Pax-5 Inhibits Breast Cancer Proliferation Through MiR-215 Up-regulation

NICOLAS LEBLANC^{1,2}, JASON HARQUAIL^{1,2}, NICOLAS CRAPOULET^{1,2},
RODNEY J. OUELLETTE^{1,2} and GILLES A. ROBICHAUD^{1,2}

¹Department of Chemistry and Biochemistry, University of Moncton, Moncton, Canada;

²Atlantic Cancer Research Institute, Moncton, Canada

Abstract. *Background/Aim:* In breast cancer, Pax-5 promotes pro-epithelial features and suppresses malignant cancer processes. However, the molecular mechanism of this antitumor activity remains largely unknown. This study aimed to identify the cellular roles of Pax-5-regulated miRNAs in breast cancer progression. *Materials and Methods:* After transient transfection of Pax-5 in MDA-MB-231 breast cancer cells, Pax-5-regulated miRNA expression was examined by next-generation sequencing. The identified Pax-5-regulated miRNAs were then validated by qRT-PCR and examined for the roles they play in breast cancer cells. *Results:* Pax-5 was shown to be an effective modulator of miR-215-5p and its target genes. MiR-215 inhibited cell proliferation and migration of breast cancer cells, but not cell invasion. More importantly, Pax-5-induced suppression of cancer cell proliferation and migration was found to be miR-215-dependent. Interestingly, miR-215 profiling in clinical tumor samples showed that miR-215 expression was lower in cancer tissues in comparison to healthy controls. *Conclusion:* Pax-5 reduces breast cancer proliferation and migration through up-regulation of the tumor suppressor miR-215. This result supports the use of miR-215 as a prognostic marker for breast cancer.

Breast cancer is the most common malignancy among women and the leading cause of cancer-related deaths worldwide (1). A better understanding of the molecular mechanisms supporting breast cancer development and progression are needed to identify novel therapeutic targets and to develop targeted treatments for breast cancer patients. Emerging evidence has recently revealed that Pax-5 is an

imperative molecular regulator of several cancers, including B cell, lung and breast cancer (2-4). Pax-5 is a member of the Paired Box (Pax) transcription factor family which plays a crucial and indispensable role in various developmental processes (5, 6). Pax-5 is normally expressed in the developing brain and in all stages of B cell development except in terminally differentiated plasma cells (7, 8). Given the requirement of Pax-5 expression to maintain B-cell lineage (9), numerous studies have shown that Pax-5 acts as a potent oncogene in many types of lymphoma and lymphocytic leukemia (10-12). In opposition to its oncogenic effects in B-cell cancers, Pax-5 has been shown to confer tumor suppressive properties in carcinomas (2, 13, 14). Intriguingly, Pax-5 suppresses cell proliferation and can modulate epithelial-mesenchymal/mesenchymal-epithelial (EMT/MET) transitioning in breast cancer cells (15, 16). Furthermore, we and others have shown that Pax-5 expression reduces colony formation and migratory capabilities by inducing epithelial characteristics in invasive breast carcinoma cells (2, 15, 17). These reports illustrate the critical role of Pax-5 in breast cancer. However, there is still a paucity of information on the molecular basis underlying its biology.

Non-coding RNAs such as microRNAs (miRNAs) have emerged as important regulators of gene expression in numerous biological processes and are involved in many diseases including cancer (18, 19). MiRNAs are approximately 22 nucleotides in lengths and regulate gene expression at the post-transcriptional level by binding to a complementary (usually the 3'UTR) region of its target mRNAs, subsequently leading to translational inhibition and/or mRNA degradation (20). MiRNAs can have oncogenic or tumor suppressor properties in cancer and are often found aberrantly expressed in many types of cancer (21, 22). Moreover, miRNAs play important roles in cellular processes closely related to cancer aggressiveness such as cell proliferation, invasiveness and motility (23, 24). Recent studies have demonstrated that miRNAs are regulated by transcription factors. Accordingly, aberrant transcription factor expression can lead to miRNA dysregulation, thus contributing to breast cancer malignancy (25). These include the transcription factors p53, MYC, and ZEB2 (25-27).

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Correspondence to: Gilles A. Robichaud, Université de Moncton, Moncton, NB, E1A 3E9, Canada. Tel: +1 5068584320, Fax: +1 5068584541, e-mail: gilles.robichaud@umoncton.ca

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In the present study, we demonstrate that Pax-5 regulates numerous microRNAs including miR-215 in aggressive breast carcinoma cells. We also show that Pax-5 consequently modulates downstream targets of miR-215. More importantly, we establish that miR-215 is a potent inhibitor of cell proliferation and migration, two processes tightly related to cancer aggressiveness. Together, our findings elucidate a non-coding RNA downstream signaling network of Pax-5. These findings are essential for a comprehensive understanding of breast carcinogenesis.

