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miR-7 and miR-218 epigenetically control tumor suppressor genes RASSF1A and Claudin-6 by targeting HoxB3 in breast cancer

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ABSTRACT

Many microRNAs have been implicated as key regulators of cellular growth and differentiation and have been found to dysregulate proliferation in human tumors, including breast cancer. Cancer-linked microRNAs also alter the epigenetic landscape by way of DNA methylation and post-translational modifications of histones. Aberrations in Hox gene expression are important for oncogene or tumor suppressor during abnormal development and malignancy. Although recent studies suggest that HoxB3 is critical in breast cancer, the putative role(s) of microRNAs impinging on HoxB3 is not yet fully understood. In this study, we found that the expression levels of miR-7 and miR-218 were strongly and reversely associated with HoxB3 expression. Stable overexpression of miR-7 and miR-218 was accompanied by reactivation of tumor suppressor genes including RASSF1A and Claudin-6 by means of epigenetic switches in DNA methylation and histone modification, giving rise to inhibition of the cell cycle and clone formation of breast cancer cells. The current study provides a novel link between overexpression of collinear Hox genes and multiple microRNAs in human breast malignancy.

1. Introduction

MicroRNAs (miRs) are small, conserved, non-coding short RNAs of 18–25 nucleotides in length that bind to target mRNAs mainly at their 3′UTR. By doing so, they importantly modulate gene expression of their mRNA targets as novel posttranscriptional regulators. A single miR can directly target several mRNAs; conversely, the expression of a single mRNA maybe controlled by several miRs. It is becoming ever clearer that such functionally versatile agents impinge on virtually all physiological and pathological processes including cancer [1,2]. Many miRs have been implicated as tumor suppressors or as oncogenes; accordingly, they have been found to be down- or up- regulated in human tumors, including breast cancer [1,3]. Cancer-linked miRs also alter the epigenetic landscape by modulating DNA methylation and histone modification in cancer cells [4,5].

Aberrations in Hox (a highly conserved subgroup of the homeobox superfamily) gene expression have been reported in abnormal development and malignancy, suggesting that altered expression of Hox genes could be important for both oncogenesis or tumor suppression, depending on context [6]. Such studies note an association of increased expression of a set of homeodomain transcription factors, including HoxB3, with poor prognosis in acute myeloid leukemia and breast cancer [7,8]. Several miRs seem to regulate Hox gene expression by mRNA cleavage or interference with the translational machinery [9]. For example, miR-181 down-regulates Hox-A11 during skeletal-muscle differentiation [10], and miR-28-5p alters HoxB3 expression in colorectal cancer [11]. miR-196a could control melanoma-associated genes by directing HoxC8 3′UTR [12].

Using the microRNA prediction tool mirBASE [13], we had identified both miR-7 and miR-218 as a potential miR for targeting HoxB3 mRNA. Pursuing this lead, we report here our discovery of an inverse correlation between the levels of endogenous miR-7 and miR-218 and the expression of HoxB3. Both miR-7 and miR-218 down-regulate the HoxB3 expression by targeting the 3′-UTR of HoxB3 mRNA. We have also defined epigenetic changes in DNA methylation and histone modification that are involved in the reactivation of HoxB3 and that presumably account for these miRNAs' inhibition of the cell cycle and clone formation of cancer cells.

2. Materials and methods

2.1. Cell lines and treatment conditions

The immortalized human mammary epithelial cell line (MCF 10A), breast cancer cell lines (MCF7 and MDA-MB-231), cervical cell line (Hela), and embryonic kidney cell line (HEK-293) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained under the cultural conditions as ATCC suggested.

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2.2. miRNA overexpression and inhibition

Precursor miRNAs pMiR-hsa-mir-7-1 (Cat# MIR-7-1) and pMiR-hsa-mir-218-1 (Cat# MIR-218-1) were obtained from Cell Biolabs (Cell Biolabs, Inc. San Diego, CA). pGreenPuro Scramble Hairpin negative control was purchased from SBI (System Biosciences, Mountain View, CA). miRNA precursors were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.3. Reporter vectors and DNA constructs

The 3′UTRs of HoxB3 were PCR-amplified from human MCF10A cDNA, and inserted downstream of CMV-driven firefly luciferase cassette in the pMIR-REPORT vector (Ambion, Austin, TX) between *Hind*III and *Spe*I sites. All inserted fragments were confirmed by direct sequencing.

