microRNA-18b is upregulated in breast cancer and modulates genes involved in cell migration

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Abstract. microRNAs are small non-coding RNAs of ~22 nucleotides that function at post-transcriptional level as negative regulators of gene expression. Aberrant expression of microRNAs could promote uncontrolled proliferation, migration and invasion of human cancer cells. In this study, we analyzed the expression of microRNA-18b (miR-18b) in breast cancer cell lines and in a set of clinical specimens. Our results showed that miR-18b was upregulated in four out of five breast cancer cell lines and also in breast tumors. In order to identify potential gene targets, we carried out transcriptional profiling of MDA-MB-231 breast cancer cells that ectopically expressed miR-18b. Our results showed that 263 genes were significantly modulated in miR-18b-deficient cells (fold change >1.5; P≤0.05). We found that knock-down of miR-18b induced the upregulation of 55 olfactory receptor (OR) genes and nine genes (NLRP7, KLK3, OLFM3, POSTN, MAGED4B, KIR3DL3, CRX, SEMG1 and CEACAM5) with key roles in cell migration and metastasis. Consistently, we found that ectopic inhibition of miR-18b suppressed the migration of two breast cancer cell models *in vitro*. In conclusion, we have uncovered genes directly or indirectly modulated by miR-18b which may represent potential therapeutic targets in breast cancer. Our data also pointed out a role of miR-18b in migration of breast cancer cells.

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Introduction

microRNAs are small non-coding RNAs of ~22 nucleotides that function at post-transcriptional level as negative regulators of gene expression. In normal cells, microRNAs maintain a balance of key cellular processes including cell growth, proliferation, differentiation and cell death. Abnormal expression of microRNAs occurs frequently in breast cancer where they act as oncogenes and tumor-suppressors. microRNAs may contribute to tumor initiation and progression by inhibiting apoptosis and promoting uncontrolled proliferation, invasion and metastasis. In consequence, they represent potential prognostic markers and novel therapeutic targets in human cancer (1). In animal cells, microRNAs are frequently transcribed together as polycistronic primary transcripts that are processed into multiple individual mature microRNAs. Particularly, microRNA-18b (miR-18b) is located at chromosome X within the miR-106a-363 cluster that is constituted by six different microRNAs (2,3). It has been proposed that expression of this microRNAs cluster is undetectable or very low in mouse (4). Diverse reports associated the aberrant expression of miR-18b to diverse human pathologies including cardiac hypertrophy (5), multiple sclerosis (6) and hepatitis B virus chronic infection (7). In human cancer, miR-18b expression is also deregulated. It has been reported that miR-18b is highly expressed in gastric cancer (8), in malignant serous tumors from borderline and benign ovarian tumors (9), in basal cell carcinoma of the skin (10), and in colonic cancer (11). Some miR-18b targets have been experimentally identified. In stem-like cells isolated from glioblastoma, it was reported that miR-18b targets the NOTCH2, NEDD9 and

MEKK1 genes (12). In breast cancer miR-18a, miR-18b, and other 21 microRNAs negatively regulate the estrogen receptor (ER)-α signaling (13). In addition, miR-18a and miR-18b showed higher expression levels in ER-α negative clinical specimens (13). In another study, a low expression of miR-18b was associated with improved survival in human epidermal receptor (HER) 2-negative breast cancer, although it was not correlated with ER-α protein levels (14). Recently, higher levels of circulating miR-18b were proposed as a potential marker of breast cancer (15). Although knowledge on the roles of miR-18b in cancer is increasing, the physiological functions still remain unclear and the number of detected miR-18b targets in cancer is scarce. Here, we studied the miR-18b expression in breast cancer cell lines and clinical tumors. In addition, we performed a genomic-wide study using DNA microarrays in order to identify genes regulated by miR-18b with putative roles in breast cancer.

Materials and methods

Tissue samples. Human primary breast tumors and adjacent non-tumoral tissues were obtained from the Institute of Breast Diseases-FUCAM, Mexico. Biopsies were obtained from breast cancer patient after selection following the regulations approved by the FUCAM Ethics Committee, including patient informed consent for research use. None of the patients recruited in this study received anti-neoplastic therapy prior to surgery. After tumor resection, specimens were embedded in Tissue-Tek and snap-frozen in liquid nitrogen at -80˚C until analysis. A pathologist analyzed samples for the amount of tumoral cells and to validate non-tumoral tissues. Tumor tissues containing at least 80% of tumor cells and non-tumoral tissues confirmed by the pathologist were used for subsequent analysis.

Cell line cultures. Human MDA-MB-231, MDA-MB-453, ZR-75, T47-D and MCF-7 breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37˚C.

RNA isolation and qRT-PCR analysis of miR-18b expression. Total RNA of tissues and cell lines was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA integrity was assessed using capillary electrophoresis system Agilent 2100 Bioanalyzer with the eukaryotic nano-chip. Only samples with a RIN value of 6 or higher were processed. The expression of miR-18b was performed using microRNA assays (Applied Biosystems, Foster City, CA, USA). Total RNA (100 ng) obtained from clinical specimens and cell lines was reverse transcribed using a looped RT primer specific for miR-18b, 0.15 μl of dNTPs (100 mM), 1.0 μl of reverse transcriptase MultiScribe™ (50 U/μl), 1.5 μl of 10X buffer, 0.19 μl of RNase inhibitor (20 U/μl) and 4.16 μl of RNase-free water. Then, diluted retrotranscription reaction (1:15) was mixed with 10 μl of master mix TaqMan (Universal PCR Master mix, No AmpErase® UNG, 2X), 7.67 μl of RNase-free water, and 1.0 μl of probe PCR. PCR reaction was done in a GeneAmp System 9700 (Applied Biosystems) as follows: 95˚C for 10 min, and 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The expression of miR-18b levels were measured by qRT-PCR using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. RNU44 and RNU48 were used as a control for normalization of data.

