

Evidence of epigenetic regulation of the tumor suppressor gene cluster flanking *RASSF1* in breast cancer cell lines

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Abbreviations: 5-Aza-dC, 5-Aza-2'-deoxycytidine; bp, base pair; *CACNA2D2*, calcium channel, voltage-dependent, alpha2/delta subunit gene; cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; chr3, chromosome 3; CpG, cytosine-guanine dinucleotide; *CYB561D2*, cytochrome b-561 domain containing 2/101F6 gene; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EGF, epidermal growth factor; *Egr-1*, early growth response; FBS, fetal bovine serum; H3K9ac, histone H3 lysine acetylation; H3K9me3, histone H3 lysine trimethylation; HA, hyaluronic acid; HAase, hyaluronidase; *HOX*, homeobox gene; HRM, high resolution melting; *HYAL1*, hyaluronidase type 1 gene; *HYAL2*, hyaluronidase type 2 gene; *HYAL3*, hyaluronidase type 3 gene; i.e., id est; iRNA, interference RNA; Kb, kilo base; LOH, loss of heterozygosity; LRES, long range epigenetic silencing; *MAGE*, melanom antigen gene; MEBM, mammary epithelial basal medium; NEAA, non-essential amino acids; *NPRL2*, nitrogen permease regulator-like2 gene; *PHDC*, protocadherin family genes; *RASSF*, ras-association domain family gene; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; *SEMA3B*, semaphorin 3B gene; SP1, Sp transcription factor; *TMEM115*, transmembrane protein 115 gene; TSA, trichostatin A; TSG, tumor suppressor gene; *TUSC2*, tumor suppressor candidate 2 gene; *ZMYND10*, zinc finger, MYND-type containing 10 gene

Epigenetic mechanisms are frequently deregulated in cancer cells and can lead to the silencing of genes with tumor suppressor activities. The isoform A of the *Ras-association domain family member 1* (*RASSF1A*) gene is one of the most frequently silenced transcripts in human tumors; however, few studies have simultaneously investigated epigenetic abnormalities associated with the 3p21.3 tumor suppressor gene cluster flanking *RASSF1* (i.e., *SEMA3B*, *HYAL3*, *HYAL2*, *HYAL1*, *TUSC2*, *RASSF1*, *ZMYND10*, *NPRL2*, *TMEM115* and *CACNA2D2*). This study aimed to investigate the role of epigenetic changes to these genes in 17 breast cancer cell lines and in three non-tumorigenic epithelial breast cell lines (184A1, 184B5 and MCF 10A) and to evaluate the effect on gene expression of treatment with the demethylating agent 5-Aza-2'-deoxycytidine and/or Trichostatin A (TSA), a histone deacetylase inhibitor. We report that, although the *RASSF1A* isoform was determined to be epigenetically silenced in 15 of the 17 breast cancer cell lines, all the cell lines expressed the *RASSF1C* isoform. Five breast cancer cell lines overexpressed *RASSF1C* when compared with the normal epithelial cell line 184A1. Furthermore, the genes *HYAL1* and *CACNA2D2* were significantly overexpressed after the treatments. After the combined treatment, *RASSF1A* re-expression was accompanied by an increase in expression levels of the flanking genes. The Spearman's correlation coefficient indicated a positive co-regulation of the following gene pairs: *RASSF1* and *TUSC2* ($r = 0.64$, $p = 0.002$), *RASSF1* and *ZMYND10* ($r = 0.58$, $p = 0.07$), *RASSF1* and *NPRL2* ($r = 0.48$, $p = 0.03$), *ZMYND10* and *NPRL2* ($r = 0.71$; $p = 0.0004$) and *NPRL2* and *TMEM115* ($r = 0.66$, $p = 0.001$). Interestingly, the genes *TUSC2*, *NPRL2* and *TMEM115* were found to be unmethylated in each of the untreated cell lines. Chromatin immunoprecipitation using antibodies against the acetylated and trimethylated lysine 9 of histone H3 demonstrated low levels of histone methylation in these genes, which are located closest to *RASSF1*. These results provide evidence that epigenetic repression is involved in the downregulation of multiple genes at 3p21.3 in breast cancer cells.

Introduction

Genetic and epigenetic alterations are well established hallmarks of human cancer that cooperate at tumor development and

can result in the silencing of key regulatory genes with demonstrated roles in tumor suppression, DNA mismatch repair and cell cycle regulation.¹⁻³ Specifically, aberrant DNA methylation content (global genome hypomethylation) and patterns of

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cytosine methylation, especially promoter-specific CpG island hypermethylation, are currently known to be associated with the development of human cancer.^{4,5}

In breast cancer, there is evidence that agglomerative epigenetic aberrations are common events.^{6,7} This observation has led to the hypothesis that DNA hypermethylation could affect not only discrete genes as a local event but could also span across a large chromosomal region, such as the one observed for the homeobox gene cluster (*HOXA* and *HOXD*)⁶ and the protocadherin family (*PHDC* genes).⁷ Long Range Epigenetic Silencing (LRES) is the term proposed to describe the coordinate suppression of neighboring genes based on the interplay between the hypermethylation of clusters of contiguous CpG islands within a genomic region and histone modifications.⁸ Aberrant DNA methylation associated with LRES has been described for several types of tumors, including colon,⁹⁻¹² bladder,¹³ prostate^{14,15} and Wilms¹⁶ and can extend over genomic regions varying from 100–2,400 Kb in size. It has been proposed that epigenetic events associated with LRES can cause the same expected effects of genetic alterations, such as deletions/microdeletions of contiguous genes, due to the functional inactivation of cancer related genes, by epigenetic modifications linked to a repressed chromatin state (such as densely methylated CpG islands and methylation of lysines 9 and 27 of histone H3).

In this context, the short-arm of human chromosome 3 has attracted special interest for harboring multiple genes that exhibit varying degrees of tumor suppressor activity. In breast cancer, allelic losses were detected along 3p by loss of heterozygosity (LOH) analysis in 80% of the cases and were associated with early tumor stages and poor prognostic parameters.¹⁷ The LOH pattern of these losses both in breast carcinomas and preneoplastic epithelial foci was revealed to be discontinuous and was characterized by at least nine small discrete intervals of common microdeletions. The 3p21.3 region, where the *RASSF1* gene is mapped, was the most frequently region involved in LOH.¹⁸