2.4. Luciferase miRNA target reporter assays

For miRNA target validation, approximately 2×10^4 HEK293 cells per well in 24-well plates were transiently transfected with 50 ng of the firefly luciferase reporter construct (Promega, Madison, WI), 12.5 to 100 ng of the precursor miRNAs (miR-7 and or miR-218), or 10 ng of pRTK-Luc (Promega) as internal control. Renilla luciferase vector was used to normalize transfection efficiency. Approximately 72 h after transfection, firefly and Renilla luciferase activities were assayed. Normalized relative light units represent firefly luciferase activity/Renilla luciferase activity.

2.5. Quantitative RT-PCR

For mRNA, total RNA was prepared using TRIzol reagent (Invitrogen) and genomic DNA was eliminated with TURBO DNA-free Kit (Ambion). Five microgram of total RNA was reverse-transcribed using Super III reverse transcriptase (Invitrogen). Omission of reverse transcriptase served as a negative control. cDNA was amplified using Platinum PCR SuperMix (Invitrogen). PCR was performed as follows: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed for 5 min at 72 °C. For real-time-PCR analysis, primers for qPCR were designed using primer 3.0 software with an optimal annealing temperature of 60 °C. Gene expression was normalized relative to 18S rRNA or ACTB using Delta–Delta CT. For isolation and expression analysis, miRNA was isolated with the RT2 qPCR- Grade miRNA Isolation kit (Qiagen) and was quantified with the qRT- PCR miRNA Detection Kit (ABI). The sequences of primers are listed in Supplementary Table S1. Experiments were performed in triplicate and results were showed as the summary of three independent experiments.

2.6. ChIP and MeDIP analysis

Cells were crosslinked with 1% formaldehyde, lysed and sonicated on ice to generate DNA fragments with an average length of 200–800 bp [14]. After pre-clearing, 1% of each sample was saved as input fraction. Immunoprecipitation was performed using antibodies specifically recognizing 5-methylcytidine (BI-MECY-0100), anti-DNMT3b (ALX-804-233-C100)(Alexis Biochemicals), H3K9Ac, H3K14Ac (Abcam) or IgG control. DNA was eluted and purified from complexes, followed by PCR amplification of the HoxB3 promoter using primers and conditions as described [8]. Before carrying out MeDIP, 10 mg of genomic DNA was sonicated to yield a size-range from 200 to 700 bp. Four microgram of gel purified DNA was used for each MeDIP assay that is described previously [15].

2.7. Western analysis and antibodies

Approximately equal amounts of protein (20–50 mg) were separated on 10% SDS–PAGE and transferred onto a nitrocellulose membrane. Antibodies anti-β-actin (A2228; Sigma) and anti-HoxB3 (ab83404, Abcam) were used for identifying protein bands.

2.8. FACS assay and soft agar assay

Flow cytometry was used to quantify cells in each phase of the cell cycle. Cells (1×10^5) were plated into 6-well plates and treated with miRNAs 72 h afterwards. Cells were harvested after an additional 72 h, washed with PBS and fixed in 70% ethanol overnight at 4 °C. To detect the fluorescent intensity of certain proteins, cells were counter-stained in the dark with 50 μg/ml phosphatidyl inositol (PI) and 0.1% ribonuclease A (RNase A) in 400 μl of PBS at 25 °C for 30 min. Stained cells were assayed and quantified using a FACSort Flow Cytometer (Becton Dickinson).

Cells suspended in 0.35% agar (1.5×10^4 cells/dish) were layered on top of 1 ml of solidified agar (0.7%) in a 35 mm dish. DMEM growth medium with a final concentration of 10% FBS was included in both layers. After 28 days of incubation, colonies were counted. Experiments were carried out in triplicates.