Materials and Methods

Cell lines and cell culture conditions. The human mammary cell line MDA-MB-231 (MB231) (mammary ductal carcinoma, HTB-26) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MB231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cell culture media and reagents were obtained from Thermo Fisher Scientific (Burlington, ON, Canada) except for the FBS which was provided by PAA Laboratories (Etobicoke, ON, Canada).

Plasmids and transfections. Transient transfections of Pax-5 were conducted using either the empty pcDNA3.1 vector; or, a vector encoding the Pax-5 mRNA as previously described (10). Briefly, MB231 cells were seeded into six-well plates (5×10^5 cells/well) and incubated overnight. Cells were then transfected with 2 µg of plasmid DNA using the X-tremeGENE 9 reagent (Roche, Brandford, CT, USA) according to the manufacturer's protocol. Stable MB231 cell lines expressing recombinant Pax-5 or empty pcDNA3.1 vector were generated by treating cells 24 h post-transfection with 500 µg/ml geneticin (Thermo Fisher). Cells were then selected for antibiotic resistance, expanded and characterized for recombinant Pax-5 expression.

Gene modulation experiments were performed using either a siRNA pool against Pax-5 transcripts (ON-TARGET plus, Dharmacon, Lafayette, CA, USA), a miR-215-5p inhibitor (Anti-miR miRNA inhibitor, Thermo Fisher) or a miR-215-5p mimic (mirVana miRNA mimic, Thermo Fisher). Cells were seeded in six-well plates (5×10^5 cells/well) and transiently transfected with 50 nM of siRNA, 36 nM of miRNA mimic, or 36 nM of miRNA inhibitor by using Lipofectamine 2000 reagent (Thermo Fisher) in Opti-MEM according to the manufacturer's recommended protocol. Control cells were transfected with a non-specific (non-targeting) siRNA (Dharmacon), scrambled non-targeting miRNA mimic (mirVana miRNA Mimic Negative Control, Thermo Fisher) or scrambled non-targeting miRNA inhibitor (Anti-miR miRNA Inhibitor Negative control, Thermo Fisher) respectively.

RNA extraction and real-time PCR. Total RNA including miRNAs was extracted using the mirVana miRNA isolation kit (Thermo Fisher) according to the manufacturer's protocol. Reverse transcription of mRNA from 1 µg of total RNA was performed using oligo-dT and SuperScript III reverse transcriptase (Thermo Fisher). Gene expression was measured with the SYBR Green 2X FastMix for iQ (Quanta, Biosciences Inc, Beverly, MA, USA). For miRNA analysis, 100 ng of RNA was converted to complementary DNA

(cDNA) using the Taqman microRNA Reverse Transcription Kit (Thermo Fisher). MiR-215-5p, miRNA-532-5p, miRNA-3619-5p, miRNA-489-3p, and miRNA-130a-3p expressions were measured with the miRNA-specific TaqMan assay (Thermo Fisher) on a CFX Connect Real-Time PCR Detection System (BioRad, Mississauga, ON, Canada). Primer sequences are listed in Table I. Relative differences in expression were determined by using the $\Delta\Delta C_t$ of Livak and Schmittgen, 2001 (28) method using HPRT and RNU48 as the endogenous standard for mRNA and miRNA respectively.

Cell viability assays. Cellular viability was monitored using the CellTiter-Blue assay (Promega, Madison, WI, USA) as previously described (29). After transfection, cells were seeded in 96 well plates (3×10^3 cells/well) where 20 µl of CellTiter-Blue substrate was added up to 100 µl/well and incubated for 1 h at 37°C. Afterwards, microplates were subjected to fluorescence analysis on a SpectraMax I3 fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) at 560_{Ex}/590_{Em}. Cell viability was measured daily on a time period of 4 days.

Western blot analysis. Cells were lysed in whole cell lysate buffer (0.125 M Tris pH 6.8, glycerol 0.2 g/ml, 4.0% SDS, 10 mg/ml PMSF, 5 µl protease inhibitor cocktail set III (Calbiochem, Etobicoke, Canada) and 100 mM sodium orthovanadate (Sigma, Oakville, Canada)). The total protein concentration for each sample was determined using the Pierce BCA protein assay kit (Thermo Fisher). Twenty micrograms of total protein from each sample was subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk and incubated overnight at 4°C with anti-Pax-5 (exon 5/6, New England Peptide, Gardner, MA, USA) or anti-G3PDH (#2275-PC-100, Trevigen, Gaithersburg, MD, USA) followed by incubation with the corresponding HRP-conjugated secondary antibodies. Immunoblots were visualized using SuperSignal Chemiluminescent Substrates (Thermo Fisher) and ChemiDoc Imaging system (Bio-Rad).

