Foreground-Background)/median, the median was the 50 percent quartile of miRNA intensity which was larger than 50 in all samples after background correction. The clustering was performed on the normalized data of miRNA. Only those miRNAs with Foreground-Back ground intensities larger than 50 in at least one sample have been included.

qRT-PCR. The SYBR Green PCR Master Mix (Applied Biosystems Foster City, CA) was used to confirm the candidate miRNAs obtained from the microarray data which changed more than 2 fold. MiRNA RT Primer and PCR primers were synthesized by Invitrogen. The template miRNA cDNA was constructed from total RNA by reverse transcriptase. The U6 gene was used for normalizing each sample.

Target genes prediction. miRNAs which had been confirmed by qRT-PCR were picked for target genes prediction. In order to enhance the reliability of predicted targets, we conducted integrated analyses of three databases including MicroCosm v5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), TargetScan 6.2 (http://www.targetscan.org/) and MICRORNA.ORG (http://www.microrna.org/microrna/home.do).

Prediction of miRNA targeting APE1 3'UTR. MiRNA targets were predicted 4 different tools: miranda, pita, rnahybrid and targetscan, all of which were pre-computed and available within the miRecord database (http://mirecords.umn.edu/miRecords/). APE1-3'UTR sequence was retrieved from TargetScan 6.2 database (http://www.targetscan.org/).

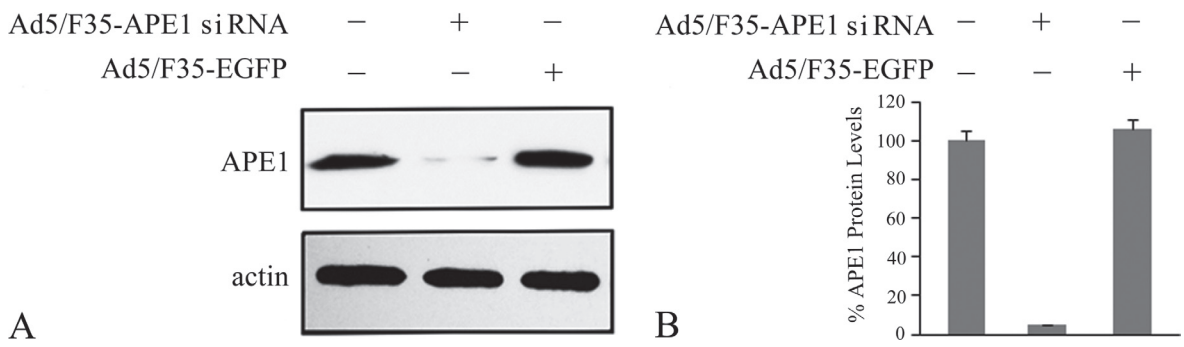


Figure 1. Knockdown of APE1 levels in HOS cells transfected with siRNA. A. Western blot. Samples were collected at 48 hours after APE1 siRNA treatment and Western blot was used to detect APE1 expression and reprobed with actin antibody as a loading control. B. Normalized APE1 levels after adjusting for loading.

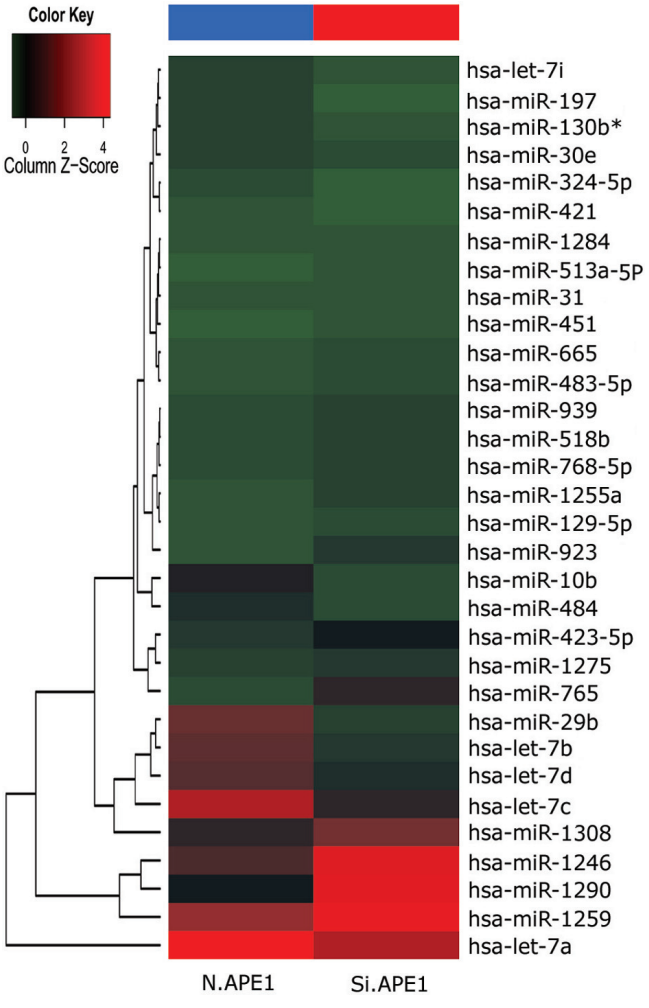


Figure 2. Hierarchical cluster analysis of miRNA expression. The heat map diagram shows the result of the two-way hierarchical clustering of genes and samples. Each row represents a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the bottom. The color scale shown at the top illustrates the relative expression level of a miRNA.

