

Full Length Research Paper

# Cloning and characterization of the 5' flanking region of microRNA *let-7a-1/let-7f-1* gene cluster in human lung cancer cell

Jian Zhao<sup>2#</sup>, Nana Ni<sup>1#</sup>, Chang Liu<sup>3</sup>, Pengju Zhang<sup>1</sup>, Yang Yu<sup>1</sup>, Zhaobo Chen<sup>1</sup>, Weiwen Chen<sup>1\*</sup> and Anli Jiang<sup>1</sup>

<sup>1</sup>Institute of Biochemistry and Molecular Biology, School of Medicine, Shandong University, 44 Wenhuxi Road, Jinan, Shandong 250012, China.

<sup>2</sup>Department of Thoracic surgery, Qilu Hospital, Shandong University, Jinan, Shandong 250012, China.

<sup>3</sup>R&D department, Shandong Freda Biopharm CO., LTD, Jinan, Shandong 250014, China.

Accepted 22 June, 2011

In order to elucidate the molecular basis of microRNA *let-7a-1/let-7f-1* gene cluster, the transcription initiation site which was determined by 5' rapid amplification of cDNA ends (5'RACE) and 2.1 kb of the 5' flanking region proximal to the *pre-let-7a-1* was isolated and characterized. The promoter activity of the 2.1 kb fragment was analyzed by a firefly luciferase-encoding gene expression vector (pGL3) transiently transfected into lung cancer cell line A549. The 2.1 kb promoter of *let-7a-1/let-7f-1* displayed a lower activity and was significantly enhanced by ectopic expression of c/EBP $\alpha$  or p53 and treatment with dexamethasone. Despite the induction of other *let-7* family members such as *let-7a-3*, *let-7c* and *let-7d*, all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA) display little enhancement effect on 2.1 kb promoter of *let-7a-1/let-7f-1*, as well as 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

**Key words:** *let-7a-1*; *let-7f-1*, 5' rapid amplification of cDNA ends (5'RACE), promoter, lung cancer.

## INTRODUCTION

MicroRNAs (miRNAs) are non-coding small RNAs found in diverse organisms (Chen, 2005; Hammond, 2006). They are encoded in long primary forms in the nucleus (pri-miR). The pri-miRs will be transported into the cytoplasm after being processed to 70 to 80 nucleotide pre-miR with a characteristic hairpin structure. The pre-miR are then processed by Dicer into the 22 to 25 nt mature forms (Lee et al., 2002). miRNAs mediate gene down-regulation by targeting mRNAs to induce RNA degradation and/or interfering with translation and thus, play key roles in regulating a wide array of cell functions

(Ambros, 2003). Concordant with this, aberrant expression of miRNA genes could lead to human disease. In recent years, many reports have shown that miRNAs regulate cell proliferation and apoptosis and play a role in cancer: microRNAs can act as oncogenes or tumor suppressors (Chen, 2005; Hammond, 2006).

There are accumulating evidences that *let-7*, the first discovered miRNA, is tumor suppressor. A number of studies have demonstrated that *let-7* was implicated in various cancers, particularly in lung cancer (Eder and Scherr, 2005; Lu et al., 2007; Sampson et al., 2007; Motoyama et al., 2008). Takamizawa et al. (2004) first reported that the expression of the *let-7* microRNA was reduced in human lung cancers and overexpression of *let-7* in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro*. Next, Johnson et al. (2005) identified that *ras* is one of the target genes of *let-7* family. Lee and Dutta (2007) also found that *let-7* repressed the HMGA2 oncogene in human lung cancer cells. Therefore, *let-7* is tumor suppressor microRNA and maybe a potential target for lung cancer therapy. Despite

\*Corresponding author. E-mail: chenweiwen@sdu.edu.cn.

**Abbreviations:** ATRA, All-trans retinoic acid; 9cRA, 9-cis-retinoic acid; DEX, dexamethasone; DMSO, dimethylsulfoxide; miRNA, microRNA; RACE, rapid amplification of cDNA ends.

#These authors contributed equally to this work.

this interest, the underlying molecular mechanisms of *let-7* reduction in lung cancer are not well understood. The qRT-PCR results from different investigators have shown the significant down-regulation of *pri-let-7* in lung cancer tissues and cells (Takamizawa et al., 2004; Yanaihara et al., 2006), suggesting the inhibition on transcription level maybe one of the mechanisms for *let-7* expression reduction in lung cancer. But our understanding of *let-7* transcription pattern in lung cancer cells is limited. Thus, cloning and characterization of the 5' flanking region of *let-7a-1* and *let-7f-1*, which are the most abundant species of *let-7* family and clustered within a few hundred bases in the human genome (Lagos-Quintana et al., 2001), may well be a start point for elucidating the transcription regulation mechanism of *let-7*.

## MATERIALS AND METHODS

### Cell culture

The human lung cancer cell line A549 (ATCC- American Type Culture Collection) were cultured with F-12K (Gibco, BRL Gaithersburg, MD, USA) medium containing 10% fetal bovine serum, plus 100 u/ml ampicillin and 100 u/ml streptomycin at 37°C with 5% CO<sub>2</sub>. Within 24 h of passage, cells with more than 70 to 80% confluence were used for transfection.

