miR-125b-1 is repressed by histone modifications in breast cancer cell lines

Fernanda Cisneros-Soberanis, Marco A. Andonegui and Luis A. Herrera*

Abstract

Purpose: Downregulation of miR-125b-1 is associated with poor prognosis in breast cancer patients. In this work we investigated the effect of histone modifications on the regulation of this gene promoter.

Methods and results: We evaluated the enrichment of two histone modifications involved in gene repression, H3K9me3 and H3K27me3, on the miR-125b-1 promoter in two breast cancer cell lines, MCF7 (luminal A subtype) and MDA-MB-231 (triple-negative subtype), compared to the non-transformed breast cell line MCF10A. H3K27me3 and H3K9me3 were enriched in MCF7 and MDA-MB-231 cells, respectively. Next, we used an EZH2 inhibitor to examine the reactivation of miR-125b-1 in MCF7 cells and evaluated the transcriptional levels of pri-miR-125b-1 and mature miR-125b by qRT-PCR. pri-miRNA and mature miRNA transcripts were both increased after treatment of MCF7 cells with the EZH2 inhibitor, whereas no effect on miR-125b-1 expression levels was observed in MDA-MB-231 and MCF10A cells. We subsequently evaluated the effect of miR-125b-1 reactivation on the expression and protein levels of BAK1, a target of miR-125b. We observed 60 and 70% decreases in the expression and protein levels of BAK1, respectively, compared to cells that were not treated with the EZH2 inhibitor. We over-expressed KDM4B/JMJD2B to reactivate this miRNA, resulting in a three-fold increase in miR-125b expression compared with the same cell line without KDM4B/JMJD2B over-expression.

Conclusion: The miR-125b-1 is repressed by different epigenetic mechanisms depending on the breast cancer subtype and that miR-125b-1 reactivation specifically eliminates the effect of repressive histone modifications on the expression of an pro-apoptotic target.

Keywords: Epigenetics, Gene promoter, Histone modifications, DNA methylation

Background

MicroRNAs (miRNAs) are short single-stranded RNAs that could regulate gene expression at the post-transcriptional level. miRNAs are typically transcribed as primary transcripts (pri-miRNAs) that are subsequently matured in a multi-step biogenesis process to generate the functional form or mature miRNA (Lee and Dutta 2009). Accumulating evidence indicates that miRNA deregulation is related to pathologies including cancer. Some miRNAs are associated with tumor suppressor or oncogene activity, depending on the downregulated target (Di Leva et al. 2014).

In particular, miR-125b-1 exhibits tumor suppressor activity in some types of cancer, including breast, ovarian and bladder cancer (Banzhaf-Strathmann 2014). miR-125b-1 may be involved in biological processes such as apoptosis, cell proliferation and cell migration because it regulates genes such as BAK1 (Zhou et al. 2010), ERBB2 (Scott et al. 2007) and ETS1 (Zhang et al. 2011), respectively. In most breast cancer tumors, miR-125b-1 downregulation is associated with a poor prognosis. miR-125b-1 can be repressed by DNA methylation in the promoter region (Zhang et al. 2011; Soto-Reyes et al. 2012). However, the miR-125b-1 promoter is within a CpG island promoter with intermediate CpG content. Promoters with intermediate CpG content are typically regulated by DNA methylation and histone modifications.

*Correspondence: HerreraM@biomedicas.unam.mx
Unidad de Investigación Biomédica en Cáncer, Instituto Nacional de Cancerosología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Avenida San Fernando 22, Mexico City 14080, Mexico

© 2016 The Author(s). This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.
(Weber et al. 2007). Thus, we were interested in evaluating the relevance of repressive histone modifications in miR-125b-1 downregulation.

In this study, we evaluated repressive histone modifications in the promoter regions of miR-125b-1 and the effect on the transcriptional regulation of this gene. Chromatin immunoprecipitation revealed H3K9me3 and H3K27me3 histone modifications associated with gene repression in the miR-125b-1 promoter in breast cancer cell lines. These histone modifications were then removed by over-expressing KDM4B/JMJD2B to remove H3K9me3 (Fodor et al. 2006) or using an EZH2 inhibitor to remove H3K27me3 (McCabe et al. 2012). Finally, we evaluated miR-125b-1 reactivation by quantitative real-time PCR (qRT-PCR) and the effect on a target of this miRNA.