3. Results

3.1. Both miR-7 and miR-218 directly inhibit HoxB3 expression via its 3′-UTR

We assessed complementarities of miR-7 and miR-218 to HoxB3 3′-UTR and 5′-UTRs, and found several seed matches in the 3′-UTR (Fig. S1). To address miR-7 and miR-218 inhibition of HoxB3, HEK-293 cells were transfected with pre-miR-7, pre-miR-218 and control-pre-miR, together with a reporter plasmid encoding luciferase prefixed to the 3′-UTR. Fig. 1A shows that both miR-7 and miR-218 inhibited the luciferase activity from the construct with the HoxB3-UTR segment in a concentration-dependent manner. There was no change in the luciferase reporter activity when the cells were co-transfected with the negative control. To clarify whether both miR-7 and miR-218 could synergistically target HoxB3 3′-UTR, we co-transfected 50 ng of pre-miR-7 and pre-miR-218 into the HEK-293 cells (Fig. 1B). Strikingly, HoxB3 expression in the cotransfected cells was decreased in comparison to either miRNA alone, indicating that miR-7 and miR-218 work synergistically on HoxB3.

To confirm the effectiveness of miR-7 and miR-218 in shutting down HoxB3, we also analyzed HoxB3 protein levels in MCF10A, MCF7, MDA-MB-231 and Hela cells (Fig. 1C). Concomitant with the decrease in expression of miR-7 and miR-218 in MDA-MB-231 and Hela cells, the levels of HoxB3 protein increased (Fig. 1D). Moreover, there was an inverse relationship between expression of the miRNAs and that of HoxB3 protein.

3.2. Reintroduction of miR-7 and miR-218 reactivated tumor suppressor genes RASSF1A and CLDN6 in MDA-MB-231 cells

RAS associated domain family 1A (RASSF1A, also known as RASSF1) silencing correlates strongly with overexpression of HOXB3 and DNMT3b [8]; Claudin 6 (CLDN6) silencing correlates with lymphoid specific helicase (LSH) and DNMT3b [14]. To address whether miR-7 and miR-218 also affect RASSF1A and CLDN6, we constructed stable expression of miR-7, miR-218 or the combination of miRNAs in MDA-MB-231 cells. Fig. 2A shows that miR-7 and miR-218, transfected singly, increased levels of