### Mapping the transcription start site of *let-7a-1/let-7f-1* gene cluster

The start of transcription of the *let-7a-1/let-7f-1* gene cluster was determined by the 5' rapid amplification of cDNA ends (5'RACE) protocol using a 5'/3' RACE kit essentially as recommended by the manufacturer (Roche Diagnostics, Laval, Quebec, Canada). Total RNA was prepared from lung cancer cell strain A549 and then treated with RNase-free DNase. Total RNA (2 µg) in a 20 µl reaction volume was reverse transcribed at 55°C with transcriptase reverse transcriptase and *let-7a-1/let-7f-1* cluster-specific primer SP1 (5'- TAATGCAGCAAGTCTACTCCTCAGGG -3'). A homopolymeric tail was appended to the 3' end of the synthesized first-strand cDNA using terminal transferase and dATP by incubation at 37°C for 20 min as described in the kit protocol. The dA-tailed cDNA was PCR amplified using an oligo (dT)-anchor primer (5'- GACCACGCGTATCGATG TCGACTTTTTTTTTTTTTT TV-3') and another *let-7a-1/let-7f-1* cluster-specific primer SP2 (5'- CCCCACAACCTATAACAATCTACTACCTCACTC- 3'). The PCR product obtained was again PCR amplified with the nested *let-7a-1/let-7f-1* cluster-specific primer SP3 (5'- CCGCCTGGATGCAGAC TTTTCTATC -3') and a kit-provided PCR anchor primer (5'- GACCACGCGTATCGATGTCGAC-3') in order to eliminate any nonspecific PCR products from the first reaction. The amplified products were purified using a high pure PCR product purification kit (Roche) and cloned into the pMDT-18 vector (Takara Biotechnology, Dalian, China). Automated DNA sequencing of the RACE inserts was performed by BGI Premier Scientific Partner, Inc. (Beijing, China).

### Amplifying and cloning a 2.1 kb fragment of the 5' flanking region of the *let-7a-1/let-7f-1* gene cluster

Human genomic DNA was extracted from white blood cells using

the method of rapid isolation of mammalian DNA. The primer pair PF (5'- CCGCTCGAGACCCAGCCATGTTTCAGTTCT -3'; with an Xho I site at its 5' end) and PR (5'- CCCAAGCTTCAGTGAA GAGAACATCCAGG-3'; with a Hind III site at its 5' end) were used to amplify the 5' flanking region of the *let-7a-1/let-7f-1* gene cluster from the extracted human genomic DNA by PCR. The PCR-amplified fragment was 2123 bp (-1999 to +124 bp) and it was excised with Xho I and Hind III (TaKaRa) and ligated into the equivalent site of the pGL3-Basic vector (Promega, Madison WI, USA) to form the *let-7a-1/let-7f-1* cluster promoter-luciferase reporter construct, designated pGL3-2123. The resulting construct was confirmed by restriction enzyme digestion and sequence analysis using the general primers Rvprimer3 and GLprimer2.

### Transient transfection

A549 cells in 70 to 80% of confluence were transfected with Fugene HD (Roche) in 24-well plates. Each well contained 0.5 µg pGL3-2123, 0.02 µg of the internal control vector pRL-TK, 1 µl Fugene HD and 500 µl F-12K medium without serum or antibiotics. For co-transfection experiments, 0.3 µg pGL3-2123 was transfected along with 0.2 µg of one of the eukaryotic expression plasmids pCMV-*p53* (Clontech, Palo Alto, CA), pcDNA3.1-*Sp1* and pcDNA3.1-NFκBp50 (gifts from Dr. Charles Young, Mayo clinic, USA), pcDNA3.1-*PPARγ2*, pcDNA3.1-*cEBPα* (gifts from Dr. Jianhua Shao, University of Kentucky, USA), pBABE <sub>puro</sub>-*ras* or pBABE<sub>hygro</sub>-*myc* (gifts from Dr. Weijing He, University of Texas, USA). All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the transfection procedure, following the protocol recommended by Promega.

