

## PAX6 Promoter Methylation Correlates with MDA-MB-231 Cell Migration, and Expression of MMP2 and MMP9

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### Abstract

**Objective:** Breast cancer is a heterogeneous disease characterized by an accumulation of genetic and epigenetic alterations that lead tumor cells to acquire characteristics like the capacity for invasion and metastasis. Metastasis remains a major challenge in cancer management and understanding of its molecular basis should result in improved prevention, diagnosis, and treatment of breast cancer patients. The aim of this study was to investigate how promoter DNA methylation regulates PAX6 gene expression and influences breast carcinoma cell migration. Methods: PAX6 promoter methylation was detected by Methyl Specific-Multiplex Ligation Probe Amplification (MS-MLPA). Gene expression was evaluated using qRT-PCR, while the effect of PAX6 on migration was assessed by wound healing assay. In addition, MMP2 and MMP9 genes were studied using different bioinformatic tools. Results: The PAX6 promoter is methylated in breast cancer cell lines and methylation in this region impacts on its expression. Migration assays revealed that PAX6 overexpression promotes cell migration, while PAX6 inhibition decreases it. More importantly, we found that migration is affected by PAX6 methylation status. Employing bioinformatic analysis, binding sites for PAX6 on the regulatory regions of the MMP2 and MMP9 genes were established, PAX6 overexpression increasing MMP2 and MMP9 expression at the mRNA level. Conclusion: Our study provides novel insights into epigenetic events that regulate PAX6 expression and molecular mechanisms by which PAX6 modifies the migration capacity of breast cancer cells.

**Keywords:** Breast cancer- metastasis- DNA methylation- PAX6- matrix metalloproteinases

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### Introduction

Breast cancer is the second most frequent cancer in the world and by far, the most frequent cancer among women, with an estimation of 1.67 million new cases occurring in 2012, according to the IARC (Ferlay et al., 2015). It is well known that breast cancer is a heterogeneous disease, that is classified in different molecular subtypes which show different features, response to treatment and outcome (Senkus et al., 2015). In the last two decades, enormous efforts have been made to characterize these subclasses at proteomic (Perou et al., 2000), genomic (Vogelstein and Kinzler, 2004) and epigenomic levels (Baylin and Ohm, 2006).

Nowadays, it is accepted that cancer harbors a genetic origin (Visvader, 2011). During breast tumorigenic process, genetic alterations known as driver mutations arise and progressively accumulate in tumoral cells deregulating multiple cell functions (Vogelstein et al., 2013). Despite the multiple existent cancer types, it has been proposed that cancer cells share similar characteristics. According to Hanahan and Weinberg (2011), these can be summarized in eight hallmarks,

among which invasion and metastasis is one of them. Cell migration and invasion represents the initial step of metastatic cascade: to disseminate and colonize distant organs, tumor cells need to degrade extracellular matrix (ECM) components and physically move to reach lymph and blood vessels. This is accomplished through the release of proteolytic enzymes known as Matrix Metalloproteinases (MMPs) (Sullu et al., 2011).

It is important to remark that even though migration and invasion are necessary to the metastatic process, released tumor cells need to acquire modifications that allow them to adapt and survive. To adapt to their new environment, cancer cells can rely on epigenetic modifications, which are more flexible and highly dynamic in comparison to genetic modifications (Jones and Baylin, 2007). It has been shown that aberrant DNA methylation affects the function of genes involved in multiple processes including DNA repair and cell cycle regulation, angiogenesis and apoptosis (Moelans et al., 2011; Lindner et al., 2013).

Our previous studies have focused on the role that aberrant DNA methylation plays in breast carcinogenesis (Branham et al., 2016), and more specifically in metastasis development. For example, we previously identified a

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CpG site in PAX6 gene that presented significant changes in the methylation status during the progression from primary breast tumor (PBT) to lymph node metastasis (LNM) (Urrutia et al., 2015). This change consisted of a methylation-to-unmethylation shift from PBT to LNM. We also established that this methylation had an impact on the expression of the PAX6 gene, which suggested that this epigenetic change induces a functional modification in metastatic lesions.

PAX6 is a transcription factor, which has been highly-conserved across evolution. Its protein structure is characterized by the presence of two DNA binding domains, and it has been described as fundamental during early development of multiple organs, including eyes, central nervous system and pancreas (Shaham et al., 2012; Engelkamp et al., 1999). Thus, given its DNA binding properties, PAX6 can regulate the expression of other genes participating in multiple processes such as cell proliferation, differentiation and migration. Recent studies have inquired about its role in malignant tumors, and evidence suggest that PAX6 could function as an oncogene during breast tumorigenesis (Zong et al., 2011).

