Biochemical and Biophysical Research Communications 457 (2015) 514–519

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

MILI, a PIWI family protein, inhibits melanoma cell migration through methylation of LINE1

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ARTICLE INFO

Article history:
Received 23 December 2014
Available online 10 January 2015

Keywords:
MILI
LINE1
MAGEA
Melanoma

ABSTRACT

MILI, a member of the PIWI/AGO gene family, has been well documented to maintain genome integrity by transposon silencing in animal germ cells. It has been reported to be selectively expressed in precancerous stem cells (pCSCs), tumor cell lines and various malignancies. However, the underlying mechanism remains largely unclear. Here, we found that MILI is expressed in the melanoma cell line B16 but not in the highly metastatic mouse melanoma model B16BL6. Interestingly, the knockdown of MILI in B16 could activate MAGEA expression and increase the cell migration ability, whereas the overexpression of MILI in B16BL6 could inhibit MAGEA expression and decrease the cell migration ability. Further investigations showed that MILI can methylate LINE1, which is crucial for MAGEA expression and melanoma cell migration. Our results provide a novel function of MILI in melanoma metastasis and tumor progression.

1. Introduction

MILI is a murine member of the PIWI/AGO gene subfamily, which is essential for germ-cell development [1,2,3]. PIWI/AGO proteins contain PIWI and PAZ domains and have multiple biological functions, including stem-cell self-renewal [4], small RNA-mediated gene silencing [5], and chromatin remodeling [6]. MILI has been shown to bind to 24- to 31-nucleotide PIWI-interacting RNAs (piRNAs) [7] and has been implicated in transposon control, DNA methylation and transcriptional repression [8]. Subsequently, Piwil2 (alias *MILI* in mouse) has been reported to be silenced in adult somatic and stem cells but is widely expressed in tumor cell lines and various types of human cancer [9]. Thus, Piwil2 may play an important role in tumor development [10,11,12].

MILI, which partners with piRNAs, has been implicated in transposon control and linked to transposon methylation in mammals [8,13,14] and is required for DNA methylation of long-interspersed elements-1 (LINE1), a member of the most abundant class of autonomous transposable elements in mammals, and for their transcriptional silencing [14,15,16]. In addition, a nonrandom organization of LINE1 repetitive sequences on the X chromosome may be responsible for its facultative heterochromatization and X inactivation [17]. Thus, these repetitive sequences have significant effects on gene organization and expression. Because the hypomethylation of LINE1 has been reported to be associated with increased cancer risk [18,19,20] and may serve as a marker of the tumor grade and lymph node metastasis [21], we hypothesize that MILI may regulate tumor development though the methylation of LINE1.

The MAGEA subfamily was the first identified cancer/testis (CT) antigen [22] and is exclusively expressed in testis or cancer cells [23]. Similar to MILI, cancer/testis (CT) antigens are predominantly expressed in human germ line cells and become aberrantly upregulated in various malignancies [24]. MAGEAs were recently found to be involved in various tumorigenesis models [25,26,27]. However, the regulatory mechanisms underlying the function of this gene family remain unclear. In this study, we found high densities of long-interspersed elements (LINE-1) in the MAGEA gene loci and that the methylation level of LINE-1 was related with the MAGEA expression pattern.

In the present study, we found that MILI can regulate melanoma cell metastasis and MAGEA expression through the methylation of LINE1. Our results suggest that the MILI-LINE1-Magea pathway may regulate melanoma cell migration.

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http://dx.doi.org/10.1016/j.bbrc.2015.01.007
0006-291X/

2. Materials and methods

2.1. Cell culture

The B16 and B16BL6 mouse melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) FBS and penicillin (100 IU/ml)/streptomycin (100 ng/ml) at 37 °C in a water-saturated atmosphere with 5% CO_2.