Cell migration and invasion assays. Migration and invasion assays were performed as previously described (30). Briefly, cells were starved in 200 µl DMEM containing 0.1% FBS for 16 h and then placed in transwell inserts (5×10^4 cells/insert) (Greiner Bio-One, Monroe, NC, USA). Following an incubation at 37°C for 1 h in DMEM 0.1% FBS, the transwell inserts were transferred into wells containing DMEM 20% and incubated for 16 h at 37°C. Inserts containing DMEM 0.1% were used as negative controls. After incubation, migrated cells on the lower surface were harvested with trypsin and submitted to a cell viability assay using CellTiter-Blue as described above. For invasion essays, inserts were pre-coated with 100 µl of Matrigel (BD Biosciences, San Jose, CA, USA) in DMEM 0.1% and incubated overnight.

Next Generation Sequencing (NGS). RNA library construction and sequencing were conducted following Ion Torrent (Thermo Fisher) protocols. Small RNAs were loaded on an Ion PI Chip v2 and sequenced with an Ion Proton Sequencer using the 260 flows parameter. Small RNA libraries were constructed from three biological replicates for each condition. Initial FASTQ files were gathered and adaptor sequences were trimmed. Sequencing data filtered by eliminating reads of <16 and of >60 nucleotides. Reads with bases showing Q scores of <20 were eliminated.

Table I. *Primer sequences.*

	Forward primers (5'→3')	Reverse primers (5'→3')
HPRT	TGA CAC TGG CAA AAC AAT GCA	GGT CCT TTT CAC CAG CAA GCT
Pax-5	GCG CAA GAG AGA CGA AGG T	CTG CTG CTG TGT GAA CAA GTC
ALCAM	AAG TTG GGT GAC TGC ATT TC	ATT ATG ACC ACC GCT CCT TC
CTNNBIP1	CCT ATG CAG GGG TGG TCA AC	CGA CCT GGA AAA CGC CAT CA
DICER1	AGC CCC AGC CCA GCG ATG AA	GTC CAG GAT TGG GGC CAA GAG TCC
DYRK3	CTC AGC ACT TTT TGG ATG GAG G	TTG GGT GCC TTG GAT GAA CTG
EFNB2	ACC AGT CCT TGT CCA GGT AGA A	TGG GGT GTT TTG ATG GTT TT
EREG	GTG ATT CCA TCA TGT ATC CCA GG	GCC ATT CAT GTC AGA GCT ACA CT
FNDC3B	CCC TCC CTA TCT GAC TCA CCA	GGT CCG GTA ACA GGT GGG TA
ZEB2	CCC TTC TGC GAC ATA AAT ACG	TGT GAT TCA TGT GCT GCG AGT

Statistical analyses. Student's *t*-test was used to analyse the significance of the differences. All data are presented as the mean±standard error. A two-sided *p*-value<0.05 was regarded as significant.

Results

Pax-5-regulated miRNA profiling in breast carcinoma. To understand the effects of Pax-5 on miRNA expression profiles in breast carcinoma cells, we sequenced small RNA in MB231 cells overexpressing Pax-5 with an Ion Torrent next-generation sequencer. These Pax-5 overexpressing MB231 cells, when compared to empty vector (pcDNA3.1) transfected control cells, revealed a list of 41 statistically significant deregulated miRNAs following fold change and abundance exclusions (Figure 1A). MiRNAs with ≥ 0.3 Log₂ fold change between samples and an average expression greater than 1.00 were retained. In order to select clinically significant miRNAs, regulated by Pax-5 in breast cancer tissues, we analyzed the expression levels of Pax-5 and the 41 miRNAs of interest using the breast cancer miRNA and mRNA sequencing dataset of The Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov>). A cohort of 113 breast cancer patients was divided into subgroups characterized with either high (≥ 1.5) or low (≤ 0.67) Pax-5 expression levels and evaluated the corresponding average and median miRNA expression (Table II). In accordance with the NGS results, average and median expression of miR-215 and miR-532 were increased; whereas, miR-3619, miR-489, and miR-130a average and median expression levels were decreased in Pax-5-high expression tumors *versus* Pax-5-low expression tumors (Figure 1B and C). These findings are in agreement with our NGS results which suggest a role for the Pax-5 transcription factor as a modulator of miRNA expression profiles in breast cancer cells.