The present study aimed to investigate the impact of PAX6 promoter methylation over gene expression and how these changes impact breast carcinoma cells migration capacity. Our findings suggest that PAX6 is epigenetically regulated by promoter methylation and its expression contributes to cell migration of breast cancer cells by targeting Matrix Metalloproteinases 2 and 9.

## Materials and Methods

### Cell lines and cell culture

Breast cancer MCF-7 and MDA-MB-231 cells were kindly provided by Dr. Lanari from the IBYME institute, Buenos Aires, Argentina. MCF-7, MDA-MB-231 and HeLa cell lines were cultured in DMEM growth medium (Gibco by Life technologies, Grand Island, NY, USA) supplemented with 10% FBS (Internegocios, BA, Argentina) 100U/ml penicillin and 100 µg/ml of streptomycin (Gibco by Life technologies, Grand Island, NY, USA). Cell lines were maintained at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. For demethylation assays, MDA-MB-231 cells were cultured in media containing 5 or 10µM of the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza) (Merck-Millipore, Billerica, MA, USA). The medium was changed once a day for 6 days with media containing fresh 5-Aza.

### Methylation analysis by Methyl Specific- Multiplex Ligation Probe Amplification (MS-MLPA)

To assess the methylation status of 27 CpG sites located on 25 cancer related genes, including PAX6, DNA of MCF-7, MDA-MB-231 and HeLa cells was analyzed by MS-MLPA methodology using the kit ME002 (MRC Holland, Amsterdam, The Netherlands). Reactions were carried out following manufacturer's instructions, including subtle modifications to avoid background signals. Fluorescent PCR products were separated by capillary electrophoresis in a Beckman CEQ8000 sequencer (Beckman Coulter Inc. Fullerton,

CA, USA) and analyzed by the GeneMarker v1.75 software (Softgenetics LLC, PA, USA). A CpG site was considered methylated when its value was higher than a pre-established cut-off of 15%, percentage which reflects the amount of methylated allele copies.

### Quantitative Real Time PCR (qRT-PCR)

Total RNA of cell lines was extracted using Trizol reagent (Ambion/Thermo Scientific, USA) following manufacturer's recommendations. cDNA synthesis was carried out by reverse transcription of 500ng of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and Random Primers (Biodynamics, BA, Argentina) during 60 minutes at 37° C. Primers used included: PAX6 (forward) 5'-CTTGGGAAATCCGAGACAGATT-3'; (reverse) 5'-GCTAGCCAGGTTGCGAAGAAC-3'; MMP2: (forward) 5'-TCTCCGACATTGACCTTGGC-3'; (reverse) 5'-CAGGGTGCTGGCTGAGTAGATC-3'; MMP9: (forward) 5'-TTGACAGCGACAAGAAGTGG-3'; (reverse) 5'-GCCATTCACGTTTCGTCTTAT-3'; GAPDH: (forward) 5'-TGGACCTGACCTGCCGTCTA-3'; (reverse) 5'-CCCTGTTGCTGTAGCCAAATT-3'. PCR reactions were performed on a Rotor Gene 6,000 thermocycler (Corbett Research, USA) using the following conditions: 40 cycles of 30s at 95° C, 30s at 56° C and 30s at 72° C. The expression of genes of interest was relativized considering GAPDH as reference gene, using the  $\Delta\Delta C_T$  method.

### Plasmids and transfections

For functional analysis, cells were transfected with a pcDNA-GFP plasmid alone or co-transfected with GFP and pMxs-PAX6 plasmid. pMxs-PAX6 plasmid was a gift from Kevin Eggen (Addgene plasmid #32932). For interference of PAX6, cells were co-transfected with GFP plasmid and scramble siRNA A or siRNA PAX6 (#sc-37007 and sc-36195, Santa Cruz Biotechnology, Dallas, TX, USA). Transfections were carried out using Lipofectamine 2000 (Invitrogen, Van Allen Way Carlsbad, CA, USA) according to manufacturer's instructions.

### Migration assays

MDA-MB-231 and HeLa cell lines were cultured and transfected as previously described. Twenty-four hours after transfection or 5-Aza treatment, cells were incubated overnight in serum-reduced medium containing 0.5% FBS. Cell surface was then scratched using a 200 µL pipet tip and washed with PBS to remove detached cells and debris. Cells were photographed every 24 hours using a TE300 Eclipse microscope equipment (Nikon, Tokyo, Japan). Images obtained were then processed using Image J software. Reduction of the scratched area was measured and expressed as migration percentage according the formula:  $((A_{\text{reat0}} - A_{\text{reat1}}) / A_{\text{reat0}}) \times 100$ , where T0 represents area at initial time point and T1 represents area at time point of interest.