2.2. Plasmids, siRNA and adenovirus construction

Mouse pcDNA4-MILI was a gift from Dr. Satomi K.-Miyagawa (Osaka University. Japan). To generate adenoviruses over-expressing MILI, the sequences of MILI were cloned from its original vectors into the pAdTrack-CMV vector. These pAdTrack-CMV vectors carrying MILI were recombined into an adenovirus backbone with the AdEasy™ System according to the manufacturer's protocol. The adenovirus vector has a GFP cassette as an infection marker. The siRNAs of MILI were purchased from Shanghai Jima Company and designed to target the following cDNA sequence of MILI-1: GGCGGGUAAUGAAACUUCUTT, MILI-2, GGACCAUUCAGUCCUUACUTT, MILI-3, GAGGCCUUGUGUUUAGAAATT. The following scrambled sequence was used as a control: CCUACGCCA CCAAUUUCGU.

2.3. RNA extraction and real time RT-PCR

Total RNA from B16 and B16BL6 cell lines was extracted using Trizol reagent (Invitrogen, CA, USA) and reversed transcribed with a PrimeScript™ RT reagent kit (Takara, Dalian, China). The resulting cDNA was used for real time PCR performed on an ABI-7300 machine (Applied Biosystems). A 20-μl reaction volume contained 200 nM primers, 1 μl of cDNA and 10 μl of SYBR Premix Ex Taq™ (Takara, Dalian, China). The relative amount of mRNA was calculated using GAPDH mRNA as the invariant control. The primers used to amplify MAGEA are shown in Table S1.

2.4. RT-PCR

Polymerase chain reaction (PCR) was performed using Taq HS (Takara, Dalian, China) according to the manufacturer's instructions to verify the expression pattern of MAGEA and PIWI in the B16 and B16BL6 cell lines. The first-strand cDNA was used as the PCR template. PCR amplification was performed for 5 min at 94 °C, 33 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a final extension for 10 min at 72 °C (Takara Thermal cycler, Takara, Japan). The PCR primers and amplification conditions are the same as those used for real time RT-PCR(Table S1).

2.5. Western blotting

B16 and B16BL6 whole-cell lysates were prepared according to previously reported standard protocols. For western blotting, equal amounts of protein from each group were resolved by 10% SDS-PAGE and transferred onto PVDF membrane (Roche Applied Science). The membranes were then incubated with the appropriate primary antibody as indicated. The bound antibody was visualized using alkaline phosphate-conjugated or HRP-labeled secondary antibodies.

2.6. DNA methylation analysis by sodium bisulfite sequencing

For sodium bisulfite conversion, 2 μg of genomic DNA was denatured in 0.3 mol/l NaOH for 30 min at 42 °C. The denatured DNA was incubated in a 600 μl solution of 3.6 mol/l sodium bisulfite, 0.5 mmol/l hydroquinone, and 1.6 mol/L NaOH (pH 5.0) for at least 16 h at 50 °C. The sodium bisulfite-treated DNA was desalted and concentrated by a column containing a silica matrix obtained from the PCR purification kit (Promega, America). Following precipitation and resuspension, 2 μl of modified DNA was amplified with primers (Table S2) specific for Line1 fragments to investigate the methylation status. For analysis of the promoter methylation patterns, the PCR products of bisulfite-treated genomic DNA were cloned into the pMD-19T (Takara, Dalian, China) vector using the TOPO TA cloning kit (Takara), and at least 10 clones were selected for sequencing analysis.

2.7. Wounded healing

B16 and B16BL6 cells were cultured in six-well plates at a density of 2×10^5 cells/well and infected with adenovirus carrying the MILI gene and control adenovirus. The cells were transfected with MILI interference RNA using Lipofectamine 2000 (Invitrogen), which was added every 48 h. After the cells reached 100% confluency, 15 μl of 1 mg/ml mitomycin C was administered. After 24 h, the cells were wounded by the formation of a line across the well with a 200 μl standard pipette tip. The wounded monolayers were then washed twice with serum-free media to remove the cell debris and incubated at 37 °C with 5% CO_2. The cell-free wound area was recorded at the indicated time points using a charge-coupled device camera connected to an inverted microscope. The image was subsequently captured by an image-analyzing frame-grabber card and analyzed using an image analysis software. The wound healing effect was calculated as the percentage of the cell-free area compared with the area of the initial wound.