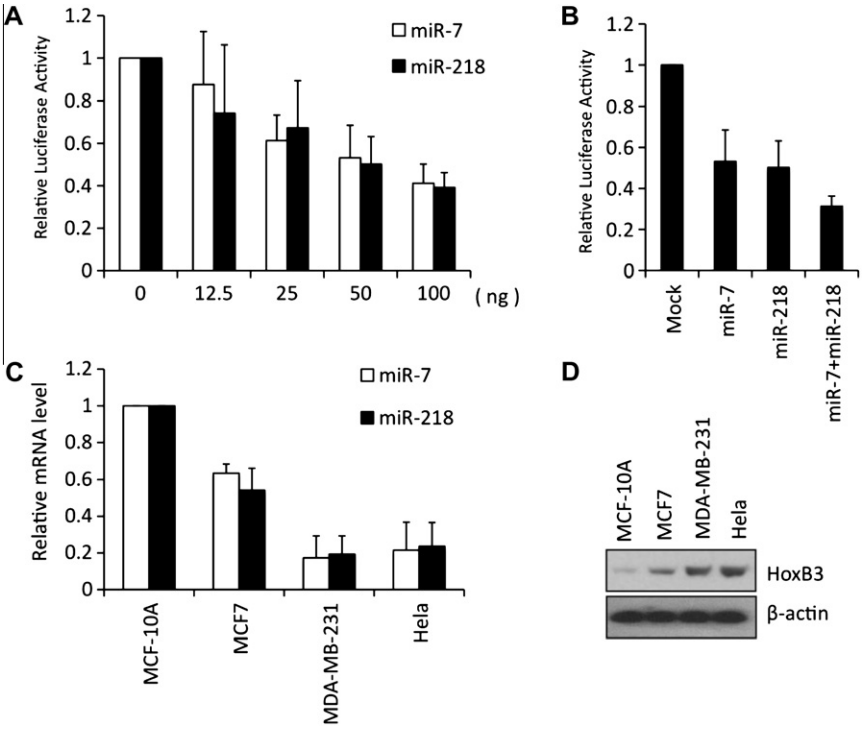

Fig. 1. miR-7 and miR-218 directly targets HoxB3. (A) Dose-dependent inhibition of HoxB3 3′UTR in the luciferase assay after transfection of the HEK293 cells with miR-7 or miR-218. (B) miR-7 and miR-218 synergistically decrease HoxB3 3′UTR-linked luciferase expression in HEK-293 cells. (C) qRT-PCR analysis of endogenous miR-7 and miR-218 expression MCF7, MDA-MB-231, and Hela cells, normalized to that in MCF10A cells. (D) Western Blot analysis of HoxB3 expression in MCF10A, MCF7, MDA-MB-231 and Hela cells.

mRNA encoding RASSF1A and CLDN6, while together they increased these mRNAs synergistically.

We also analyzed HoxB3 protein levels in MDA-MB-231 cells after stable expression of miR-7 and miR-218, singly and in combination. Again, while each miRNA alone attenuated the HoxB3 protein expression, the combination of the two dramatically decreased the levels of HoxB3 protein (Fig. 2B).

Since epigenetic silencing of RASSF1A and CLDN6 in other contexts is known to involve DNA methylation, we hypothesized that DNA methylation may also play a role in silencing these genes by miR-7 and miR-218. Indeed, MeDIP analysis demonstrated that DNA methylation decreased significantly in the promoter regions of RASSF1A (Fig. 2C) and CLDN6 (Fig. 2D) following introduction of miR-7 and miR-218 into MDA-MB-231 cells, indicating an addition mode of action of these miRNAs, namely reactivation of the expression RASSF1A and CLDN6 via epigenetic alterations.

3.3. Epigenetic alterations coinciding with miR-7- and miR-218-mediated activation of HoxB3

DNA methyltransferase 3b (DNMT3b) is usually overexpressed in a variety of cancers, where it contributes to the silencing of tumor suppressor genes [8,14,16]. To determine whether DNMT3b is involved in hypermethylation of the RASSF1A and CLDN6 promoters, we carried out a series of chromatin immunoprecipitation (ChIP) to look for evidence of the direct binding of DNMT3b to these promoter regions.

The ChIP results indicate that DNMT3b is directly associated with the RASSF1A promoter (Fig. 3A) and CLDN6 promoter (Fig. 3B). Reintroduction of miR-7 and miR-218 substantially decreased the association of DNMT3b with the RASSF1A and CLDN6 promoters.

To explore possible additional epigenetic alterations exerted by miR-7 and miR-218, we measured levels of histone acetylation at the promoter-proximal regions of the RASSF1A and CLDN6. The data show that both acetylation of H3 at the RASSF1A promoter (Fig. 3C) and CLDN6 promoter (Fig. 3D) increased in the presence of miR-7 and miR-218, pointing to histone acetylation as a further likely mode of action of miR-7 and miR-218 into MDA-MB-231 cells.

3.4. Both miR-7 and miR-218 suppress cell growth in MDA-MD-231 cells

Additional experiments were undertaken to understand the physiological role of miR-7 and miR-218 in breast cancer. First, FACS was performed with MDA-MB-231 cells stably expressing miR-7 and miR-218. The data shown in Fig. 3A demonstrate that the fraction of cells in G1 increased from 51.0% in the control group to 58.9%, 66.6% and 69.0%, respectively, in the miR-7, miR-218 and doubly transfected groups. The fraction of cells in S phase, accordingly, decreased from 35.1% in the control group to 25.5%, 23.3%, and 19.5%, respectively, in the miR-7, miR-218 and combination groups. However, the fraction of cells in G2/M phase was changed from 13.9% in the control group to 15.6%, 10.1%, and 11.5% in the miR-7, miR-218 and the combination groups, respectively.

Stable reintroduction of miR-7 and miR-218 also decreased formation of colonies in soft-agar colonies (Fig. 4B). These results, all together, imply that miR-7 and miR-218 exert their effects on cell cycle progression mainly by prolonging the G1/S phase.

