Biochemical and Biophysical Research Communications 446 (2014) 267–271

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transcriptional regulation of miR-146b by C/EBPβ LAP2 in esophageal cancer cells

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ARTICLE INFO

Article history:
Received 15 February 2014
Available online 28 February 2014

Keywords:
microRNA
miR-146b
C/EBPβ
ESCC

ABSTRACT

Recent clinical study indicated that up-regulation of miR-146b was associated with poor overall survival of patients in esophageal squamous cell carcinoma. However, the underlying mechanism of miR-146b dysregulation remains to be explored. Here we report that miR-146b promotes cell proliferation and inhibits cell apoptosis in esophageal cancer cell lines. Mechanismly, two C/EBPβ binding motifs are located in the miR-146b promoter conserved region. Among the three isoforms of C/EBPβ, C/EBPβ LAP2 positively regulated miR-146b expression and increases miR-146b levels in a dose-dependent manner through transcription activation of miR-146b gene. Together, these results suggest a miR-146b regulatory mechanism involving C/EBPβ, which may contribute to the up-regulation of miR-146b in esophageal squamous cell carcinoma.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) has been generally considered as one of the most aggressive cancers with poor prognosis, characterized by high incidence and mortality rate [1]. MicroRNA (miRNA) is a small, non-coding RNA that negatively regulates gene expression mainly via translational repression. Recently, the association of miRNAs with cancer progression has been established in many cancers including ESCC [2–5].microRNA profiling has shown that miR-146b is overexpressed in many cancer types, such as thyroid tumor, breast cancer, colorectal cancer and melanoma [6–12]. In lung cancer, the expression patterns were extraordinary conflicting by two different research groups [13,14]. In ESCC, miR-146b expression is independent risk factor for prognosis. Patients with higher miR-146b expression levels had significantly poorer overall survival compared with those with lower miR-146b levels [2,15]. Although miR-146b has great diagnostic value in predicting outcome of ESCC for miR-146b up-regulation was associated with poor overall survival rate, the biological role for miR-146b in esophageal cancer cells and the underlying mechanism of miR-146b dysregulation are still obscure.

The CCAAT/enhancer binding protein β (C/EBPβ) is a transcription factor implicated in many biological activities including tumor progression. In our prior study, we found C/EBPβ was aberrantly expressed in ESCC tissues and was associated with metastasis (unpublished papers). C/EBPβ exists in three isoforms: LAP1, LAP2 and LIP. LAP1 and LAP2 are transcriptional activators, whereas LIP frequently acts as a dominant-negative inhibitor of transcription [16].

In the present study, we investigated the role of miR-146b on proliferation and apoptosis in esophageal cancer cells. Next, we explored the regulatory role of C/EBPβ LAP2 on miR-146b gene expression.

2. Materials and methods

2.1. Cell culture and transfection

Human esophageal cancer cell line EC109 was purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. Cells were maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C in the 5% CO_2 humidified atmosphere. Cells were transfected with plasmid DNA, miR-146b-3p mimics (GeneCopoeia, Guangzhou, China) by

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http://dx.doi.org/10.1016/j.bbrc.2014.02.096
0006-291X/

using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

2.2. Plasmid construction

Expression vectors encoding pcDNA3-LAP1, pcDNA3-LAP2 and pcDNA3-LIP were constructed as previously described [17]. For reporter assay, the non-coding upstream regulatory region (nt-1786/-1384, including the predicted C/EBPβ recognition sites) of *mir-146b* gene was PCR-amplified from human genomic DNA and cloned into pGL3-promoter dual-luciferase reporter plasmid (Promega, Madison, WI, USA) to generate pGL3-promoter-146b (CNS-Luc). The regulatory element mutant reporter, designated as mCNS-Luc, was created by mutating the core binding region of the predicted C/EBPβ sites (CCATTGCACAATAC to CCATTATATAATAC, TCCTTTCCCAACTC to TCCATTACCAATTC, respectively) by nested PCR. All primers are listed in Table 1.