Transfection of the miR-18b inhibitor. MDA-MB-231 and MCF-7 cells were transfected with miR-18b inhibitor (#MH10466; Life Technologies) and scrambled at indicated final concentrations using siPORT™ amine transfection agent (Ambion, Inc., Austin, TX, USA). The miR-18b inhibitor was diluted in 25 μl of Opti-MEM® to 50-200 nM concentrations, and individually added to wells containing cultured cells in 450 μl of DMEM. Knock-down of miR-18b expression was evaluated by qRT-PCR at 48 h post-transfection.

DNA microarray analysis. To analyze the effects of miR-18b inhibition on gene expression we compared the gene expression profiles of MDA-MB-231 cells transfected with miR-18b inhibitor and with siPORT amine transfection agent (control) using DNA microarrays. Total RNA (1 μg) was employed for cDNA synthesis and labeling using Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion) following manufacturer's instructions. Total RNA obtained 48 h after transfection with empty transfection agent was labeled with Cy3, whereas cDNA from cells transfected with miR-18b inhibitor was Cy5 labeled. Dye incorporation efficiency was analyzed measuring specific absorbance. Samples (500 pmol) were hybridized onto Human Exonic Evidence Based Oligonucleotide (HEEBO) arrays obtained from the Stanford Functional Genomics Facility (Stanford, CA, USA). Each microarray contained 43,000 spots, including 85.8% I.M.A.G.E. consortium clones from the Research Genetics sequence verified clone set and 4.4% control spots, corresponding to 18,141 mapped human genes. Hybridization was performed at 42˚C for 18 h. Following hybridization, arrays were washed and scanned using a GenePix 4100A Axon scanner (Axon Instruments, Inc., Union City, CA, USA). Fluorescence ratios were extracted using GenePix 5.0 software (Axon Instruments, Inc.). We defined well-measured spots by: i) having a ratio of normalized signal intensity to background noise of >2 for either the Cy5 signal derived from miR-18b inhibitor transfected cells, or the Cy3 signal derived from empty transfected cells and, ii) with no flag assignation by GenePix software which corresponds to poor spot quality caused by non-reliable hybridization and corruption by noise. We performed an algorithm on Microsoft Excel to accomplish both conditions. Background-subtracted fluorescence log2 ratios were globally normalized for each array, and then mean-centered for each gene. Each microarray experiment was repeated as technical replicates for statistical robustness. Finally, to avoid bias caused by different properties of the two cyanine dyes we balanced the intensity level of both channels using Lowess normalization method included on ArrayNorm software (16).

MTT cell viability assay. To evaluate the cell viability after inhibition of miR-18b in MDA-MB-231 and MCF-7 cells a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed. Cells ($2x10^4$) transfected with miR-18b inhibitor (50 nM), scramble

Table I. Clinicopathological features of breast cancer patients and tumors analyzed for miR-18b expression.

Patient no.	Age (years)	Tumor size (mm)	Clinical stage	Tumor grade	HER2	ER	PR	Classification	Histological subtype
15	45	35	ND	2	+	-	+	HER2-positive	Infiltrating ductal carcinoma
30	41	20	0	2	+	+	+	Luminal B	*In situ* ductal carcinoma
75	50	25	IIB	2	+	-	-	HER2-positive	Infiltrating ductal carcinoma
76	49	20	IIB	3	+	-	-	HER2-positive	Infiltrating ductal carcinoma
78	81	47	IIIB	3	+	-	-	HER2-positive	Infiltrating ductal carcinoma
79	56	15	IIA	2	+	-	-	Luminal A	Infiltrating ductal carcinoma
82	59	25	IIB	ND	+	-	-	Luminal A	Infiltrating ductal carcinoma
85	47	27	IIA	3	+	-	+	HER2-positive	Infiltrating ductal carcinoma
98	65	30	IIA	ND	+	-	-	Luminal A	Infiltrating ductal carcinoma
105	39	23	IIIC	2	+	-	+	HER2-positive	Infiltrating ductal carcinoma
139	44	30	IIB	3	-	-	-	Triple negative	Infiltrating ductal carcinoma
169	65	40	IIB	ND	-	-	-	Triple negative	Infiltrating medular carcinoma
191	55	39	IIB	2	+	-	-	HER2-positive	Infiltrating ductal carcinoma

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal receptor 2; ND, not determined.

sequence, and siPORT transfection reagent as controls were seeded in 48-well culture dishes and incubated in MTT (1 mg/ml) at 37˚C for 4 h. The medium was removed and formazan dye crystals were solubilized with 500 μl isopropanol, 4 mM HCl, NP-40 0.1% for 5 min. Absorbance was measured in a spectrophotometer at 540 nm wavelength. Assays were performed in triplicate.