2.8. Migration assay

The migration of melanoma cells was determined through a Boyden chamber assay using migration chambers with 8 μm pores. The adenoviruses carrying MILI and MILI siRNA were transfected using the same protocol as that described for the wound healing assay. A total of 2×10^4 cells were added to the top chambers. The chambers were then transferred into wells of 24-well plates, each of which contained different treatments. After 48 h, the non-migrated cells on the top chamber were removed with a cotton swab. The migrated cells on the bottom of the chamber were fixed with 4% glutaraldehyde and stained with 0.5% crystal violet. The migrated cells were photographed and quantified by a light microscope by counting the stained cells in four randomly selected fields.

3. Results

3.1. MILI inhibits melanoma cell line migration

MILI has been reported to be expressed in tumor cell lines and various types of human cancer [28]. In this study, we found that MILI is expressed in the melanoma cell line B16 but not in the highly metastatic mouse melanoma model B16BL6 (Fig. 1A), which indicates that MILI may be involved in melanoma cell migration. To express MILI in B16BL6 cells and silence MILI expression in B16 cells, we constructed the pcDNA4-MILI plasmid and designed three pair of siRNA oligonucleotides targeting the MILI gene. To determine the efficiency of transfection and MILI knockdown in B16BL6 and B16 cells, respectively, the MILI protein and mRNA levels were measured by western blot and Q-PCR analyses (Fig. 1B and C).

To assess the potential effect of MILI knockdown on the migration of B16, we used wounded healing and transwell assays. As shown in Fig. 1D, E and 1H, the migration of the MILI-