### Bioinformatic assays

MMP2 and MMP9 sequences were analyzed by means of bioinformatic tools to detect promoters, evolutionary

conserved regions and potential binding sites for PAX6 protein. A two species non-coding alignment was performed using mVISTA (<http://genome.lbl.gov/vista/>) with a window of 50 base pairs and 70% identity. The alignment was performed among human MMP2 (Accession #NG\_008989.1) and MMP9 (Accession #NG\_011468.1), and their respective mouse orthologues sequences: Mmp2 (Accession #NM\_0088610) and Mmp9 (Accession #NM\_013599). Detection of MMP2 and MMP9 promoter regions and first exon was carried out with First EF prediction software (<http://rulai.cshl.org/tools/FirstEF/>) using default parameters. Potential PAX6 paired domain binding sites were identified using the Transfac based rVISTA program (<http://genome.lbl.gov/vista/>) and the JASPAR CORE database with matrices M00979 and MA0069. Nucleotides ranging from -3,000 to +500 to the TSS of MMP2 and MMP9 sequences were tested, using an 80% matrix similarity cutoff.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.03 Software. Results are expressed as the mean  $\pm$  SD of three independent experiments. Statistical analysis of mean differences between two groups was performed by Student's t-test. P values  $<0.05$  were considered as

statistically significant.

## Results

### *PAX6 is methylated in MCF-7 and MDA-MB-231 breast cancer cell lines*

To determine the impact of PAX6 promoter methylation over its expression, we analyzed by MS-MLPA the methylation status of a CpG site located in PAX6 gene promoter, in breast cancer cell lines MCF-7 and MDA-MB-231. Previous data from our group has shown that the cervix cell line HeLa does not present methylation at the studied CpG site, therefore we included them in our analysis to further evaluate the relation of lack of methylation at promoter and PAX6 gene expression. MS-MLPA assays showed that MCF-7 and MDA-MB-231 cell lines present high levels of methylation in the PAX6 CpG site assessed (90% and 100% of the allele copies, respectively), while HeLa cells did not show methylation at this site (Figure 1A). Since promoter methylation is an important mechanism implicated in gene expression regulation during tumorigenesis, we assessed the expression of PAX6 mRNA by qRT-PCR on MCF-7 and MDA-MB-231 breast cancer cell lines and HeLa cell line. We found that PAX6 expression on