Prediction of transcription factor putative binding sites in the miRNA promoters. Previous data have demonstrated that APE1 functions as a reduction-oxidation (redox) factor maintaining the DNA binding activity of transcription factors such as p53, NF-κB, HIF-1α, HLF, PEBP2, ATF/CREB, Myb, Pax, Egr-1 and AP-1. The aberrant APE1 elevation was strongly correlated with the activation of these transcription factors. Therefore, we tried to identify the putative transcription factor binding regions in promoters of miRNAs.

Promoters (from 2500bp upstream to 500 bp downstream of TSS, transcription start site) [20,21] of differentially expressed miRNAs were retrieved from UCSC genome browser(http://genome.ucsc.edu/). The binding sites of transcription factors HIF-1a and PEBP2 were predicted using EMBOSS (http://helixweb.nih.gov/emboss/html/fuzznuc.html). The consensus sequences for HIF-1a and PEBP2 were [AG]CGTG and [AGT]ACC[AG]CA, respectively. For transcription factors other than HIF-1a or PEBP2, TRANSFAC 7.0 public database and the patser software (http://ural.wustl.edu/resources.html) were used for transcription factor binding site (TFBS) prediction. The cutoff of patser score was set to 0.9.

Network analysis. The network is generated by integrating TFBS and miRNA target prediction results. The results were saved in data files describing links between transcription factors and miRNAs and then handled in cytoscape software (http://cytoscape.org).

Pathway analysis. The predicted miRNA targets found to be up- or down-regulated by APE1 were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY Database (http://www.genome.jp/kegg/pathway.html) to uncover their association with molecular interaction and reaction networks.

Statistical analysis. Experimental results are expressed as mean values ± standard error. Statistical analysis for microarray data and qRT-PCR was performed with Student's *t* tests for comparison of two groups (SPSS software, version 13.0). Differences with $p < 0.05$ were considered significant.

Results

Confirmation of siRNA-mediated down-regulation of APE1 in HOS cells. The expression of APE1 protein after APE1 siRNA infection was examined by Western blot analysis. As shown in Fig.1, APE1 levels were decreased by 95% after siRNA infection for 48h as observed previously [10].

Down-regulating APE1 in HOS cells changes the miRNA expression profile. In this study, miRNA microarray was used to profile the changes of miRNA expression. The cluster analysis and a volcanl plot were applied to quickly identify the most-meaningful changes of miRNA and effectively target the most significant candidates for follow-up studies (Fig. 2 and Fig. 3). There were 32 miRNAs that changed >2-fold in the APE1 knocked-down HOS cells (Table 1). qRT-PCR was used to verify the results. For this approach, RNA was isolated from HOS cells treated with Ad5/F35-APE1 siRNA and Ad5/F35-EGFP in three independent experiments. The quantitative analysis demonstrated that 13 miRNAs showed statistically significant changes (>2 fold) in expression levels following knock-down of APE1; 7 of them were up-regulated (hsa-miR-451, hsa-miR-1290, hsa-miR-765, hsa-miR-483-5p, hsa-miR-513a-5p, hsa-miR-129-5p and hsa-miR-31) and the other 6 miRNAs were down-regulated (hsa-miR-29b, hsa-miR-197, hsa-let-7b, hsa-miR-324-5p, hsa-let-7i and hsa-miR-484) (Table 2).

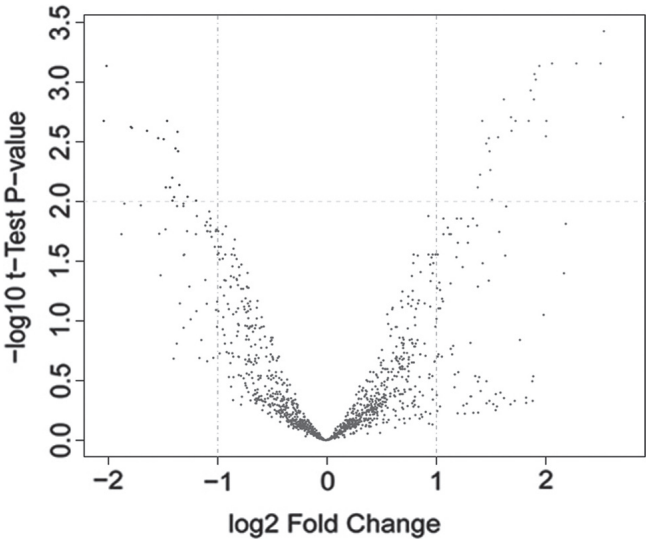


Figure 3. A volcano plot shows the significance miRNAs in APE1 knockdown HOS cells. The horizontal axis represents the fold change between normal HOS cells and APE1 knockdown HOS cells. The vertical axis represents the p-value of the t-test for the differences between samples (p<0.01).