Cell migration assays. To evaluate the effect of miR-18b inhibition in MDA-MB-231 and MCF-7 cell migration a scratch/wound-healing assay was performed. Briefly, cells transfected with the miR-18b inhibitor (50 nM), scramble sequence, and siPORT transfection reagent as controls were seeded in triplicate in a 6-well plate and grown to 80% confluence. Twenty-four hours post-transfection a vertical wound was traced in the cell monolayer with a sterile plastic tip. After 4 and 24 h cells were fixed with 4% paraformaldehyde in PBS pH 7.0 and monolayer restoration was imaged through an inverted phase contrast microscope (magnification, x10). The scratched area was quantified at each time point and the resulting values were graphed. To quantify migratory capacity of cells, transwell chambers (Corning) with 6.5-mm diameter and 8-μm pore size polycarbonate membrane were used. MDA-MB-231 and MCF-7 cells ($1x10^5$) transfected with miR-18b inhibitor, scramble or siPORT transfection reagent (mock) were transferred to 0.5 ml serum-free medium and placed in the upper chamber, whereas the lower chamber was loaded with 0.8 ml medium containing 10% FBS. The total number of cells that migrated into the lower chamber was counted after 24 h incubation at 37˚C.

Statistical analysis. Each experiment was performed at least three times, and the results are presented as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test were used to compare the differences between means. $P<0.05$ was considered as statistically significant.

Results

miRNA-18b expression is upregulated in breast cancer cell lines and clinical tumors. In our previous studies, we analyzed a limited set of clinical breast tumors and identified 54 microRNAs that exhibit a significant differential expression between tumor and non-tumor tissues (our unpublished data). Of these, miR-18b was overexpressed in breast cancer and it was selected for further analysis. Here, we studied the miR-18b levels in an independent set of clinical specimens and in five human breast cancer cell lines. We extended our analysis by assaying the miR-18b expression using qRT-PCR TaqMan microRNA assays in clinical specimens obtained from 14 human breast cancer patients who did not receive anti-neoplastic therapy prior to surgery. Tumors were stratified based on clinical subtypes as luminal, HER2-positive and triple negative. Clinical features of breast tumors including hormonal receptors status, tumor size, histology, clinical stage, and tumor grade are summarized in Table I. Our results showed that miR-18b expression was increased in breast tumors in comparison to non-tumoral tissues (Fig. 1A). Particularly, the miR-18b expression average was augmented in luminal (2.5-fold), HER2-positive (4.6-fold), and triple negative (17-fold) tumor subtypes relative to non-tumoral specimens (Fig. 1B). We found no relationship between the expression of miR-18b and clinical characteristics of the patients such as tumor grade, clinical stage, tumor size or histological subtype. Then, we analyzed the miR-18b expression in breast cancer cell lines by qRT-PCR using TaqMan microRNA assays. Results showed that miR-18b levels were significantly increased (2- to 7-fold) in MDA-MB-231, MDA-MB-453, ZR-75 and T47-D cell lines in comparison to non-tumoral breast tissues used as control samples (Fig. 1C). In contrast, miR-18b expression was similar in MCF-7 cell line and normal breast tissues. Interestingly, the triple negative MDA-MB-231 cell line and triple negative breast tumors exhibit the highest miR-18b

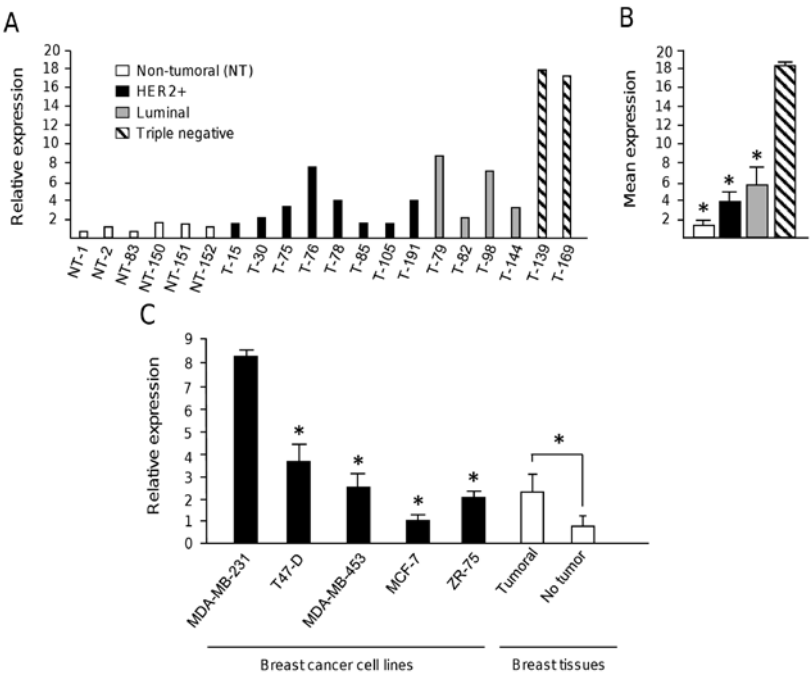

Figure 1. miR-18b is overexpressed in clinical tumors and breast cancer cell lines. (A) TaqMan microRNA assay for miR-18b expression in non-tumoral (NT) and tumor (T) breast tissues. (B) Graphical representation of mean average data in A. Breast tumors were analyzed to compare to NT tissues by measuring the relative expression and normalized with endogenous small-nucleolar RNU-44. (C) TaqMan microRNA assay for miR-18b expression in diverse breast cancer cell lines, NT and T tissues. Bars represent the means of at least three independent experiments ± SD. *P<0.005. Experiments were repeated at least twice with similar results.

expression levels. We decided to use the MDA-MB-231 cell line as model for searching potential miR-18b targets.