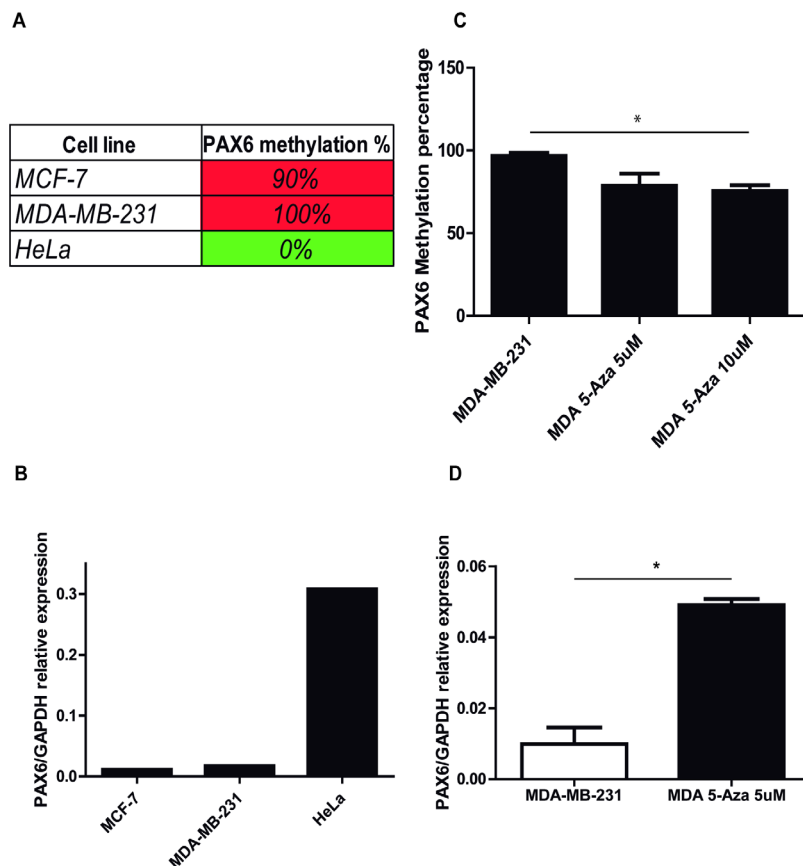


Figure 1. PAX6 Methylation Status and Expression in Breast Cancer Cell Lines. A, The PAX6 gene methylation status on cancer cell lines is shown in the table. Red boxes represent methylated status; green boxes represent unmethylated status. Methylation at the assessed CpG site is expressed as a percentage, representing the amount of methylated allele copies in the sample; B, PAX6 mRNA expression relative to GAPDH gene for MCF-7, MDA-MB-231 and HeLa cell lines is represented in the histogram; C, The effect of 5-Aza treatment on PAX6 methylation was tested at increasing concentrations. \*P  $<0.05$ ; D, The effect of 5-Aza treatment on PAX6 mRNA expression was assayed by qRT-PCR. Histogram shows PAX6 expression before and after (left and right respectively) treatment at 5uM. \*P  $<0.02$



MCF-7 and MDA-MB-231 cells do not differ significantly between both breast cancer cell lines. This observation suggested that high methylation on PAX6 promoter have similar effect over PAX6 mRNA expression level (Figure 1B), while lack of methylation correlates with higher expression of PAX6, as observed in HeLa cell line.

*5-Aza-2'-deoxycytidine treatment reverses methylation and increases PAX6 expression*

Since PAX6 promoter was methylated on both breast cancer cell lines, and methylation is associated with gene repression, we asked whether demethylation of the PAX6 promoter could restore PAX6 gene expression. To answer this question, we chose to work with the MDA-MB-231 cell line, which presented the highest methylation level of PAX6. MDA-MB-231 cells were treated with the demethylating agent 5-Aza at concentrations of 5 and 10  $\mu$ M and subjected to methylation assays by MS-MLPA. We observed that 5-Aza treatment reduced PAX6 methylation significantly, from 100% to 78% ( $\pm$  7,3%) and 75,5% ( $\pm$  3,5%) in the 5 and 10  $\mu$ M groups respectively (P= 0.01) (Figure. 1C). After confirming that treatment with 5-Aza reduces

PAX6 methylation, we evaluated whether treatment with 5  $\mu$ M of 5-Aza induced changes in PAX6 expression in MDA-MB-231 cells. We observed by qRT-PCR, that PAX6 expression in the 5-Aza treated cells was increased when compared with control MDA-MB-231 cell line (P< 0.02) (Figure 1D).

Our results suggest that methylation at this specific CpG site located on PAX6 promoter downregulates its expression, and treatment with the drug 5-Aza-2'-deoxycytidine increases PAX6 expression through promoter demethylation.

*PAX6 expression regulates MDA-MB-231 cell migration ability*

Since cell migration is one of the initial steps of the metastatic cascade and we previously reported that PAX6 is unmethylated and expressed in cells from metastatic breast cancer lesions, we asked if PAX6 expression could be contributing to the migration potential of breast cancer cells. To answer this, we transfected MDA-MB-231 cells with a GFP encoding plasmid (GFP) or co-transfected with GFP plus PAX6 encoding vector (GFP/PAX6). Next, we performed wound healing assay. As shown in Figure.