Validation of Pax-5-modulated miRNAs. In order to validate the modulation of the selected five miRNAs by Pax-5, qRT-PCR in MB231 cells transiently transfected with the Pax-5 recombinant gene was performed (Figure 2A). First, we made use of Western blots to confirm recombinant expression of

Pax-5 in the MB231 cells. miR-215, miR-532, and miR-489 relative miRNA expression was 3.01, 1.70, and 0.31 times higher, respectively, in Pax-5-bearing cells compared to empty vector-transfected control cells, in accordance to NGS and TCGA results. MiR-130a and miR-3619 also showed differential levels although not significant when compared to control cells. To further validate the role of Pax-5 in the modulation of miR-215, -532, and -489, MB231 stably expressing either Pax-5 or pcDNA empty vector were transfected with a silencing siRNA pool targeting Pax-5 to reverse these effects. As expected, siRNA suppression of Pax-5 was minimal in MB231-pcDNA control cells given the low endogenous Pax-5 levels in these cells (15). On the other hand, Pax-5 mRNA levels in Pax-5 transfected cells were reduced by 92% following siPax-5 transfection in comparison to control cells treated with a non-specific siRNA (non-targeting siRNA control) (Figure 2B). Of the three miRNAs validated in Figure 2A (*i.e.* miR-215, -532 and -489), only the expression of miR-215 was rescued following Pax-5 inhibition in the MB231 breast cancer cell line (Figure 2C). Our results indicated that miR-215 is tightly regulated by Pax-5, and for this reason, investigation of the function of miR-215 in breast cancer cells was performed.

Identification of miR-215 targets. Reports have shown that elevated levels of miR-215 are associated with better prognostic and clinical outcomes for breast cancer patients (31, 32). However, the mechanistic role of miR-215 in breast cancer remains to be elucidated. Thus, four different algorithms that predict miRNA targets to uncover the downstream signaling targets of miR-215: DIANNA (<http://diana.imis.athena-innovation.gr/DianaTools/>), TargetScan (http://www.targetscan.org/vert_71/), RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>), and microRNA.org (<http://www.microrna.org/microrna/home.do>) were used. This prediction approach indicated 439 miRNA common targets (Figure 3A). Then the DAVID functional annotation tool was used for a functional analysis of this list

Figure 1. Pax-5 miRNA profiling in breast carcinoma cells and tissues. (A) MB231 cells were transfected with either recombinant Pax-5 or the empty vector (pcDNA3.1) and 48 h later were submitted to Ion Torrent Next-Generation Sequencing. Data are represented as differential miRNA expression between Pax-5 and pcDNA3.1-transfected cells of three biological experiments ($p < 0.05$). Average (B) and median (C) expression of miR-215-5p, miR-532-5p, miR-3619-5p, miR-489-3p, and miR-130a-3p in TCGA patients presenting a 1.5-fold upregulation (Pax-5 high) or downregulation of Pax-5 (Pax-5 low) in tumours are shown as a fold change of the tumor relative to the marginal healthy tissue.

of the 439 predicted miR-215-targeted genes (33). Intriguingly, several of the top categories in the KEGG pathway analysis were found to be associated with known and reported Pax-5 breast cancer processes (Figure 3B and

Table III). Among these pathways in cancer, regulation of actin cytoskeleton and MAPK signaling pathways were the most enriched. Then, genes that were highlighted in this analysis were selected to assess whether miR-215 could

Table II. TCGA data dividing a cohort of 113 breast cancer patients into subgroups characterized with either high (≥ 1.5) or low (≤ 0.67) Pax-5 expression levels and the corresponding average and median miRNA expression profiles.