Transcriptional profiles of miR-18b-deficient MDA-MB-231 cells. In order to obtain a comprehensive analysis of cellular transcripts that may represent potential targets of miR-18b in triple negative breast cancer, we studied the effects of its inhibition on the transcriptome of MDA-MB-231 cells. We confirmed the effective knock-down of miR-18b expression by qRT-PCR assays using antagomiRs (data not shown). Total RNA was extracted from cells transfected with miR-18b inhibitor (50 nM) and siPORT amine transfection agent (control) cells, and hybridized to Human Gene Expression 12x135 K DNA microarrays (NimbleGen, Roche). We performed data extraction, normalization of replicates and controls, and selection of spots with a significant modulation (cut-off of ≥1.5-fold) as described in Materials and methods. Our microarray analysis identified 263 genes with a significant modulation (adjusted P-value = 0.05). Of these, 43 genes were downregulated and 220 were upregulated. These results indicated that repression of miR-18b had positive effects in global gene expression of MDA-MB-231 cells, as expected for a negative regulator of gene expression. In Tables II and III we show an overview of the top 20 modulated genes in miR-18b-deficient MDA-MB-231 cells.

Olfactory receptor (OR) genes were upregulated in miR-18b-deficient breast cancer cells. Surprisingly, we evidenced the upregulation of 55 OR genes in MDA-MB-231 cells deficient for miR-18b (Table IV). The OR proteins are members of a large family of G-protein-coupled receptors which are expressed not only in the sensory neurons of the olfactory epithelium, but also in various other non-chemosensory tissues (17) and in tumor tissues including prostate cancer where their activation inhibits cell proliferation (18,19). However, the relevance and function of OR genes is still unknown in breast cancer. Here, we found that ten upregulated ORs genes were clustered in 11q12.1 chromosome region and six were grouped in 11q24.2 locus. To understand the coordinated upregulation of these genes, we searched for miR-18b binding sites in the 3'UTR of OR genes using miRanda software (20). Intriguingly, results showed that none of modulated OR genes presented potential miR-18b binding sites. This raises the possibility that OR gene expression could be regulated by a miR-18b-mediated indirect mechanism. To test this hypothesis, we searched for miR-18b binding sites in the 3'UTRs of transcription factors leading to activation of OR genes (21). Interestingly, we found that pancreatic and duodenal homeobox 1 (PDX1), a transcription factor regulating ORs, has potential binding sites for miR-18b. In agreement, we found that PDX1 gene was also up-regulated in our microarray assays (fold change 1.98; P=0.039). These data suggested that OR gene expression could be regulated through a miR-18b-indirect mechanism by targeting transcription factors leading to OR activation. However, further experiments are needed to support these assumptions.

miR-18b modulates genes involved in tumorigenesis. Further analysis of microarray data leads us to the identification of diverse genes involved in cancer whose expression was modulated when miR-18b expression was inhibited in breast cancer cells. In Table V we show an overview of upregulated genes which have been reported previously in different malignancies. These genes have been implicated in tumor growth (CHRM2 and POSTN), cell proliferation (NLRP7 and

Table II. Top 20 of upregulated genes in miR-18b-deficient human MDA-MB-231 breast cancer cells.

Gene symbol[a]	Protein name[b]	Fold change	P-value
OR8D2	Olfactory receptor, family 8, subfamily D, member 2	7.10	0.039
OR8G2	Olfactory receptor, family 8, subfamily G, member 2	6.44	0.011
OR8D1	Olfactory receptor, family 8, subfamily D, member 1	6.36	0.017
REXO1L1	REX1, RNA exonuclease 1 homolog-like 1	5.94	0.013
KRTAP13-1	Keratin associated protein 13-1	5.87	0.013
ERC2	ELKS/RAB6-interacting/CAST family member 2	5.77	0.044
OR11H1	Olfactory receptor, family 11, subfamily H, member 1	5.65	0.032
OR9G1	Olfactory receptor, family 9, subfamily G, member 1	5.63	0.021
KIR3DL3	Killer cell immunoglobulin-like receptor	5.46	0.047
OR52E2	Olfactory receptor, family 5, subfamily E, member 2	5.46	0.046
NLRP7	NLR family, pyrin domain containing 7	5.33	0.018
OR52J3	Olfactory receptor, family 52, subfamily J, member 3	5.33	0.013
KCND2	Potassium voltage-gated channel	5.33	0.040
PRAMEF5	PRAME family member 5	5.32	0.031
REG1B	Regenerating islet-derived 1 β	5.26	0.014
OR4A47	Olfactory receptor, family 4, subfamily A, member 47	5.19	0.006
OR8H3	Olfactory receptor, family 8, subfamily H, member 3	5.18	0.041
LOC391747	Similar to hCG1807616; TBP-associated factor 11	5.04	0.017
OR4A15	Olfactory receptor, family 4, subfamily A, member 15	5.00	0.006
OR10G8	Olfactory receptor, family 10, subfamily G, member 8	4.98	0.001

[a]GenBank databases; [b]UniProt database (recommended name).

Table III. Top 20 of downregulated genes in miR-18b-deficient human MDA-MB-231 breast cancer cells.