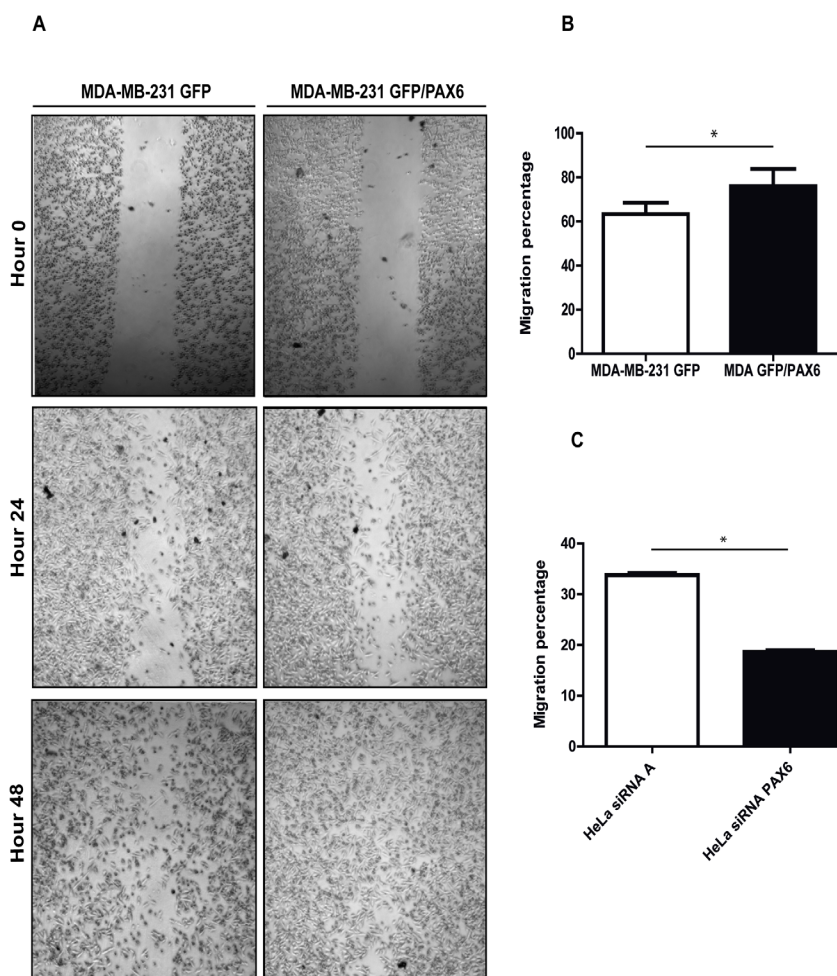


Figure. 2 Effect of Ectopic PAX6 Overexpression and PAX6 Inhibition by siRNA Transfection on Cancer Cell Migration. A, Representative photographs of migration assays performed on MDA-MB-231 cells transfected either with GFP alone or GFP/PAX6 (left and right respectively); B, Bar graph representation of wound healing assays. Cell migration is expressed as percentage of wound closure. Columns represent the mean  $\pm$  SD of at least 3 independent experiments. \*P <0.05; C, Histogram representation of wound healing assays performed on HeLa cell line. Cell migration is expressed as percentage of wound closure. Columns represent the mean  $\pm$  SD of at least 2 independent experiments. \*P <0.05

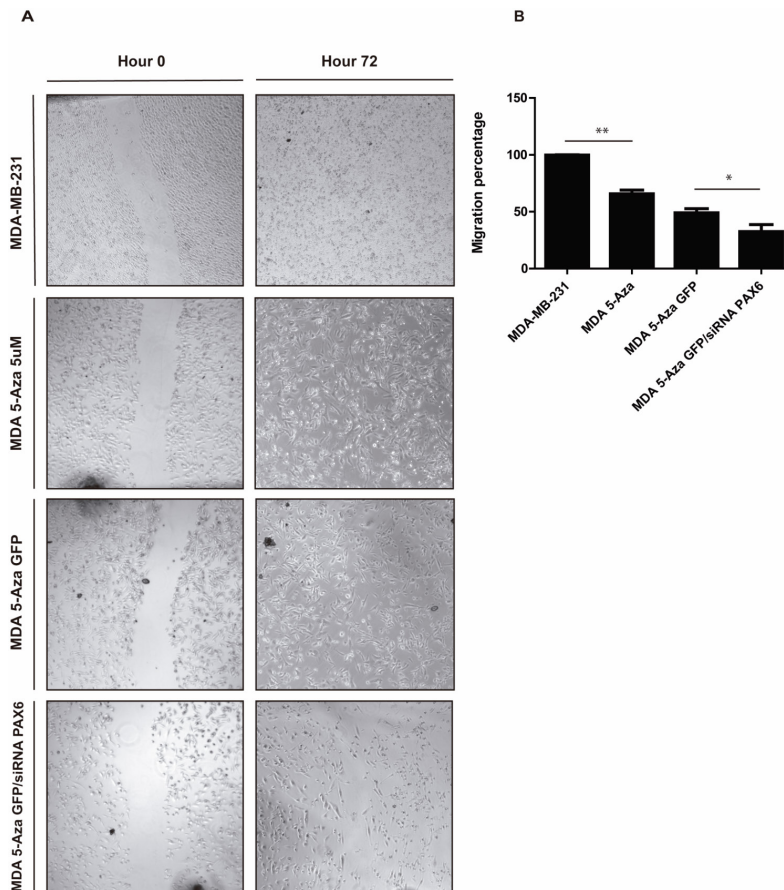


Figure 3. Effect of PAX6 Demethylation and Inhibition by siRNA on Cell Migration Capacity. A, Representative photographs of migration assays performed on MDA-MB-231 cells after treatment with 5-Aza at 5µM and transfection with either GFP or GFP/siRNA PAX6; B, Histogram representation of wound healing assays performed on MDA-MB-231 cell line. Cell migration is expressed as percentage of wound closure. Columns represent the mean  $\pm$  SD of at least 3 independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$ .