Methods

Human breast cell lines and treatment

The human breast cancer cell lines MCF7 and MDA-MB-231 were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (GIBCO). MCF10A, a non-transformed cell line, was cultured in DMEM/F12 (3:1) supplemented with 10% fetal bovine serum (GIBCO), 2 mM glutamine (GIBCO), 10 ng/mL EGFr (Invitrogen), 120 mU/mL human recombinant insulin (Insulinex) and 1 µg/mL hydrocortisone (SIGMA).

To eliminate the H3K9me3 histone modification, MCF10A and MDA-MB-231 were transfected with a plasmid encoding KDM4B/JMJD2B-GFP (Fodor et al. 2006) using Lipofectamine LTX (Invitrogen). As a control, the same cell lines were transfected with empty plasmid. Plasmid-carrying cells were selected by cell sorting using a FACSAria III cytometer (BD). To eliminate the H3K27me3 histone modification, the breast cell lines were incubated with 200, 500, 1000 or 2000 nM GSK126 for 4 days.

RNA isolation and qRT-PCR expression analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). pri-miR-125b-1 and pri-miR-125b-2 expression levels were evaluated by qRT-PCR using SYBR Green (ThermoFisher Scientific). Mature miR-125b expression levels were quantified by qRT-PCR using a TaqMan assay according to the manufacturer’s protocol (Applied Biosystems). One hundred nanograms of total RNA were reverse transcribed using specific stem-loop RT primers. Next, the products were amplified and detected by PCR with specific primers and TaqMan probes (miR-125b, Applied Biosystems). U6 snRNA served as an internal normalized reference.

The amplification and detection of specific products were calculated by the 2^(-ΔΔCt) method.

Western blot analysis

Cells were grown in 10-cm² dishes and lysed using Cell Lysis Buffer (Cell Signaling) with protease inhibitor cocktail (Cell Signaling) and 100 µM PMSF (SIGMA). Protein samples were resolved on 12% Tris–glycine gels and transferred to PVDF membranes. The membranes were then incubated with α-H3K27me3 (1:1000; Millipore, 07-449), α-H3 (1:1000; SIGMA, H0164), α-BAK1 (Santa Cruz, sc-832) and α-GAPDH (Santa Cruz, sc-25778). After washing with TBS, the membranes were incubated with peroxidase-conjugated goat anti-rabbit antibody (1:20,000; Novus, NB7187) followed by chemiluminescence staining (Millipore).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performing using the OneDay ChIP kit (Diagenode) according to the manufacturer’s protocol. To immunoprecipitate H3K9me3 and H3K27me3, we used 4 µg each of α-H3K9me3 (Abcam, ab-8898) and α-H3K27me3 (Millipore, 07-449), respectively. The immunoprecipitated DNA was analyzed by PCR using primers specific for the miR-125b1 promoters. As positive ChIP controls, we used SOX-9 and MYT-1 promoters.

Statistical analysis

Data from at least three independent experiments are expressed as the mean ± standard deviation. Differences between groups were analyzed using Student’s t test. Data were considered significant at p < 0.05.

Results

miR-125b-1 is downregulated in breast cancer cell lines

miR-125b (mature miRNA) is transcribed from two different genes: miR-125b-1 (chromosome 11) and miR-125b-2 (chromosome 21). However, the transcriptional activity of miR-125b-2 is low (Additional file 1: Fig. 1). Thus, most miR-125b is derived from the miR-125b-1 gene. To determine miR-125b-1 expression levels, we evaluated pri-miRNA and mature miRNA levels in the breast cancer cell lines MCF7 and MDA-MB-231 by qRT-PCR compared with the non-transformed breast cell line MCF10A. pri-miR-125b-1 levels in MCF7 and MDA-MB-231 cells were reduced by 99 and 72%, respectively, compared with MCF10A cells. However, mature miR-125b levels were reduced only in MCF7 cells. In MDA-MB-231 cells, mature miR-125b levels were increased by nearly threefold (Fig. 1). This increment of miR-125b in
MDA-MB-231 cells may be associated with the accumulation of miRNA transcripts.

**Histone modification marks in miR-125b-1 promoter regions**

Two promoters are associated with miR-125b-1 transcriptional regulation. The first is in a CpG island close to the miR-125b-1 sequence (Wang et al. 2013). This CpG island has an intermediate CpG content, and thus the transcriptional regulation of miR-125b-1 may be associated with DNA methylation and histone modifications (Weber et al. 2007; Marson et al. 2008). The second promoter is 55 kb upstream from the miR-125b-1 sequence. This promoter, which may regulate the transcription of the miR-125b-1, let-7a-2 and miR-100 genes (Chien et al. 2011), is not in a CpG island, and thus the regulation of these genes may be associated with histone modifications (Fig. 2).