Gene symbol[a]	Protein name[b]	Fold change	P-value
PELP1	Proline, glutamate and leucine rich protein 1	-8.664	0.007
FBXW4	F-box and WD repeat domain containing 4	-5.560	0.041
MR1	Major histocompatibility complex, class I-related	-4.16	0.044
MARCH1	Membrane-associated ring finger (C3HC4) 1, E3 ubiquitin protein ligase	-4.065	0.042
MAK	Male germ cell-associated kinase	-4.009	0.047
MAGED4B	Melanoma-associated antigen D 4B	-3.451	0.043
EXOC6	Exocyst complex component 6	-3.089	0.042
PLEKHH2	Pleckstrin homology domain containing, family H member 2	-2.919	0.012
FAM113B	Family with sequence similarity 113, member B	-2.783	0.031
BAIAP2L2	BAI1-associated protein 2-like 2	-2.63	0.043
THAP9	THAP domain containing 9	-2.547	0.009
KCNK7	Potassium channel, subfamily K, member 7	-2.429	0.044
C17orf57	Chromosome 17 open reading frame 57	-2.321	0.035
BEND2	BEN domain containing 2	-2.300	0.016
TMEM27	Transmembrane protein 27	-2.284	0.038
SOX4	SRY (sex determining region Y)-box 4	-2.281	0.040
BCAT1	Branched chain aminotransferase 1, cytosolic	-2.20	0.031
TMEM19	Transmembrane protein 19	-2.148	0.023
ADPGK	ADP-dependent glucokinase	-2.12	0.032
SPN	Sialophorin	-2.07	0.043

[a]GenBank databases; [b]UniProt database (recommended name).

Table IV. Upregulated olfactory receptor genes in miR-18b-deficient MDA-MB-231 breast cancer cells.

Gene symbol	Fold change	P-value	Locus
OR8D2	7.10	0.039	11q24.2
OR8G2	6.44	0.011	11q24.2
OR8D1	6.36	0.017	11q24.2
OR11H1	5.65	0.032	22q11.1
OR9G1	5.63	0.021	11q12.1
OR52E2	5.46	0.046	11p15.4
OR52J3	5.33	0.013	11p15.4
OR4A47	5.19	0.006	11p11.2
OR8H3	5.18	0.041	11q12.1
OR4A15	5.00	0.006	11q11
OR10G8	4.98	0.001	11q24.2
OR2T8	4.97	0.035	1q44
OR4K2	4.92	0.049	14q11.2
OR1C1	4.71	0.036	1q44
OR5J2	4.56	0.036	11q12.1
OR1S1	4.49	0.040	11q12.1
OR4Q3	4.44	0.001	14q11.2
OR10H4	4.36	0.050	19p13.12
OR10H3	4.24	0.014	19p13.12
OR5D16	4.23	0.020	11q11
OR8H2	4.20	0.007	11q12.1
OR6K2	4.17	0.015	1q23.1
OR6N1	4.15	0.025	1q23.1
OR7C2	4.12	0.010	19p13.12
OR10K2	3.95	0.038	1q23.1
OR4C13	3.95	0.004	11p11.12
OR56A4	3.93	0.026	11p15.4
OR2T27	3.89	0.036	1q44
OR52K2	3.84	0.022	11p15.4
OR1E1	3.77	0.016	17p13.2
OR2J3	3.69	0.019	6p22.1
OR6N2	3.66	0.043	1q23.1
OR8B3	3.45	0.046	11q24.2
OR4F17	3.36	0.029	19p13.3
OR8H1	3.49	0.011	11q12.1
OR10Q1	3.35	0.023	11q12.1
OR5M8	3.31	0.017	11q12.1
OR5M11	2.69	0.010	11q12.1
OR5AR1	2.74	0.028	11q12.1
OR14I1	3.31	0.040	1q44
OR1J4	3.23	0.026	9q33.2
OR5AU1	3.11	0.034	14q11.2
OR2T29	3.00	0.004	1q44
OR8B8	2.40	0.009	11q24.2
OR4C46	2.20	0.003	11p11.12
OR6K3	2.16	0.001	1q23.1
OR2T5	2.10	0.015	1q44
OR10S1	2.09	0.042	11q24.1
OR3A2	2.02	0.020	17p13.3
OR2J2	1.98	0.036	6p22.1
OR2AK2	1.88	0.035	1q44
OR5D14	1.82	0.044	11q11
OR1L6	1.79	0.025	9q33.2
OR13C5	1.60	0.028	9q31.1
OR6Y1	1.52	0.035	1q23.1

Genes clustered in 11q24.2 chromosomal region are underlined. Genes clustered in 11q12.1 chromosomal region are shown in bold.

CHMR2), anoikis (OLFM3), apoptosis (REG1B, SCN3B and POSTN), and angiogenesis (KLK3, CHRM2 and POSTN). Interestingly, eight genes (KIR3DL3, NLRP7, KLK3, OLFM3, SEMG1, CRX, POSTN and CEACAM5) have been shown to be involved in invasion and metastasis of cancer cells. To determine whether the overexpression of these genes was related to direct binding of miR-18b, we searched for potential miR-18b binding sites in their 3'UTR region. Eleven genes were predicted to have binding sites for miR-18b, nine of them with one site and two with more than one binding site. Among these, four genes (KIR3DL3, KLK3, CRX and POSTN) were implicated in invasion and metastasis of cancer cells. For instance, the KLK3 gene encodes prostate-specific antigen (PSA), which is a serin protease that has been established as a tumor marker of prostatic adenocarcinoma. This protein was found elevated in women with renal cell carcinoma (22), it has been shown to exert anti-angiogenic properties, and to present mutations in breast tumors (23). Another upregulated gene, POSTN encodes periostin, a secretory protein which has been suggested to function as a cell adhesion molecule participating in osteoblast recruitment, attachment and spreading (24,25). POSTN was found overexpressed in several cancers with enhanced invasiveness, and correlated with metastasis in colorectal and liver cancers, as well in oral cancer cell lines (26).