2A and B, PAX6 overexpression increased migration in GFP/PAX6 transfected cells as compared with the control GFP cells at 48 hs ( $76\% \pm 7,8\%$  vs.  $63\% \pm 5\%$ ,  $P = 0.04$ ).

From this observation, we hypothesized that PAX6 expression could be promoting cell migration in these breast carcinoma cells. To test our hypothesis, we decided to evaluate migration on HeLa cells, on which we showed that do not present PAX6 promoter methylation and express PAX6 (Figure 1 A, B). We performed co-transfection with a GFP encoding plasmid and scramble siRNA (GFP/siRNA A) or with GFP and siRNA PAX6 (GFP/siRNA PAX6). Cells were then subjected to wound healing migration assay. As shown in Figure 2C, we found that HeLa cells migration ability was significantly reduced in the siRNA PAX6 transfected cells when compared with the siRNA A control cells ( $33,8\%$  vs.  $18,6\%$ ,  $P = 0.03$ ).

Taken together, the results obtained by migration assays allowed us to conclude that PAX6 plays a role in cell motility, promoting cell migration.

#### *PAX6's role in MDA-MB-231 cell migration is epigenetically regulated*

Given that PAX6 expression is regulated by DNA methylation and since ectopic expression and interference assays showed that PAX6 contributes to breast cancer cell migration potential, the role of PAX6 methylation in cell migration was investigated. We treated MDA-MB-231

cells with the demethylating agent 5-Aza and then performed transfection with GFP or GFP/siRNA PAX6 followed by migration assays. As shown in Figure 3B first and second bars, 5-Aza treatment reduced migration when compared to control cells at 72hs ( $65,8\% \pm 3,2\%$  vs.  $100\%$  respectively,  $P < 0.01$ ), as expected from an antineoplastic drug.

Given that 5-Aza has unspecific effect over global DNA methylation, to assess the individual contribution of PAX6 demethylation to migration we compared 5-Aza MDA-MB-231 treated cells after transfection with GFP and GFP/siRNA PAX6. PAX6 siRNA inhibition after 5-Aza treatment significantly reduced cell migration when compared with 5-Aza + GFP alone transfected cells ( $32,5\% \pm 6,2\%$  vs.  $49\% \pm 3,6\%$ ,  $P = 0.034$ ) (Figure 3b, third and fourth bars).

These data indicate that PAX6's contribution to cell migration is epigenetically regulated, since the methylation of its promoter decreases the migration ability of breast cancer cells.

#### *MMP2 and MMP9 genes present PAX6 specific binding sites at promoter regions*

Since cell migration is associated with tissue invasion as the initial steps of the metastatic cascade and given that PAX6 is a transcription factor with well-known DNA binding properties, we hypothesized that PAX6 could be