4. Discussion

Although recent studies have suggested that HoxB3 are abundant in breast cancer, the putative function of the miRs at HoxB3 is not yet fully understood. In this study, we found that the expression levels of miR-7 and miR-218 are strongly and reversely asso-

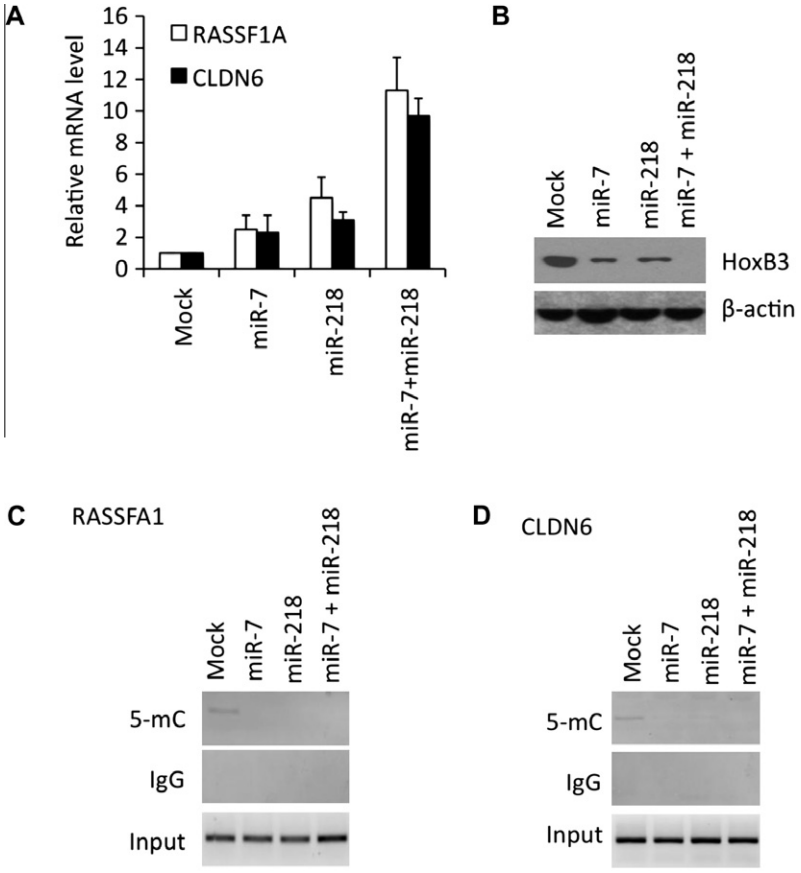

Fig. 2. Both RASSF1A and CLDN6 are reactivated after stable expression of miR-7 and miR-218 in MDA-MB-231 cells. qRT-PCR analysis of RASSF1A and CLDN6 expression (A) and Western Blot analysis of HoxB3 expression (B) in MDA-MB-231 cells after stable expression of miR-7 and miR-218. MeDIP analysis of the RASSF1A promoter (C) and CLDN6 promoter (D) in MDA-MB-231 cells treated with a control, or miR-7 and miR-218.

ciated with HoxB3 expression. Stable overexpression of miR-7 and miR-218 is accompanied by the reactivation of multiple tumor suppressor genes by epigenetic switches in DNA methylation and histone modification. To our knowledge, this is the first article to show concurrent overexpression of collinear Hox genes and multiple microRNAs in human breast malignancy.

The Hox genes comprise a family of homeodomain-containing transcription factors that determine cellular identity during development. In humans, four Hox clusters containing 39 Hox genes have been identified, and dysregulation of their expression is observed in various malignancies. Although the role of Hox genes in cancer is not fully understood, aberrant expression of (various of) them is thought to affect pathways that promote tumorigenesis and metastasis in many types of cancer including lung, ovarian, breast, prostate cancers and leukemia [6]. Hox genes such as HoxA9, HoxA10 and HoxA11 are expressed in different epithelial ovarian cancer subtypes according to the pattern of mullein-like differentiation of cancers [17], suggesting that Hox genes have a role in determining ovarian cancer histotypes.