miRNA	NGS log2 relative miRNA count	AVG Exp. in Pax-5 Up-regulated tumors	AVG Exp. in Pax-5 Down-regulated tumors	MEDIAN Exp. in Pax-5 Up-regulated tumors	MEDIAN Exp. in Pax-5 Down-regulated tumors
hsa-miR-4690-3p	-3.042	N/A	N/A	N/A	N/A
hsa-miR-4667-3p	-2.485	N/A	N/A	N/A	N/A
hsa-miR-3130-3p	-2.093	1.326	1.447	0.946	0.748
hsa-miR-3939	-1.848	N/A	N/A	N/A	N/A
hsa-miR-194-3p	-1.763	1.604	1.231	1.344	1.116
hsa-miR-548a-5p	-1.746	N/A	N/A	N/A	N/A
hsa-miR-197-5p	-1.337	1.223	1.174	0.974	0.809
hsa-miR-522-3p	-1.050	4.633	10.042	1.000	1.000
hsa-miR-4454	-0.822	N/A	N/A	N/A	N/A
hsa-miR-521	-0.773	1.660	2.872	1.000	1.000
hsa-miR-3619-5p	-0.760	6.112	9.478	1.000	4.793
hsa-miR-450b-5p	-0.711	2.603	2.443	2.063	1.484
hsa-miR-4448	-0.690	N/A	N/A	N/A	N/A
hsa-miR-7111-5p	-0.673	N/A	N/A	N/A	N/A
hsa-miR-628-5p	-0.630	2.700	2.658	2.326	1.870
hsa-miR-489-3p	-0.585	2.241	2.832	0.588	1.000
hsa-miR-20b-5p	-0.522	4.797	3.815	2.788	1.450
hsa-miR-744-3p	-0.508	1.422	1.956	1.323	1.194
hsa-miR-589-3p	-0.502	1.814	1.327	1.515	1.114
hsa-miR-92b-5p	-0.500	3.513	3.495	2.782	2.192
hsa-miR-130a-3p	-0.458	0.728	0.767	0.488	0.544
hsa-miR-424-3p	0.332	2.716	2.868	1.733	1.612
hsa-miR-30d-5p	0.356	0.840	1.214	0.715	0.769
hsa-miR-27a-5p	0.359	1.312	1.535	1.154	1.210
hsa-miR-532-5p	0.367	1.487	0.997	1.316	0.848
hsa-miR-24-2-5p	0.628	1.192	1.251	0.963	0.947
hsa-miR-642a-3p	0.745	4.171	9.055	1.926	1.630
hsa-miR-642b-5p	0.745	1.266	1.537	1.000	1.000
hsa-miR-122-5p	0.773	1.215	1.491	1.000	1.000
hsa-miR-215-5p	0.776	1.389	0.910	0.461	0.335
hsa-miR-148a-5p	0.781	2.058	2.947	1.761	1.955
hsa-miR-642b-3p	0.788	1.266	1.537	1.000	1.000
hsa-miR-576-5p	0.925	1.828	2.062	1.416	1.212
hsa-miR-146a-3p	0.997	2.146	1.125	1.517	0.791
hsa-miR-3591-3p	1.145	N/A	N/A	N/A	N/A
hsa-miR-4284	1.165	1.000	1.107	1.000	1.000
hsa-miR-186-3p	1.168	1.053	1.242	0.927	0.998
hsa-miR-125b-2-3p	1.446	0.351	0.318	0.303	0.206
hsa-miR-548a-5p	1.727	N/A	N/A	N/A	N/A
hsa-miR-556-5p	2.557	4.474	16.809	1.727	3.118
hsa-miR-4483	2.725	N/A	N/A	N/A	N/A

modulate their expression. Thus, a breast cancer model of miR-215 overexpression through the transfection of miRNA-215 mimics was developed that showed elevated miR-215 levels in a MB231 cells 24 h and 48 h post transfection (Figure 3C). Next, the mRNA levels of predicted miR-215 target genes was evaluated (*i.e.* ALCAM, CTNNBIP1, DICER1, DYRK3, EFNB2, EREG, FNDC3B, and ZEB2) using TaqMan PCR. As expected, all mRNA targets of miR-215 were found to be decreased in miR-215 transfected cells

when compared to transfected controls (Figure 3D). Given that Pax-5 is a positive regulator of miR-215, modulation of these miR-215 target genes by Pax-5 in MB231 cells was examined. Interestingly, all miR-215 targets (with the exception of EREG) were found to be downregulated in Pax-5-transfected cells (Figure 3E), suggesting that these latter genes are modulated by Pax-5 *via* miR-215. Altogether, these results demonstrate that the Pax-5-miR-215 axis can modulate key molecular mediators of cancer biological processes.

Figure 2. Validation of Pax-5-modulated miRNAs. (A) MB231 cells were transfected with either Pax-5 or pcDNA3.1 and evaluated for miR-215-5p, miR-532-5p, miR-3619-5p, miR-489-3p, and miR-130a-3p expression using RT-qPCR (lower panel). Western blots were also performed on Pax-5 and GAPDH on these treated cells (upper panel). (B) Pax-5 expression and (C) miR-215-5p, miR-532-5p, and miR-489-3p expression levels were also determined by RT-qPCR in MB231 cells transfected with a Pax-5 silencing siRNA (siPax-5) or a control scrambled siRNA (siCtrl) for 72 h. MiRNA and mRNA levels were normalized against the HPRT and the small-nucleolar RNA 48 (RNU48), respectively. Data are presented as means±SEM of independent experimental triplicates (* p <0.05, ** p <0.01, ns: No significance).