### Treatment of the transfected cells with hormones

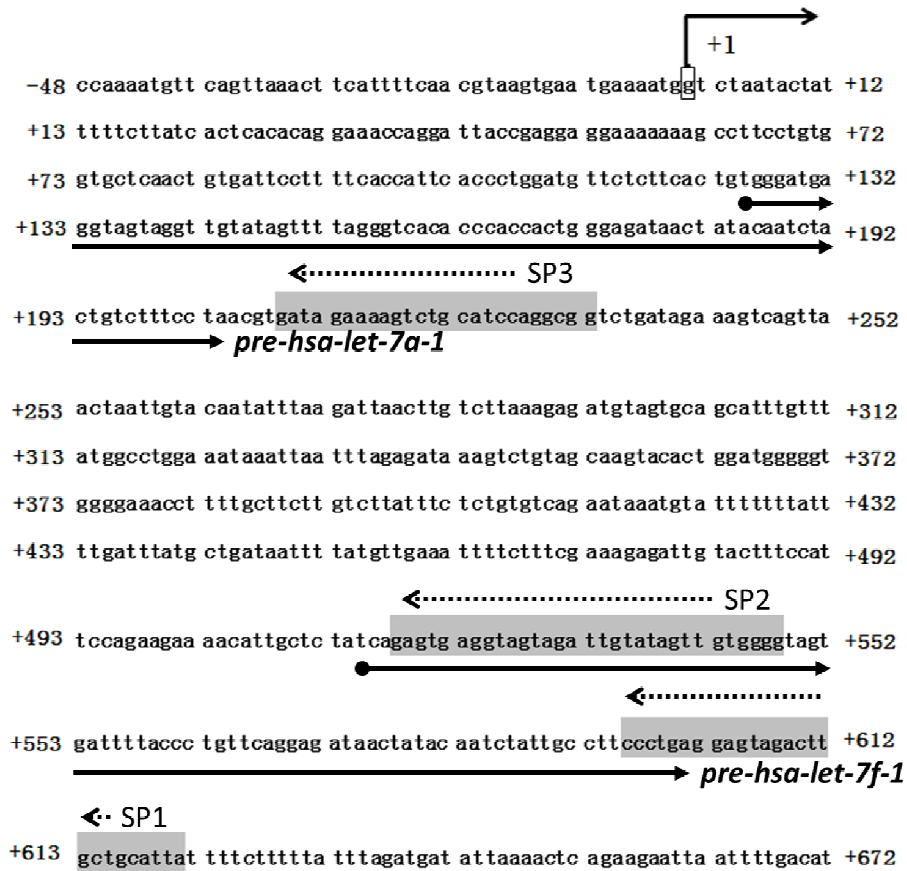
The stocks of all-trans retinoic acid (ATRA), 9-cis-retinoic acid (9cRA) and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) were prepared in dimethylsulfoxide (DMSO); the stock of dexamethasone (DEX) were prepared in ethanol. All of the hormones were purchased from Sigma (St. Louis, MO, USA). After the transfection of pGL3-2123 in 24-well plates, the cells were treated for 48 h with 10<sup>-6</sup>~10<sup>-8</sup> M of ATRA, 9cRA, 1,25(OH)<sub>2</sub>VitD<sub>3</sub> or DEX in 10% FBS-F-12K medium, respectively. The controls received the DMSO or ethanol vehicle at a concentration equal to that of the treated cells. All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the treatment procedure.

### Dual-luciferase reporter assay

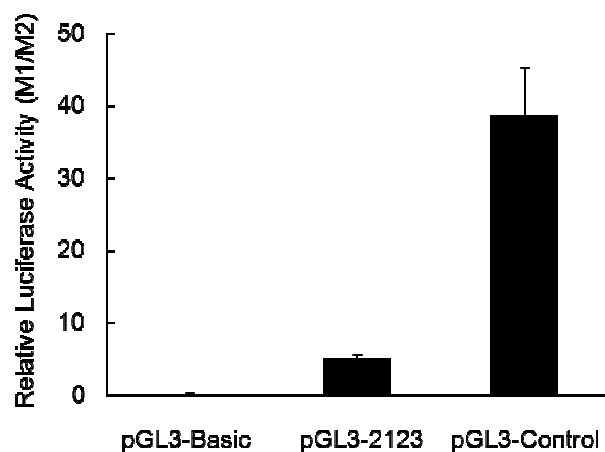
The activities of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK (Promega) were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed with PBS after harvest and cell lysates were prepared by manually scraping the cells from the culture plates in the presence of 1×PLB (passive lysis buffer). 20 µl of cell lysate was transferred into luminometer tubes containing 100 µl LAR. Firefly luciferase activity (M1) was measured first and then Renilla luciferase activity (M2) was measured after the addition of 100 µl of Stop and Glo Reagent.

### Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. Statistical significance of differences between groups



**Figure 1.** Map the 5' end of primary transcript of the *let-7a-1/let-7f-1* cluster. The transcription start site detected by 5' RACE is boxed and numbered +1. The primers used for 5'RACE (SP1-3) are shaded in gray. The lines and arrows indicate the pre-miR sequence of *let-7a-1* (80nt) and *let-7f-1* (87nt).



**Figure 2.** Activity assay of pGL3-2123 in A549 cells. pGL3-2123 was transfected into A549 cells along with a negative control pGL3-Basic and a positive control pGL3-Control. The promoter activities were determined via the dual-luciferase reporter assay. The results are expressed as the relative luciferase activities (M1/M2), that is, the ratio of firefly luciferase activity (M1) in the pGL3 plasmid and Renilla luciferase activity (M2) in the pRL-TK plasmid.

was analyzed by unpaired Student's t test and  $P < 0.05$  was considered to be statistically significant.

## RESULTS AND DISCUSSION

### Mapping the transcription start site of *let-7a-1/let-7f-1* gene cluster

Many evidences have indicated that microRNA genes are transcribed by RNA polymerase II (Tam, 2001; Johnson et al., 2003; Xie et al., 2005) and a handful of miRNA promoters in *Homo sapiens* have been identified so far (Lee et al., 2004; Houbaviv et al., 2005). According to MiRBase data, the hsa-let-7 family has 11 members that distribute on different chromosomes. Searching result from Entrez gene indicate that the *let-7a-1* and *let-7f-1* gene is an intergenic cluster located within chromosome 9q22.32 (Gene ID: 406881) (Figure 1). To determine the transcription initiation site of *let-7a-1/let-7f-1* cluster, 5'RACE was performed using RNA isolated from A549 cells and three *let-7a-1/let-7f-1* cluster-specific primers (SP1-3). As shown in Figure 1, *let-7a-1/let-7f-1* cluster has single transcription initiation site, located at 124 bp upstream the *pre-let-7a-1*.