Bioinformatics analysis of predicted target genes. To discover the effect of APE1 on miRNA expression profiles in osteosarcoma HOS cells, we predicted and analyzed the target genes of significant miRNAs by bioinformatics. In our study, there were 3617 predicted target genes of up-regulated miRNAs and 1781 predicted targets of down-regulated miRNAs (Table 3).

Transcription factors binding sites. Generally, TFBS are 8-15 bp long and located in upstream regions near the TSS. We searched for potential TFBS in the flanking regions of

Table 1. miRNAs differentially expressed in APE1 siRNA transfected HOS cells and normal controls

Up regulation of miRNAs	Fold(>2)	p-value	Down regulation of miRNAs	Fold (<0.5)	p-value
hsa-miR-923	7.68	0.0000	hsa-miR-29b	0.29	0.0000
hsa-miR-451	7.21	0.0000	hsa-miR-197	0.31	0.0000
hsa-miR-1290	5.50	0.0000	hsa-let-7b	0.36	0.0004
hsa-miR-765	5.19	0.0002	hsa-miR-324-5p	0.39	0.0011
hsa-miR-483-5p	4.21	0.0000	hsa-miR-421	0.43	0.0013
hsa-miR-513a-5p	3.02	0.0005	hsa-miR-10b	0.44	0.0005
hsa-miR-1246	2.61	0.0012	hsa-let-7i	0.44	0.0000
hsa-miR-768-5p	2.61	0.0006	hsa-miR-130b*	0.45	0.0021
hsa-miR-665	2.53	0.0000	hsa-let-7c	0.46	0.0000
hsa-miR-518b	2.41	0.0026	hsa-let-7a	0.47	0.0000
hsa-miR-129-5p	2.39	0.0019	hsa-let-7d	0.47	0.0001
hsa-miR-1255a	2.35	0.0021	hsa-miR-484	0.49	0.0006
hsa-miR-423-5p	2.25	0.0000			
hsa-miR-1284	2.13	0.0000			
hsa-miR-939	2.08	0.0002			
hsa-miR-1275	2.06	0.0033			
hsa-miR-1259	2.05	0.0001			
hsa-miR-1308	2.01	0.0000			
hsa-miR-31	2.01	0.0002			
hsa-miR-30e*	2.00	0.0018			

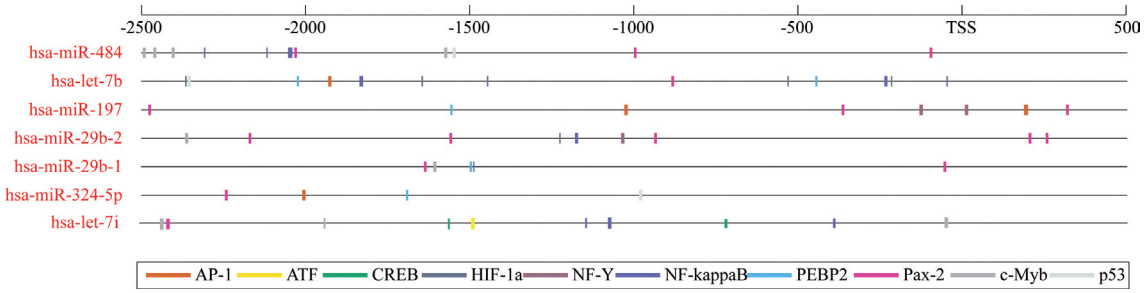


Figure 4. Putative transcription factors binding sites in down-regulated miRNAs promoters.

clustered and unclustered miRNAs to define their positional preferences and their class representation. TFBS can also be used to locate the core promoters of miRNA genes. In order to investigate the potential function of transcription factors mediated by APE1 in the regulation of miRNAs expression, the potential binding sites of miRNA promoters were analyzed using a bioinformatics approach. Because down-regulation of APE1 was strongly correlated with the decreased activation of transcription factors, down-regulated miRNAs after APE1 siRNA were chosen to identify the putative binding sites. Intriguingly, in six of the down-regulated miRNAs (Table 2), we found there are putative binding sites of NF-κB, p53, HIF-1α, AP-1, PEBP2, ATF, NF-Y, Pax-2,CREB and c-Myb in the promoters of several down regulated miRNAs (Fig 4 and Supplement Table 1).