2.3. ChIP assay

Quantitative ChIP assays were performed using a commercially available Chromatin Immunoprecipitation Kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, cells (2.5×10^6/immunoprecipitation) were treated with 1% (w/v) formaldehyde for 10 min at room temperature, and cross-linking was halted with 0.125 M glycine. The cross-linked chromatin was then sonicated and immunoprecipitated using rabbit polyclonal anti-C/EBPβ antibody or rabbit polyclonal anti-IgG antibody. After the reversal of cross-linking, the precipitated DNA was analyzed by PCR (35 cycles) with two primer pairs (R1 and R2) for the regions of the miR-146b loci containing two potential C/EBPβ binding sites and with two negative primer pairs (Neg.1 and Neg.2) for the regions without C/EBPβ binding sites. All primers are listed in Table 1.

2.4. Luciferase reporter assay

For luciferase reporter assay, HEK 293FT cells (2.0×10^5 cells per well) were plated in a 24-well plate (Corning) 24 h before transfection. Cells were cotransfected with 1 ng of Renilla luciferase reporter pRL-TK, 100 ng of either the CNS-Luc or mCNS-Luc, and 20 ng of either pcDNA3-LIP or empty vector pcDNA3.1. After a transfection of 24 h, cells were lysed in Passive Lysis Buffer (Promega) and activities of Firefly and Renilla luciferase were measured with a GloMax20/20 Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocols.

Table 1
Primers used in this study.

Primer name	Sequence
For ChIP assay	
R1 forward	AGAGACCAGCCATCCCTTTC
R1 reverse	CAGGGCAGCTCTACTGAGGA
R2 forward	CTGAGCTTTCAAGGGAGGAG
R2 reverse	GTGTCTGTGGGGTTCCCTCT
Neg.1 forward	TTGGAGCAGTGCCTGATACATT
Neg.1 reverse	TCAAAGTCCAACAGCCAGAAAA
Neg.2 forward	TCTATGTTTTTGGGGAGGGAGA
Neg.2 reverse	AATGAATGGGATTGGCCATAAG
For reporter assay	
CNS-Luc forward	GAGGCAGCAGAGACCGAGAG ACCAGCCATCCCTTTC
CNS-Luc reverse	CGAACAGAGAGAGACCGCAG GGCAGCTCTACTGAGGA
mCNS-Luc-55 M reverse	CATCAGTATTATATAATGGACA GGGGGAAGCAG
mCNS-Luc-65 M forward	CATTATATAATACTGATGACTC TTCTCTCCCTACC
mCNS-Luc-296 M reverse	AGTTTGGGACTCCCCCTAAGAAGTCTT
mCNS-Luc-309 M forward	GAGTCCCAAACTCTGGGGAGTCCCA
For miR-146b overexpression	
miR-146b-3p mimics sense	UGCCCUGUGGACUCAGUUCUGG
miR-146b-3p mimics antisense	AGAACUGAGUCCACAGGGCAUU
Mimic control sense	UUCUCCGAACGUGUCACGUTT
Mimic control antisense	ACGUGACACGUUCGGAGAATT

2.5. RNA isolation and miRNA detection

Total RNA was extracted from the adherent cultured EC109 cells using RNAiso Plus reagent (TaKaRa, Dalian, China) according to the manufacturer's directions. The concentration of total RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific).

Expression of mature miR-146b was assayed using the All-in-One miRNA qRT-PCR detection kit according to the manufacturer's instructions (GeneCopoeia). Relative miRNA expression level was analyzed using the comparative Ct method [18], with U6 snRNA (GeneCopoeia) as the internal control for normalization.

2.6. EdU proliferation assay

Proliferating EC109 cells were determined by using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) according to the manufacturer's protocol. Briefly, cells were incubated with 10 μM EdU for 3 h before fixation, permeabilization, and EdU staining. Cell nuclei were stained with Hoechst 33342 (Invitrogen) at a concentration of 5 μg/ml for 30 min.

2.7. Statistical analysis

The results were presented as the mean ± standard error (S.E.M.), and each experiment was performed at least three times. Student's *t*-test analysis was used to compare the difference between two different groups. A probability value of $P < 0.05$ was defined as statistical significance.