The gene *RASSF1* is a member of the Ras-Association Domain Family (*RASSF*) and encodes seven distinct transcripts generated by the differential usage of two promoters and alternative splicing. However, only two of these transcripts (*RASSF1A* and *RASSF1C*) have been attributed with a relevant biological role in cancer cells.¹⁹ These isoforms are transcribed from the upstream and downstream promoter, respectively, and each one is located within a CpG island. Previous studies have demonstrated that only the upstream promoter is frequently hypermethylated in different tumor types and cancer cell lines.²⁰ Interestingly, the *RASSF1* gene is found in the central position of the 3p21.3 tumor suppressor cluster,^{21,22} identified by the overlapping regions of minimal deletions detected by LOH analysis as well as by the occurrence of homozygous deletions in both primary tumors and cancer cell lines.^{23,24} These studies have indicated that at least 19 genes are located in the approximately 630 Kb region at 3p21.3. An approximately 120 Kb long subregion within this area contains the following eight genes flanking *RASSF1*: *CACNA2D2* (*calcium channel, voltage-dependent, α 2/delta subunit 2*), *TMEM115* (*transmembrane protein 115* or *placental protein 6*, *PL6*), *CYB561D2* (*cytochrome b-561 domain containing*

2/101F6), *NPRL2* (*nitrogen permease regulator-like 2, primed named tumor suppressor candidate 4* or *TUSC4*), *ZMYND10* (*zinc finger, MYND-type containing 10*, *BLU* or *FLU*), *RASSF1*, *TUSC2* (*tumor suppressor candidate 2*, also named *FUS1*), *HYAL2* and *HYAL1* (*hyaluronidase type 2* and *type 1*) and the genes *HYAL3* and *SEMA3B* (*semaphorin 3B*, originally named *LUCA1*) immediately adjacent to them. Genetic analysis of lung and breast tumors indicated that none of these candidate tumor suppressor genes (TSG) were frequently mutated.

These putative tumor suppressor genes may affect and regulate many important biological processes, such as cell proliferation, cell cycle kinetics, signal transduction, ion exchange and apoptosis and could have a synergistic effect in cancer cells. Therefore, and considering that the genes in 3p21.3 tumor suppressor cluster are rarely inactivated in human cancer by somatic mutations, we have designed this study to investigate whether alternative epigenetic mechanisms may play a role in the downregulation/inactivation of resident candidate TSG flanking *RASSF1* in breast cancer cell lines.

Results

Gene transcript expression, HRM and DNA copy number analysis in untreated breast cell lines. To achieve our goal, we initially evaluated the expression levels of transcripts from *RASSF1* and the neighboring genes mapped within a genomic region of approximately 236 Kb, flanked by the *SEMA3B* (position chr3: 50,305,040–50,314,570, UCSC Genome Browser on Human, GRCh37/hg19 Assembly) and *CACNA2D2* (position chr3: 50,400,233–50,540,892, UCSC Genome Browser on Human, GRCh37/hg19 Assembly) genes, which contains nine genes (*SEMA3B*, *HYAL3*, *HYAL1*, *HYAL2* and *TUSC2*, located upstream of *RASSF1* and *ZMYND10*, *NPRL2*, *TMEM115* and *CACNA2D2*, located downstream) (Fig. 1A). These genes were evaluated in three non-malignant cell lines derived from human breast epithelial cells (184A1, 184B5) and fibrocystic disease (MCF 10A) as well as in a series of 17 breast cancer cell lines. The expression levels of the *RASSF1* gene were assessed by real-time PCR using a set of primer and probes targeting specifically the exons 5 and 6 (common to all isoforms) and the *RASSF1A* isoform. Although only two breast cancer cell lines expressed the *RASSF1A* isoform (MDA-MB-415 and Hs 578T), transcripts of the *RASSF1* gene were detected in all of the cell lines analyzed. Interestingly, when the non-tumorigenic and tumorigenic cell lines were compared, a trend of high *RASSF1* transcript levels was observed in the breast cancer cell lines compared with the normal epithelial cell line 184A1 (Mann Whitney test, $p = 0.0720$) (Fig. 1B). Expression levels of the *RASSF1* gene were found to be elevated in five cell lines (BT-20, BT-549, MDA-MB-231, MDA-MB-468 and T-47D), by at least 2-fold, compared with the normal epithelial cells 184A1. In addition, among the other nine genes evaluated, only the *HYAL2*, *TUSC2* and *CACNA2D2* exhibited differential expression levels in the malignant compared with the non-malignant cell lines; these transcripts were detected at higher levels in cancer cell lines (Mann Whitney test, $p = 0.0199$, $p = 0.0443$ and $p = 0.0262$, respectively) (Fig. 1B).