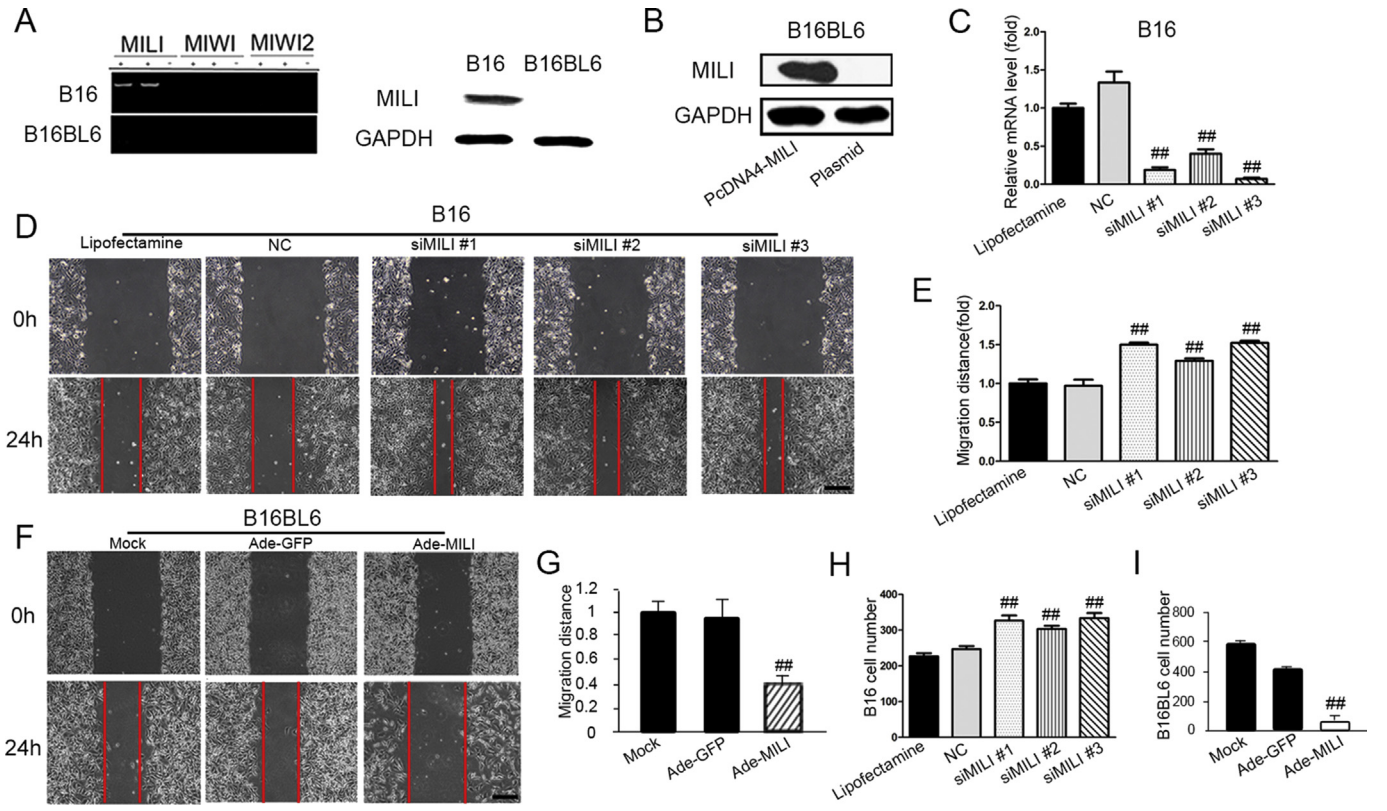

Fig. 1. MILI inhibits melanoma cell line migration. (A) RT-PCR of MILI mRNA and western blot of MILI protein in B16 and B16BL6 melanoma cell lines. (B) Transfection efficiency of PcDNA4-MILI and empty vector into B16BL6 cells. The MILI mRNA level (C) decreased in B16 cells after si-MILI transfection. Straight wounds in B16 cells transfected with si-MILI (D) and B16BL6 cells infected with Ade-MILI (F) were generated after 48 h of incubation. (E and G) Statistical results of the analysis of the migration distance. The invasion of B16 (H) and B16BL6 cells (I) was examined using the Transwell assay. The experiments were repeated three times. ##P < 0.01. Scale bar:200 μm.

knockdown B16 cell lines was significantly increased. The migration of B16 cells transfected with the nonspecific-siRNA and the control cells was not markedly different. We further overexpressed MILI in B16BL6 cells and found that their migration ability was significantly attenuated compared with that of the control cells, whereas the migration of the empty vector cells and control cells was similar (Fig. 1F, G, and 1I). Collectively, these results show that MILI can inhibit melanoma cell line migration.

3.2. Identification of MAGEA subfamily expression pattern in B16 and B16BL6 cells

The MAGEA subfamily, the first identified cancer/testis (CT) antigen, is expressed in testis or cancer cells [23] and has been reported to be involved in various tumorigenesis models. The mouse MAGEA genes are located on the X chromosome, similarly to the human MAGEA genes. To establish comprehensive expressions