Knockdown of miR-18b suppresses cell migration in vitro. Our transcriptional profiling of MDA-MB-231 cells with reduced miR-18b expression revealed that a set of modulated genes have key roles in migration and invasion of cancer cells. In order to evaluate whether miR-18b could be implicated in the regulation of migration properties of breast cancer cells, we performed scratch/wound-healing and Transwell assays in MDA-MB-231 cells with reduced expression of miR-18b. We first evaluated the effect of increasing concentrations of antagomiR-18b in cell viability by MTT assay. No significant changes in cell viability were observed using a range of concentrations (25-200 mM) of miR-18b inhibitor in metastatic MDA-MB-231 cells (Fig. 2A). Similar results were obtained in the MCF-7 breast cancer cell line (Fig. 2E). Then, we performed a scratch/wound-healing assay to evaluate whether the suppression of miR-18b expression could have effects on cancer cell migration. Monolayers of MDA-MB-231 and MCF-7 cells non-transfected and transfected with miR-18b

Table V. The genes modulated by miR-18b inhibition with a role in human cancers.

Gene symbol[a]	Fold change	P-value	Description	Gene function related to cancer	Refs.	miR-18b binding sites[b]
ERC2	5.77	0.044	ELKS/RAB6-interacting/ CAST family member 2	Promotes oncogenesis in papillary thyroid carcinoma by fusion of ELKS to RET.	(40)	1
KIR3DL3	5.46	0.047	Killer cell immunoglobulin-like receptor	Interactions with MHC-I on melanoma inhibit tumor cells lysis by NK and T cells. Interactions between KIR and HLA in NK cell-controls uveal melanoma metastasis.	(41,42)	1
NLRP7	5.33	0.018	NLR family, pyrin domain containing 7	Cell proliferation and tumorigenesis in testicular germ cell tumor. Promote tumor invasion and poor prognosis in endometrial cancer.	(43,44)	None
KCND2	5.33	0.040	Potassium voltage-gated channel subfamily D2	Participates in brain cancer-induced cachexia.	(45)	1
REG1B	5.26	0.014	Regenerating islet-derived 1 β	Regulates IL-6 and enhance the chemo- and radio-sensitivity on squamous esophageal cancer.	(46,47)	1
KIR2DS2	4.85	0.020	Killer cell immunoglobulin-like receptor	Associated with better response to treatment and prolonged survival in non-small cell lung carcinoma. Activated KIR genes are associated with reduced risk for developing acute lymphoblastic leukemia.	(48,49)	None
KLK3	4.52	0.013	Prostate-specific antigen (PSA). Kallikrein-related peptidase 3	Contribute to bone metastasis in prostate cancer. Modulates cell invasion of prostate cancer cells. High levels of PSA may slow down prostate cancer progression by inhibiting angiogenesis. Regulates androgen receptor in prostate cancer. Decreased expression in ovarian tumors. Favorable indicator of prognosis in breast cancer.	(50-54)	1
PRAMEF4	4.49	0.012	PRAME family member 4	Correlates with poor prognosis in head and neck cancer.	(55)	None
CHRM2	4.47	0.005	Muscarinic acetylcholine receptor M2	Involved in proliferation of murine adenocarcinoma cell lines. Involved in growth, proliferation and angiogenesis in breast cancer cells. Involved in inhibition of glioma cell proliferation. Involved in cell proliferation of chronic myelogenous leukemia.	(56-60)	1
OLFM3	4.44	0.037	Olfactomedin 3/noelin-3	Contributes to anoikis-resistance in squamous lung carcinoma. Olfml3 targeting inhibits endothelioma cell migration, tumor growth and sprouting. OLFM3 is pro-angiogenic.	(61,62)	None
PCDH15	4.12	0.014	Protocadherin 15	Potential marker for NK/T cell lymphomas.	(63)	1

Table V. Continued.