3. Results

3.1. Overexpression of miR-146b promotes cell proliferation and inhibits cell apoptosis

Since miR-146b was closely related with clinicopathologic factors and survival, we first investigated the biological function of miR-146b in ESCC. To determine whether miR-146b could modulate cell proliferation and apoptosis, ESCC cell line EC109 cells were transfected with miRNA mimic control or miR-146b mimics 24 h before harvested. EdU incorporation experiments were performed to assess cell proliferation. As shown in Fig. 1A, miR-146b mimics increased miR-146b level in EC109 cells. Importantly, miR-146b mimics accelerated proliferation of EC109 cells (Fig. 1C, left). Quantitative analysis demonstrated that this change is statistically significant (Fig. 1C, right). Next, effect of miR-146b on cell apoptosis was analyzed by flow cytometry. ESCC cells transfected with miR-146b mimics showed decreased apoptosis rate, compared to mimic control group (Fig. 1B). These results indicated that miR-146b promoted proliferative activity and suppressed apoptosis in ESCC cells.

3.2. C/EBPβ directly binds to miR-146b promoter conserved region

In silico analyses revealed that two C/EBPβ-binding motifs were located 2 kb upstream away from the transcription start site of miR-146b. Both sites (nt -1736/-1722 and -1492/-1478) were in

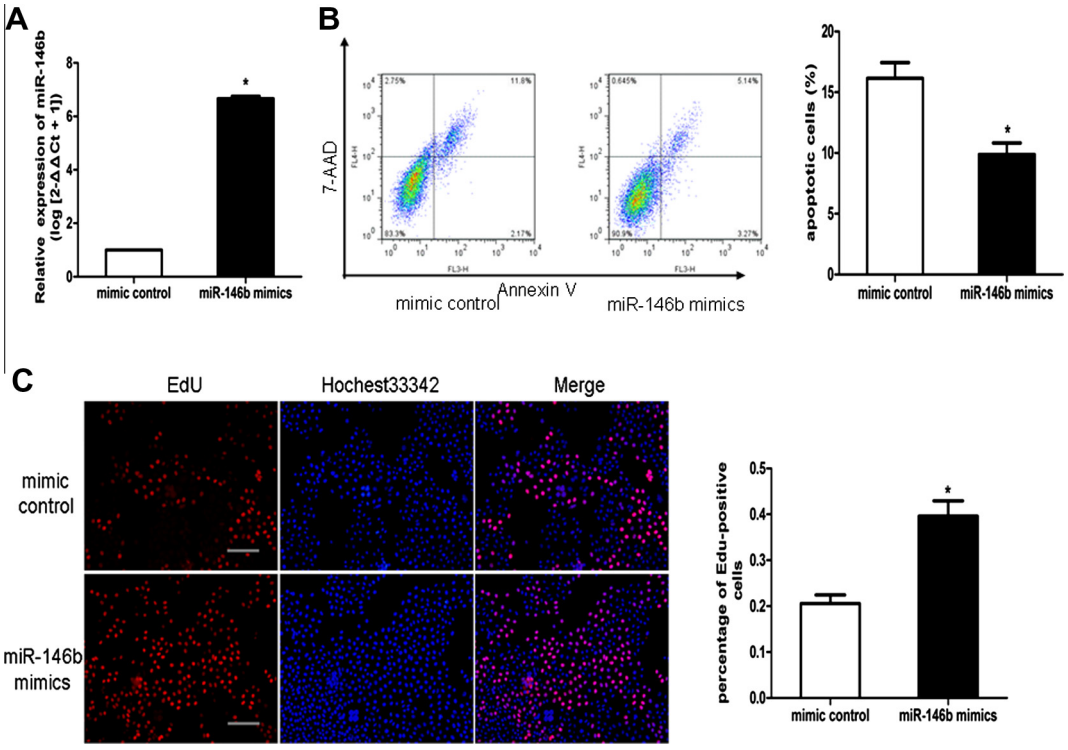

Fig. 1. Effects of miR-146b overexpression on esophageal cancer cell proliferation and apoptosis. (A) Relative miR-146b expression after mimic control or miR-146b mimics transfection. The miR-146b levels were measured by real-time PCR and normalized by the U6 snRNA levels. The log ratio of the relative miR-146b level relative to the U6 snRNA level is plotted on the *y*-axis. Significant differences are shown by asterisks (*$P < 0.05$). (B) EC109 cells were transiently transfected with either mimic control or miR-146b mimics 24 h before flow cytometric analysis of apoptosis were performed. (C) EC109 cells were transiently transfected with either mimic control or miR-146b mimics 24 h before EdU proliferation assays were performed. Scale bars represent 100 μm.