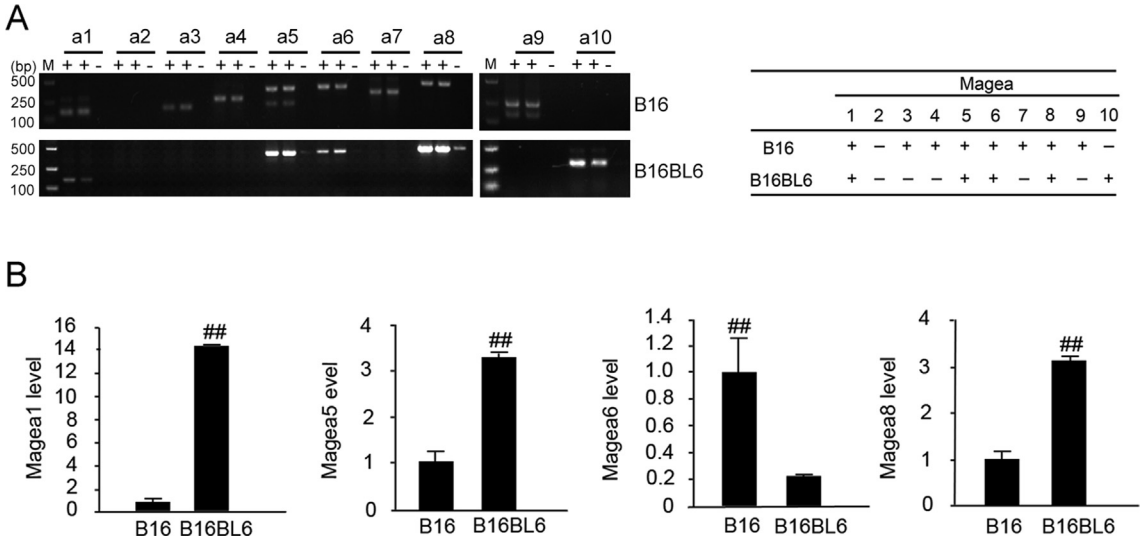

Fig. 2. Identification of MAGEA subfamily expression pattern in B16 and B16BL6 cells. (A) The expression levels of MAGEA members in B16 and B16BL6 cell lines were determined by RT-PCR; the table presents the statistical analysis. (B) The MAGEA expression level in two melanoma cell lines was determined by real-time PCR. The experiments were repeated three times. ##P < 0.01.

profile for all 10 MAGEA subfamily members in the B16 and B16BL6 melanoma cell lines, we detected the expression levels of all 10 MAGEA members by RT-PCR. The product size of Magea1, 2, 3, 4, 5, 6, 7, 8, 9, 10 was 155, 219, 193, 270, 384, 396, 328, 442, 244, 285 bp, respectively. Eight members were found to be expressed in the B16 cell line, and five members were expressed in the B16BL6 cell line (Fig. 2A). We found that MAGEA1, 5, 6, and 8 are expressed in both the B16 and B16BL6 cell lines. In addition, compared with those found in B16 cells, the expression levels of MAGEA1, 5, and 8 in B16BL6 cells were higher, whereas that of MAGEA6 was lower (Fig. 2B).

3.3. MILI can regulate the expression of MAGEA subfamily members

We further explored whether MILI could regulate MAGEA expression and found that the MAGEA expression levels can be affected by altering the MILI expression level. As shown in Fig. 3A, the expression levels of the MAGEA subfamily members rapidly decreased after MILI was overexpressed in B16BL6 cells. In contrast, the MAGEA expression level increased after the transfection of MILI small interfering RNA into B16 cells (Fig. 3B). These results indicate that MILI inhibits melanoma cell line migration likely through regulation of MAGEA expression.

3.4. MILI can methylate LINE1

The hypomethylation of LINE1 has been shown to be associated with increased cancer risk. We found a nearly 20% LINE1 content in mouse and a high LINE1 content in the human MAGEA region (data not shown). We analyzed LINE1 in the region of the MAGEA subfamily members and found three conserved LINE1 truncated sequences located in the MAGEA subfamily region (Chromosome X: 151413987–151414382; 151483599–151483914; 151514905–151515252). Thus, we hypothesized that the methylation of LINE1 was associated with the expression of MAGEAs and cell metastasis. In the present study, we found that the methylation level of conserved LINE1 among the MAGEA subfamily members increased when MILI is overexpressed (Fig. 4A) in B16BL6 but decreased when after the transfection of MILI siRNA into B16 cells (Fig. 4B). These findings revealed that MILI inhibits cell migration through the methylation of LINE1.

4. Discussion

In this study, we found that MILI is expressed in the melanoma cell line B16 but not in the highly metastatic mouse melanoma model B16BL6. The knockdown of MILI in B16 could increase the cell migration ability, whereas the overexpression of MILI in B16BL6 could decrease the cell's migration ability. This finding revealed that MILI can inhibit melanoma cell metastasis. However, our results were contrary to those reported by other researchers, who found that Piwil2 (alias *MILI* in mouse) is widely expressed in metastatic cancers, such as breast, and promotes tumorigenesis [9,28,29]. These results demonstrated that the regulation of MILI is complex and suggest that MILI plays different roles in various tumors.

