Specific activation of microRNA-127 with downregulation of the proto-oncogene *BCL6* by chromatin-modifying drugs in human cancer cells

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Summary

Expression profiling of T24 cells revealed that 17 out of 313 human miRNAs were upregulated more than 3-fold by simultaneous treatment with the chromatin-modifying drugs 5-aza-2′-deoxycytidine and 4-phenylbutyric acid. One of these, miR-127, is embedded in a CpG island and is highly induced from its own promoter after treatment. miR-127 is usually expressed as part of a miRNA cluster in normal cells but not in cancer cells, suggesting that it is subject to epigenetic silencing. In addition, the proto-oncogene *BCL6*, a potential target of miR-127, was translationally downregulated after treatment. These results suggest that DNA demethylation and histone deacetylase inhibition can activate expression of miRNAs that may act as tumor suppressors.

Introduction

MicroRNAs (miRNAs) are ~22 nucleotide (nt) noncoding RNAs that can downregulate various gene products by translational repression when partially complementary sequences are present in the 3′ untranslated regions (3′ UTR) of the target mRNAs or by directing mRNA degradation (He and Hannon, 2004). miRNAs are expressed in a tissue-specific manner and are considered to play important roles in cell proliferation, apoptosis, and differentiation during mammalian development (Ambros, 2004; Bartel, 2004; Sempere et al., 2004). Moreover, recent studies have shown a link between patterns of miRNA expression and the development of cancer (Meltzer, 2005).

The profiling of miRNA expression has revealed that most of them have lower expression levels in tumors compared to normal tissues, while some miRNAs are upregulated or unchanged (Lu et al., 2005). Recent studies have also shown that some miRNAs are downregulated in various human cancers, indicating that they may act as tumor suppressors (Calin et al., 2002; Michael et al., 2003; Takamizawa et al., 2004). Indeed, let-7, which is downregulated in lung cancers, targets a critical oncogene, *RAS*, and miR-15 and 16, which are downregulated in chronic lymphocytic leukemias, target an antiapoptotic factor, *BCL2* (Cimmino et al., 2005; Johnson et al., 2005).

Although the biological importance of miRNAs is becoming increasingly apparent, regulation of miRNA expression is not fully understood. DNA methylation and histone modification play critical roles in chromatin remodeling and general regulation of gene expression in mammalian development and human diseases, such as cancer (Egger et al., 2004). To investigate whether miRNAs can be controlled by these epigenetic alterations, we analyzed miRNA expression in human cancer cells and normal fibroblasts treated with the DNA-demethylating agent 5-aza-2′-deoxycytidine (5-Aza-CdR) and/or the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA). Expression profiling revealed that 17 out of 313 human miRNAs examined were upregulated more than 3-fold by simultaneous treatment of T24 human bladder cancer cells with 5-Aza-CdR and PBA. In particular, we focused on miR-127, because it is embedded in a CpG island, and its expression was highly induced by 5-Aza-CdR and PBA treatment. Recent studies with mice have shown that miR-127 is located in a miRNA cluster

SIGNIFICANCE

Little is known about the regulation of microRNA (miRNA) expression, although the biological importance of these noncoding RNAs is becoming increasingly apparent. We show that a subset of miRNAs is upregulated in cancer cells by treatment with 5-aza-2′-deoxycytidine and 4-phenylbutyric acid, which inhibit DNA methylation and histone deacetylase, respectively. In particular, miR-127, which is located within a CpG island and silenced in human cancer cells, is highly induced after treatment from its own promoter with downregulation of its potential target, the proto-oncogene *BCL6*. Our study suggests that induction of some miRNAs by epigenetic treatment may be a novel strategy for anticancer therapy.

A

microRNA[a]	Cy5/Cy3 ratio[b]			
	T24			LD419
	PBA	AZA	AZA+PBA	AZA+PBA
34a	8	–	–	–
126	–	18	3	–
127	–	–	49	–
132	–	4	3	–
134	–	–	4	–
139	6	–	–	–
146a	3	–	–	27
146b	5	–	–	19
148a	7	–	–	–
182	3	–	4	–
183	3	–	3	–
192	–	–	6	–
193b	–	–	6	–
205	–	9	–	–
368	–	–	4	7
377	–	–	44	–
494	–	–	36	–
495	–	–	31	4
498	–	–	4	–
512-3p	–	–	8	–
516-5p	–	–	26	–
517a	–	–	27	–
517b	–	–	10	–

B

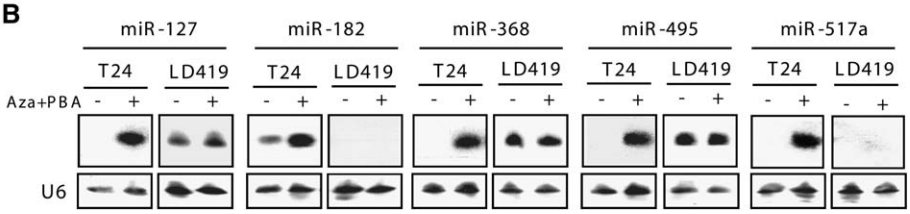


Figure 1. Expression profiling of human miRNAs in T24 and LD419 cells after 5-Aza-CdR and PBA treatment

A: miRNAs upregulated over 3-fold by treatment of T24 and LD419 cells with 5-Aza-CdR and PBA. [a]A total of 313 human miRNAs were analyzed by miRNA microarray (LC Sciences; http://www.lcsciences.com/). [b]Untreated cells and cells treated with 5-Aza-CdR (3 μM) and/or PBA (3 mM) for 6 days were labeled with Cy3 and Cy5, respectively. These values represent the mean of seven redundancies on the chip for each miRNA, while the dashes indicate that the difference between Cy5 and Cy3 was not significant or Cy5/Cy3 ratio was under 3.

B: Northern blot analyses of miR-127, -182, -368, -495, and -517a in T24 and LD419 cells untreated or treated with 5-Aza-CdR (3 μM) and PBA (3 mM). U6 RNA expression was used as a loading control.

of an imprinted locus on mouse chromosome 12, whereas the imprinted expression of these miRNAs has not been demonstrated in human (Seitz et al., 2003, 2004). Although the human miR-127 gene is located within a cluster with miR-136, miR-431, miR-432, and miR-433 on chromosome 14q32.31 (Altuvia et al., 2005), recent studies have revealed that miR-127 and miR-136 have different expression patterns in human cancers (Iorio et al., 2005; Lu et al., 2005). This indicates that miR-127 and miR-136 have different regulatory mechanisms for their expression, at least in human cancers. These findings encouraged us to study the potential epigenetic regulation of miR-127 expression in human cancer cells. In addition, we examined the potential correlation between miR-127 and *BCL6*, which is a proto-oncogene and a predicted target of miR-127.