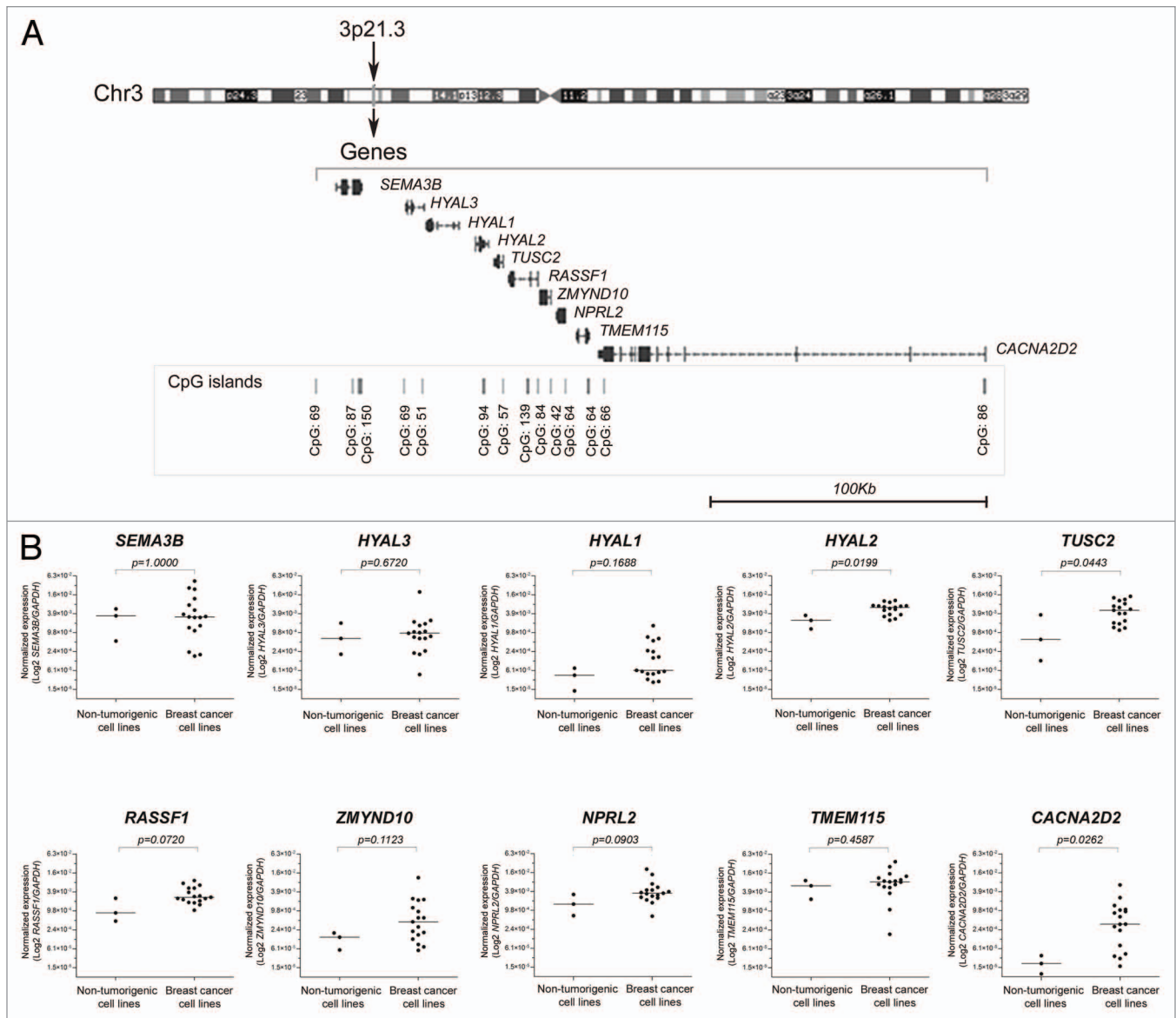


Figure 1. (A) Scheme of the genes and CpG islands organized according to their physical location on 3p21.3 (UCSC Genome Browser on Human, assembly GRCh37/hg19, <http://genome.ucsc.edu>). (B) Differential gene expression among breast cancer cell lines and non-tumorigenic breast cells. The *HYAL2*, *TUSC2* and *CACNA2D2* genes were differentially expressed in breast cancer compared with non-tumorigenic cell lines. Global *RASSF1* transcripts showed a trend to overexpression in breast cancer cells lines compared with non-tumorigenic epithelial breast cells 184A1. All quantifications were performed by real-time PCR using specific hydrolysis probes for each target gene normalized to the endogenous control expression levels (*GAPDH* gene).

The DNA methylation status of the *RASSF1A* promoter region was measured by High Resolution Melting (HRM) analysis. As expected, the silencing of this isoform was correlated with DNA hypermethylation of CpG island 1 (CpG island 84, nucleotide position chr3: 50377804–50378540, UCSC Genome Browser on Human, assembly GRCh37/hg19): concordant results were observed in 13/17 (76%) breast cancer cell lines, which showed 100% methylation and an absence of *RASSF1A* gene expression. On the other hand, approximately 50% of methylation was detected in the MDA-MB-453 and 25% in the BT-20 cell lines. Furthermore, the two cell lines expressing the A isoform of *RASSF1* showed 0% and 25% of methylation (MDA-MB-415

and Hs 578T, respectively). Interestingly, despite the lack of methylation observed in 184A1 and 184B5 cells and the 25% of methylation in MCF 10A cells, these cells did not express *RASSF1A*. When the copy number of the *RASSF1* locus was evaluated, 11/20 cell lines showed allelic losses. The comparison between the expression level of the *RASSF1* transcript and gene copy number revealed a significant correlation ($r^2 = 0.44$, $p = 0.0015$). In contrast, the two breast cell lines that expressed *RASSF1A*, MDA-MB-415 and Hs 578T, presented no alterations in the copy number at this locus.

Gene transcript expression analysis in 5-Aza-dC and TSA treated breast cell lines. In a second step, the panel of 20 epithelial

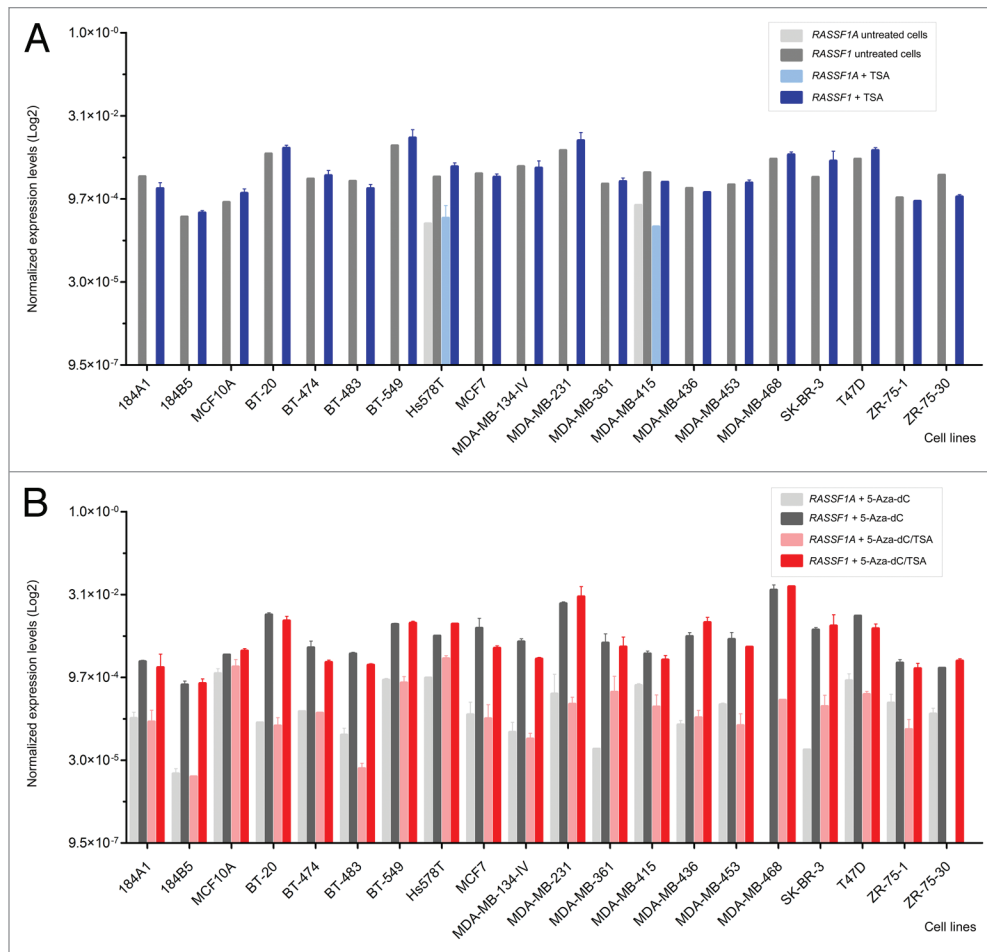


Figure 2. Drug induced expression levels of global *RASSF1* transcripts and A isoform of *RASSF1* gene in breast cancer cell lines. (A) Expression levels in untreated cell lines and after treatment with trichostatin A (TSA). Only two cell lines, Hs 578T and MDA-MB-415, expressed the A isoform of *RASSF1* gene. As expected, the TSA treatment alone did not affect DNA methylation and as a result the expression levels of this isoform. (B) The treatment with 5-Aza-dC, isolated or combined with TSA, resulted in the reactivation of A isoform of *RASSF1* gene. Gene expression levels were normalized to *GAPDH* gene. Error bars indicate standard deviation from duplicates.

breast cell lines was treated with the demethylating nucleoside analog 5-Aza-2'-deoxycytidine (5-Aza-dC) and the histone deacetylase inhibitor Trichostatin A (TSA), either alone or in combination. These treatments were used to evaluate whether these drugs were able to induce the reactivation of the *RASSF1A* isoform and to evaluate the drugs' effect on the expression levels of the neighboring genes. The treatment with TSA alone was not able to significantly modulate the expression levels (Fig. 2A). The treatment with 5-Aza-dC alone or in combination with TSA led to the re-expression of *RASSF1A* in 18 breast epithelial cell lines (Fig. 2B). However, the two cell lines expressing *RASSF1A* showed differential responses to the treatments, as *RASSF1A* was upregulated in Hs 578T cells and remained unchanged in MDA-MB-415 (Fig. 2B) compared with the untreated controls (Fig. 2A). The non-tumorigenic 184A1 cell line showed the lowest variation in the expression levels of all transcripts evaluated after treatments with 5-Aza-dC and 5-Aza-dC/TSA.