MiRNA target to APE1-3'UTR. We then performed analysis of potential binding sequences for these ten miRNAs, miR-484, miR-513a-5p, miR-765, miR-324-5p, miR-451, miR-483-5p, miR-197, miR-let-7i, miR-let-7b and miR-29b, in the 3'-UTR of APE1 mRNA and identified one putative binding site. site (the data not yet published). The „seed match" sequence of miR matched 4/9 nucleotides in positions 21-1112. The seed match sequence is important in guiding miRNA to specifically bind to the 3'complementary sequences in mRNA, and some upstream sequences may be also involved in binding. (Supplement Table 2)

Gene network analysis. We used the bioinformatics soft to generate a network from experimentally verified interactions of the biologically active, validated, down regulated miRNAs, transcription factors and APE1 (Supplement Figure 1). We further explored the regulation of the differentially regulated miRNAs by examining the interactions with the validated transcription factors at the predicted TFBS (Supplement Table 1), and found that 10 transcription factors interacted with the experimentally validated miRNAs (Supplement Figure 1). The interaction networks demonstrated a complex role for miRNAs in human osteosarcoma cells. Since miRNAs usually inhibit mRNA expression of their target genes, we expected that target mRNAs of the miRNAs down regulated in human osteosarcoma cells would be upregulated; several gene targets exhibited expression levels consistent with this expectation (shown in Table 4).

Table 2. Identification of differentially regulated miRNAs by qRT-PCR

miRNAs	Sequence 5'→3'	qRT-PCR (APE1 siRNA/N)	p value
Upregulated miRNAs			
hsa-miR-451	AAACCGTTACCATTACTGAGTT	8.01	0.0000
hsa-miR-1290	tGGAtttttGGAtCAGGGA	4.62	0.0000
hsa-miR-765	tGGAGGAGAAGGAAGGtGAtG	6.28	0.0002
hsa-miR-483-5p	AAGACGGGAGGAAAGAAGGGAG	5.54	0.0000
hsa-miR-513a-5p	ttCACAGGGAGGtGtCAt	2.89	0.0001
hsa-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC	2.88	0.0019
hsa-miR-31	AGGCAAGAtGCtGGCAtAGCt	2.31	0.0000
Downregulated miRNAs			
hsa-miR-29b	TAGCACCATTTGAAATCAGTGTT	0.39	0.0000
hsa-miR-197	ttCACCACCttCtCCACCCAGC	0.44	0.0003
hsa-let-7b	tGAGGtAGtAGGttGtGtGGtt	0.46	0.0005
hsa-miR-324-5p	CGCAtCCCCtAGGGCAttGGtGt	0.48	0.0008
hsa-let-7i	TGAGGTAGTAGTTTGTGCTGTT	0.49	0.0006
hsa-miR-484	tCAGGCtCAGtCCCCtCCCGAt	0.43	0.0000

Supplement Table 1. Putative transcription factors binding sites in down-regulated miRNAs promoters