Results

miR-127 is located within a CpG island and is highly induced by DNA demethylation and histone deacetylase inhibition

miRNA microarray analysis was done on T24 human bladder cancer cells and LD419 human normal fibroblast cells treated with 5-Aza-CdR and/or PBA. The combination treatment with 5-Aza-CdR and PBA showed that 17 out of 313 (5.4%) human miRNAs examined were upregulated more than 3-fold in T24 cells (Figure 1A). Normal fibroblasts (LD419 cells) also showed more than 3-fold induction of 17 miRNAs after the treatment. However, the pattern was quite different from those in T24 cells (Figure 1A and data not shown). To confirm the miRNA microarray data, we used Northern blot analysis of T24 and LD419 cells before and after the treatment (Figure 1B). The Northern blot results were consistent with the microarray data for the subset of miRNAs examined.

One of these, miR-127, is embedded in a CpG island, as determined by the CpG Island Searcher Program (http://cpgislands.usc.edu/; Takai and Jones, 2002), and was upregulated 49-fold after the combination treatment with 5-Aza-CdR and PBA but showed no significant difference with either one of the two drugs, indicating that miR-127 can be regulated directly by the synergistic effects of DNA demethylation and histone deacetylase inhibition.

miR-127 is silenced in cancer cells but is expressed as a part of a miRNA cluster in normal fibroblasts

For further examination of the regulation of miR-127 by 5-Aza-CdR and PBA, we extended our study to a panel of seven human cancer cell lines (HCT116, HeLa, NCCIT, Ramos, CFPAC-1, MCF7, and CALU-1) as well as two normal human fibroblast cell lines (LD98 and CCD-1070Sk). Expression patterns of miR-127 in these cell lines were analyzed by Northern blot and

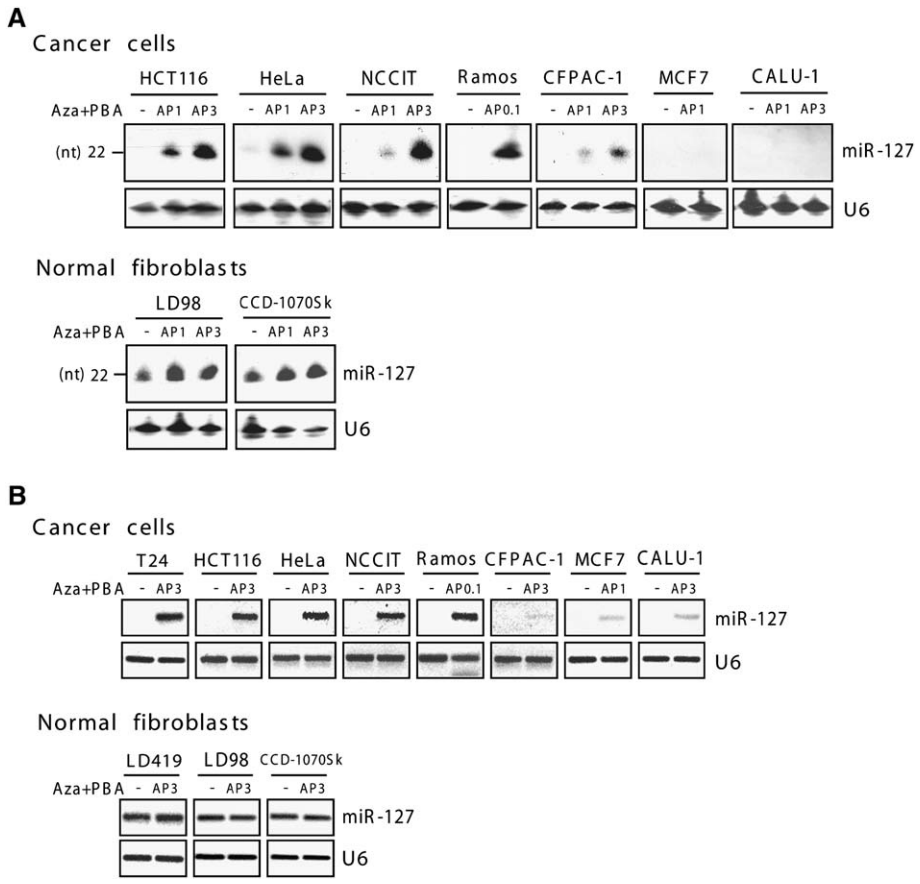


Figure 2. Expression patterns of miR-127 in cancer cells and normal fibroblasts treated with 5-Aza-CdR and PBA

A: Northern blot analysis of miR-127 expression before and after treatment with different doses of 5-Aza-CdR and PBA in a panel of cancer cell lines (HCT116 colon carcinoma, HeLa cervical carcinoma, NCCIT embryonic carcinoma, Ramos lymphoma, CFPAC-1 pancreatic carcinoma, MCF7 breast carcinoma, and CALU-1 lung carcinoma cells) and normal fibroblasts (LD98 and CCD-1070Sk). U6 RNA expression was used as a loading control. AP0.1, combination of 0.1 μM 5-Aza-CdR and 1 mM PBA; AP1, combination of 1 μM 5-Aza-CdR and 1 mM PBA; AP3, combination of 3 μM 5-Aza-CdR and 3 mM PBA.

B: Confirmation of expression patterns of miR-127 in cancer cells and normal fibroblasts treated with 5-Aza-CdR and PBA by stem-loop RT-PCR analyses. U6 RNA expression was used as an internal control.