Figure 3. Identification of miR-215 targets. (A) The number and identification of predicted mRNA targets of miR-215 were determined by software tools (i.e. DIANNA, TargetScan, RNAhybrid, and miRNA.org). (B) Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of miR-215 target genes was performed with DAVID bioinformatics. (C) MiR-215 and (D) miR-215 target genes expression was evaluated by qRT-PCR in MB231 cells transiently transfected with miR-215 mimic (miR-215) or scrambled non-targeting mimic (miR-control). (E) Transcriptional expression of miR-215 target genes was also monitored in MB231-Pax5 and MB231-pcDNA stable cell lines. MiRNA and mRNA expression levels were normalized against HPRT and RNU48, respectively. Data is presented as means $\pm$ SEM of independent experimental triplicates (* p <0.05, ** p <0.01).

miR-215 inhibits breast cancer cell proliferation. We have previously reported that Pax-5 inhibits breast cancer cell growth (15). Our findings herein also reveal that miR-215 is a modulator of genes implicated in cellular growth and proliferation (*i.e.* EFNB2, EREG, and FNDC3B). We thus investigated whether the modulation of miR-215 levels could result in changes in breast cancer cell proliferation. To study this, MB231 cells were transfected with miR-215 mimics and subsequently cell viability was measured over time using a colorimetric microplate assay. An inhibition of cell growth by 44% in miR-215-transfected cells at day 4 post-transfection in comparison to scramble transfected controls was observed (Figure 4A). To further determine the involvement of miR-215 on Pax-5-mediated suppression of cell growth rescue assays on MB231-Pax-5 cells were performed by transfecting anti-miR-215. First, we confirmed that the transfection of anti-miR-215 resulted in the inhibition of miR-215 levels by at least 50% in MB231 cells (Figure 4B). Next, as revealed in Figure 4C, we show that transient inhibition of miR-215 in MB231-Pax-5 cells rescued cell growth to the level of control cells. Together, these results indicate that the attenuation of breast cancer growth by Pax-5 is in partially mediated by the downstream effects of miR-215.

miR-215 suppresses cell migration. Given that Pax-5 has been shown to suppress breast cancer aggressive features (2, 15) the ability of miR-215 to modulate breast cancer malignant processes was explored (*i.e.* cell migration and invasion). To this direction, MB231 cells were transfected with miR-215 mimics and compared to cells treated with non-specific microRNA (scramble RNA). Elevated expression of miR-215 significantly decreased the migration capacity of MB231 cells by 69% when compared to miR-control cells (Figure 5A). To assess whether Pax-5 mediated suppression of breast cancer migration (2, 15) was attributable to miR-215, migration experiments on MB231 cells stably expressing Pax-5 and transfected anti-miR-215 were performed. As expected, MB231-Pax-5 cells migrated less than the control MB231-pcDNA cells by 46% (Figure 5B). More importantly, the reduction in migration capacity of MB231-Pax-5 cells was completely abolished by miR-215 silencing using anti-miR-215 transfection. Next, the role of miR-215 in regulating cell invasion was examined using matrigel-filled transwell inserts. MiR-215 did not influence invasive capacity of MB231 cells (Figure 5C). Collectively, these results demonstrate that miR-215 is a modulator of cell migration, an essential process in breast cancer progression. More importantly, miR-215 is a pivotal mediator of Pax-5-induced suppression of breast cancer migration.

Clinical relevance of miR-215 in breast cancer. To evaluate the clinical relevance of miR-215, the expression levels of miRNA in breast cancer tissues and non-tumorous breast

Table III. KEGG pathway analysis of the list of 439 predicted miR-215-targeted genes.

Term	Count	%
Pathways in cancer	16	3.7
Regulation of actin cytoskeleton	11	2.5
MAPK signaling pathway	11	2.5
Insulin signaling pathway	10	2.3
Focal adhesion	10	2.3
Insulin resistance	9	2.1
Thyroid hormone signaling pathway	8	1.8
Vascular smooth muscle contraction	8	1.8
Neurotrophin signaling pathway	8	1.8
cGMP-PKG signaling pathway	8	1.8
Prostate cancer	7	1.6
Hepatitis B	7	1.6
Gap junction	6	1.4
Estrogen signaling pathway	6	1.4
Glucagon signaling pathway	6	1.4
Amphetamine addiction	5	1.1
Chronic myeloid leukemia	5	1.1
Aldosterone synthesis and secretion	5	1.1
Prion diseases	4	0.9
Endocrine and other factor-regulated calcium reabsorption	4	0.9
Cocaine addiction	4	0.9

All *p*-Values<0.1.

tissues of 100 breast cancer patients was analyzed using the available breast cancer dataset of The Cancer Genome Atlas (TCGA). When comparing miR-215 profiles in cancer tissues to healthy marginal samples, miR-215 levels were found to be deregulated in a total of 91% of all tumor samples. Specifically, miR-215 was downregulated in 70% of cases while was over-expressed in 21% of cases (± 1.25 -fold difference between breast cancer tissue and healthy marginal tissue) (Figure 6A). More precisely, the expression of miR-215 is significantly lower in tumor samples, as compared with non-tumour tissue (Figure 6B). Our results suggest that miR-215 is clinically relevant in breast cancer tissues where its expression levels are apparently lower.