To evaluate the effects of the demethylating agent and the histone deacetylase inhibitor on the transcription of the genes flanking *RASSF1*, the expression levels were determined as fold-change

relative to the respective untreated controls. In addition, an unsupervised hierarchical clustering analysis was performed based on the Euclidean distance to calculate the average linkage. A single cluster was obtained with the untreated controls and the cell lines treated only with TSA and another cluster obtained separated those with the cell lines treated with 5-Aza-dC or 5-Aza-dC/TSA (Fig. 3). The gene expression profiles in Hs 578T, MDA-MB-415 (25% methylated and unmethylated at the *RASSF1A* locus, respectively) and BT-20 cells (25% methylated) did not significantly change upon the treatments and were distinct, compared with the other cell lines. A group of five genes were found to be more similar to the *RASSF1A/RASSF1* expression levels before and after the treatments, including *SEMA3B*, *TMEM115*, *HYAL2*, *TUSC2* and *NPRL2* (Fig. 3). Although all genes were positively regulated by the epigenetic drugs, their effect was highest for the *HYAL1* and *CACNA2D2* genes (Fig. 4 and Table S1).

A pairwise Spearman correlation coefficient was then calculated to create a correlation coefficient matrix for each pair of genes (Table 1). After 5-Aza-dC treatment, a positive correlation was observed between *RASSF1*, *NPRL2* and *TMEM115* and

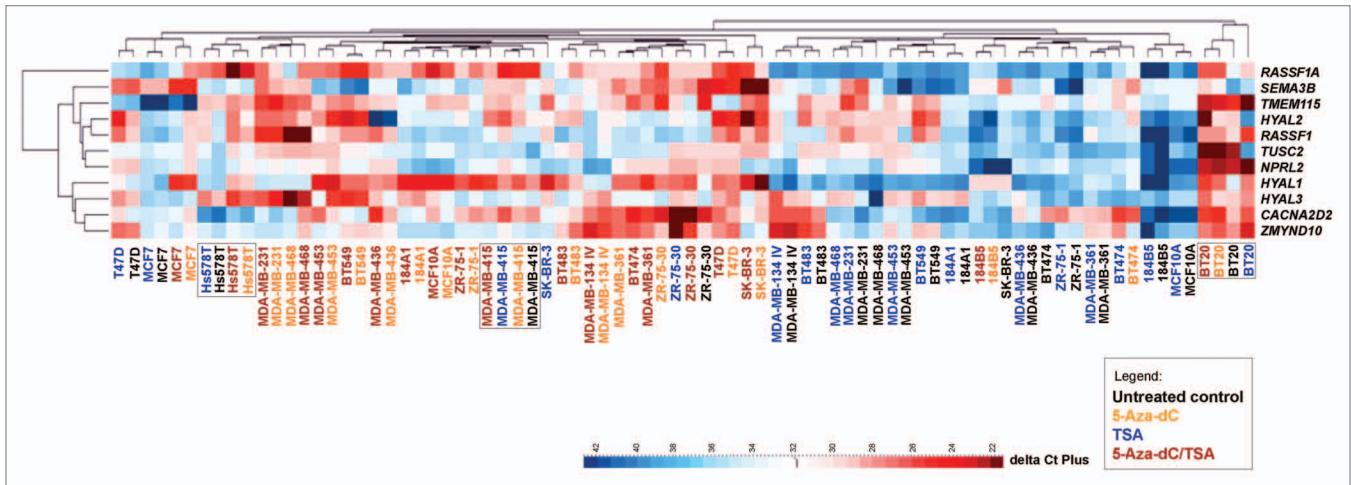


Figure 3. Expression profile summarized in a heat map. Effect of treatments with 5-Aza-dC and with 5-Aza-dC combined with TSA leading to the increase in the expression levels of the tumor suppressor cluster, except in the cell lines that were unmethylated at CpG island 1 of *RASSF1A* promoter (MDA-MB-415) or showing low levels of methylation (Hs 578T and BT-20).

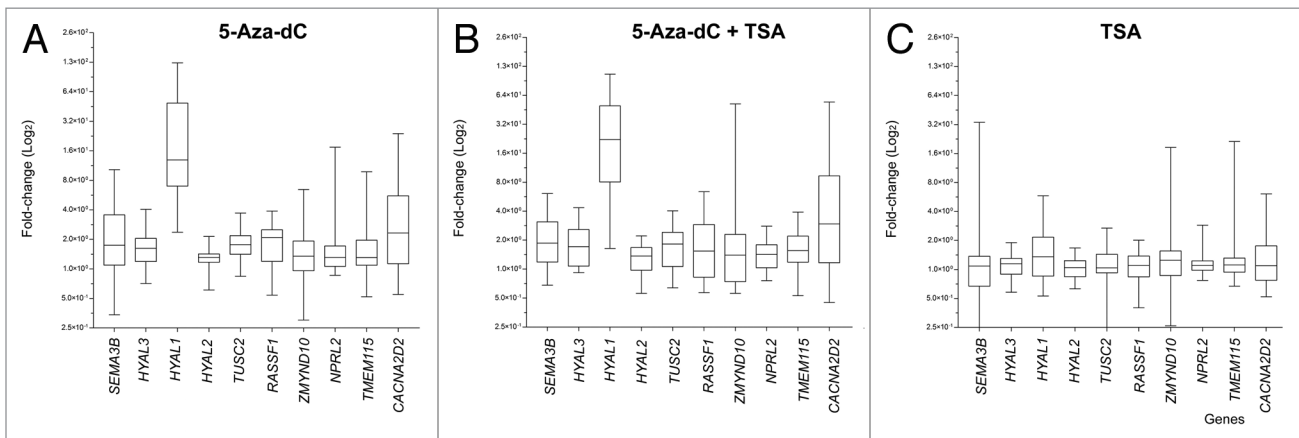


Figure 4. Effect of epigenetic drugs (5-Aza-dC and trichostatin A, TSA) on gene expression. (A and B) All genes were upregulated by treatment with 5-Aza-dC, isolated and combined with TSA. *HYAL1* and *CACNA2D2* genes showed the highest expression levels induced by these drugs. (C) Effects on gene transcript levels after isolated treatment with TSA. For each gene, the results are expressed as fold change relative to the reference (respective untreated control cell line) calculated using the $\Delta\Delta Ct$ -method normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression levels and represent the mean of real-time PCR results of independent biological replicas.