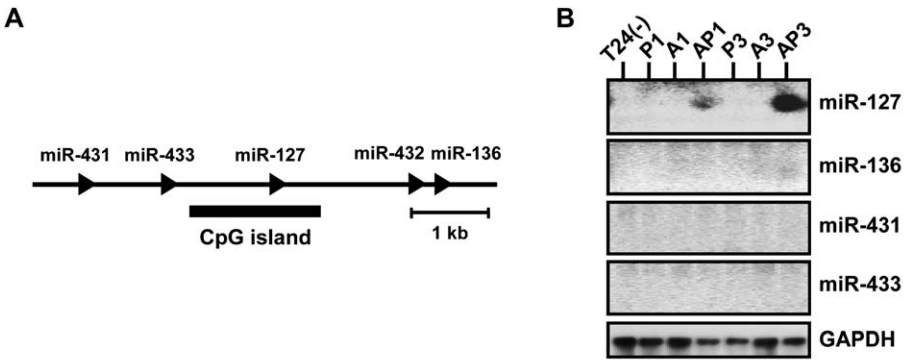
stem-loop reverse transcription (RT)-PCR analyses (Chen et al., 2005). miR-127 was silenced in all the cancer cell lines examined but was substantially induced after 5-Aza-CdR and PBA treatment in a dose-dependent manner in HCT116, HeLa, NCCIT, and Ramos cells (Figures 2A and 2B). CFPAC-1 showed only a slight induction of miR-127 after treatment. MCF7 and CALU-1 showed no induction of miR-127 by Northern blot analyses, whereas stem-loop RT-PCR showed a slight induction, probably due to its higher sensitivity (Figures 2A and 2B). On the other hand, miR-127 was expressed substantially in normal fibroblasts and was slightly upregulated after treatment (Figures 2A and 2B).

As shown in Figure 3A, miR-127 is embedded in a CpG island and is part of a miRNA cluster with miR-136, miR-431, miR-432, and miR-433, which shows imprinted expression in the mouse (Seitz et al., 2003, 2004). Interestingly, the miRNA microarray data clearly demonstrated that only miR-127 showed prominent upregulation, while the other miRNAs in the cluster, miR-136, -431, -432, and -433 (Figure 3A), showed no significant upregulation after the combination treatment with 5-Aza-CdR and PBA. Moreover, the Northern blot analyses also demonstrated that neither 5-Aza-CdR nor PBA alone activated miR-127 expression, but the combination treatment with both drugs substantially induced miR-127 expression in a dose-dependent manner (Figure 3B). On the other hand, we could not detect any induction of miR-136, -431, and -433 after treatment (Figure 3B). In contrast, all the miRNAs in the cluster (miR-127, -136, -431, -432, and -433) were expressed in LD419 cells, as determined by the microarray data and the Northern blot analyses (Figure 3C). Similar to T24 cells, only miR-127 was significantly upregulated in LD419 cells after 5-Aza-CdR and PBA treatment, and the induction of miR-127 expression was more pronounced in T24 cells than in LD419 cells. These results suggest that miR-127 is expressed as a part of the cluster in normal fibroblasts as previously observed in mice (Davis et al., 2005), whereas all miRNAs in the cluster seem to be downregulated and/or silenced in cancer cells. Only miR-127 expression is inducible by 5-Aza-CdR and PBA treatment in both normal fibroblasts and cancer cells (see also Figure 7).

Alterations in DNA methylation and histone modification around the promoter region of the miR-127 gene by 5-Aza-CdR and PBA treatment

Our data obtained from the microarray and the Northern blot indicate that miR-127 is induced from its own promoter after 5-Aza-CdR and PBA treatment in both LD419 and T24 cells. Therefore, we determined the potential transcription start sites for the primary transcripts of miR-127 after 5-Aza-CdR and PBA treatment by 5′ rapid amplification of cDNA ends (RACE; Cai et al., 2004) in both cell lines. As shown in the diagram in Figure 4A, we were able to identify three different start sites in treated T24 cells and one single start site in treated LD419 cells.

Next, we examined the DNA methylation status of the promoter region of the miR-127 gene before and after 5-Aza-CdR and PBA treatment by bisulfite genomic sequencing (Figures 4A and 4B). The promoter region of the miR-127 gene was heavily methylated in LD419 cells (97%), and the methylation level was decreased to 81% after 5-Aza-CdR and PBA



C

microRNA	LD419				T24			
	microarray[a]		Northern[b]		microarray[a]		Northern[b]	
	expression	upregulation	expression	upregulation	expression	upregulation	expression	upregulation
127	+	1.5	+	+	–	49	–	+++
136	+	–	ND	ND	–	–	–	–
431	+	–	+	–	–	–	–	–
432	+	–	ND	ND	–	–	ND	ND
433	+	–	+	–	–	–	–	–

Figure 3. Expression patterns of miRNAs in the cluster in T24 and LD419 cells before and after treatment with 5-Aza-CdR and PBA

A: Schematic representation of the locations of the miRNA genes (miR-127, -136, -431, -432, and -433) on human chromosome 14q32.31. The positions of these miRNA genes are indicated by triangles. The miR-127 gene is embedded in a CpG island.

B: Northern blot analyses of miR-127, -136, -431, and -433 in T24 cells untreated or treated with PBA and/or 5-Aza-CdR for 6 days. GAPDH mRNA expression was used as a loading control. P1, 1 mM PBA; A1, 1 μM 5-Aza-CdR; AP1, combination of 1 μM 5-Aza-CdR and 1 mM PBA; P3, 3 mM PBA; A3, 3 μM 5-Aza-CdR; AP3, combination of 3 μM 5-Aza-CdR and 3 mM PBA.

C: Summary of expression patterns of miRNAs in the cluster in LD419 and T24 cells before and after 5-Aza-CdR and PBA treatment as determined by microarray and Northern blot analyses. [a]Untreated cells and cells treated with 5-Aza-CdR (3 μM) and PBA (3 mM) were labeled with Cy3 and Cy5, respectively. Expression: + or –, mean Cy3 signal was over or under 30 for untreated cells. Upregulation: this value represents mean Cy5/Cy3 ratio of seven redundancies on the chip. –, no significant difference between Cy5 and Cy3. [b]Expression levels were determined by Northern blot analyses as described in Figures 1B and 3B. ND, not determined. Expression: + or –, miRNA was visibly expressed in untreated cells or not. Upregulation: + or +++, degree of upregulation of miRNA expression after treatment with 5-Aza-CdR (3 μM) and PBA (3 mM). –, no observable change.

treatment (Figure 4A). On the other hand, the methylation level of the same region in T24 cells was only 60%, and it was further decreased to 41% after treatment (Figure 4B). The high levels of methylation in LD419 cells did not block expression of miR-127 as a part of the cluster (see Figure 7) but were associated with low levels of induction of miR-127 after 5-Aza-CdR and PBA treatment (Figure 3C).