Transcription factors	score	pval	hit_start	Down regulated miRNAs	hit_seq
Pax-2	0.95238	0.000225	1614(+)		CCGAAACCC
p53	9.64E-01	1.14E-05	143(-)		AGACAAGTCT
NF-kappaB	0.90741	1.14E-05	2262(-)		GGGGCTCCCC
NF-kappaB	9.41E-01	9.56E-07	665(-)		TGGGAAGTCCCT
AP-1	0.90265	6.19E-05	570(-)		CCTCAGTCACC
HIF-1a	1	0	135(+)		GCGTG
HIF-1a	1	0	854(+)	hsa-let-7b	ACGTG
HIF-1a	1	0	1053(+)		GCGTG
HIF-1a	1	0	1967(+)		GCGTG
HIF-1a	1	0	2282(+)		ACGTG
HIF-1a	1	0	2451(+)		ACGTG
PEBP2	1	0	475(+)		AACCACA
PEBP2	1	0	2052(+)		GACCGCA
Pax-2	0.98214	3.04E-05	861(-)		GAGTTTGTG
Pax-2	0.99405	1.51E-05	2442(-)		GGGTTTATT
c-Myb	9.38E-01	6.84E-05	890(+)	hsa-miR-29b-1	GATAACTGGC
HIF-1a	1	0	1011(+)		ACGTG
PEBP2	1	0	1001(+)		AACCACA
Pax-2	0.95833	0.000145	328(-)		GTGTTTAAT
Pax-2	0.95833	0.000145	939(+)		CTTAAACAC
Pax-2	0.95833	0.000145	1562(+)		ATTAAACAC
NF-Y	0.9026	3.72E-05	1461(-)	hsa-miR-29b-2	TTGATTGGTTG
NF-kappaB	9.33E-01	9.54E-06	1321(+)		AGGAATTTCC
c-Myb	9.38E-01	6.84E-05	135(+)		ACTAACTGGC
HIF-1a	1	0	1273(+)		ACGTG
Pax-2	0.97619	6.07E-05	256(-)		GAGTTTCTT
p53	9.16E-01	7.05E-05	1516(-)	hsa-miR-324-5p	GGACAAGTCC
AP-1	9.20E-01	2.67E-05	491(-)		TTTTAGTCACT
PEBP2	1	0	807(+)		GACCACA
Pax-2	0.95238	0.000225	2400(-)		GAGTTTTTT
Pax-2	0.96429	0.000126	467(-)		GGGTTTATC
Pax-2	0.96429	0.000126	1500(-)		GAGTTTATA
p53	9.13E-01	8.19E-05	949(+)		TGGCTTGTCT
NF-kappaB	0.94167	1.50E-06	448(-)		CGGGAAAGTCCCTA
c-Myb	9.07E-01	0.000218	6(-)	hsa-miR-484	TCCAGTTGAG
c-Myb	9.07E-01	0.000218	38(+)		GCCAACTGGG
c-Myb	9.07E-01	0.000218	94(-)		GGCAGTTATT
c-Myb	9.79E-01	8.63E-06	923(-)		GCCAGTTGGG
HIF-1a	1	0	192(+)		ACGTG
HIF-1a	1	0	382(-)		ACGTG
Pax-2	0.96429	0.000126	23(-)		GAGTTTCAG
Pax-2	0.97024	7.26E-05	2132(+)		CTCAAACTC
NF-Y	0.9026	3.72E-05	2369(+)	hsa-miR-197	GAACCAATAAG
AP-1	9.12E-01	3.83E-05	1471(+)		TGTGACTCAAT
PEBP2	1	0	942(+)		AACCACA
Pax-2	0.9881	2.28E-05	2402(-)		GAGTTTAAT
NF-kappaB	0.91358	6.66E-06	1188(+/-)		GGGAATTCCC
NF-kappaB	1	9.56E-07	371(+)		GGGGATTCCC
NF-kappaB	0.91589	2.08E-05	1150(+)		GGGAAGTCCC
NF-kappaB	0.93333	2.13E-06	1148(-)		CCGGGAAGTCCCC
c-Myb	0.90722	0.00021822	1949(-)	has-let-7i	GGAAGTTAAC
c-Myb	0.91753	0.00014923	2427(+)		AATAACTGGC
c-Myb	0.91753	0.00014923	31(-)		GCCAGTTCTG
ATF	0.91346	1.51E-05	1472(-)		GGCAGGCGTCAGCG
CREB	0.90099	0.00015225	739(-)		GTACGTCA
CREB	0.94262	1.35E-05	1525(-)		CCACGTCACCAG

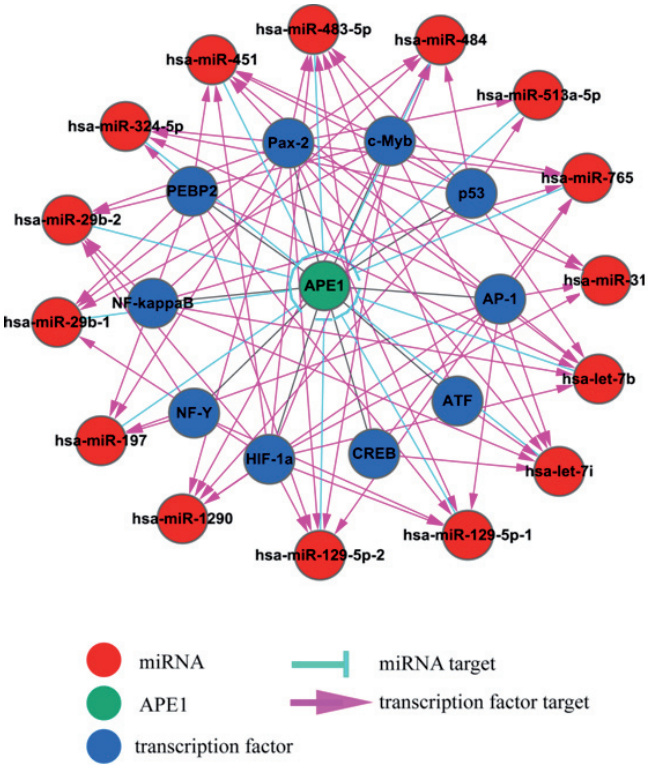
Supplement Table 2. Putative differentially miRNAs binding sites in APE1