between *ZMYND10* and *NPRL2*. Moreover, after the combined treatment with 5-Aza-dC and TSA, *RASSF1* expression levels were positively correlated with *TUSC2* and *ZMYND10*. Because *TUSC2* expression was positively correlated with *ZMYND10*, which in turn correlated with *NPRL2*, and this gene also positively correlated with *TMEM115*, the contiguous genes mapped in the central portion of the 3p21.3 cluster flanking *RASSF1* (i.e., from telomere to centromere, *TUSC2*, *RASSF1*, *ZMYND10*, *NPRL2* and *TMEM115*) were selected for DNA methylation analysis and chromatin immunoprecipitation (ChIP).

In contrast to the high hypermethylation frequencies observed in *RASSF1A* after the HRM approach, the CpG islands in the promoter regions of the *CACNA2D2*, *TUSC2*, *NPRL2* and *TMEM115* genes were unmethylated in all 20 cell lines analyzed. For *ZMYND10*, two cell lines were 100% methylated (MDA-MB-436 and ZR-75-30) and one was 25% methylated

(MDA-MB-453). In contrast, the non-CpG promoter of *HYAL1* gene was 25% methylated in all cell lines with exception of three (BT-20, MCF7 and MDA-MB-415) that were unmethylated. **Figure 5** shows the enrichment status over the input fraction for the five genes evaluated after ChIP using antibodies to detect two modifications of histone H3 (H3K9ac and H3K9me3). Marked differences in the levels of histone H3 acetylation and methylation at the promoters of the *TUSC2*, *RASSF1*, *ZMYND10*, *NPRL2* and *TMEM115* genes were detected, with a clear predominance of acetylation. H3K9 acetylation and the absence of trimethylation at the same site were observed at five loci in the two cell lines with unmethylated *RASSF1A* (**Fig. 5**). In contrast, the non-tumorigenic cell lines (184A1, 184B5 and MCF 10A) manifested enrichment of H3K9me3, which could explain the inactivation of the *RASSF1A* gene despite the absence or low levels of DNA methylation.

Table 1. Pair-wise correlation coefficient matrix of expression levels of contiguous genes mapped across 3p21.3 after treatments with 5-Aza-dC and 5-Aza-dC/TSA

| After treatment with 5-Aza-dC | | | | | | | | | | |
|---------------------------------------|-----------------------------------|----------------------------------|---------------------------------|---------------------------------|-----------------------------------|----------------------------------|-----------------------------------|----------------------------------|----------------|-----------------|
| | <i>SEMA3B</i> | <i>HYAL3</i> | <i>HYAL1</i> | <i>HYAL2</i> | <i>TUSC2</i> | <i>RASSF1</i> | <i>ZMYND10</i> | <i>NPRL2</i> | <i>TMEM115</i> | <i>CACNA2D2</i> |
| <i>SEMA3B</i> | | | | | | | | | | |
| <i>HYAL3</i> | -0.02 | | | | | | | | | |
| <i>HYAL1</i> | 0.16 | 0.16 | | | | | | | | |
| <i>HYAL2</i> | 0.02 | 0.14 | 0.43 ^{p = 0.05} | | | | | | | |
| <i>TUSC2</i> | -0.02 | 0.62 ^{p = 0.003} | 0.05 | 0.51 ^{p = 0.02} | | | | | | |
| <i>RASSF1</i> | 0.02 | 0.36 | 0.07 | 0.24 | 0.45 ^{p = 0.04} | | | | | |
| <i>ZMYND10</i> | 0.37 | 0.38 | -0.25 | -0.15 | 0.39 | 0.18 | | | | |
| <i>NPRL2</i> | 0.42 ^{p = 0.06} | 0.32 | -0.13 | 0.27 | 0.69 ^{p = 0.0008} | 0.56 ^{p = 0.09} | 0.58 ^{p = 0.007} | | | |
| <i>TMEM115</i> | 0.39 | 0.41 | 0.02 | 0.24 | 0.42 ^{p = 0.06} | 0.57 ^{p = 0.008} | 0.16 | 0.46 ^{p = 0.04} | | |
| <i>CACNA2D2</i> | -0.18 | 0.27 | 0.50 ^{p = 0.02} | 0.48 ^{p = 0.03} | 0.38 | 0.23 | 0.07 | 0.10 | -0.25 | |
| After treatment with 5-Aza-dC and TSA | | | | | | | | | | |
| | <i>SEMA3B</i> | <i>HYAL3</i> | <i>HYAL1</i> | <i>HYAL2</i> | <i>TUSC2</i> | <i>RASSF1</i> | <i>ZMYND10</i> | <i>NPRL2</i> | <i>TMEM115</i> | <i>CACNA2D2</i> |
| <i>SEMA3B</i> | | | | | | | | | | |
| <i>HYAL3</i> | 0.05 | | | | | | | | | |
| <i>HYAL1</i> | 0.18 | 0.14 | | | | | | | | |
| <i>HYAL2</i> | 0.03 | 0.15 | 0.12 | | | | | | | |
| <i>TUSC2</i> | 0.01 | 0.56 ^{p = 0.01} | -0.05 | 0.36 | | | | | | |
| <i>RASSF1</i> | 0.08 | 0.49 ^{p = 0.02} | -0.06 | 0.38 | 0.64 ^{p = 0.002} | | | | | |
| <i>ZMYND10</i> | 0.23 | 0.57 ^{p = 0.009} | -0.19 | -0.12 | 0.58 ^{p = 0.007} | 0.54 ^{p = 0.01} | | | | |
| <i>NPRL2</i> | 0.51 ^{p = 0.02} | 0.30 | -0.11 | 0.04 | 0.35 | 0.48 ^{p = 0.03} | 0.71 ^{p = 0.0004} | | | |
| <i>TMEM115</i> | 0.69 ^{p = 0.0008} | 0.17 | -0.01 | 0.15 | 0.24 | 0.24 | 0.29 | 0.66 ^{p = 0.001} | | |
| <i>CACNA2D2</i> | -0.42 ^{p = 0.06} | 0.48 ^{p = 0.03} | 0.24 | 0.34 | 0.51 ^{p = 0.02} | 0.49 ^{p = 0.02} | 0.21 | 0.10 | -0.06 | |

Statistically significant correlation coefficients (r) are indicated in bold, p < 0.05.