Discussion

Emerging evidence indicates that Pax-5 is a critical regulator of the multistep processes involved in breast cancer malignancy. Despite the importance of Pax-5 in breast carcinogenesis, the association between this transcription factor and downstream miRNA regulation remains largely unknown. In this study, miR-215-5p is identified as a tumor suppressive miRNA that is up-regulated by Pax-5 in breast cancer cells. Accordingly, it is demonstrated that miR-215 is under-expressed in clinical breast cancer tumor tissues.

In an attempt to identify the downstream miRNA expression profile of the Pax-5 transcription factor in malignant breast

Figure 4. *miR-215 inhibits cell proliferation.* (A) MB231 cells transfected either with miR-215 mimics (miR-215), scrambled non-targeting mimic (miR-control) or non-transfected (NT) were monitored for cell viability over time (1 to 4 days) using the CellTiter-Blue viability assay. (B) MiR-215 expression was evaluated by qRT-PCR in MB231 cells transfected with miR-215 inhibitors (anti-215) or scramble miRNA controls (anti-control) at 24h and normalized against RNU48. (C) Cell viability assays were then performed in MB231-Pax-5 and MB231-pcDNA cells transfected with anti-miR-215 (anti-215) or scramble non-targeting inhibitor (anti-control). Data is presented as means $\pm$ SEM of independent experimental triplicates (* p <0.05, ** p <0.01).

cancer cells, microRNA sequencing on Pax-5-transfected MB231 breast cancer cells was performed. Our experiments identified 41 miRNAs significantly deregulated by Pax-5 expression when compared to vector-transfected controls. This study is the largest compilation of Pax-5-regulated miRNA expression profile in a breast cancer cell line. Of the 41 Pax-5-regulated miRNAs, at least 5 were found to be correlated

with Pax-5 expression levels in breast cancer clinical tissues. In validation, the use of a TCGA analysis and approach inherently assures a clinical relevance for the latter miRNAs through their respective aberrant expression profiles in clinical samples. Interestingly, the selected five miRNAs (*i.e.* miR-215; -532; -3619; -489 and -130a) found to influence breast cancer cell processes in the same fashion as does Pax-5. For example,

Figure 5. *miR-215 inhibits cell migration.* (A) Cell migration was monitored in MB231 cells transfected either with miR-215 mimics (miR-215), transfected with scrambled non-targeting mimic (miR-control) or non-transfected (NT). (B) Cell migration was then performed on MB231-Pax-5 or MB231-pcDNA cells transfected with either an anti-miR-215 (anti-215) or a scramble miRNA control (anti-control). Data are presented as means $\pm$ SEM of independent experimental triplicates (* $p < 0.05$).

miR-130a, miR-489 and miR-215 have been found to be implicated in cell proliferation and invasion of breast cancer cells (34, 35) whereas miR-3619 and miR-532 have been shown to modulate these cancer processes in other types of solid tumors (36-39). Unfortunately, only miR-215 was validated in our breast cancer cell models conditionally overexpressing Pax-5.

Our analysis of TCGA database revealed that miR-215 expression is frequently down-regulated in tumor samples from breast cancer patients when compared to their healthy counterpart tissue. More specifically, in a cohort of 100 patients, 70 patients were found with attenuated levels of miR-215 in comparison to control tissues. These results are in agreement with previously published data indicating that low miR-215 expression in breast cancer tissue is associated with higher tumor grade and lymph node metastasis (32). Furthermore, a study from Madhavan *et al.*, 2016 has reported that the levels of miR-215 in blood plasma from

breast cancer patients is a significant predictor of overall and progression-free survival (31). Altogether, these findings strongly suggest that miR-215 may act as a tumor suppressor and inhibitor of malignant breast cancer features. Furthermore, while we and others have characterized aberrant miR-215 expression in human breast tumors (40), Pax-5 transcription factor is identified as a potent regulator of miR-215 in breast cancer cells.