Furthermore, we analyzed alterations in chromatin structure in four regions around the transcription start sites of the miR-127 gene by chromatin immunoprecipitation (ChIP) assay and quantitative real-time PCR in T24 cells (Figure 4C). Both acetylated histone H3 and methylated histone H3-lysine 4 (K4) are associated with open chromatin structure and active gene expression. Levels of both acetylated histone H3 and methylated histone H3-K4 were increased in T24 cells by all types of treatments compared to untreated cells in all the regions examined (Figure 4C). Remarkably, regions 2 and 3, which contain the transcription start sites of miR-127, showed a pronounced increase in both active marks after the high-dose combination treatment. Moreover, we confirmed the real-time PCR data by conventional PCR and gel analyses (Figure 4D). Both levels of acetylated histone H3 and methylated histone H3-K4 in region 2 were increased in T24 cells by all types of treatments. These results indicate that the induction of miR-127 after 5-Aza-CdR and PBA treatment is accompanied by a prominent decrease in DNA methylation levels and an increase in active histone marks around the transcription start sites of miR-127.

miR-127 is downregulated in primary human tumors

To test whether our observations in cell lines are relevant to human cancer, we examined miR-127 expression in matched sets of normal tissues and primary tumors of prostate, bladder, and colon. As shown in Figure 5A, miR-127 was highly expressed in normal prostate and bladder tissues but was remarkably downregulated or silenced in the corresponding tumors in all cases examined. Normal colon tissues showed weak or no expression of miR-127, and one case (case 1) showed downregulation in the corresponding tumor (Figure 5A). We also examined expression levels of the other miRNAs that are adjacent to miR-127 in the cluster, and they showed the same expression patterns as miR-127 in the normal tissues and primary tumors (data not shown). These results indicate that all the miRNAs in the cluster, including miR-127, are expressed together in normal tissues of specific organs but are downregulated or silenced in most tumors.

Next, we analyzed DNA methylation levels in several embryonic and somatic tissues and primary tumors by methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) (Gonzalgo and Jones, 2002). The promoter region of miR-127 was unmethylated in sperm, roughly 30% and 50% methylated in testis and placenta, respectively, and heavily methylated in all other human embryonic and somatic tissues examined (Figure 5B). In addition, the prostate, bladder, and colon tissues and the corresponding tumors that we examined for miR-127 expression in Figure 5A were also heavily methylated; there was no significant difference between normal tissues and tumors (Figure 5B). These results indicate that miR-127 can be expressed as a part of the cluster even though it is highly methylated, supporting the fact that miR-127 is processed from a large transcript including all miRNAs in the cluster in normal tissues. However, activation of miR-127 from its own promoter in cancer cells is dependent on DNA demethylation and histone deacetylase inhibition.

Expression of BCL6, a target of miR-127, is suppressed after 5-Aza-CdR and PBA treatment

Computational predictions for human miRNA targets show that BCL6 is a potential target of miR-127. Detailed information is available at Human microRNA Targets (http://www.microrna.org/) and miRBase Targets (http://microrna.sanger.ac.uk/). To

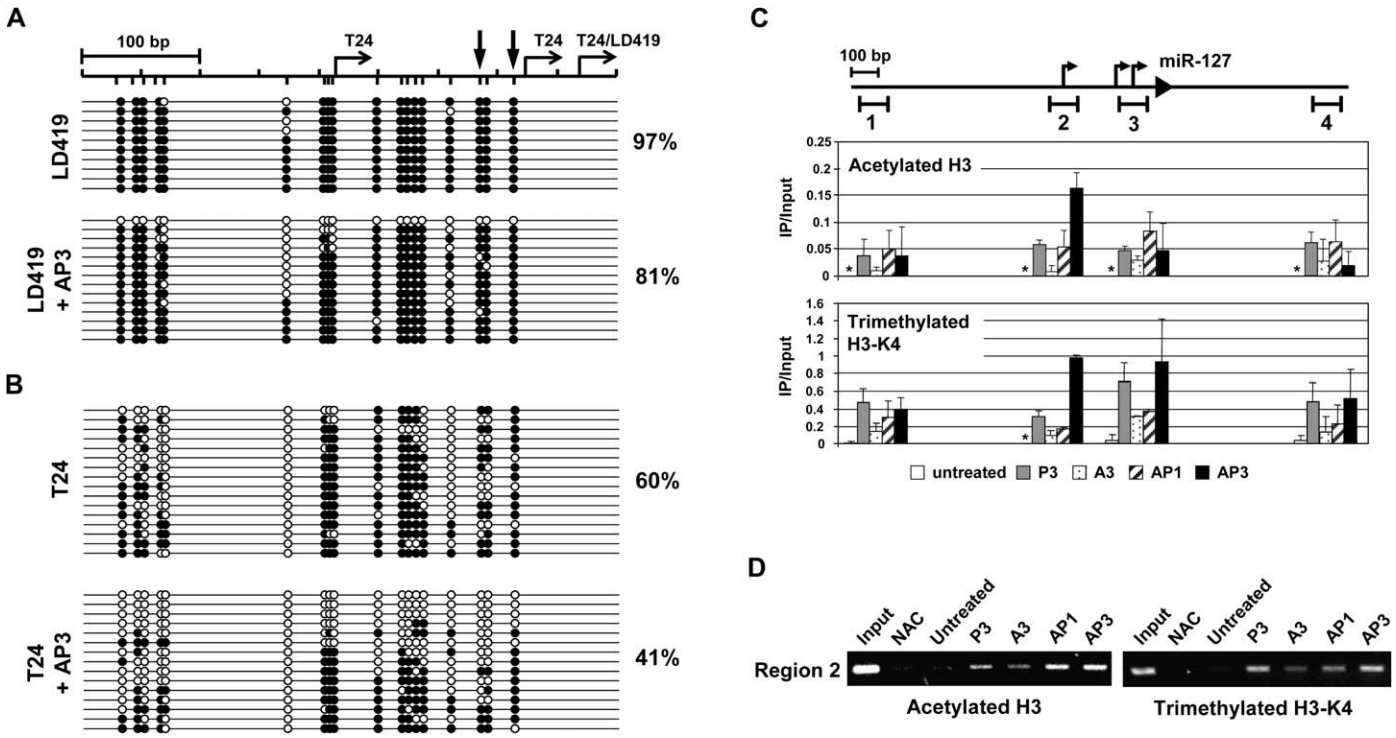


Figure 4. Alterations in DNA methylation and histone modifications around the promoter region of the miR-127 gene by 5-Aza-CdR and PBA treatment