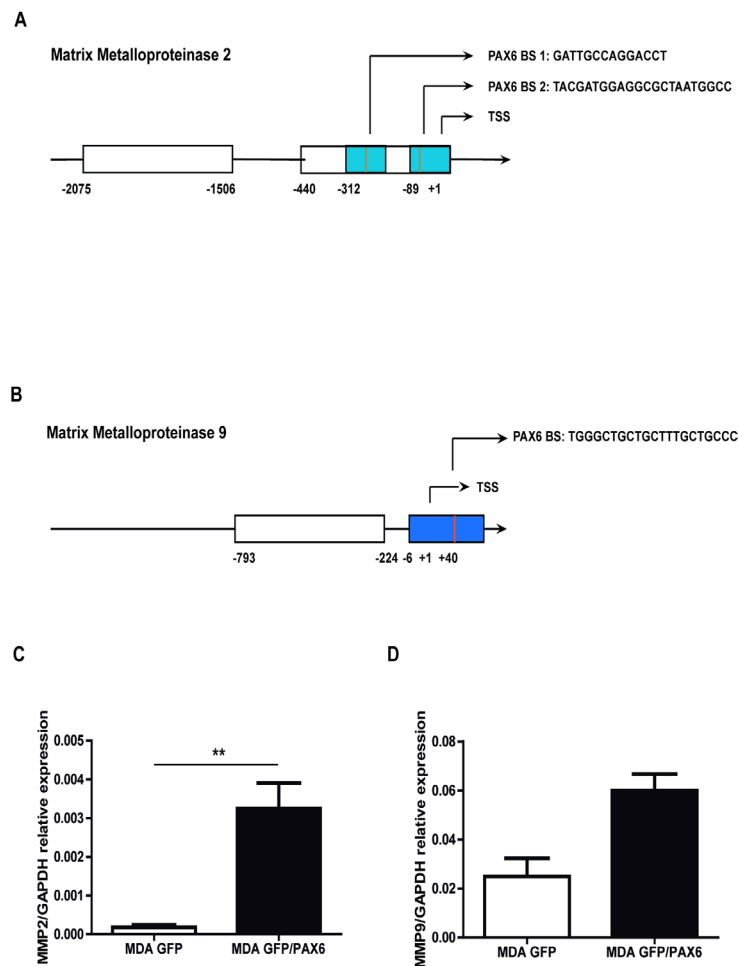


Figure 4. A Structure of the MMP2 gene. Promoter regions are shown as white boxes. At the distal promoter, evolutionary conserved regions (ECR) A and B are shown as blue boxes. Two potential binding sites for PAX6 protein (BS1 and 2) were detected inside the ECRs A and B using JASPAR database (orange stripes). PAX6 binding site sequences are shown above. B Structure of the MMP9 gene. An ECR (blue box) is shown flanking promoter region (white box). PAX6 binding site (orange stripe) is located at +40 bp of TSS. PAX6 binding site sequence is shown. C Graph bar representation of MMP2 mRNA expression relative to GAPDH for MDA-MB-231 cells transfected either with GFP or GFP/PAX6. \*\*P< 0.01. D Graph bar representation of MMP9 mRNA expression relative to GAPDH for MDA-MB-231 cells transfected either with GFP or GFP/PAX6. P= 0.06.

contributing to the initial events of the metastatic process by regulation of other genes. To test this, we analyzed the DNA sequences of Matrix Metalloproteinases 2 and 9 genes, which encode two important enzymes involved in cell migration and invasion of surrounding tissues, to identify the presence of potential binding sites for PAX6 protein.

First, we sought for the presence of promoter regions by using the FirstEF software. We identified the presence of two promoters in the 5' regulatory region of MMP2 gene (-2,075 bp to -1506 bp and -440 bp to +229 bp) and one promoter within the MMP9 gene (-793 bp to -224 bp). Given that regulatory elements often reside in evolutionary conserved regions (ECRs), we used the mVISTA software to perform non-coding alignment among MMP2 and MMP9 sequences and their corresponding murine orthologues *Mmp2* and *Mmp9*. Within the MMP2 gene, two ECRs were identified, A: 184 bp (from -312 bp to -128 bp) and B: 304 bp (from -89 bp to +215 bp). Analysis on the MMP9 gene revealed the presence of an ECR ranging from -6 bp to +180 bp of the major transcription start

site (TSS). Next, we analyzed the MMP2 and MMP9 5' non-coding DNA sequences searching for PAX6 paired domain binding sites, using mVISTA program and the JASPAR CORE matrices. Using these tools, we identified 4 putative binding sites in the MMP2 regulatory DNA and focused on two that reside in the ECRs of the distal, as shown in Figure 4A. Within the MMP9 regulatory DNA, we found two putative binding sites of which we focused in the first one, located 40 bp downstream of the TSS, within the ECR (Figure 4B).

Based on these findings, we hypothesized that PAX6 could be contributing to initial steps of metastatic cascade on breast cancer cells by regulating the expression of MMP2 and MMP9 genes, possibly binding to their promoter regions.

#### *PAX6 overexpression increases MMP2 and MMP9 expression*

Based in the in-silico assays, we decided to test our previous hypothesis whether MMP2 and MMP9 expression is regulated by PAX6, by modulating the



expression of PAX6 on MDA-MB-231 cell line. To do this, we evaluated by qRT-PCR the expression of both MMPs on MDA-MB-231 cells transfected with GFP or GFP/PAX6. We found that PAX6 overexpression on the GFP/PAX6 transfected cells increased both MMPs transcripts when compared to the MDA-MB-231 GFP cells. The increase in expression was significant for MMP2 ( $P < 0.01$ , Figure 4C). MMP9 also increased mRNA expression, although it did not reach statistical significance ( $P = 0.06$ , Figure 4D).

Taken together, the changes observed after modulation of PAX6 expression evidenced the existence of a positive regulation of PAX6 over the MMP2 and MMP9 genes, by increasing their expression.