### Cloning and activity assay of the 2.1 kb promoter fragment of *let-7a-1/let-7f-1* cluster

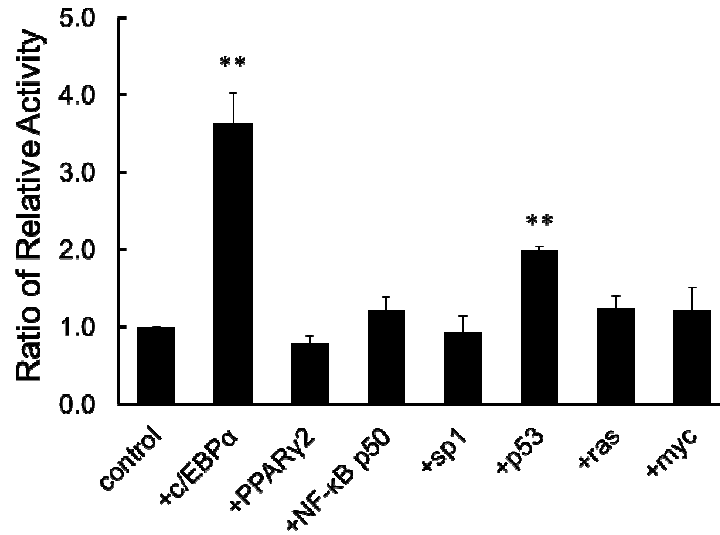
Based on the result of 5'RACE, starting 2.1 kb upstream of pre-let-7a-1, a genomic fragment including positions -1999 to +124 relative to the transcription start site was cloned into the pGL3-Basic to form pGL3-2123, which was confirmed by DNA sequencing. To evaluate the promoter activity, the pGL3-2123 was transfected into lung cancer cell line A549, pGL3-Control and pGL3-Basic were also introduced into parallel wells, respectively, as positive and negative controls. The luciferase activity was assayed and normalized to pRL-TK (Figure 2). After 48 h of transfection into A549 cells, pGL3-2123 yielded a result of 5.24 by dual-luciferase reporter assay (M1/M2). Comparing with respective 38.76 of pGL3-Control and 0.26 of pGL3-Basic, the 2.1 kb promoter of *let-7a-1/let-7f-1* cluster display a lower activity in A549 cells, which was consistent with the fact that A549 cell line has the reduced expression of endogenous *let-7a-1/let-7f-1* (Takamizawa et al., 2004), suggesting the inhibition of promoter may be one of the reasons for reduced expression of *let-7a-1/let-7f-1* in lung cancer cells.

### The effect of some tumor-associated transcription factors on promoter activity of pGL3-2123

As class-II genes, expressions of many microRNAs are also regulated by enhancers (Johnson et al., 2003;

Lagos-Quintana et al., 2001) or hormones (Lee et al., 2002). Computer-based analysis of this 2.1 kb 5'-flanking fragment sequence with MatInspector 2.0 software (<http://www.genomatix.de>), three putative TATA-boxes (-414/-398, -366/-344 and -110/-94) and two CCAAT-boxes (-118/-104 and -314/-300) were searched within the -1000/+1 region. In addition, more than 700 other putative transcription factor binding sites (Matrix similarity > 0.75) were searched by MatInspector within the 2.1 kb fragment. Because *let-7a-1/let-7f-1* cluster is tumor suppressor miRNAs, we are interested in the tumor-associated transcription factor binding sites. The computer search result displayed four *c/EBP* sites (-1726/-1712, -1683/-1669, -722/-708 and -163/-149), two *PPAR* sites (-1434/-1412 and -533/-511), one *NF-κB* site (-1255/-1243), two *Sp1* sites (-1953/-1933 and -1008/-988) and two *P53* sites (-1170/-1148 and -1159/-1137). In order to investigate whether the putative *cis*-elements mentioned earlier have functions, the eukaryotic expression plasmids of *c/EBPα*, *PPARγ<sub>2</sub>*, *NF-κB p50*, *Sp1* and *p53*, were co-transfected with pGL3-2123 into A549 cells and the corresponding empty eukaryotic expression vectors of themselves were co-transfected with pGL3-2123 into parallel wells as a control. In addition, because the oncogenes *ras* and *myc* are targets of *let-7* (Boominathan, 2010), the co-transfection of *ras/myc* expression vector with pGL3-2123 were also performed to test whether the *let-7* and *ras/myc* share a feedback loop. The relative luciferase activities from pGL3-2123 co-transfected with eukaryotic expression plasmids was divided by the relative luciferase activity from pGL3-2123 co-transfected with control vector from the same cell line and plotted in Figure 3. Our results indicate that ectopic expression of *PPARγ<sub>2</sub>*, *NF-KappaB*, *Sp1*, *ras* and *myc* have no significant effect on pGL3-2123 promoter activity, whereas *c/EBPα* and *p53* increases the promoter activity of pGL3-2123 about 3.6-fold and 2.0-fold, respectively.