DNA methylation status of LD419 (**A**) and T24 (**B**) cells untreated or treated with 5-Aza-CdR and PBA were determined by bisulfite genomic sequencing. The bent arrows indicate the transcription start sites of primary transcripts of miR-127 determined by 5′-RACE. The vertical arrows indicate the primers used for Ms-SNuPE analyses in Figure 5B. Each row represents a single cloned allele. Open circle, unmethylated CpG; filled circle, methylated CpG; AP3, combination of 3 μM 5-Aza-CdR and 3 mM PBA. Percentage of methylated CpG sites is shown for each analysis. **C:** The levels of acetylated histone H3 and trimethylated histone H3-K4 in four regions around the miR-127 gene determined by ChIP assay in T24 cells untreated or treated with PBA and/or 5-Aza-CdR. The bent arrows indicate the transcription start sites of primary transcript of miR-127 determined by 5′-RACE. IP/Input = (immunoprecipitated DNA with each antibody − nonspecific antibody control [NAC])/(input DNA − NAC). Values represent mean + SD of two or three experiments from two independent ChIP samples. P3, 3 mM PBA; A3, 3 μM 5-Aza-CdR; AP1, combination of 1 μM 5-Aza-CdR and 1 mM PBA; AP3, combination of 3 μM 5-Aza-CdR and 3 mM PBA. Asterisks indicate that values were not detectable. **D:** Confirmation of ChIP assay for acetylated histone H3 and methylated histone H3-K4 in region 2 by conventional PCR and gel analyses.

validate that miR-127 can target BCL6, expression levels of BCL6 in T24 cells and a lymphoma cell line, Ramos, treated with 5-Aza-CdR and/or PBA were assessed by Western blot analysis of nuclear protein extracts and by RT-PCR of mRNA. Ramos cells are derived from a human B cell lymphoma and strongly express BCL6 (Phan and Dalla-Favera, 2004). We treated Ramos cells with 0.1 μM of 5-Aza-CdR because they are more sensitive to this drug compared to T24 cells, which caused a reduction of DNA methylation at the promoter region of miR-127 from 75% to 57% (data not shown). We confirmed that miR-127 was substantially induced by the low-dose combination treatment (Figures 2A and 2B). We found a decrease in BCL6 protein expression in T24 and Ramos cells treated with 5-Aza-CdR and PBA (Figure 6A). On the other hand, there was no difference in the mRNA expression levels of *BCL6* before and after treatment as examined by conventional RT-PCR (Figure 6A) and quantitative real-time RT-PCR (data not shown), suggesting translational inhibition of BCL6 by treatment and not mRNA degradation. Furthermore, Ramos cells were transfected with miR-127 precursor molecules, which are designed to directly enter the miRNA processing pathway and mimic endogenous miR-127 in the cells. Overexpression of miR-127 induced a reduction of BCL6 protein level (Figure 6B).

To further confirm target specificity between miR-127 and BCL6, we performed a luciferase reporter assay with a vector containing the putative *BCL6* 3′ UTR target site downstream of a luciferase reporter gene. Base pairing between miR-127 and wild-type (WT) or mutant (MUT) putative target site in the 3′ UTR of *BCL6* mRNA is shown in Figure 6C. These vectors were transfected into LD419 or HeLa cells that express or don't express miR-127, respectively. Luciferase activity of LD419 cells transfected with BCL6-WT was significantly lower than LD419 cells transfected with BCL6-MUT ($p = 0.0001$). In contrast, luciferase activities of HeLa cells transfected with BCL6-WT and BCL6-MUT showed no measurable difference ($p = 0.1481$; Figure 6D). These data suggest that BCL6 is one of the targets of miR-127 and that miR-127 can downregulate BCL6 by translational repression.

Discussion

Understanding the regulatory mechanisms controlling miRNA expression is very important, since miRNAs can have large-scale effects through regulation of a variety of genes during mammalian development and carcinogenesis. Here we show, to our knowledge for the first time, that some miRNAs can be activated by chromatin-modifying drugs such as inhibitors of DNA methylation and histone deacetylation in human cancer cells. Our microarray data indicated that a small percentage of known miRNAs examined were upregulated by 5-Aza-CdR and PBA

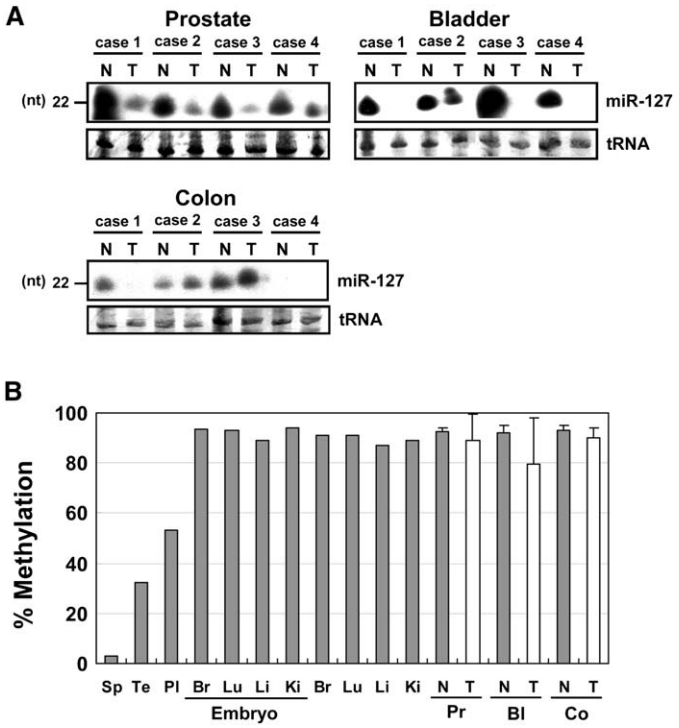


Figure 5. Expression levels and DNA methylation status of miR-127 in normal tissues and primary tumors

A: Northern blot analyses of miR-127 in matched sets of normal tissues and primary tumors of prostate, bladder, and colon. Lower panels indicate loading controls of tRNA obtained from methylene blue staining of membranes. N, normal tissues; T, the corresponding tumors.

B: The average methylation levels of the promoter region of miR-127 in human embryonic and somatic tissues and primary tumors determined by Ms-SNuPE analysis. The primers used for Ms-SNuPE analysis are shown in Figure 4A. Sp, sperm; Te, testis; Pl, placenta; Br, brain; Lu, lung; Li, liver; Ki, kidney; Pr, prostate; Bl, bladder; Co, colon; N, normal tissues; T, the corresponding tumors. The average methylation levels of four cases of prostate, bladder, and colon tissues and the corresponding tumors that we examined for miR-127 expression in Figure 5A are shown as mean + SD.

treatment in both T24 and LD419 cells. These upregulated miRNAs were quite different between these two cell lines, indicating that DNA methylation status and chromatin structure around miRNA genes are different between cancer cells and normal fibroblasts. As shown in Figure 1B, miR-127, -368, -495, and -517a, which were silenced in T24 cells, were highly induced by the treatment. All of them were also highly expressed in LD419 cells except miR-517a. In addition, a recent study predicted BCL2 as a target of miR-182 (Cimmino et al., 2005). These results suggest that inhibitors of DNA methylation and histone deacetylation can induce expression of some miRNAs that may act as tumor suppressors.