miRNA	Position	Site
hsa-miR-484	21-27 of APE1 3'UTR	5' ...CCCCUAAAUCACUUUGAGCCUGG... | | | | | | | 3' UAGCCCUCCCCUGACUCGGACU
hsa-miR-513a-5p	129-135 of APE1 3'UTR	5' ...UCCUCCAACCAGGCUCCUGUGAU... | | | | | | | 3' UACUGUGGAGGGACACUU
hsa-miR-765	114-120 of APE1 3'UTR	5' ...GUAUAAAACUAGGAAUCCUCCAA... | | | | | | 3' GUAGUGGAAGGAAGAGGAGGU
hsa-miR-324-5p	1038-1042 of APE1	5' ...AGCGCCAAGGCUUCGGGGAAUUACUGCA... | | | | | 3' UGUGGUUACGGGAUCCCCUACGC
hsa-miR-451	285-289 of APE1	5' ...CUUCGGUGGGUGACGCGGUA... | | | | 3' UUGAGUCAUUACCAUUGCCAAA
hsa-miR-483-5p	93-102 of APE1	5' ...ACCCUUCUUUGUGCUCGG... | | | | | | | | | 3' GAGGGAAGAAAGGAGGGCAGAA
hsa-miR-197	340-345 of APE1	5'...AGCGUGGGAAAAAGGGAGCGGUGGCGGAAG... | | | | | 3' CGACCCACCUCUUCCACCACUU
hsa-let-7i	298-302 of APE1	5' ...CGCGGUACAGCUGCCCAA... | | | | 3' UUGUCGUGUUUGAUGAUGGAGU
hsa-let-7b	1107-1112 of	5' ...ACACACCCUAUGCCUACACCUUU... | | | | | 3' UUGGUGUGUUGGAUGAUGGAGU
hsa-miR-29b	125-130 of	5' ...GGCUGCCAUCGGGCCGGUGCA... | | | | | 3' UUGUGACUAAAGUUUACCACGAU

Table 3. Validated and predicted target genes of differentially regulated miRNAs

miRNAs	Location	Validated Target	Predicted Target
hsa-miR-451	chr 17: 27188387-27188458	ABCB1, MIF,CAB39	20
hsa-miR-1290	chr 1:19221565-19225642	/	133
hsa-miR-765	chr 1:156905923-156906036	NTRK3	364
hsa-miR-483-5p	chr 10:28246127-28246150	/	149
hsa-miR-513a-5p	chr X: 146294981-146295109	CD274	904
hsa-miR-129-5p	chr 7:127845925-127849996	NOTCH1, EIF2C3, CAMTA1	390
has-miR-31	chr 9:21510114-21514184	RHOA, LATS2, PPP2R2A, CXCL12, KLF13, FIH, DMD, APRC5, MPRIP, MMP16, FOXP3, HOXC13, TIAM1, JAZF1, NUMB, RET, NFAT5, FZD3, RDX, YY1, ITGA5	368
hsa-miR-29b	chr 7:130560218-130564298	DNAJB11, SFPQ, CL1A, MCL1, CDK6, SP1, BACE1, DNMT3A, DNMT3B, NID1, COL3A1, TET1, S100B, VEGFA, DNMT1, COL1A1, MMp15, MMP24, FGG, COL4A1, ADAM12, GRN, MMP2, BCL2, FGA, FGB	725
hsa-miR-197	chr 1:110139515-110143589	FUS1	217
hsa-let-7b	chr 22:46507566-46511648	LIN28B, CDK6, CCND1, CDC25A, RPIA, HMGA2, CCND2, PRDM1, LIN28A, NRAS, CCNA2, ACTG1, CDC34, IFNB1, NR2E1, RDH10	577
hsa-miR-324-5p	chr 17:7124616-7128698	GLI1, SMO	141
hsa-let-7i	chr 12:62995466-62999549	TLR4	24
hsa-miR-484	chr 16:15735151-15739229	/	97

Target genes pathway analysis. Pathway analysis by KEGG was used to uncover their association with molecular interaction and reaction networks. According to the results of data mining, we obtained lots of up-regulated pathways and down-regulated pathways ($p<0.01$) (Table 4). Among all the differentially regulated pathways, several signaling pathways (e.g., TGF-β, Wnt, MAPK and p53) including cancer pathways appeared to be most affected. It suggested that APE1 might participate in these signaling pathways and the development of cancer by interaction with miRNAs. This represents novel evidence for the regulation by APE1 of signaling pathways via miRNAs.



Supplement Figure 1 The network among APE1, transcription factors and differentially regulated miRNAs. Red circle, the differentially regulated miRNAs; Green circle, APE1 protein; Blue circle, the transcription factors regulated by APE1; Blue line, miRNAs were predicted to target APE1; Red line, the transcription factors have been indentified to regulate miRNAs; black line, transcription factors regulated by APE1