*c/EBPα*, one member of the *c/EBP* family, is a gene thought to be a tumor suppressor (Timchenko et al., 1996; Lekstrom-Himes and Xanthopoulos, 1998; Pabst et al., 2001). As a transcription factor, *c/EBPα* is discovered controlling the transcription of some tumor suppressor microRNA. Recently, the *c/EBPα* is reported to directly interacted with the *mir-122* (hepatocyte-specific microRNA) promoter and transactivate it (Zeng et al., 2010). Studies in acute myeloid leukemia (AML) have shown that *mir-34a* and *mir-223* were transcriptional targets of *c/EBPα* (Pulikkan et al., 2010, 2010). In A549 and H1299 lung cancer cells, the ectopic *c/EBPα* expression increases expression of *mir-1* 6.1-fold and 4.92-fold, respectively (Nasser et al., 2008). *mir-661*, which can inhibit metastatic tumor antigen 1, is another *c/EBPα* target (Reddy et al., 2009). In our experiment, the expression of ectopic *c/EBPα* significantly increase the promoter activity of *let-7a-1/let-7f-1* cluster (3.6-fold) in A549 cells with little endogenous *c/EBPα* expression (Li



**Figure 3.** The effects of some transcription factors on promoter activities of *let-7a-1/let-7f-1* cluster. A549 cells were co-transfected with pGL3-2123 and eukaryotic expression plasmids of *c/EBPα*, *PPARγ<sub>2</sub>*, *NF-κB p50*, *Sp1*, *p53*, *ras* or *myc*. The control cells were co-transfected with pGL3-2123 and corresponding empty eukaryotic expression plasmids. All the cells were harvested for the dual-luciferase reporter assay after 48 h of transfection. The results were expressed as ratio of relative activity, that is, the M1/M2 value from pGL3-2123 co-transfected with eukaryotic expression plasmids was divided by the M1/M2 value from controls. \*\*P < 0.01, compared with the control.

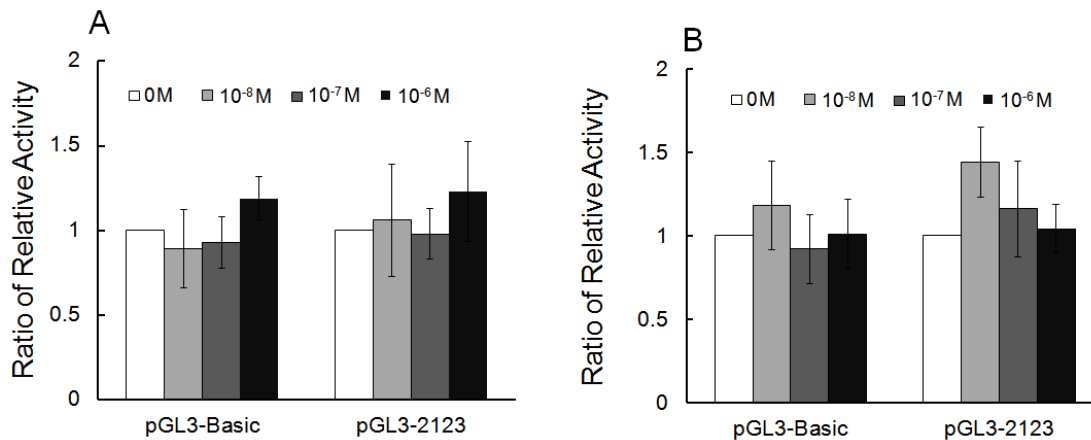
et al., 1995), suggesting that the *let-7a-1/let-7f-1* cluster is a new transcriptional target of *c/EBPα*.

*p53* is a broad-spectrum tumor suppressor gene that plays very important roles in various cancers. The *P53* protein functions as transcription factor to transactivate expression of a number of target genes to promote tumor suppression and genome integrity. Recently, a number of studies have shown that many miRNAs are also members of *p53*-network. For example, *mir-34*, *mir-192/215*, *mir-107* and *mir-145*, all of which function as tumor suppressors and play a key role in control of tumor progression, angiogenesis and metastasis, are shown to be direct transcriptional targets of *p53* (Hermeking, 2007; Tarasov et al., 2007; Boominathan, 2010). Besides *mir-34a*, Tarasov et al. (2007) also observed that activation of *p53* in lung cancer cells results in increased expression of *mature-let-7c* (2.7-fold), *mature-let-7e* (2.1-fold) and *mature-let-7a* (1.9-fold). Our results further suggest that *p53* increase the expression of *let-7a-1/let-7f-1* at transcriptional level, but maybe due to the endogenous expression of wt *p53* by A549 cells (Jia et al., 1997), the ectopic expression of *p53* had a weaker increase effect on *let-7a-1/let-7f-1* promoter than that of *c/EBPα*.

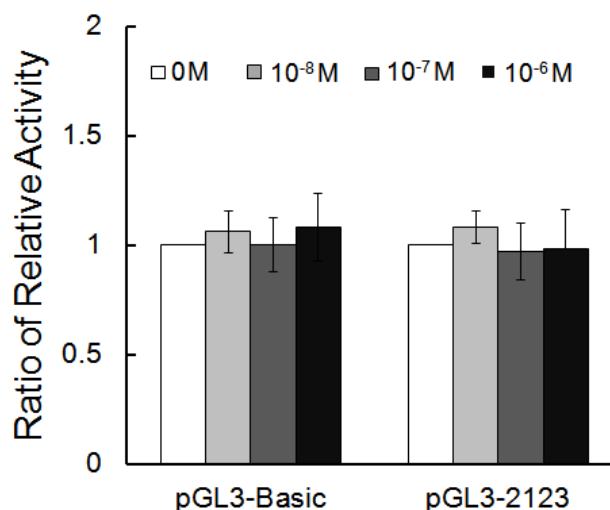
#### The effects of hormones on promoter activity of *let-7a-1/let-7f-1* cluster