## Discussion

In the last years, many authors have contributed to understand the role of aberrant DNA methylation during tumorigenesis, demonstrating its involvement in human malignancies including breast and others (Esteller, 2005). Despite its importance, a limited number of studies have assessed the specific contribution of this epigenetic mark to the molecular events that lead to metastasis establishment. The present study demonstrates that PAX6 expression is epigenetically regulated by promoter methylation and that PAX6 over-expression enhances cell migration and expression of MMP2 and MMP9, two well-known proteases involved in cancer cells migration and invasion during metastasis development. Furthermore, we also found that PAX6 promoter methylation negatively impacts on cell migration, supporting the notion that PAX6 has a pro-oncogenic role on mammary carcinogenesis.

PAX6 has been demonstrated to be involved in development of eye, central nervous system and pancreas (Shaham et al., 2012; Engelkamp et al., 1999). Besides its function during development, PAX6 has also been reported to play different roles in malignant tumors (Shyr et al., 2010; Salem et al., 2000). However, there is a controversy about PAX6 and its specific role in human cancers. For instance, in neoplasms of the CNS such as gliomas and glioblastomas, it has been postulated as a tumor suppressor gene, whose down-regulation enhances cell invasiveness (Mayes et al., 2006; Cheng et al., 2014). In other cancer types, an opposite function is proposed: In lung cancer cell lines PAX6 inhibition reduces cell proliferation (Zhao et al., 2014), while in retinoblastoma, overexpression of PAX6 has been reported (Wang et al., 2013). Globally, these findings support the notion that PAX6 role during tumorigenesis could be dual and tissue specific and influenced by multiple variables including the tissue microenvironment and other epigenetic factors.

In breast cancer, Zong et al., (2011) suggested that PAX6 promotes cancer cell proliferation and tumorigenesis. However, the role that PAX6 plays during metastatic cascade as well as the underlying molecular mechanisms, remain unknown. Our group has reported that the CpG site of PAX6 evaluated in this study changed its methylation status during progression from PBT to LNM. We also established that this mark has

an impact on the expression of PAX6 gene in primary tumor and LNM, suggesting that this change induces a functional modification in the metastasis (Urrutia et al., 2015). We tested this hypothesis by evaluating migration after modulation of PAX6 expression, finding that overexpression of PAX6 on MDA-MB-231 cell line increases migration. To confirm the participation of PAX6 expression on cell migration, we evaluated migration on HeLa cell line after cell transfection with an siRNA for PAX6. Our results showed that interfering PAX6 expression reduced migration of HeLa cells. More importantly, we confirmed that PAX6 promoter methylation reduces cell migration by performing migration assays on MDA-MB-231 cells after 5-Aza treatment and siRNA/PAX6 transfection. Our results obtained by MS-MLPA and qPCR indicate that 5-Aza treatment can reduce PAX6 methylation and increase its expression on MDA-MB-231 cells. Subsequent inhibition of PAX6 after 5-Aza treatment showed greater reduction of migration when compared with control and 5-Aza alone treated cells. This observation reveals the importance of methylation in cell migration and supports our previous theory that cells without PAX6 methylation have greater chances of leaving primary tumor and establishing on distant organs like lymph nodes.

In addition, the present study demonstrates that mRNA levels of MMP2 and MMP9 are increased in PAX6 overexpressed cells. MMP2 and MMP9 are two proteases secreted by tumor cells, that degrade components of basal membrane and extracellular matrix, thus linking cancer cells with invasion and metastasis. Our results here described are in line with those informed by Li et al., (2014) who found that PAX6 promotes invasion in colon cancer by upregulation of both MMPs. In a similar manner, Meng et al., (2015) reported that PAX6 down-regulation by miRNA-335 reduces cell invasion in MCF-7 cells. Besides our functional assays, our bioinformatic assays provide evidence of the existence of specific binding sites for PAX6 in the regulatory regions of both MMPs genes. Mayes et al., (2006) previously reported the presence of a 245 bp PAX6 responsive region ranging from -177 to +68 bp of the MMP2 TSS, suggesting the existence of a PAX6-specific binding site. This PAX6 responsive region overlaps with the ECR B region and the second PAX6 binding site we described in the MMP2 gene promoter, supporting the hypothesis that PAX6 binding to this region is the mechanism involved in regulation of MMP2 expression. In a similar way, Sivak et al., (2004) previously confirmed the activation of MMP9 promoter by PAX6. However, the binding site described in this study is not the same as previously reported by Sivak. Therefore, the functionality of the PAX6 binding site we describe in this report must be further confirmed.

Overall data from this study supports the pro-migratory role of PAX6 in breast carcinoma and delineate one of its possible mechanisms: we postulate PAX6 as an epigenetically regulable transcription factor that promotes migration and increases expression of the pro-metastatic proteins MMP2 and MMP9.

### Conflict of interest

The authors declare no conflict of interest.

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