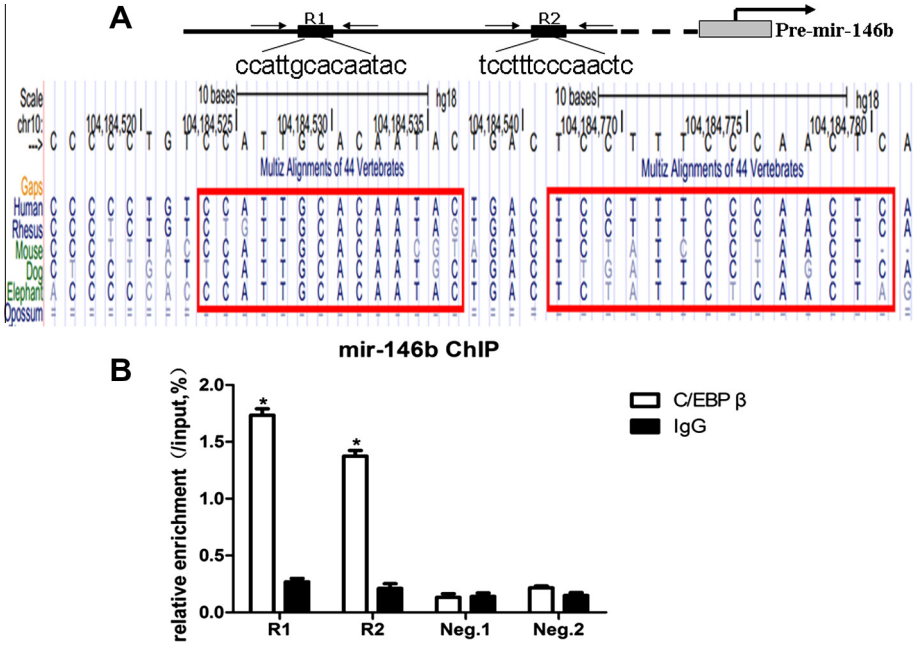

Fig. 2. C/EBPβ directly binds to miR-146b promoter conserved region. (A) Schematic description of the putative miR-146b promoter with potential C/EBPβ binding sites in a vertebrate conserved non-coding region (CNS) and pre-miR-146b start site (an arrow). (B) EC109 cells were subjected to quantitative ChIP analysis for the C/EBPβ occupancy, rabbit monoclonal anti-C/EBPβ antibody (Abcam, Cambridge, MA) or rabbit polyclonal anti-IgG antibody (Upstate Biotechnology, Lake Placid, NY, USA) were used to immunoprecipitate chromatin. R1 and R2 were primer pairs for the regions of miR-146b loci containing two potential C/EBPβ binding sites. Two negative primer pairs (Neg.1 and Neg.2) were for the regions without C/EBPβ binding sites in genomic sequence. Data are presented as percentages of input. Error bars indicate the SD from two independent experiments performed in triplicate.