Besides the transcription factors mentioned earlier, two

RAR-RXR heterodimer binding sites (-46/-22 and +90/+114), four VDR-RXR heterodimer binding sites (-1955/-1931, -1691/-1667, -1600/-1576 and -1592/-1568), three glucocorticoid responsive and related elements (GRE) (-1877/-1859, -1588/-1561 and -1416/-1398) were detected by MatInspector 2.0 software. The RAR (retinoic acid receptor), RXR (retinoid X receptor) and VDR (Vitamin D3 receptor) belong to the nuclear receptor superfamily of ligand-inducible transcription (Stunnenberg, 1993). The RAR and RXR mediate the effect of retinoids signals such as all-trans-RA (ATRA) and 9-cis-RA (9cRA), which influence processes such as growth and differentiation by regulation of target gene expression at the cellular level (Gudas, 1994). Retinoids suppress carcinogenesis in diverse epithelial tissues including lung. Clinical trials have demonstrated the efficacy of retinoids in suppressing lung cancer (Aapro, 1995). Several lines of evidence have shown that treatment with retinoic acid not only down-regulate miRNAs that function as oncogenes, but also up-regulate tumor suppressor miRNAs including members of *let-7* family (Garzon et al., 2007; Careccia et al., 2009; Weiss et al., 2009). For example, acute promyelocytic leukemia (APL) successfully treated with ATRA showed upregulation of *let-7c* (Careccia et al., 2009), as well as Garzon et al. (2007) found up-regulation of *let-7a-3*, *let-7c* and *let-7d* by miRNA microarrays and qRT-PCR in APL patients and cell lines during ATRA treatment. To explore whether the retinoic acids are implicated in



**Figure 4.** The effects of 9cRA and ATRA on promoter activity of *let-7a-1/let-7f-1* cluster. A549 cells transfected with pGL3-2123 were treated with  $10^{-8}$ ~ $10^{-6}$  M of 9cRA and ATRA for 48 h, as well as parallel wells transfected with pGL3-Basic as negative control. The cells were harvested for the dual-luciferase reporter assay to detect the effects of hormones on promoter activity of the *let-7a-1/let-7f-1* cluster. The results were expressed as ratio of relative luciferase activity.

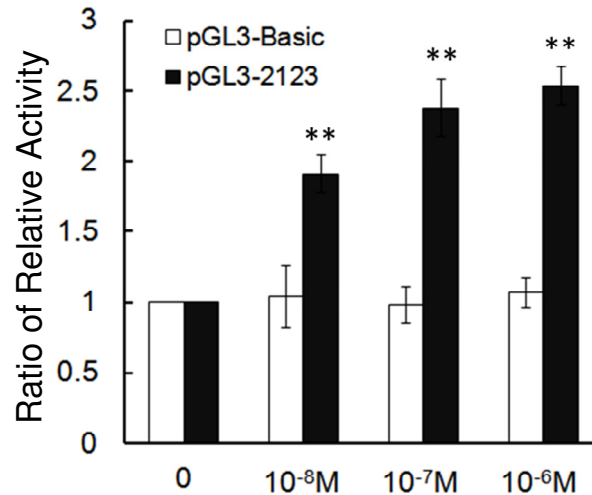


**Figure 5.** The effects of  $1,25(\text{OH})_2\text{D}_3$  on promoter activity of *let-7a-1/let-7f-1* cluster. A549 cells transfected with pGL3-2123 were treated with  $10^{-8}$ ~ $10^{-6}$  M of  $1,25(\text{OH})_2\text{D}_3$  for 48 h, as well as parallel wells transfected with pGL3-Basic as negative control. The cells were harvested for the dual-luciferase reporter assay to detect the effects of hormones on promoter activity of the *let-7a-1/let-7f-1* cluster. The results were expressed as ratio of relative luciferase activities.

controlling expression of *let-7a-1/let-7f-1* cluster, the A549 cells transfected with pGL3-2123 were treated with  $10^{-8}$  ~  $10^{-6}$  M of 9cRA or ATRA for 48 h. As shown in Figure 4, the 2.1 kb promoter activity of *let-7a-1/let-7f-1* cluster did not display significant increase after treatment with 9cRA as well as ATRA.

The VDR, upon activation by  $1,25(\text{OH})_2\text{D}_3$ , forms a heterodimer with the RXR and binds to corresponding hormone response elements on DNA resulting in expression of specific gene products (Stunnenberg,

1993).  $1,25(\text{OH})_2\text{D}_3$  exerts important effects on cellular proliferation and differentiation and has been shown to decrease the growth of many cancers (Walters, 1992; Campbell et al., 1997). To date, a few studies have revealed the interaction between  $1,25(\text{OH})_2\text{D}_3$  signal pathway and miRNAs (Mohri et al., 2009; Essa et al., 2010; Xi et al., 2010). In our study,  $1,25(\text{OH})_2\text{D}_3$ , like retinoic acids, have no significant effect on 2.1-kb promoter of *let-7a-1/let-7f-1* cluster (Figure 5), suggesting the little possibility that the expression of *let-*



**Figure 6.** The effects of DEX on promoter activity of *let-7a-1/let-7f-1* cluster. A549 cells transfected with pGL3-2123 were treated with  $10^{-8}$ M~ $10^{-6}$  M of DEX for 48 h, as well as parallel wells transfected with pGL3-Basic as negative control. The cells were harvested for the dual-luciferase reporter assay to detect the effects of hormones on promoter activity of the *let-7a-1/let-7f-1* cluster. The results were expressed as ratio of relative luciferase activities (M1/M2). \*\*P < 0.01, compared with the control.