MAGEA proteins belong to Class I cancer/testis (CT) antigen and are normally expressed in male germ cells and aberrantly upregulated in a variety of cancers, such as melanomas. It has been reported that MAGEA expression is associated with higher-grade lesions and advanced cancer [27,30], which is consistent with our result that the expression levels of MAGEA1, 5, and 8 were higher in the highly metastatic cell line B16BL6 compared with B16 cells. However, five MAGEA members are expressed in the highly metastatic cell line B16BL6, whereas eight are expressed in B16 cells. This result is in agreement with the previous finding that different members of MAGEA may play different roles in cell migration. In addition, we found the MAGEA expression is activated after the

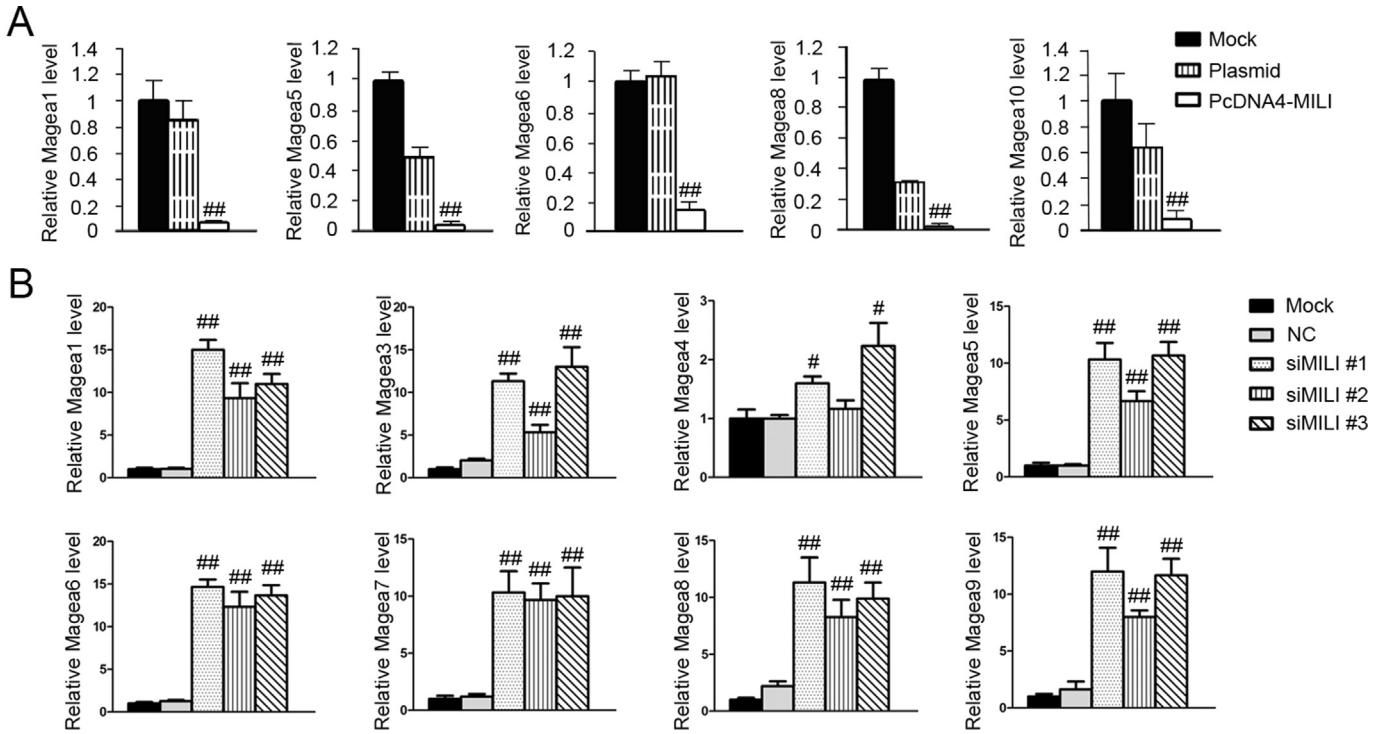

Fig. 3. MILI can regulate the expression of MAGEA subfamily members. (A) The MAGEA expression levels after B16BL6 cells transfected with PcDNA4-MILI. (B) MAGEA expression levels after si-MILI transfection in B16 cells. The experiments were repeated three times. #P < 0.05, ##P < 0.01.