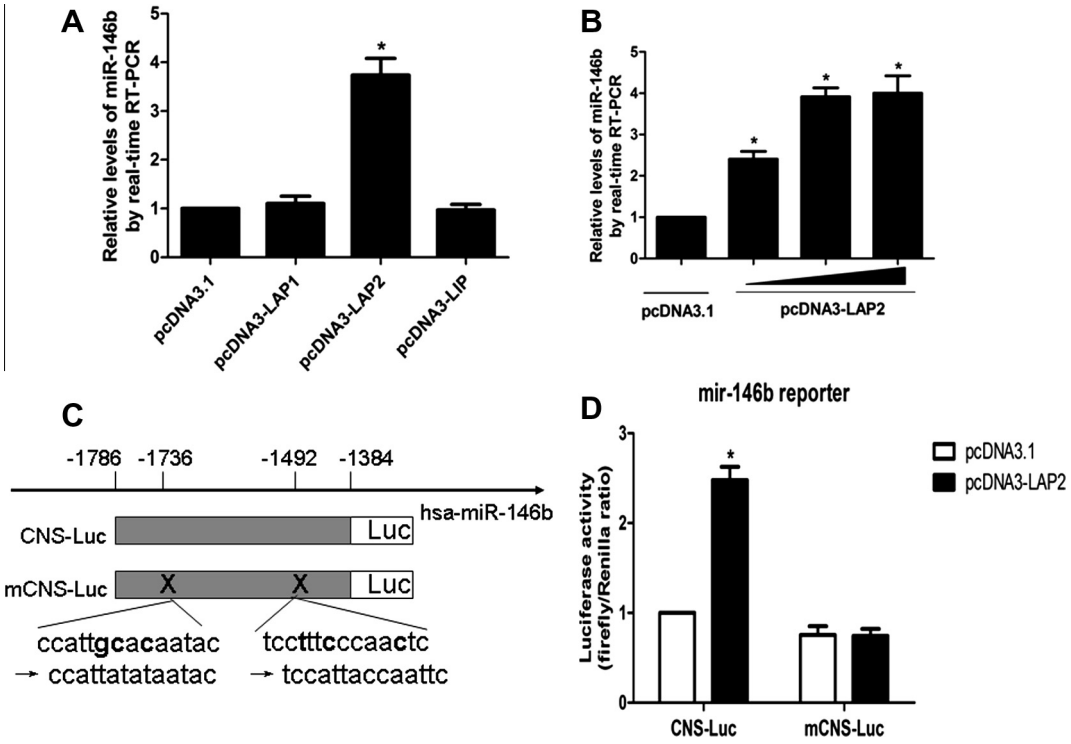

Fig. 3. MiR-146b is up-regulated by C/EBPβ LAP2 transcriptional activation. (A) EC109 cells were seeded in a 6-well culture plates and transfected with 1 μg of empty vector pcDNA3.1 or equal amount of expression vectors pcDNA3-LAP1, pcDNA3-LAP2, pcDNA3-LIP, respectively. MiR-146b levels were measured by real-time RT-PCR 24 h after transfection. (B) EC109 cells were seeded in a 6-well culture plates and transfected with 1 μg of empty vector pcDNA3.1 or 0.5, 1, 2 μg of expression vector pcDNA3-LAP2, respectively. MiR-146b expression were measured by real-time RT-PCR 24 h after transfection. Relative expression levels of miR-146b were normalization to U6 snRNA. (C) Schematic description of the wild-type miR-146b conserved regulatory element construct containing C/EBPβ binding sites and construct with the mutated core binding sequences, which were designated as CNS-Luc and mCNS-Luc, respectively. (D) Luciferase activity of the wild-type (CNS-Luc) or mutant (mCNS-Luc) reporter gene in HEK 293FT cells tranfected with empty vector pcDNA3.1 or expression vector pcDNA3-LAP2. Data were analyzed using Student's *t*-test (*$P < 0.05$).

a conserved non-coding sequence (CNS), as suggested by UCSC TFBS track, which predicted the conserved TF binding sites using the tfloc program using data from Transfac Matrix and Factor databases (Fig. 2A). The binding motifs of the evolutionarily conserved transcription factors tend to reside in the CNS within the regulation regions of their putative target genes.

To determine whether C/EBPβ regulate the expression of miR-146b by binding directly to the corresponding genomic sequences, we performed chromatin immunoprecipitation (ChIP) experiments with EC109 cells. We observed that C/EBPβ protein bound directly to two conserved CNS encompassing canonical C/EBPβ-binding sites (R1 and R2) in the mir-146b locus, but did not bind to unrelated negative regions (Neg.1 and Neg.2, Fig. 2B).

3.3. MiR-146b is up-regulated by C/EBPβ LAP2 transcriptional activation

Emerging evidence has shown that C/EBPβ exists in three isoforms: LAP1, LAP2 and LIP. To ascertain which isoform has a role in regulating miR-146b, EC109 cells were transfected with expression vectors encoding pcDNA3-LAP1, pcDNA3-LAP2, pcDNA3-LIP or an empty vector pcDNA3.1 24 h before harvested for miR-146b detection. We observed that ectopic overexpression of C/EBPβ LAP2 dramatically elevated miR-146b expression, compared to pcDNA3.1 group (Fig. 3A). Besides, miR-146b levels were up-regulated by C/EBPβ LAP2 in a dose-dependent manner (Fig. 3B).