To further investigate the repressive mechanism of miR-125b-1 in breast cancer cell lines, we evaluated two histone modifications involved in gene repression: H3K9me3 and H3K27me3. H3K9me3 and H3K27me3 are associated with constitutive and facultative heterochromatin, respectively. We observed enrichment of H3K9me3 in both promoters in MDA-MB-231 cells but not MCF7 and MCF10A cells (Fig. 2a). However, in MCF7 cells, we observed twofold enrichment of H3K27me3 compared with MCF10A cells. We did not observe this enrichment on miR-125b-1 promoters in MDA-MB-231 cells (Additional file 2: Fig. 2; Fig. 2b).

**miR-125b-1 reactivation in MDA-MB-231 by KDM4B/JMJD2B over-expression**

To determine the role of H3K9me3 in MDA-MB-231 cells, we over-expressed KDM4B/JMJD2B to reduce global levels of H3K9me3 histone modification in MCF10A and MDA-MB-231 cells. We specifically selected transfected cells over-expressing KDM4B/JMJD2B by cell sorting (Fig. 3a). Subsequent qRT-PCR analysis revealed a three-fold increase in miR-125b levels in KDM4B/JMJD2B-transfected MDA-MB-231 cells compared with MDA-MB-231 cells transfected with empty vector. However, no significant differences in miR-125b levels were observed between KDM4B/JMJD2B-transfected MCF10A cells and empty vector-transfected MDA-MB-231 cells (Fig. 3b).

**miR-125b-1 is reactivated by an EZH2 inhibitor in MCF7 cells**

To determine if H3K27me3 is responsible for miR-125b-1 repression, we treated the breast cell lines with the EZH2 inhibitor GSK126. GSK126 is more than 1000-fold selective for EZH2 versus 20 other human methyl transferases (McCabe et al. 2012). To choose an effective GSK126 concentration without altering other methyltransferases, we constructed a dose curve to determine the minimum GSK126 concentration at which global H3K27me3 was decreased. We estimated global H3K27me3 levels by Western blotting and determined that 1000 nM was the minimum effective GSK126 concentration to evaluate miR-125b-1 reactivation in breast cancer cell lines.
Subsequent qRT-PCR analysis revealed that the expression levels of pri-miR-125b-1 (primary transcript) and miR-125b (mature transcript) were increased ninefold (Fig. 4c) and 16-fold (Fig. 4d), respectively, in MCF7 cells compared to untreated MCF7 cells. Interestingly, no changes in pri-miR-125b-1 (Fig. 4c) and mature miR-125b (Fig. 4d) transcription levels were observed in MDA-MD-231 and MCF10A cells. Hence, we conclude that H3K27me3 is responsible for miR-125b-1 repression in MCF7 cells.

miR-125b-1 reactivation affects the expression levels of BAK1

miR-125b is involved in biological processes such as apoptosis, cell proliferation and cell migration-regulating genes such as BAK1 (Zhou et al. 2010), ERBB2 (Scott et al. 2007) and ETS1 (Zhang et al. 2011), respectively. We therefore sought to evaluate the effects of miR-125b-1 reactivation on the expression and protein levels of its targets. Using the ENCODE database, we evaluated the presence of H3K27me3 on some miR-125b target gene promoters in the MCF7 cell line (Additional file 3: Fig. 3). This histone modification was absent only in BAK1 (Fig. 5a). This analysis is important because GSK126 treatment of MCF7 cells may alter the expression and protein levels of target genes via an miR-125b-independent mechanism.

We therefore evaluated BAK1 transcription levels in MCF7 cells treated with and without GSK126 by qRT-PCR. We observed a 71 % reduction in BAK1 expression levels in MCF7 cells treated with GSK126 (Fig. 5b). Next, we determined the proteins levels of BAK1 by Western blotting. We observed a 53 % reduction in BAK1 protein levels in MCF7 cells treated with GSK126 (Fig. 5c, d). We therefore conclude that miR-125b-1 reactivation affects the expression and protein levels of BAK1, an miR-125b target.