Discussion

miRNAs are an abundant class of small non-protein-coding RNAs that function as negative gene regulators. With our growing understanding of biogenesis and biological functions of miRNAs, more evidence has indicated that they can repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer [22]. Although only a few reports have investigated the role of miRNA in osteosarcoma, previous studies using different osteosarcoma tissue samples and miRNA microarray assay identified several differentially expressed miRNAs, including miR-99b [23], miR-143 [24], miR-652 [25] that were identified to be down-regulated and related to metastasis, apoptosis, and tumorigenesis in osteosarcoma. In the osteosarcoma cell line U2OS, exogenous overexpression of miR-31 inhibited proliferation and induced apoptosis [26]. In addition, miR-99b and miR-31 were shown to target pathways with importance to tumor behaviors, including TGF-β, Wnt, MAPK, and the p53 pathways [23]. Hence, these studies suggest that miRNAs could be considered as novel osteosarcoma markers and potential therapeutic targets [23, 27]. We found similar results in our microarray profile. miR-99b, miR-143 and miR-652 were down-regulated 1.89 fold, 1.61 fold, and 1.56 fold, respectively and miR-31 up-regulated more than 2 fold in HOS cells after APE1 knockdown. In contrast, we analyzed the global miRNA expression profile in APE1 knockdown human cervical cancer HeLa cells. The above miRNAs, except for miR-143, were down-regulated more than 2 fold in the APE1 knockdown HeLa cells when compared to wild type HeLa cells (unpublished data). These results suggest that miR-143 exhibits tissue-specific expression, while miR-99b, miR-31 and miR-652 were APE1 regulated miRNAs. We interpreted the association between the APE1 expression level and osteosarcoma-specific miRNAs to be due to a critical role that APE1 plays in osteosarcoma development and progression. Therefore, these results provide more evidence to validate new functions for APE1 in osteosarcoma.

Increasing evidence has indicated that miRNAs are involved deeply in gene regulation. The potential influence of miRNAs may impact almost every genetic pathway [28]. For investigating the interaction between APE1 and miRNAs, we used a miRNA high-throughput screening assay to analyze the differentially expressed miRNAs in the presence and absence of APE1. As APE1 expression is ubiquitous in almost every human tissue and organ, the APE1 knock-down model of HOS cells by RNAi was designed for this approach. By controlling the expression of the APE1 gene, we observed 13 miRNA expression changes and we further confirmed these findings by quantitative real-time RT-PCR. In general, the up-regulated miRNAs may target genes to reduce their protein expression. In our study, among the seven up-regulated miRNAs, miR-451 was the most elevated, more than 7 fold in APE1 knock-down HOS cells. Previous studies have found that miR-451 can increase sensitivity of cells to doxorubicine in ovarian cancer [29] and breast cancer [30] by targeting the multidrug resistance gene MDR1. Another study reported by Chattopadhyay et al showed that acetylated APE1 enhanced Y-box-binding protein 1 (YB-1) expression, leading to the activation of MDR1 [31]. According APE1 has the miRNA cleaving properties [19], therefore, the result indicated that APE1 may directly or indirectly mediate miR-451 to establish a novel pathway in overcoming cell resistance, making it a potential target for the

Table 4. Significant pathways analysis of miRNAs target genes (partial data)

Pathway title	gene in pathway	p value	Symbol
regulated by up-regulated miRNAs			
TGF-β signaling pathway	24	0.0000	EP300, TNF, CREBBP, BMP2, CDKN2B, ID2, BMPR2, E2F5, SMAD6, BMPR1, AGDF6, RPS6KB1, PPP2R1B, RHOA, SMAD7, TGFBR1, BMP4, ID4, TFDP1, CHRD, THBS3, NOG, GDF5, PPP2R1A
Adherens junction	22	0.0000	EP300, FYN, CREBBP, CSNK2A2, WAS, TCF7, NLK, SRC, CTNND1, ACTN1, PVRL4, PVRL1, PTPRB, WASF2, SSX2IP, TGFBR1, CTNNA2, WASF3, PVRL2, IGF1R, RHOA FGFR1
Axon guidance	30	0.0001	EFNB1, EPHA4, RGS3, EPHB3, EPHB1, SEMA3F, NRP1, EFNA5, NGEF, EPHA6, SEMA4C, SRGAP1, PLXNB1,EFNA3, PAK6, EPHA8, EPHB2, ABLIM1, DPYSL2, FYN, ABLIM2, GNAI2, RHOA, SRGAP2, DPYSL5, SEMA6A, SEMA4G, NFATC2, FES, PAK7
Wnt signaling pathway	31	0.0009	EP300, SFRP2, CREBBP, WNT5A, CTBP2, CSNK2A2, CER1, PPARD, CTNNBIP1, NLK, PPP2R5B, NFATC2, VANGL1, CTBP1, TBL1XR1, PRICKLE2, PPP2R1B, CAMK2D, WIF1, AMK2G, PPP2R1A, PLCB1, TBL1X, LRP6, AXIN2, TCF7, APC, FRAT1, SIAH1, FZD5RHOA
Long-term potentiation	17	0.0020	EP300, ARAF, CREBBP, PPP1CB, GRIN2A, CALM2, CALM1, RPS6KA3, CACNA1C, CAMK4, CAMK2D, CAMK2G, PLCB1, GRM1, RAP1B, GNAQ, CALM3
Regulated by down-regulated miRNAs			
Small cell lung cancer	19	0.0000	LAMA2, PTEN, CYCS, CCNE1, RARB, CDK6, PIAS4, BIRC2, LAMC1, COL4A2, COL4A4, BCL2L1, COL4A1, LAMC2, TRAF4, IK3R1AKT3, COL4A6, E2F2
Pathways in cancer	42	0.0000	MMP2, IGF1, LAMA2, PTEN, CYCS, FGF11, CCNE1, FAS, TPM3, ARNT, RARB, NRAS, CDK6, PDGFRB, TGFBR1, CDC42, ACVR1C, EGFA, BIRC2, AMC1, CASP3, PPARD, FASLG, WNT1, COL4A2, TGFB3, COL4A4, FZD5, FOS, TRAF4, IGF1R, BCL2L1, E2F2, FZD4, COL4A6, COL4A1, ACVR1B, AMC2, PIAS4, IK3R1, AKT3, DAPK1
TGF-β signaling pathway	14	0.0016	GDF6, ID1, ACVR2A, TGFBR1, ACVR1C, E2F5, TGFB3, SP1, IFNG, ZFYV, E16, ACVR2B, ACVR1B, CHRD, SMAD6
p53 signaling pathway	11	0.0054	IGF1, PTEN, CYCS, BBC3, CCNE1, FAS, STEAP3, CDK6, CASP3, CND2, PPM1D
Pancreatic cancer	11	0.0074	CDK6, TGFBR1, CDC42, ACVR1C, VEGFA, TGFB3, BCL2L1, E2F2, ACVR1B, PIK3R1, AKT3