In non-malignant tissues Hox genes have crucial roles in development, regulating numerous processes including apoptosis, receptor signaling, differentiation, motility and angiogenesis [6].

In this study, we have shown that HoxB3 can control growth of transformed cell lines by epigenetic silencing some tumor suppressor genes such as RASSF1A and CLDN6, which confirms similar observations by Palakurthy et al. [8].

An association between various miRs and Hox expression in development and cancer has also been reported. miR-196-directed cleavage of HoxB8 mRNA was detected in mouse embryos [18]. miR-181 down-regulates the homeobox protein Hox-A11 (a repressor of the differentiation process), thus establishing a functional link between miR-181 and the complex process of mammalian skeletal-muscle differentiation [10]. miR-196a controls melanoma-associated genes by regulating HoxC8 expression [12], and strand-specific miR-28-5p and miR-28-3p are down-regulated in colorectal cancer cells [11]. Here we have demonstrated two additional miRNAs - miR-7 and miR-218 - can target HoxB3 genes, and do so in a synergistic manner.

A number of studies have implicated miR-7 in malignancies including breast, gastric, and lung tumors [19–21]. miR-7 may directly target the 3′UTR regions of several genes such as epidermal growth factor receptor [21,22], phosphoinositide 3-kinase catalytic subunit delta [23], BCL-2 [24], SATB1 [25], p21-activated kinase 1 (Pak1) [26] and associated cdc42 kinase 1 (Ack1) [27]; miR-7 targeting of these genes leads to inhibition of cell cycle progression, tumor growth and metastasis, all pointing to miR-7's function as a possible tumor suppressor. But the mechanistic links between miR-7 and Hox expression in cancer have been unclear.

This study expands the list of affected genes to HoxB3, and thereby sheds light on a possible mechanism, since HoxB3 plays a critical role in the epigenetic silencing of tumor suppressor gene RASSF1A in breast cancer cells. Increasing evidence indicates that miR-218, too, is a tumor suppressor microRNA [28], and, as such, might serve as a tumor biomarker, for example, for clear cell renal cell carcinoma [29]. The laminn-5 beta3 (LAMB3) gene is targeted by miR-218, and that gene is reportedly under-regulated in human cervical carcinoma cell infected with human papillomavirus type 16 [30]. Single nucleotide polymorphisms in pre-miR-218 and LAMB3 contribute