Discussion

miR-125b is an miRNA that may be involved indirectly in biological processes such as apoptosis, cell proliferation and cell migration (Banzhaf-Strathmann 2014). miR-125b-1 downregulation has been associated with increased cell proliferation (Scott et al. 2007), metastasis (Zhang et al. 2011) and drug resistance (Zhou et al. 2010; Wang et al. 2013; Vilquin et al. 2015) in breast cancer. miR-125b-1 repression has been associated with DNA methylation on its promoter region (Zhang et al. 2011; Soto-Reyes et al. 2012). However, in this study, we were interested in evaluating the relationship between
Fig. 3  JMJD2B over-expression reactivates miR-125b in MDA-MB-231 breast cancer cells. To determine if H3K9me3 is involved in miR-125b-1 repression, we over-expressed JMJD2B in MCF10A and MDA-MB-231 breast cells and then selected cells over-expressing JMJD2B by sorting (a). After the selection, we evaluated the expression levels of miR-125b (b).

Fig. 4  An inhibitor of EZH2 reactivates miR-125b-1 in MCF7 breast cancer cells. To determine the dose of GSK126 that reduces H3K27me3 levels, we treated the breast cell lines with different GSK126 concentrations and performed Western blot analysis (a). b Densitometric analysis of triplicate Western blots. We chose the 1000-nM dose to evaluate pri-miR-125b-1 (c) and mature miR-125b expression levels (d).
miR-125b-1 repression and repressive histone modifications such as H3K9me3 and H3K27me3. As a study model, we used two breast cancer cell lines, MCF7 (luminal A subtype) and MDA-MB-231 (triple-negative subtype), and the non-transformed breast cell line MCF10A.

We detected H3K9me3 and H3K27me3 in the miR-125b-1 promoter region in MDA-MB-231 and MCF7 cells, respectively. These histone modifications were mutually exclusive and did not coexist. These repressive histone modifications are related to different silencing mechanisms. The H3K9me3 repressive histone modification is associated with constitutive heterochromatin. Histone methyltransferases, such as G9a and SUV39H1, are involved in “writing this mark.” H3K9me3 is commonly localized in repeat regions but is not usually observed in gene-rich regions (Kim 2012; Chase and Cross 2011). In breast cancer tumors, H3K27me3 has been related to clinical stage and estrogen-receptor positive tumors (Healey et al. 2014). Some groups have reported an increase in the transcriptional levels of EZH2 (a H3K27 histone methyltransferase) in the luminal breast cancer subtype (Healey et al. 2014), and an association between increased EZH2 activity and poor prognosis has been reported (Jene-Sanz et al. 2013; Holm et al. 2012). These analyses are relevant to our work because miR-125b-1 is an EZH2 target in the luminal breast cancer cell line MCF7. miR-125b-1 repression by EZH2 may be involved in drug resistance because miR-125b can downregulate BAK1, an anti-apoptosis gene (Zhou et al. 2010). We demonstrated that the reactivation of miR-125b-1 decreases BAK1 expression and protein levels (Figs. 4, 5). This result suggests that a decrease in BAK1 may increase the sensitivity of cells to Taxol treatment (Zhou et al. 2010).

We conclude that miR-125b-1 is transcriptionally regulated by histone modifications such as H3K9me3 and H3K27me3, depending on the breast cancer cell subtype. In the luminal breast cancer cell line, we demonstrated that the reactivation of this miRNA affects the expression and protein levels of BAK1, a target involved in anti-apoptosis.
Author’s contributions
FCS participated in the design of the study, carried out the molecular genetic studies, performed the statistical analysis and drafted the manuscript. MA carried out the Western blot analysis. LAH conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
FCS was a fellow of the Programa de Doctorado en Ciencias Biomedicas, Universidad Nacional Autonoma de Mexico (UNAM). This work was partially supported by the Programa de Apoyo a Proyectos de Investigacion e Innovacion Tecnologica, UNAM, Grant Number IN208815.

Competing interests
The authors declare that they have no competing interests.