drug sensitization in osteosarcoma. Currently, further studies attempting to discover the mechanism of action on miR-451 and APE1 are underway in our laboratory.

In addition, based on previous studies which have demonstrated that down expression of APE1 was strongly correlated with the depressed activation of transcription factors [32], we presumed that the down-regulated miRNAs may be regulated by transcription factors via binding in promoters regions of miRNAs. The potential binding sites of miRNA promoters analyzed by bioinformatics suggested that APE1 may indirectly control the down-regulated let-7b, miR-324-5p, let-7i and miR-484 via AP-1, NF-κB and c-Myb, respectively. Based on the bioinformatics tool, miR-29b which has been reported to be suppressed by NF-κB and c-Myc via promoter region binding [33] was not predicted in our study. Moreover we still need more evidence to confirm the possible effect of APE1 on expression of let-7b, let-7i, miR-324-5p and miR-484 via transcription factors. Network showed there were also several genes known to be regulated by these miRNAs whose expression was decreased in human osteosarcoma cells (Supplement Figure 1), such as κB, p53, c-Myb, HIF-1α and AP-1 et al.,which are involved in tumorigenesis and tumor progress [34] .

In order to understand the function of miRNAs, bioinformatics analysis of the 13 miRNAs on their target genes was used as evidence to support the APE1-mediated regulation of miRNAs. KEGG pathway annotation was applied to the target gene pool. The results showed that important proliferative (MAPK, Wnt and ErbB), survival (p53, TGF-β and mTOR), adhesive (adherens junction, focal adhesion and ECM-receptor interaction), oncogenic (prostate cancer, glioma, small cell lung cancer, colorectal cancer, melanoma and pancreatic cancer) and neural development (axon guidance, long-term potentiation and neurotrophin signaling pathway) signaling pathways were abundant among the significantly enhanced pathways. Some of them have already been reported to take part in an APE1-genes-network. For example, APE1 has been identified to take part in the p53 signaling pathway [35,36] and in cancer development [10,37-40]. Small interfering RNA (siRNA) knockdown of endogenous APE1 impaired HMGB1-mediated cytokine expression and MAPK activation in THP-1 cells [41]. Furthermore, noteworthy, Wang et al have reported

the altered gene profile of HOS cells by gene array after knockdown of APE1 and verified that MAPK14 and TGF-β1 were increased significantly [42]. Likewise, and interestingly, our study is the first to show that APE1 contributes to the Wnt and mTOR signaling pathways. These results not only support earlier findings, but also provide a new explanation of APE1-related gene regulation pathways.

In conclusion, our data provide evidence that APE1 appears to have a direct influence on global miRNA expression, especially with regard to the tissue specific miRNAs in human osteosarcoma cells. It seems that APE1 may down regulate miRNAs *via* interaction with transcription factors. Certainly, there are several issues to be resolved in further studies. For instance, miRNAs which target APE1 should be identified and the binding sites of NF-κB, p53, HIF-1α, AP-1, PEBP2, ATF, NF-Y, Pax-2, CREB and c-Myb in miRNA promoters also should be investigated. In addition, more experiments should be carried out to fully explain the relationship between APE1 and its potentially targeted pathways.

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