*7a-1/let-7f-1* cluster is regulated by retinoic acid or 1,25-(OH)<sub>2</sub>D<sub>3</sub> at transcriptional level.

Despite the 1,25-(OH)<sub>2</sub>D<sub>3</sub> and retinoic acids, the glucocorticoid signal dexamethasone enhanced the promoter activity of *let-7a-1/let-7f-1* 1.91-fold, 2.38-fold and 2.54-fold at concentration of  $10^{-8}$ M,  $10^{-7}$ M and  $10^{-6}$ M, respectively (Figure 6). Dexamethasone, as a confirmed induction agent for apoptosis, has broad application in treating cancers (De Bosscher et al., 2000; Riccardi et al., 2000; Almawi et al., 2002). Dexamethasone act transcriptionally by binding the glucocorticoid receptor (GR) and subsequent binding to the promoter region of target genes on sites compatible with GRE motifs, which in turn directly or indirectly regulated gene expression (Almawi et al., 2002). Now the relationship between the glucocorticoid signal pathway and miRNAs has aroused the interest of investigators. For example, Liao and Lonnerdal (2010) found increase of mir-30e in dexamethasone-induced IEC-6 cells. Smith et al. (2010) studied the microRNA expression and processing during lymphocyte apoptosis induced by dexamethasone and found that mir-17-92 was repressed significantly (Smith et al., 2010). But so far, there is no evidence to support that dexamethasone directly activates the transcription through GRE within the promoter of microRNA. Thus, in our experiment, whether the dexamethasone enhance the promoter activity of *let-7a-1/let-7f-1* by activate the GRE within its promoter need further work to confirm.

In summary, we determined the transcription start site of *let-7a-1/let-7f-1* cluster and cloned its 2.1 kb 5' flanking

fragment into the pGL3-Basic vector to form the *let-7a-1/let-7f-1* promoter-luciferase reporter construct pGL3-2123, which represented lower promoter activity that consistent with the expression level of *let-7a-1/let-7f-1* in A549 cells. Preliminary function analysis on pGL3-2123 revealed that this 2.1 kb promoter of *let-7a-1/let-7f-1* was transactivated by ectopic expression of c/EBP $\alpha$  or p53. Treatment with 9-cis-retinoic acid, all-trans retinoic acid or 1,25-(OH)<sub>2</sub>D<sub>3</sub> resulted in little induction of *let-7a-1/let-7f-1* promoter, whereas  $10^{-8}$ ~ $10^{-6}$  M of dexamethasone significantly increase the promoter activity of *let-7a-1/let-7f-1*, further research should be done to identify the responsive functional cis-elements within the *let-7a-1/let-7f-1* promoter and elucidate their regulatory mechanisms, thus, providing a foundation for understanding the transcription pattern of *let-7a-1/let-7f-1* and their application in target therapy for lung cancer.

## ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (81071720), Natural Science Foundation of Shandong Province (ZR2009CM044) and Foundation for Outstanding Young Scientist in Shandong Province (BS2009SW042).

## REFERENCES

Aapro MS (1995). Retinoids in oncology. Eur. J. Cancer. 31: 834-835.