In particular, we focused our study on the induction of miR-127, which is embedded within a CpG island methylated in most tissues except sperm, and which shows imprinted expression in the mouse (Seitz et al., 2003, 2004). In agreement with a previous study with mice (Davis et al., 2005), miR-127 is expressed as a part of the cluster with miR-136, -431, -432, and -433 in normal tissues and cultured fibroblasts. In contrast, the expression of the whole cluster is downregulated or completely silenced in primary tumors and various cancer cell lines. However, combination treatment with 5-Aza-CdR and PBA leads to a specific and prominent reactivation of miR-127 in various cancer cell lines (Figure 7).

The synergistic effect of DNA demethylation and HDAC inhibition was investigated in greater detail by Cameron et al. and Suzuki et al. (Cameron et al., 1999; Suzuki et al., 2002), who showed that silenced genes with unmethylated promoters can often be activated by HDAC inhibitors, whereas these agents are largely ineffective for genes with methylated promoters. In contrast, DNA methylation inhibitors are highly effective not only at removing cytosine methylation but also at rapidly reversing chromatin structural changes (Nguyen et al., 2002). To maximally achieve gene reactivation, it may be necessary to simultaneously inhibit both DNA methylation and histone deacetylation. The fact that the combined effect of DNA demethylation and HDAC inhibition could induce miR-127 expression of cancer cells, but neither alone, suggests that the transcription of the miR-127 gene is strongly suppressed in cancer cells.

The DNA-demethylating agent 5-Aza-CdR was effective in removing cytosine methylation in the promoter region of miR-127 in both normal fibroblasts and cancer cells. The difference of methylation levels between LD419 and T24 cells is quite interesting and might be attributed to the fact that cancer cells have a higher proliferation rate and more efficient incorporation of 5-Aza-CdR. The decrease of methylation level in the promoter region of miR-127 was higher in T24 cells (32%) compared to LD419 cells (16%), which is consistent with the fact that the induction of miR-127 expression after treatment was much higher in T24 cells compared to LD419 cells, although induction of miR-127 expression needs both DNA demethylation and histone deacetylase inhibition.

The histone deacetylase inhibitor PBA acts to increase the level of gene expression in combination with 5-Aza-CdR through chromatin structural changes. ChIP assays showed that the levels of histone H3 acetylation and H3-K4 methylation, which are associated with active gene expression, were increased around the transcriptional start sites of the miR-127 gene in T24 cells treated with the high-dose combination of 5-Aza-CdR and PBA. A previous study from our laboratory has also shown distinct localization of histone H3 acetylation and H3-K4 methylation to transcription start sites in the human genome (Liang et al., 2004). These results indicate that decreased cytosine methylation and increased histone H3 acetylation and H3-K4 methylation in the promoter region of the miR-127 gene by 5-Aza-CdR and PBA treatment are associated with its activation.

Since miR-127 is constitutively expressed in normal fibroblasts and normal tissues but silenced in cancer cells and downregulated in 9/12 (75%) primary tumors, we have focused on a potential role of miR-127 as a tumor suppressor. The human proto-oncogene *BCL6* encodes a BTB/POZ-zinc finger transcriptional repressor that is necessary for germinal center formation and is implicated in the pathogenesis of B cell lymphoma (Phan and Dalla-Favera, 2004; Polo et al., 2004). Moreover, BCL6 suppresses p53 expression and modulates DNA damage-induced apoptotic responses in germinal center B cells (Phan and Dalla-Favera, 2004). Our results in this study suggest that miR-127 can downregulate BCL6 by translational repression. Thus, the induction of miR-127 expression by 5-Aza-CdR and PBA treatment in cancer cells may have an anticancer effect through downregulation of its targets such as BCL6. In addition, the specific induction of miR-127 is more

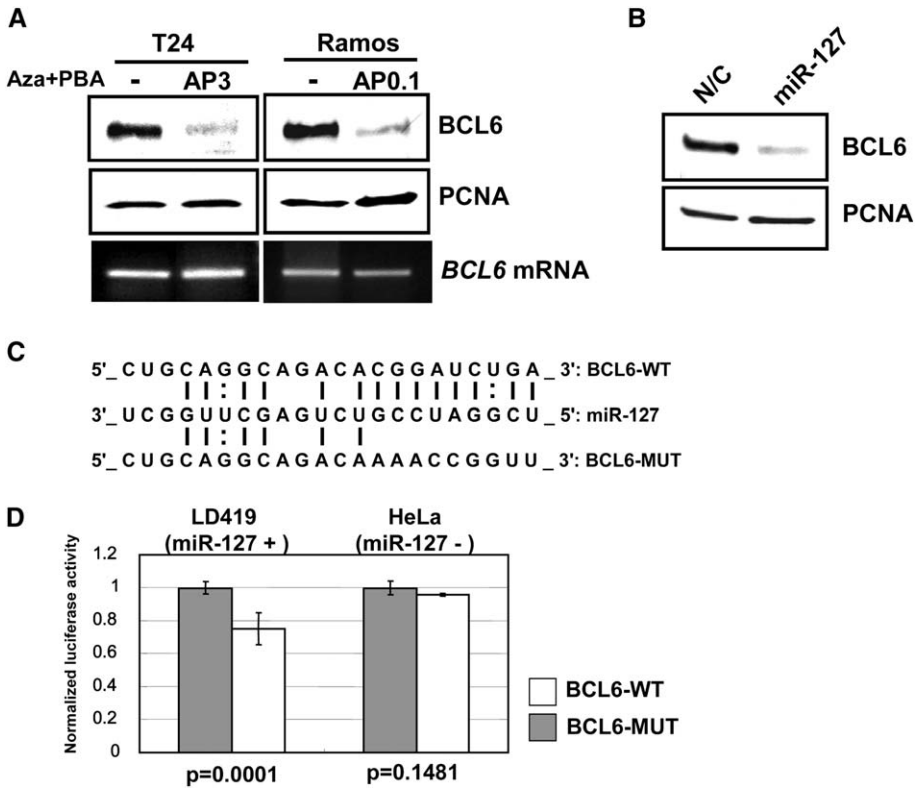


Figure 6. Identification of BCL6 as a target of miR-127

A: Western blots of nuclear extracts sequentially probed with antibodies against BCL6 (top panel) and PCNA as a loading control (middle panel), and RT-PCR analysis of *BCL6* mRNA in T24 and Ramos cells untreated or treated with 5-Aza-CdR and PBA. AP0.1, combination of 0.1 μM 5-Aza-CdR and 1 mM PBA; AP3, combination of 3 μM 5-Aza-CdR and 3 mM PBA.