Discussion

Recently, “omics”-based integrative analysis has predicted regions of the human genome that are probably subjected to mechanisms of coordinated epigenetic suppression in different tumor types.^{13,15,25} In fact, some of these predictions have been experimentally validated on the chromosome regions 2p14.2,^{9,11,12} 3p22.3,^{13,26} 5q31,^{7,16} 7p15.2,⁶ 7q31.1-q31.3¹⁵ and 16p11.2.²⁵ These studies have provided new approaches for the characterization of discrete regions of LRES, supporting the hypothesis that this alternative mechanism may be a common phenomenon in cancer⁸ and could lead to the identification of new genes or genomic regions that are involved in tumor progression and can be potentially targeted for epigenetic therapy.

Genetic and epigenetic abnormalities at 3p21.3 are frequently observed in several tumor types, and a particular focus has been given to *RASSF1A* promoter hypermethylation, which is one of the most frequent epigenetic alterations in human cancers. However, few studies have been dedicated to the investigation of the DNA methylation patterns of the other genes mapped to the 3p21.3 tumor suppressor cluster and only few reports have

simultaneously analyzed the epigenetic status of more than two genes at this locus.²⁶⁻³⁰

In the present study, the expression levels of ten contiguous genes mapped to the 3p21.3 tumor suppressor cluster were compared among non-tumorigenic and tumorigenic epithelial breast cell lines, before and after treatment with the epigenetics drugs 5-Aza-dC and TSA (alone or in combination). Initially, the expression levels of each gene were compared among the different breast cell lines studied. The *RASSF1* gene, and specifically the *RASSF1A* isoform, received a special focus because epigenetic modification at this isoform, i.e., DNA methylation of the promoter-associated CpG island 1, has frequently been reported in breast cancer.³¹ Our data showed that, although epigenetic silencing of *RASSF1A* was detected in 18/20 epithelial breast cell lines, all of them expressed the *RASSF1* gene. This gene encodes seven isoforms (*RASSF1A-G*), of which *RASSF1A* and *RASSF1C* are ubiquitously expressed in normal tissues and are controlled by two distinct promoter regions associated with CpG islands.¹⁹ *RASSF1B* isoform is expressed in hematopoietic cells and has a different 5' exon (exon 1β); *RASSF1D-G* isoforms, which are splice variants of *RASSF1A*, are transcribed from the promoter associated with the first CpG island. The biological function of

these additional transcripts is not clear; however, all the *RASSF1* isoforms that are transcribed from the first CpG island are frequently missing in a variety of tumors as a result of epigenetic inactivation of the *RASSF1A* promoter. Additionally, because the second CpG island (CpG island 139, nucleotide position chr3: 50,374,265–50,375,629, UCSC Genome Browser on Human, assembly GRCh37/hg19) associated with the *RASSF1C* promoter did not present abnormal DNA methylation in cancer cells,¹⁹ the *RASSF1* transcripts detected in the panel of 20 epithelial breast cells are probably from the *RASSF1C* isoform. Thus, this present study has demonstrated that, in breast cell lines, although *RASSF1A* is commonly epigenetically silenced, *RASSF1C* remains expressed. Interestingly, although all cell lines expressed *RASSF1C*, this transcript was differentially expressed in the non-tumorigenic and tumorigenic cell lines, with 5/17 breast cancer cell lines (BT-20, BT-549, MDA-MB-231, MDA-MB-468 and T-47D) overexpressing this isoform compared with the 184A1 expression levels. Our findings are in accordance with those recently reported for the MDA-MB-231 and T-47D cell lines, in which the downregulation of caspase 3 via overexpression of *RASSF1C* was demonstrated to reduce the sensitivity of breast cancer cells to apoptosis. *RASSF1C* overexpression also enhanced T-47D cell invasion/migration in vitro.³² A study from Estrabaud et al. demonstrated that, when the balance of *RASSF1C* and *RASSF1A* is disrupted by the absence of *RASSF1A* or by *RASSF1C* overexpression, the function of SCF^{BTICP} is inhibited, leading to the accumulation of the β -catenin protein. In this sense, it is possible that *RASSF1C* could exert an opposite effect than *RASSF1A* in the process of carcinogenesis. Although previous reports have attributed *RASSF1C* with tumor suppressor activity, other studies have suggested that *RASSF1C* could activate osteoblast cell proliferation.¹⁹ Together, these data suggest that *RASSF1C*, unlike *RASSF1A*, is not a tumor suppressor but instead may play a role in stimulating proliferation and invasion in breast cancer cells.

HYAL1 and *CACNA2D2* were the most positively modulated genes after treatments with 5-Aza-dC alone or with TSA. Non-tumorigenic cell lines were found to have even higher expression levels of these genes after the treatments. *HYAL1* belongs to the hyaluronidase (HAases) family of enzymes that degrade hyaluronic acid (HA). There are six hyaluronidase genes in the human genome: three are arranged at the 3p21.3 suppressor tumor cluster (*HYAL1*, *HYAL2* and *HYAL3*). HAase levels were demonstrated to be elevated in breast tumors, and RT-PCR analysis has detected the expression of *HYAL2* and *HYAL3* in breast cancer tissues,³⁴ suggesting an association between HAase and the tumor invasive/metastatic phenotype.^{35,36} In accordance with the data from the present study demonstrating higher levels of *HYAL2* in breast cancer cell lines compared with non-tumorigenic cell line 184A1, previous reports have shown that, whereas less invasive breast cancer cells expressed *HYAL3*, highly invasive cells expressed *HYAL2*.³⁴ Although the promoter region of these genes is characterized by a CpG island, their expression levels were not significantly increased after the treatments with the demethylating agents in our study. However, it is of note that the promoter region of the *HYAL1* gene has a CpG-rich promoter that does not meet the established criteria for a CpG island,