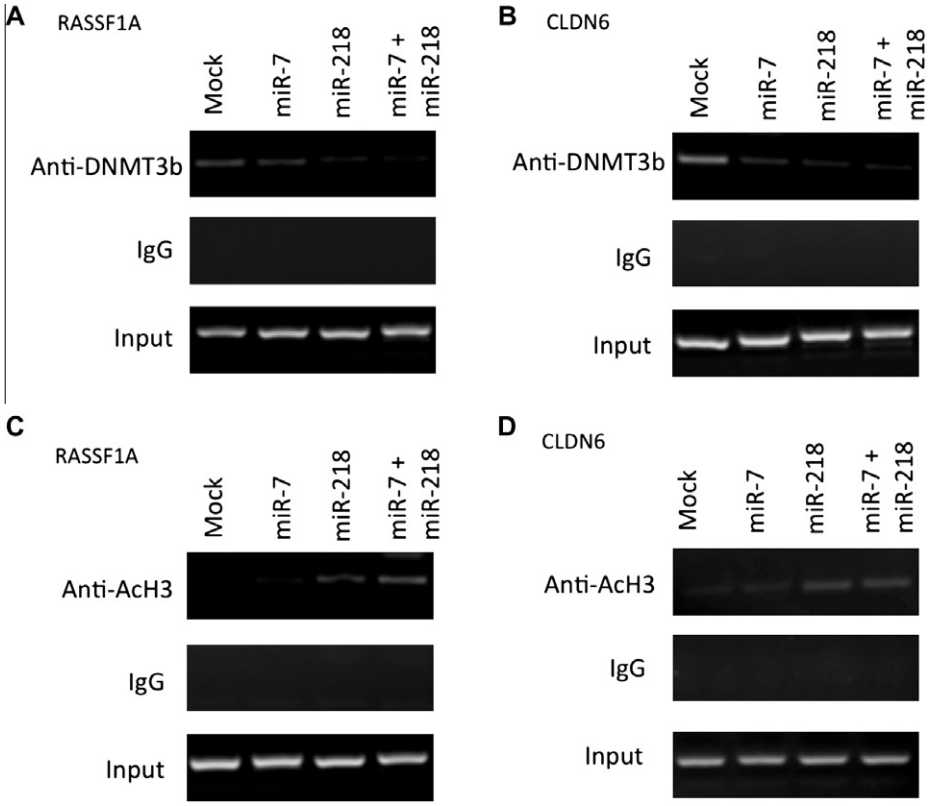

Fig. 3. Epigenetic alterations coinciding with miR-7- and miR-218- mediated activation of HoxB3. ChIP analysis was used with MDA-MB-231 cells treated with miR-7 and miR-218 to monitor the binding of DNMT3b to the RASSF1A (A) or CLDN6 (B) promoter, or the binding of acetylation Histone H3 (Ac-H3) to the RASSF1A (C) or CLDN6 (D) promoter.

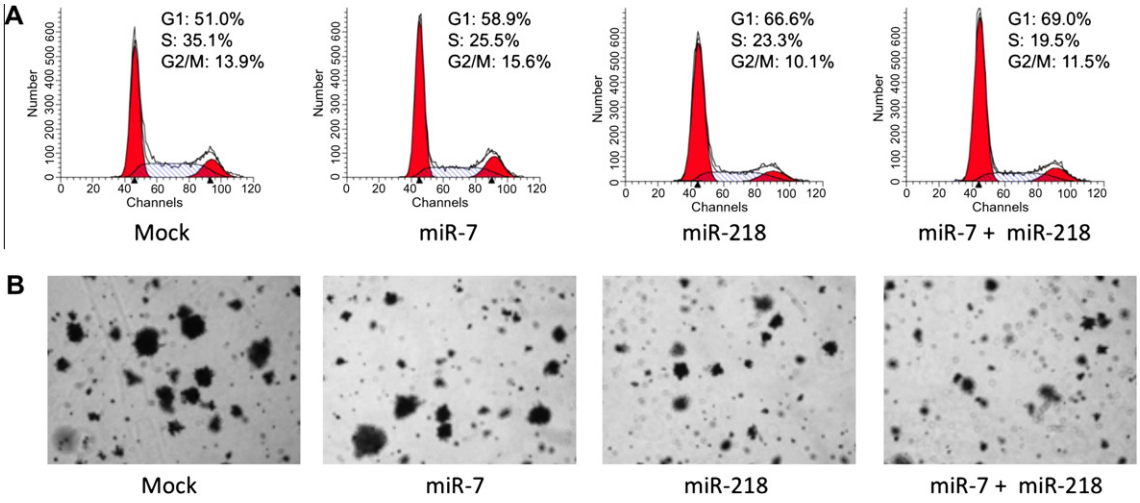

Fig. 4. Both miR-7 and miR-218 suppressed cell growth in MDA-MD-231 cells. (A) FACS analysis depicting cell-cycle progression of MDA-MB-231 cells after overexpression of miR-7 and miR-218 in these cells. (B) MDA-MB-231 cells expressing a mock or miR-7 and miR-218 were analyzed for their ability to grow in soft agar.

to cervical cancer carcinogenesis [31].miR-218 inhibits invasion and metastasis of gastric and nasopharyngeal cancers by targeting the Robol receptor [32,33]. Another newly discovered target of miR-218 is the gene encoding paxillin; this gene is deleted and down-regulated in lung squamous cell carcinoma [34,35].

Expression of both miR-7 and miR-218 corresponds with estrogen receptor status in breast cancer [19,36–38], implicating that these two miRs may involve in estrogen receptor-positive cancers. Paradoxically HoxB3 is strongly linked with estrogen receptor-negative cancer. Resolution of this paradox awaits further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.028.

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