B: Western blots of nuclear extracts isolated from Ramos cells transfected with miR-127 precursor molecules (miR-127) or negative control precursor miRNAs (N/C) sequentially probed with antibodies against BCL6 (top panel) and PCNA as a loading control (bottom panel).

C: Base pairing comparison between mature miR-127 and the wild-type (WT) or mutant (MUT) putative target site in the 3′ UTR of *BCL6* mRNA. The mutant sequence is identical to the wild-type construct except for mutations disrupting base pairing at the 5′ end of miR-127.

D: Dual luciferase assay of LD419 or HeLa cells transfected with firefly luciferase constructs containing the wild-type (WT, open bar) or mutant (MUT, filled bar) target site of the *BCL6* 3′ UTR for 48 hr. The firefly luciferase activity was normalized to the *Renilla* luciferase activity as an internal transfection control. Then, the luciferase values of wild-type transfections were normalized to the average values for the corresponding mutant transfections. Values represent mean ± SD of six experiments from three independent transfections.

pronounced in cancer cell lines compared to normal fibroblasts, which might be beneficial for cancer therapy. However, as it is possible that BCL6 expression is additionally controlled by other miRNAs or transcriptional regulators during 5-Aza-CdR and PBA treatment, further studies such as conditional overexpression models of an exogenous miR-127 in lymphoma cells that overexpress BCL6 are needed to reveal the regulatory mechanism of BCL6 expression.

Taken together, our results suggest an effect of epigenetic treatment that couples inhibitors of DNA methylation and histone deacetylation to induction of some miRNAs. These miRNAs can potentially regulate expression of target genes that are important in human carcinogenesis. Further studies on epigenetic regulation of miRNA expression are necessary, and regulation of miRNA expression by epigenetic treatment may be a novel strategy for prevention or treatment of human cancer.

Experimental procedures

Cell lines

T24 (bladder transitional carcinoma cells), HCT116 (colon carcinoma cells), NCCIT (embryonic carcinoma cells), Ramos (lymphoma cells), HeLa (cervical carcinoma cells), CFPAC-1 (pancreatic carcinoma cells), MCF7 (breast carcinoma cells), CALU-1 (lung carcinoma cells), and CCD-1070Sk (human normal fibroblast cells) were obtained from the American Type Culture Collection (Rockville, MD). LD98 and LD419 (human normal fibroblast cells) were established in our laboratory. T24, HCT116, LD98, and LD419 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). NCCIT and Ramos cells were cultured in RPMI1640 medium supplemented with 10% FBS. HeLa and MCF7 cells were cultured in MEM medium supplemented with 10% FBS. CALU-1 was cultured in McCoy's 5A medium supplemented with 10% FBS and 1× glutamine. CFPAC-1 cells were cultured in IMDM medium supplemented with 10% FBS and 1× glutamine. CCD-1070Sk cells were cultured in MEM medium supplemented with 10% FBS, 1× sodium pyruvate, and 1× MEM nonessential amino acids.

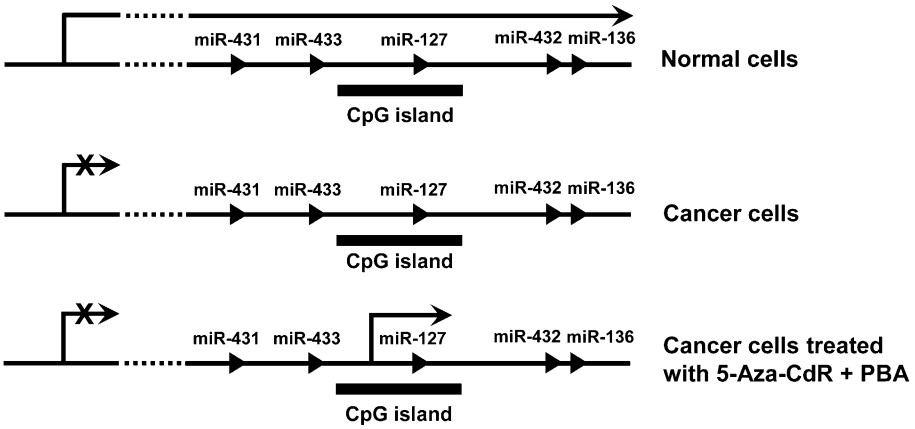


Figure 7. A model summarizing expression patterns of miRNAs in the cluster in normal and cancer cells

Schematic representations of the locations of the miRNA genes (miR-127, -136, -431, -432, and -433) in the cluster on human chromosome 14q32.31 are shown. The miR-127 gene is embedded in a CpG island. This locus is imprinted in the mouse. miR-127 is expressed as a part of the cluster with miR-136, -431, -432, and -433 in normal cells. In contrast, the expression of the whole cluster is downregulated or completely silenced in cancer cells. However, combination treatment with 5-Aza-CdR and PBA leads to a specific and prominent reactivation of miR-127 in cancer cells.

RNA and DNA extraction from primary tissues

Matched sets of primary prostate, bladder, and colon tumors and adjacent normal tissues from the same patients were obtained through the USC/Norris Tissue Procurement Core Resource after informed consent and Institutional Review Board (IRB) approval (IRB #886005 and #926041) at the USC/Norris Comprehensive Cancer Center. Fresh frozen blocks of tumor and corresponding adjacent normal tissue identified by a pathologist were used for nucleic acid extraction. RNA was extracted using the ToTALLY RNA Isolation Kit (Ambion, Austin, TX), and DNA was isolated from the same blocks as previously described (Wu et al., 1995).