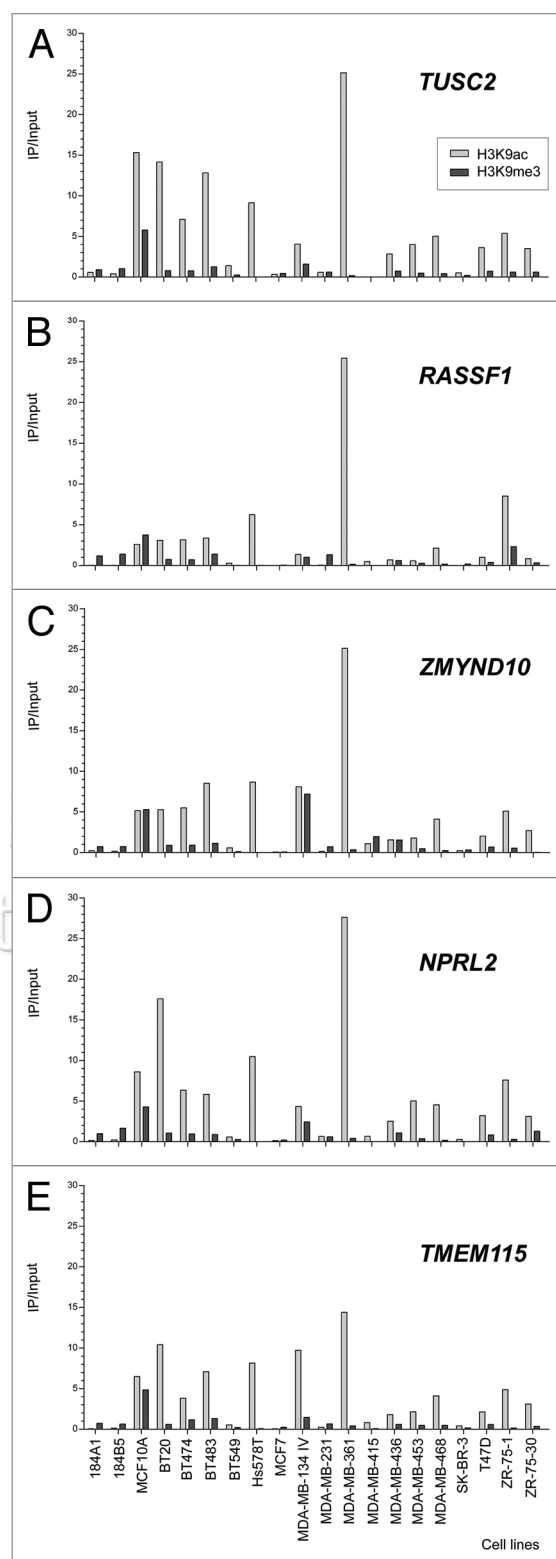


Figure 5. Analysis of Chromatin Immunoprecipitation (ChIP) of genes close to *RASSF1* in breast cancer and epithelial mammary breast cell lines. The amount of immunoprecipitated target (using antibodies against H3K9 acetylation and H3K9 trimethylation) was quantified by real time PCR and plotted as the ratio of immunoprecipitated DNA to input.

similar to that observed in the *MASPIN* gene (which encodes a serine protease inhibitor with tumor-suppressor activity) and the *MAGE* family (for which promoter hypermethylation is responsible for the restricted expression of the tumor-associated MAGE antigens).³ Like *MASPIN* and *MAGE*, the present study suggests that the *HYAL1* gene is clearly upregulated by epigenetic therapy. Recently, it was described that *HYAL1* expression is regulated by methylation of cytosine at positions -71 and -59, which constitute portions of the binding sites for the transcription factors SP1/Egr-1.³⁷ Thus, in the present study we have included the methylation analysis by melting curve of the non-CpG promoter region of *HYAL1* gene. Low levels of DNA methylation (approximately 25%) were detected in 17/20 cell lines.

The *HYAL1* gene encodes the major tumor-derived HAase. In bladder, prostate and head and neck carcinomas, higher levels of HA and HAases were found in tumor cells, tissues and related blood fluids and serve as biomarkers to detect high-grade bladder tumors.³⁸ In breast cancer cells, HAase levels were associated with the ability to invade through matrigel, and silencing of *HYAL1* gene expression by iRNA induced cell cycle arrest and inhibition of proliferation in vitro. Tan et al. showed that *HYAL1* was overexpressed in the breast cancer cell lines MDA-MB-231 and MCF7, in invasive ductal cancer tissues and in metastatic lymph nodes, compared with the nonmalignant breast cell line HBL-100 and normal breast tissues. In our study, although slightly higher levels of *HYAL1* transcript were detected in the MDA-MB-231 and MCF7 cell lines, the majority of breast cancer-derived cells showed lower expression levels of this gene. Furthermore, after treatments with 5-Aza-dC and TSA, the non-tumorigenic cell lines showed a 50–150 fold increase in the expression of *HYAL1* mRNA, whereas the breast cancer cell lines manifested a change of up to 50-fold relative to untreated controls. These data are relevant and indicate that epigenetic modifications of *HYAL1*, like the methylation levels found in our study, could affect *HYAL1* expression in epithelial breast cells. At present, it is unknown whether *HYAL1* functions as a tumor-promoter or tumor-suppressor gene. These controversial cellular roles are highlighted by previous studies demonstrating that silencing of *HYAL1* reduced tumor growth and invasion, suggesting a tumor promoting function. In contrast, the overexpression of *HYAL1* in tumor cells induced apoptosis and inhibited tumor formation.³⁸ More studies are clearly necessary to establish a correlation between the regulation of tissue-specific expression and methylation at non-CpG islands and to explain how this correlation plays a role in breast cancer cells.

The *CACNA2D2* gene was also downregulated in breast cancer cells, and expression levels were reversed by treatment with 5-Aza-dC both in the presence or absence of TSA. Nevertheless, all cell lines were unmethylated at the CpG island promoter of *CACNA2D2*. This gene encodes a member of the α -2/delta subunit family, a protein of the voltage-dependent calcium channel complex. Initial studies demonstrated frequent loss of *CACNA2D2* expression in lung cancer cell lines.^{20,21} In nasopharyngeal and glioma cell lines, loss of *CACNA2D2* expression occurred concomitantly with DNA methylation. However, DNA methylation was less frequently observed in primary tumors.^{39,40}

Overexpression of *CACNA2D2* induced apoptosis in lung cell lines, in which the levels of intracellular free Ca^{2+} were elevated in Ad*CACNA2D2*-transduced cells compared with the controls, followed by depolarization of the mitochondrial membrane.⁴¹ These results led to the suggestion that re-expression of this gene could induce apoptosis. The findings of the present study provide additional evidence that *CACNA2D2* may be a putative tumor suppressor gene that is epigenetically regulated in breast cancer cells.

Among the genes located between *HYAL1* and *CACNA2D2*, expression of three genes (*TUSC2*, *ZMYND10* and *NPRL2*) positively correlated with *RASSF1* or with the closest gene (i.e., *ZMYND10* positively correlated with *NPRL2* and *NPRL2* with *TMEM115*) after the combined treatment with the DNA demethylating and the histone deacetylase inhibitor drugs. These findings indicate that the genes flanking *RASSF1* were simultaneously upregulated by the epigenetic treatment. As previously suggested, epigenetic changes in cancer cells could occur at the neighborhood level, in which the expression of genes residing in the same vicinity could be affected by hypermethylation of a flanking gene, leading to coordinate downregulation or inactivation mediated by chromatin modifications. However, in our study, it was observed that the promoter-associated CpG islands of the *TUSC2*, *NPRL2* and *TMEM115* genes were unmethylated and showed varying degrees of enrichment of the H3K9me3 fraction after ChIP in the untreated cells. Similar findings were first reported by Frigola et al. in colorectal cancer in which genes located at an approximately 4 Mb cluster on chromosome 2q14.2 were epigenetically suppressed even though the involved CpG islands remained unmethylated. Despite the unmethylated CpG islands, the repression of these genes on 2q14.2 was relieved by demethylating drug treatment. The effect of the 5-Aza-dC on global gene expression showed that this drug can result in variable changes in gene expression depending on the functional categories of the genes and that most genes that demonstrated altered expression levels were not regulated by promoters displaying DNA methylation prior to the treatment.⁴²