Gene symbol[a]	Fold change	P-value	Description	Gene function related to cancer	Refs.	miR-18b binding sites[b]
SCN3B	3.96	0.029	Sodium channel subunit β-3	Mediates a p53-dependent apoptotic pathway (pro-apoptotic) in colorectal carcinoma cell line HCT116.	(64)	1
SEMG1	3.86	0.005	Semenogelin I	Overexpressed in OH-1 SCLC lung cancer cell lines and associated with cell surface adhesion complexes. Mutated in colorectal cancer. Inhibits motility of sperm cells. SEMG1 inhibits prostate-tumor growth.	(65-69)	None
CRX	3.82	0.012	Cone-rod homeobox protein	Overexpressed in medulloblastomas. Upregulated in retinoblastoma tumors. Metastatic retinoblastoma marker.	(70-72)	2
KLK15	3.78	0.003	Kallikrein-related peptidase 15	Upregulated by steroid hormones in the LNCaP prostate cancer cell line; associated with more aggressive forms of prostate cancer. Marker of unfavorable prognosis in ovarian cancer. Prognostic marker for progression-free survival in patients with radical prostatectomy. Favorable prognostic marker for breast cancer.	(73-76)	None
POSTN	3.77	0.043	Periostin	Enhances invasion, angiogenesis and metastasis in oral cancer and head and neck squamous cell carcinoma. Promotes the metastatic growth of colon cancer. It is required for cancer stem cell maintenance, and blocking its function prevents metastasis. Reduces cell migration and suppressed metastasis *in vivo*. Inhibits breast cancer progression and metastasis by anti-periostin in a murine model.	(77-80)	1
PCDHB2	2.39	0.019	Protocadherin beta 2	Mutation of PCDHB2 (cell adhesion protein) in pancreatic cancers.	(81)	None
C1QTNF4	2.16	0.010	C1q and tumor necrosis factor related protein 4	Stimulates the STAT3 and NF-κB pathways and promotes cell survival in human cancer cells.	(82)	None
FOXD4L4	1.95	0.010	Forkhead box D4-like 4	FOX4DL4 gene amplification in pilocytic astrocytoma. Hypermethylated in ovarian tumors.	(83-85)	3
CEACAM5	1.78	0.034	Carcinoembryonic antigen-related cell adhesion molecule 5	Methylated in non-small cell lung cancer tumors. Implied in bad prognosis in patients with adenocarcinoma. Overexpression enhances metastasis in coloncarcinoma. Brain metastasis development and poor survival associated with CEA level in NSCLC.	(86-88)	None

[a]GenBank databases. [b]As prediceted by TargetScan. Cell migration and invasion-related genes are denoted in bold.

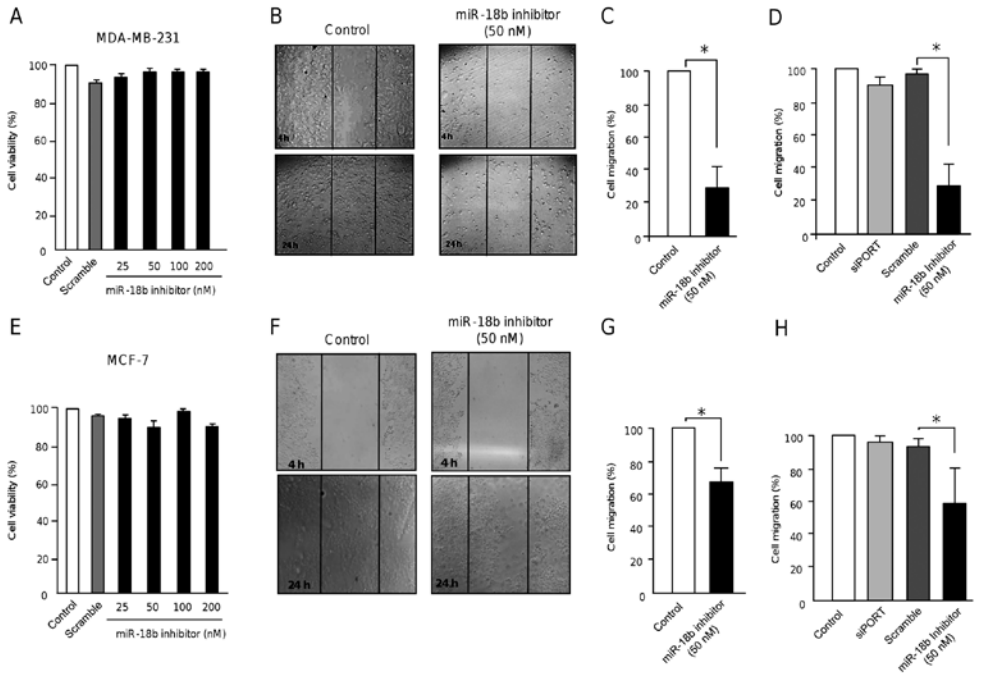

Figure 2. Knockdown of miR-18b suppresses cell migration. MTT cell viability assays of (A) MDA-MB-231 and (E) MCF-7 cells transfected with different concentrations (50-200 nM) of miR-18b inhibitor. Scratch/wound-healing assays of (B) MDA-MB-231 and (F) MCF-7 cell monolayers transfected with miR-18b inhibitor (50 nM) or treated with transfectant agent as control. Graphical representation of migrating (C) MDA-MB-231 and (G) MCF-7 breast cancer cells after wound-healing. Graphical representation of cell migration in transwell chambers of (D) MDA-MB-231 and (H) MCF-7 cells. Percent of cell migration is shown with bars for each experiment. Bars represent the means of three independent experiments ± SD. *P<0.005.

inhibitor were grated, and the wounded areas of the cell monolayer were analyzed after 24 h. Results showed that the formation of monolayers was significantly delayed about 70 and 30% in MDA-MB-231 and MCF-7 cells transfected with miR-18b inhibitor, respectively, in comparison with control cells (Fig. 2B and F). By using Transwell chamber assays, we found that the number of migratory cells that crossed the membrane after 4 and 24 h was reduced in MDA-MB-231 (75%) and MCF-7 (40%) cells in comparison with controls confirming that miR-18b abolishment has negative effects in cell migration (Fig. 2D and H). These findings suggested that miR-18b is able to modulate the expression of genes that are important for migration of cancer cells *in vitro*.