Received: 27 May 2016 Accepted: 30 May 2016
Published online: 02 July 2016

References
Banzhaf-Strathmann JE (2014) Good guy or bad guy: the opposing roles of microRNA-125b in cancer. Cell Commun Signal 12:30
Chase A, Cross NC (2011) EZH2 in cancer. Clin Cancer Res 17:2613–2618
Chien CH, Sun YM, Chang WC, Chiang-Hsieh PY, Lee TY, Tsai WC, Horng JT, Tsou AP, Huang HD (2011) Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Res 39:9345–9356
Di Leva G, Garofalo M, Croce CM (2014) MicroRNAs in cancer. Annu Rev Pathol 9:287–314
Fodor BD, Kubicek S, Yonezawa M, O’Sullivan RJ, Sengupta R, Perez-Burgos L, Di Leva G, Garofalo M, Croce CM (2014) MicroRNAs in cancer. Annu Rev Pathol 4:199–227
Graves AP, Della Pietra A, 3rd, Diaz E, LaFrance LV, Mellinger M, Duquenne C, Tian X, Kruger RG, McHugh CF, Brandt M, Miller WH, Dhanak D, Verma SK, Tummino PJ, Creasy CL (2012) EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature 492:108–112
Graves AP, Della Pietra A, 3rd, Diaz E, LaFrance LV, Mellinger M, Duquenne C, Tian X, Kruger RG, McHugh CF, Brandt M, Miller WH, Dhanak D, Verma SK, Tummino PJ, Creasy CL (2012) EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature 492:108–112
Healey MA, Hu R, Beck AH, Collins LC, Schmitt SJ, Tamimi RM, Hazra A (2014) Association of H3K27me3 and H3K27me3 repressive histone marks with breast cancer subtypes in the Nurses’ Health Study. Breast Cancer Res Treat 147:639–651
Holm K, Grabau D, Lovgren K, Aradottir S, Gruberger-Saal S, Howlin J, Saal LH, Ethier SP, Bendahl PO, Stal O, Malmstrom P, Ferno M, Ryden L, Hegardt C, Borg A, Ringner M (2012) Global H3K27 trimethylation and EZH2 abundance in breast tumor subtypes. Mol Oncol 6:494–506
Jene-Sanz AV, Vilкова AV, Khramtsova GF, Khramtsov AI, Olapade OI, Lopez-Bigas N, Benvenolenskaia EY (2013) Expression of polycomb targets predicts breast cancer prognosis. Mol Cell Biol 33:3951–3961
Kim JKH (2012) Recruitment and biological consequences of histone modification of H3K27me3 and H3K9me3. ILAR J 53:232–239
Lee YS, Dutta A (2009) microRNAs in cancer. Annu Rev Pathol 4:199–227
Marson A, Levine SS, Cole MF, Frampton GM, Bramb tink BR, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hennett N, Sharp PA, Bartel DP, Jaenisch R, Young RA (2008) Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134:521–533
McCabe MT, Ott HM, Ganji G, Koenen chuk S, Thompson C, Van Aller GS, Liu Y, Graves AP, Della Pietra A, 3rd, Diaz E, LaFrance LV, Mellinger M, Duquenne C, Tian X, Kruger RG, McHugh CF, Brandt M, Miller WH, Dhanak D, Verma SK, Tummino PJ, Creasy CL (2012) EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature 492:108–112
Scott GK, Goga A, Bhau mik D, Berger CE, Sullivan CS, Benz CC (2007) Coordinate suppression of ERBB2 and ERBB3 by enforced expression of microRNA-miR-125a or miR-125b. J Biol Chem 282:1479–1486
Soto-Reyes E, Gonzalez-Barrion R, Cisneros-Soberanis F, Herrera-Goepfert R, Perez Y, Cantu D, Prada D, Castro C, Recillas-Targa F, Herrera LA (2012) Disruption of CTCF at the miR-125b locus in gynecological cancers. BMC Cancer 12:40
Vilquin F, Donini CF, Villedieu M, Grisard E, Corbo L, Bachelot T, Vendrell JA, Cohen PA (2015) MicroRNA-125b upregulation confers aromatase inhibitor resistance and is a novel marker of poor prognosis in breast cancer. Breast Cancer Res 17:13
Wang YD, Cai N, Wu XL, Cao HZ, Xie LL, Zheng PS (2013) OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. Cell Death Dis 4:e760
Webber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schuberler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39:457–466
Zhang Y, Yan LX, Wu QN, Du ZM, Chen J, Liao DZ, Huang MY, Hou JH, Wu QL, Zeng MS, Huang WL, Zeng YX, Shao YJ (2011) microRNA-125b is methylated and functions as a tumor suppressor by regulating the ETS1 proto-oncogene in human invasive breast cancer. Cancer Res 71:3552–3562
Zhou M, Liu Z, Zhao Y, Ding Y, Liu H, X Y, Xiong W, Li G, Lu J, Liu J, Fodstad O, Riker AI, Tan M (2010) MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. J Biol Chem 285:21496–21507

Submit your manuscript to a SpringerOpen journal and benefit from:

► Convenient online submission
► Rigorous peer review
► Immediate publication on acceptance
► Open access: articles freely available online
► High visibility within the field
► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com