- Almawi WY, Abou Jaoude MM, Li XC (2002). Transcriptional and post-transcriptional mechanisms of glucocorticoid antiproliferative effects. *Hematol. Oncol.* 20: 17-32.
- Ambros V (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell.* 113: 673-676.
- Boominathan L (2010). The guardians of the genome (p53, TA-p73, and TA-p63) are regulators of tumor suppressor miRNAs network. *Cancer Metastasis Rev.* 29: 613-639.
- Campbell MJ, Elstner E, Holden S, Uskokovic M, Koeffler HP (1997). Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. *J. Mol. Endocrinol.* 19: 15-27.
- Careccia S, Mainardi S, Pelosi A, Gurtner A, Diverio D, Riccioni R, Testa U, Pelosi E, Piaggio G, Sacchi A, Lavorgna S, Lo-Coco F, Blandino G, Levrero M, Rizzo MG (2009). A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytes. *Oncogene.* 28: 4034-4040.
- Chen CZ (2005). MicroRNAs as oncogenes and tumor suppressors. *N. Engl. J. Med.* 353: 1768-1771.
- De Bosscher K, Vanden Berghe W, Haegeman G (2000). Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J. Neuroimmunol.* 109: 16-22.
- Eder M, Scherr M (2005). MicroRNA and lung cancer. *N. Engl. J. Med.* 352: 2446-2448.
- Essa S, Denzer N, Mahlknecht U, Klein R, Collnot EM, Tilgen W, Reichrath J (2010). VDR microRNA expression and epigenetic silencing of vitamin D signaling in melanoma cells. *J. Steroid. Biochem. Mol. Biol.* 121: 110-113.
- Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, Cimmino A, Wang H, Sun H, Volinia S, Alder H, Calin GA, Liu CG, Andreeff M, Croce CM (2007). MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene.* 26: 4148-4157.
- Gudas LJ (1994). Retinoids and vertebrate development. *J. Biol. Chem.* 269: 15399-15402.
- Hammond SM (2006). MicroRNAs as oncogenes. *Curr. Opin. Genet. Dev.* 16: 4-9.
- Hermeking H (2007). p53 enters the microRNA world. *Cancer Cell.* 12: 414-418.
- Houbaviy HB, Dennis L, Jaenisch R, Sharp PA (2005). Characterization of a highly variable eutherian microRNA gene. *RNA.* 11: 1245-1257.
- Jia LQ, Osada M, Ishioka C, Gamo M, Ikawa S, Suzuki T, Shimodaira H, Niitani T, Kudo T, Akiyama M, Kimura N, Matsuo M, Mizusawa H, Tanaka N, Koyama H, Namba M, Kanamaru R, Kuroki T (1997). Screening the p53 status of human cell lines using a yeast functional assay. *Mol. Carcinog.* 19: 243-253.
- Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005). RAS is regulated by the let-7 microRNA family. *Cell.* 120: 635-647.
- Johnson SM, Lin SY, Slack FJ (2003). The time of appearance of the *C. elegans* let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. *Dev. Biol.* 259: 364-379.
- Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001). Identification of novel genes coding for small expressed RNAs. *Science.* 294: 853-858.
- Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002). MicroRNA maturation: stepwise processing and subcellular localization. *EMBO. J.* 21: 4663-4670.
- Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO. J.* 23: 4051-4060.
- Lee YS, Dutta A (2007). The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes.* 21: 1025-1030.
- Lekstrom-Himes J, Xanthopoulos KG (1998). Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* 273: 28545-28548.
- Li F, Rosenberg E, Smith CI, Notarfrancesco K, Reisher SR, Shuman H, Feinstein SI (1995). Correlation of expression of transcription factor C/EBP alpha and surfactant protein genes in lung cells. *Am. J. Physiol.* 269: L241-247.
- Liao Y, Lonnerdal B (2010). Beta-catenin/TCF4 transactivates miR-30e during intestinal cell differentiation. *Cell Mol. Life. Sci.* 67: 2969-2978.
- Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007). Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. *Cancer Res.* 67: 10117-10122.
- Mohri T, Nakajima M, Takagi S, Komagata S, Yokoi T (2009). MicroRNA regulates human vitamin D receptor. *Int J Cancer.* 125: 1328-1333.
- Motoyama K, Inoue H, Nakamura Y, Uetake H, Sugihara K, Mori M (2008). Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family. *Clin. Cancer Res.* 14: 2334-2340.
- Nasser MW, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, Wang B, Suster S, Jacob ST, Ghoshal K (2008). Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1. *J. Biol. Chem.* 283: 33394-33405.
- Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG (2001). Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat. Genet.* 27(3): 263-270.
- Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G (2010). Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood.* 115: 1768-1778.
- Pulikkan JA, Peramangalam PS, Dengler V, Ho PA, Preudhomme C, Meshinchi S, Christopheit M, Nibourel O, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G (2010). C/EBP{alpha}-regulated microRNA-34a targets E2F3 during granulopoiesis and is downregulated in AML with CEBPA mutations. *Blood.*
- Reddy SD, Pakala SB, Ohshiro K, Rayala SK, Kumar R (2009). MicroRNA-661, a c/EBPalpha target, inhibits metastatic tumor antigen 1 and regulates its functions. *Cancer Res.* 69: 5639-5642.
- Riccardi C, Zollo O, Nocentini G, Bruscoli S, Bartoli A, D'Adamo F, Cannarile L, Delfino D, Ayroldi E, Migliorati G (2000). Glucocorticoid hormones in the regulation of cell death. *Therapie.* 55: 165-169.
- Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, Petrelli NJ, Dunn SP, Krueger LJ (2007). MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res.* 67: 9762-9770.
- Smith LK, Shah RR, Cidlowski JA (2010). Glucocorticoids modulate microRNA expression and processing during lymphocyte apoptosis. *J. Biol. Chem.*
- Stunnenberg HG (1993). Mechanisms of transactivation by retinoic acid receptors. *Bioessays.* 15: 309-315.
- Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 64: 3753-3756.
- Tam W (2001). Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. *Gene.* 274: 157-167.
- Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A, Meister G, Hermeking H (2007). Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle.* 6: 1586-1593.
- Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ (1996). CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes Dev.* 10: 804-815.
- Walters MR (1992). Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* 13: 719-764.
- Weiss FU, Marques IJ, Woltering JM, Vlecken DH, Aghdassi A, Partecke LI, Heidecke CD, Lerch MM, Bagowski CP (2009). Retinoic acid receptor antagonists inhibit miR-10a expression and block metastatic behavior of pancreatic cancer. *Gastroenterology.* 137: 2136-2145.
- Xi Q, Wang S, Ye Z, Liu J, Yu X, Zhu Z, Su S, Bai J, Li C (2010).



- Adenovirus-delivered microRNA targeting the vitamin D receptor reduces intracellular Ca<sup>2+</sup> concentrations by regulating the expression of Ca-transport proteins in renal epithelial cells. *BJU. Int.* [Epub ahead of print]
- Xie Z, Allen E, Fahlgren N, Calamar A, Givan SA, Carrington JC (2005). Expression of Arabidopsis MIRNA genes. *Plant Physiol.* 138: 2145-2154.
- Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell.* 9: 189-198.
- Zeng C, Wang R, Li D, Lin XJ, Wei QK, Yuan Y, Wang Q, Chen W, Zhuang SM (2010). A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. *Hepatology.* 52: 1702-1712.