Discussion

microRNA expression is frequently deregulated in breast cancer where they play key roles in tumorigenesis acting as oncogenes and tumor suppressors. In this study, we showed that miR-18b is commonly overexpressed in breast cancer cell lines and in clinical tumors, particularly in triple negative clinical specimens (17-fold). We found an inverse correlation between miR-18b expression and hormonal receptors and HER2/neu status in breast cell lines and tumors (Fig. 1). These data are in agreement with a previous study which reports that ER-α is a target of miR-18a and miR-18b suggesting that inhibition of this receptor in triple negative breast cancer may be due, at least in part, by miR-18b (13). Moreover, in a study of 204 lymph node-negative breast cancers, authors found an association among higher expression of miR-18b with negative expression of ER and strongly associated with basal-like breast cancer features (27), in agreement with our results. We showed that miR-18b is overexpressed in triple negative breast tumors. Triple negative breast cancer refers to a heterogeneous group of tumors that do not express estrogen, progesterone or HER2/neu receptors, accounting for approximately 12-15% of breast cancer diagnoses (28). It represents the most aggressive subtype of breast cancer, and is associated with high rates of mortality and poor survival. Notably, no specific therapeutics targets have been identified in triple negative tumors, thus treatment is restricted to conventional chemotherapy. Therefore, the search for novel potential therapeutic targets represents today a challenge for oncology community.

In order to identify potential targets of miR-18b that could represent novel therapeutic targets in triple negative breast cancer, we suppressed its expression in MDA-MB-231 cells using antagomiRs and performed a genome-wide analysis using DNA microarrays. Our analysis showed that several genes with key roles in cancer were significantly modulated (Table V). In addition, a set of 55 OR genes were also found as regulated in miR-18b-deficient cells. The OR gene family is the largest family in the genome with more than 800 genes located in clusters that are dispersed across almost all chromosomes. Although the majority of OR members are expressed on the olfactory sensory neurons in the olfactory epithelium, they are also expressed in a large number of different tissues, but their functions remain unclear. It has been suggested that they may control cell positioning during embryogenesis (29). Of interest, the expression of ORs has been also reported in cancer. Previous studies detected the expression of ORs in human primordial germ cells and prostate cancer (30,31). In a recent study it was reported that the activation of an OR (PSGR/OR51E2) inhibits proliferation of

prostate cancer cells (18). In addition, four microRNA:mRNA modules containing the olfactory transduction pathway, are over-represented in metastatic prostate cancer (19). Most of the 55 upregulated ORs detected in this study were clustered in 11q12.1 and 11q24.2 chromosome regions, suggesting that a gain or loss of these genomic regions could explain the changes in their expression. The 11q12.1 locus is defined as an overlapping region of imbalance involved in testicular germ cell tumors (32), whereas homozygous loss of 11q24.2 region was observed in uterine leiomyosarcoma (33). OR genes located on 11q12.1 have been already reported as overexpressed in breast tumors. Of a set of 862 differentially expressed genes, 34 ORs were found to have elevated expression in breast tumors with CHEK2 1100delC mutation, where 16 ORs resided on chromosome 11, and 5 in a region flanking the centromere region 11.p11.2-11.q12.1 (34). Our results on the identification of OR genes modulated in miR-18b-deficient cells indicate that they could be involved in tumorigenesis, however additional experiments are needed to clarify their role in breast cancer. The absence for potential miR-18b binding sites in the OR genes suggested that they could be modulated by an miR-18b-driven indirect mechanism. Plessy *et al* (21) found binding sites for several transcription factors on OR promoters that may control their transcription. In particular, a binding site motif for PDX1 homeobox transcription factor was found in the promoter of mouse and human olfactory epithelium (MOE). PDX1 participates during branching morphogenesis in the developing mouse embryonic pancreas (35). In cancer, PDX1 is activated at various stages of gastric carcinogenesis (36), and it was suggested as a novel tumor suppressor in gastric cancer (37). It was also involved in pancreas development, in pediatric solid pseudopapillary tumors (38), and in precursor lesions of ductal adenocarcinomas (39). In our study, we found that PDX1 was upregulated after inhibition of miR-18b. From these findings, we suggested that miR-18b could indirectly regulate OR genes by direct modulation of PDX1, however, additional experimentation is needed to corroborate this hypothesis.

We have shown that the inhibition of miR-18b induces the modulation of genes with key roles in cancer. These genes have been implicated in tumor growth (CHRM2 and POSTN), cell proliferation (NLRP7 and CHMR2), anoikis (OLFM3), apoptosis (REG1B, SCN3B and POSTN), and angiogenesis (KLK3, CHRM2 and POSTN). Notably, eight genes (KIR3DL3, NLRP7, KLK3, OLFM3, SEMG1, CRX, POSTN and CEACAM5) are involved in invasion and metastasis of cancer cells (Table V). Moreover, these genes contain potential binding sites for miR-18b which strongly suggested that they could be targets of miR-18b. Our findings using wound-healing assays in miR-18b depleted cells showed that cell migration was significantly affected. By Transwell chamber assay, we corroborated the impact of miR-18b inhibition on the capacity of migration in MDA-MB-231 and MCF-7 cells. In both assays, results showed that the formation of monolayer was delayed about 40-75% in miR-18b-deficient cells in comparison to control migrating cells. Taken together, our findings strengthened the view that miR-18b is able to modulate the expression of genes that are important for migration of triple negative breast cancer cells *in vitro*.

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