5-Aza-CdR and PBA treatment

Cells were seeded at 5×10^5 cells per 100 mm dish 24 hr prior to treatment with 5-Aza-CdR (1 μM or 3 μM; Sigma-Aldrich, St. Louis, MO) and/or PBA (1 mM or 3 mM; Sigma-Aldrich). 5-Aza-CdR was removed after 24 hr, while PBA was continuously administered by replacing the medium containing PBA every 24 hr for 5 or 6 days. Ramos cells were seeded at 2×10^6 cells per 100 mm dish 24 hr prior to treatment with 5-Aza-CdR (0.1 μM) and/or PBA (1 mM). Treatments were done as described above for 4 days.

miRNA microarray analysis

miRNA microarray analysis was carried out by LC sciences (http://www.lcsciences.com/; Houston, TX). In brief, poly-A tails were added to the RNA sequences at the 3′ ends using a poly(A) polymerase, and nucleotide tags were then ligated to the poly-A tails. For each dual-sample experiment, two sets of RNA sequences were added with tags of two different sequences. The tagged RNA sequences were then hybridized to the miRNA microarray chip containing 313 human miRNA probes. The probe sequences are available upon request. The labeling reaction was carried out during the second hybridization reaction using tag-specific dendrimer Cy3 and Cy5 dyes. RNAs from untreated cells and cells treated with 5-Aza-CdR and/or PBA were labeled with Cy3 and Cy5, respectively. The human miRNA chip includes seven redundancies for each miRNA. The data were corrected by subtracting the background and normalizing to the statistical median of all detectable transcripts. Background was calculated from the median of 5% to 25% of the lowest-intensity cells. The data normalization balances the intensities of Cy3- and Cy5-labeled transcripts so that differential expression ratios can be correctly calculated. All data were submitted to the ArrayExpress database, and the accession number is E-MEXP-706.

Northern blots

Total RNA was extracted from cells with the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA (30 μg) was loaded onto a denaturing gel and transferred to a nylon membrane. The StarFire radiolabeled probes (Integrated DNA Technologies, Coralville, IA) were prepared by incorporation of [α-^{32}P] dATP 6000 Ci/mmol following the manufacturer's recommendation. The sequences of the probes are available upon request. Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (Clontech, Mountain View, CA). As a loading control, random primed labeling probes specific for U6 RNA and GAPDH mRNA were generated using High Prime (Roche, Indianapolis, IN).

Stem-loop RT-PCR for miRNAs

Stem-loop RT for mature miR-127 was performed as previously described (Chen et al., 2005). All reagents for stem-loop RT were obtained from Applied Biosystems (Foster City, CA). PCR products were analyzed on 3% agarose gels. U6 RNA was used as an internal control. The primers used for stem-loop RT-PCR for miR-127 are as follows: miR-127 RT, 5′-GTCGTATCCAGTGC AGGGTCCGAGGTATTCGCACTGGATACGAAGCCAA-3′; miR-127 forward, 5′-GCGGCTCGGATCCGTCTGAGCT-3′; miR-127 reverse, 5′-GTGCAGGG TCCGAGGT-3′; U6 forward, 5′-CTCGCTTCGGCAGCACA-3′; U6 reverse, 5′-AACGCTTCACGAATTTGCGT-3′.

Bisulfite genomic sequencing

Genomic DNA (2 μg) was converted with sodium bisulfite as previously described (Frommer et al., 1992). After amplification of the bisulfite-converted DNA with specific primers for the miR-127 gene, DNA methylation levels were analyzed by bisulfite genomic sequencing as previously described (Daskalakis et al., 2002).

Ms-SNuPE analysis

Genomic DNA (2 μg) was converted with sodium bisulfite as described above. After amplification of bisulfite-converted DNA with specific primers for the miR-127 gene, DNA methylation levels were analyzed by Ms-SNuPE as previously described (Gonzalgo and Jones, 2002). The percentage of methylation at each CpG site was determined by the C:T signal ratio. The primers used for Ms-SNuPE are available upon request. A genomic DNA panel of several human tissues was purchased from the BioChain Institute (Hayward, CA).

ChIP assay

The ChIP assay was performed as previously described (Nguyen et al., 2002). Ten microliters of anti-trimethylated histone H3-K4 (Upstate Biochemistry, Lake Placid, NY) and 10 μl of anti-acetylated histone H3 antibodies (Upstate Biochemistry) were used. Quantitative analysis was performed by real-time PCR with CYBR green using the DNA Engine Opticon System (MJ Research, Waltham, MA). The specific primer sequences used for the ChIP assay are available upon request. The fraction of immunoprecipitated DNA was calculated as follows: (immunoprecipitated DNA with each antibody − nonspecific antibody control [NAC])/(input DNA − NAC). Signals were also confirmed by conventional PCR and gel analyses.

Western blot with nuclear protein extracts and RT-PCR of BCL6

Nuclear protein extracts were separated by SDS/polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were hybridized with antibodies against BCL6 (N-3, Santa Cruz Biotechnology, Santa Cruz, CA) and PCNA (Santa Cruz Biotechnology). Total RNA (5 μg) was used for reverse transcription. After incubation with DNase I (Invitrogen) to eliminate DNA contamination, Superscript III (Invitrogen) and random hexamers (Promega, Madison, WI) were added for first strand cDNA synthesis. Then PCR was performed with primers specific for *BCL6* mRNA (forward, 5′-CGGAAGATGAGAGATTGCCCTGC-3′; reverse, 5′-GCCTGGAGGATGCA GGCATT-3′).

Transfection with miR-127 precursor molecules

miR-127 precursor molecules and negative control 1 precursor miRNAs were purchased from Ambion. They were transfected into Ramos cells at final concentrations of 100 nM each using oligofectamine (Invitrogen) according to the manufacturer's instruction. Three days after transfection, cells were collected and BCL6 expression was analyzed by Western blot as described above.

Luciferase assay

Luciferase constructs were made by ligating oligonucleotides containing the wild-type or mutant target site of the *BCL6* 3′ UTR into the XbaI site of the pGL3-control vector (Promega). LD419 or HeLa cells were transfected with 0.4 μg of firefly luciferase reporter vector containing a wild-type or mutant target site and 0.02 μg of the control vector containing *Renilla* luciferase, pRL-CMV (Promega), using Lipofectamine 2000 (Invitrogen) in 24-well plates. Luciferase assays were performed 48 hr after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Databases and GenBank accession number

The miRNA sequences were analyzed using miRBase (http://microrna.sanger.ac.uk/), University of California at Santa Cruz Human Genome Browser (http://genome.cse.ucsc.edu/), and CpG Island Searcher Program (http://cpgislands.usc.edu/; Takai and Jones, 2002). Detailed information of base pairing comparison between miR-127 and its target site in the 3′ UTR of *BCL6* mRNA is available at Human microRNA Targets (http://www.microrna.org/) and miRBase Targets (http://microrna.sanger.ac.uk/). The GenBank accession number of *BCL6* mRNA is NM_001706.

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Accession numbers

All microarray data have been submitted to the ArrayExpress database (accession number E-MEXP-706). The GenBank accession number for the *BCL6* mRNA is NM_001706.