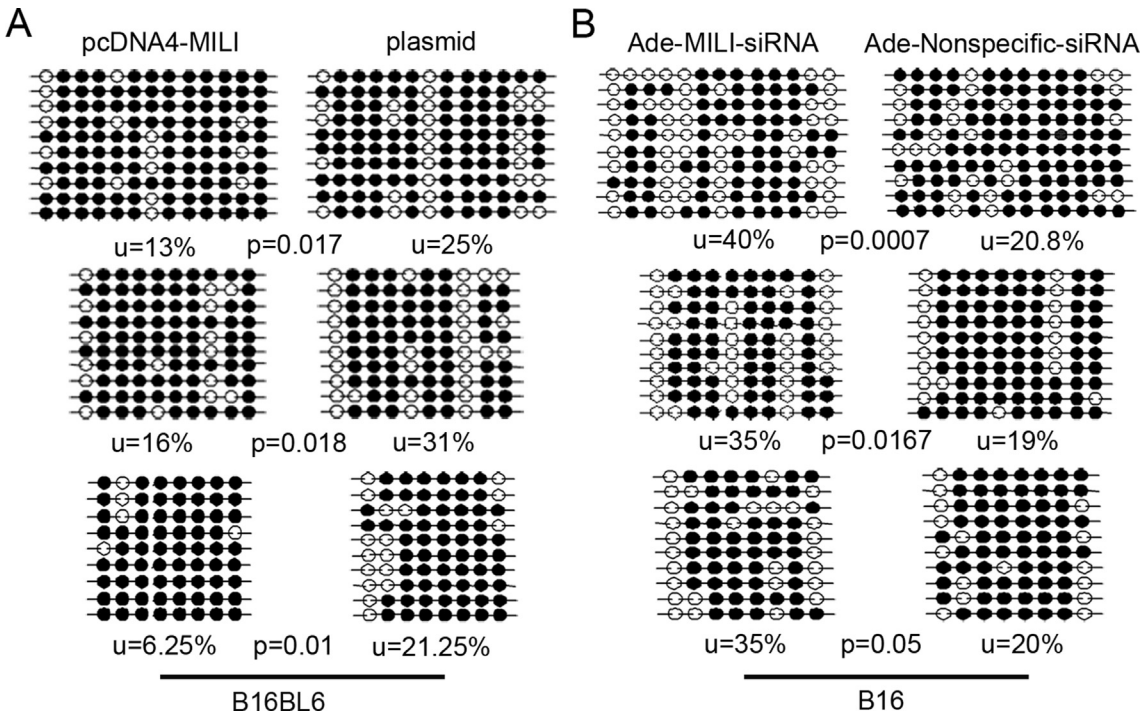

Fig. 4. MILI can methylate LINE1. Measurement of the methylation of LINE1 truncated conserved sequence in B16BL6 cells transfected with PcDNA4-MILI (A) and in B16 infected with Ade-si-MILI (B).

knockdown of MILI in B16 cells but inhibited after the overexpression of MILI in B16BL6 cells, which revealed that MILI can regulate MAGEA expression and thus play an important role in cell migration.

In the present study, we analyzed all MAGEA subfamily member regions and found three conserved LINE1 truncated sequences. The methylation level of the three LINE1 regions was significantly decreased in the melanoma cell lines compared with NIH3T3 cells (data not shown). This finding is similar to the results of a previous report, which showed that the hypomethylation of LINE1 is associated with increased cancer risk [18]. MILI in the presence of piRNAs has known functions in transposon silencing in the male germline of fetal and newborn mice [8,14]. In this study, we found that MILI can methylate LINE1 in cancer, which may contribute to the regulation of MAGEA expression. Thus, we hypothesized that MILI inhibits melanoma cell line metastasis through the methylation of LINE1. However, we remain far from our ultimate goal of identifying the relationship between MILI, LINE1 and MAGEA, particularly the regulation of MAGEA expression by LINE1 DNA sequence epigenetic modifications.

In summary, we provide the first demonstration that MILI has an inhibitory function in melanoma cell migration, and we provide a potentially novel mechanism in which MILI regulates gene expression through an epigenetic modification of LINE1.

Conflict of interests

None.

Acknowledgments

This work was supported by Chinese National Program on Key Basic Research Project (973 Program) (2012CB524900) awarded to Prof. Chao-Jun Li.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.007.

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