In our evaluation of miR-215 downstream targets, four mRNA-miRNA prediction tools were used and functional analysis was performed using the Kyoto Encyclopedia of genes and Genomes (KEGG) on 439 genes which were common and predicted by all four algorithms. Interestingly, amongst the top cellular signaling themes highlighted, "Pathways in cancer" and "MAPK signaling pathway" revealed a list of genes that have previously been associated to Pax-5 signaling in cancer cells (2, 13, 15). Other functional terms such as "Neurotrophin signaling pathway",

Figure 6. Clinical relevance of miR-215 in breast cancer patients. (A) MiRNA expression profiles from cancer tissues and marginal non-cancerous tissues were obtained from the breast cancer cohort through The Cancer Genome Atlas (TCGA) and plotted to display the percentage of patients presenting a 1.25-fold upregulation or downregulation of miR-215 in tumor tissue compared to healthy marginal tissue (left panel). The relative expression of miR-215 (tumor/healthy tissue) is plotted in all of 100 breast cancer patients (right). (B) Expression levels of miR-215 in tumour and non-tumour tissue (Normal) are shown by box blot. (**** $p < 0.0001$, Wilcoxon test).

"Focal adhesion" and "Regulation of actin cytoskeleton" suggest the implication of miR-215 in morphological plasticity and cellular motility. Based on these analyses, several putative downstream transcriptional targets known for their involvement in cancer processes were selected and it was demonstrated that miR-215 negatively regulates the expression of ALCAM, CTNNBIP1, DICER1, DYRK3,

EREG, FNDC3B, and ZEB2 in breast cancer cells. Furthermore, given the capacity of Pax-5 to regulate miR-215, Pax-5 was shown to consequently inhibit transcription of the majority of these genes. These findings are consistent with the ability of Pax-5 to positively regulate miR-215.

It is important to note that most of the miR-215-targeted genes have oncogenic properties which corresponds to the

tumor suppressive role of Pax-5 in breast cancer cells (41-46). For example, Fibronectin type III domain containing 3B gene (*FNDC3B*) located at the 3q26 locus is amplified in 20% of tumors and plays an oncogenic role in many cancers (41, 47). Additionally, activated leukocyte cell adhesion molecule (ALCAM) is implicated in cell to cell interactions and inhibits breast cancer apoptosis (43). ZEB2, a repressor of E-cadherin, has largely been characterized for its implication in EMT transitions and aggressivity in cancer (42, 48). Accordingly, we have also recently reported that Pax-5 is a potent inducer of E-cadherin and a concomitant inhibitor of ZEB expression in breast cancer cells leading to epithelial-dominant features (15). Indirectly, miR-215 may have a pivotal role in the pro-epithelialization processes mediated by Pax-5. Altogether, these findings align with the observations that miR-215 suppresses gene expression profiles governing pro-aggressive features in breast cancer. Consequently, miR-215 is also a potent downstream effector of the tumor suppressor activities of Pax-5 in breast cancer carcinogenesis.

Our study on the impact of miR-215 in malignant processes has shown that miR-215 is a strong regulator of breast cancer cell proliferation and migration activities. Accordingly, we demonstrate that miR-215 is critical effector for Pax-5-mediated suppression of these processes. These findings also corroborate with the genetic signatures and phenotypes highlighted in the KEGG analysis. Based on the literature, the ability of miR-215 to modulate these processes seems strongly dependant on cancer type. For example, miR-215 has been identified to enhance migration and invasion in high-grade glioma by targeting retinoblastoma tumor suppressor gene 1 (49). On the other hand, miR-215 has been found to inhibit growth and cell invasion in epithelial ovarian cancer cells by targeting NOB1 (35). Since miRNAs target numerous genes, the cumulative effect of a miRNA will dictate its tumor suppressive or oncogenic role in that particular context. In our study, miR-215 was found to reduce proliferative and migratory capacities of breast cancer cells. More importantly, rescue experiments demonstrated that the reduction of cell migration and proliferation by Pax-5 is dependant of miR-215 activity. Given that Pax-5 restoration in breast cancer cells is associated with pro-epithelialisation and up-regulation of the tumor-suppressive features of miR-215, strategies targeting the Pax-5/miR-215 axis may be used as potential therapeutic or diagnostic approaches in breast cancer patients. Surprisingly, although miR-215 inhibited MB231 cell migration, it did not alter the cells' invasive capacity. Given that cell migration and invasion processes are closely related by distinct activities, it may be possible that miR-215 targets a pivotal factor necessary for migration and is not related to cell invasion processes (ex: metalloproteinases). Further studies are warranted to elucidate these observations.

In summary, this study demonstrated that Pax-5 regulates numerous miRNAs including miR-215 which is aberrantly under-expressed in breast cancer tumors. Pax-5 found to inhibit aggressive features of breast cancer cells in a miR-215-dependant manner. Restoration of this tumor-suppressive miRNA may represent a potential therapeutic approach against breast cancer malignancy and disease progression. Further studies are, thus, warranted to evaluate the therapeutic potential of miR-215 restoration in breast cancer.

Conflicts of Interest

None of the Authors have any conflicts of interest to disclose regarding this study.

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