Having confirmed C/EBPβ binding to the putative motifs by ChIP analysis and the positive regulatory effect of C/EBPβ LAP2 on miR-146b, we further investigated the functional role of C/EBPβ LAP2 on the conserved regulatory elements of miR-146b locus. Reporter constructs were performed as shown in Fig. 3C. Reporter assays showed that C/EBPβ LAP2 increased the activity of a luciferase reporter fused to the wild-type mir-146b conserved non-coding sequence (CNS) but not a mutant CNS (mCNS), the latter carried altered nucleotides in the C/EBPβ-binding motifs (Fig. 3D). Hence, the up-regulation of miR-146b by C/EBPβ LAP2 depends directly on these cognate recognition sites in the conserved responsible element of mir-146b locus.

4. Discussion

MiRNAs have been shown to be a significant gene modulator during esophageal cancer progression and global miRNA expression profiles have been performed using microarray, real-time PCR or next generation sequencing approaches [4,15,19,20]. There is a significant amount of information on aberrantly expressed miRNAs and their tumorigenic effects in esophageal cancers, but the detailed control mechanisms of such miRNA dysregulation remain poorly understood.

The biological functions of miR-146b are quite different according to tumor types. In gloma, by targeting MMP16, miR-146b is basically identified as anti-tumor miRNA which inhibits tumor growth, migration and invasion [21,22]. In pancreatic cancer and breast cancer, miR-146b overexpression significantly reduced the abilities of cellular migration and invasion [23,24]. On the contrary, there was other evidence demonstrating that overexpression of miR-146b significantly increased cell migration and invasiveness in papillary thyroid carcinoma [25], and suppression of miR-146b significantly decreasing the proliferation rate [8]. Consistently, we found that overexpression of miR-146b significantly promotes proliferation and inhibits apoptosis of esophageal cancer cell lines (Fig. 1). These inconsistent results from different research groups

may attribute to different tumor backgrounds and cellular contexts. Our observation would enlarge the understanding on the overall role of miR-146b in esophageal squamous cell carcinoma progression.

As miR-146b was reported to exert strong diagnostic value in predicting outcome of ESCC and further, we demonstrated miR-146b plays critical roles in modulating proliferation and apoptosis of esophageal cancer cell lines, the mechanism of miR-146b dysregulation became apparently important. In breast cancer, breast cancer metastasis suppressor 1 (BRMS1) up-regulates miR-146 in MDA-MB-435 cell lines, which suppresses breast cancer metastasis [24]. Our results demonstrate that C/EBPβ LAP2 directly regulates miR-146b expression in esophageal cancer cells (Figs. 2 and 3).

In our study, C/EBPβ levels were confirmed to aberrantly expressed in ESCC, resulting in a more aggressive pathological phenotype. C/EBPβ was reported to exist in three isoforms, each isoform exserts different regulatory role on corresponding downstream molecules. In our results, C/EBPβ LAP2 up-regulated miR-146b expression but C/EBPβ LIP repressed miR-203 expression (unpublished papers), demonstrating that different isoforms even showed opposing effect on target genes. Apart from our results, the dichotomy of C/EBPβ transcriptional activity, whereby it acts as both an activator and a suppressor, was also reported previously in various cell lines [26–28]. Therefore, C/EBPβ has diverse activities across various cell types, these activities are likely to be cell context-dependent involving different target genes. Recently, cumulative evidence suggests that C/EBPβ protein does not work alone but rather depends on requisite partners for target specificity and combinatorial control for gene regulation, this working mechanism could at least, in part, explain the dichotomy of C/EBPβ on gene transcriptional regulation [17,29,30].

In summary, we found that miR-146b overexpression promoted cell proliferation and inhibited apoptosis in human ESCC cell lines. Additionally, we uncovers a novel mechanism for miR-146b regulation at the transcriptional level, C/EBPβ LAP2 is a direct positive regulator of miR-146b. Although our understanding of the complicated mechanisms underlying ESCC progression are still limited, our data may provide a strategy for targeting miR-146b as a novel therapeutic application to treat advanced ESCC patients.

Acknowledgment

This work was supported by the National Natural Science Foundation of China [Grant number 81172378].

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