Furthermore, the fact that the expression levels of the unmethylated genes located close to *RASSF1* were upregulated in tumor cell lines treated with 5-Aza-dC suggests an effect of this nucleoside analog on histone modification.⁴³ In light of the results obtained from the ChIP assays in our study, which showed less enrichment of the H3K9me3 in the breast cancer cells when compared with non-malignant cell lines, it is possible that other histone repressive marks could modulate the expression levels that were detected before the set of breast cancer cell lines studied were treated. Distinct repressive histone modifications have been frequently investigated in human cancer, H3K9me2, H3K9me3 and H3K27me. Besides the interactions between DNA methylation and H3K9me, which may establish and reinforce a silencing loop, polycomb group proteins can modify the methylation status of histone H3 on lysine 27 and recruit other members of repressor complex, including PRC1, which is able to prevent transcriptional activation.⁴⁴ In a recent report by Mayo et al.⁴⁵ the epigenetic profile of two chromosomal regions undergoing long range epigenetic silencing was investigated in colorectal cancer

(2q14.2 and 5q35.2). After the treatment with 5-Aza-dC or TSA, alone or in combination, the authors classified the genes into three groups according the expression levels and the epigenetic states of their promoters: (1) silenced genes exhibiting methylated CpG island promoters and retention of bivalent chromatin epigenetic marks (i.e., H3K4me3 and H3K27), termed MBV genes (methylated and bivalent); (2) downregulated unmethylated CpG island-promoter genes and (3) low expressed and non-CpG island promoter. Interestingly, six MBV genes were reactivated after treatment with 5-Aza-dC or TSA, and a combination of both. However, the expression of these genes was restored at low levels, similar to findings observed in the present study. Alternatively, it is possible that changes in gene expression observed in the treated cells could be the result of a secondary effect, instead of direct promoter demethylation. According to this idea, genes encoding transcription factors could become demethylated and as result upregulated. In turn, these transcription factors could initiate a cascade of transcriptional changes.⁴²

Previous studies have identified genomic regions showing coordinated epigenetic deregulation of neighboring genes in cancer cells as an event independent from copy number alterations. However, allelic losses and deletions at the 3p21.3 region are frequently observed in the most common human tumors, including breast cancer. Therefore the parallel analysis of epigenetic and copy number alterations, as it was performed in this study, is imperative and have demonstrated for our cell lines that DNA copy number changes are implicated in the impairment of the corresponding expression levels of the genes studied. However, although not completely characterized, epigenetic changes at 3p21.3 can also contribute to the repression of several TSG. Altogether, these data suggest that genetic and epigenetic changes in this tumor suppressor cluster can affect a wide spectrum of relevant biological pathways. Further studies could lead to the characterization of the new deregulated networks in human cancer.

Materials and Methods

Cell lines and cell culture. Seventeen human breast cancer cell lines (BT-20, BT-474, BT-483, BT-549, Hs 578T, MCF7, MDA-MB-134-IV, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436, MDA-MB-453, MDA-MB-468, SK-BR-3, T-47D, ZR-75-1 and ZR-75-30) and three non-tumorigenic epithelial breast cell (184A1, 184B5 and MCF 10A) were obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC. Cell lines were cultured at 37°C in a humidified environment containing 5% CO₂. DMEM medium containing 10% fetal bovine serum (FBS) was used for the MDA-MB-134-IV, SK-BR-3, MDA-MB-453, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-436 and MDA-MB-468 cell lines. This medium was supplemented with 1% Non-Essential Amino Acids (NEAA) for the BT-20 and MCF7 cells and with 10 µg/ml insulin for the Hs 578T cells. RPMI medium containing 10% FBS was used in cultures of T-47D cells and was supplemented with 1% pyruvate for the ZR-75-1 and ZR-75-30 cells. The BT-549, BT-483 and BT-474

cells were maintained in RPMI medium containing 10% FBS supplemented with 10 µg/ml insulin and 1% pyruvate. The 184A, 184B5 and MCF 10A cells were cultured in serum-free Mammary Epithelial Basal Medium (MEBM) supplemented with Epidermal Growth Factor (EGF), hydrocortisone, L-glutamine and insulin using Singlequot reagent packs from Lonza, along with 0.005 mg/mL transferrin for 184A1 and 5% horse serum for MCF 10A.

5-Aza-2'-deoxycytidine and trichostatin A treatment of cells. The breast cell lines were seeded at a density of 1 x 10⁶ cells in 25 cm³ culture flask. After 24 h of growth, 1 µM 5-Aza-2'-deoxycytidine (5-Aza-dC; Sigma) was added to the medium, and the cells were incubated for 96 h. Due to its chemical instability, 5-Aza-dC was added to the fresh medium every 24 h. Alternatively, the cells were treated with 150 ng/mL trichostatin A (TSA; Sigma) for 24 h. To assess the combined effect of both drugs, we performed the co-treatment of cells with 5-Aza-dC and TSA as follows: 5-Aza-dC was added initially for 96 h, after which it was removed, and TSA was added for an additional period of 24 h. All experiments were performed in duplicate. A mock treatment was done with DMSO. The cells were then allowed to recover for 24 h prior to harvesting. The genomic DNA and total RNA were extracted from the cell lines after the drug treatments and were used for HRM analysis and reverse transcription-PCR (RT-PCR).

Total RNA extraction and quantitative real-time RT-PCR. The expression of the *SEMA3B*, *HYAL3*, *HYAL1*, *HYAL2*, *TUSC2*, *RASSF1*, *ZMYND10*, *NPRL2*, *TMEM115* and *CACNA2D2* genes in the breast cell lines were analyzed by quantitative real-time RT-PCR. Total RNA was extracted from the untreated and 5-Aza-dC and TSA treated cultured breast cell lines using the RNeasy Mini Kit (Qiagen) and subsequently treated with DNase I to degrade any contaminating genomic DNA. mRNA was reverse transcribed from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction was primed with random primers in a 20 µL volume, according to the manufacturer's instructions. The reverse transcription reaction was diluted 1:20 with sterile H₂O before addition to the RT-PCR reaction. The diluted reverse transcription reaction (1 µL) was used in a 10 µL reaction with 2x TaqMan Universal PCR Master Mix and 0.5 µL of the 20X TaqMan Gene Expression Assay (primer/probe sets) (Applied Biosystems) for *SEMA3B* (Hs01090156_m1), *HYAL3* (Hs00185910_m1), *HYAL1* (Hs00201046_m1), *HYAL2* (Hs00186841_m1), *TUSC2* (Hs00200725_m1), *RASSF1* (Hs00200394_m1 all transcripts and Hs00945257_m1 for isoform A), *ZMYND10* (Hs00210720_m1), *NPRL2* (Hs00198012_m1), *TMEM115* (Hs00183656_m1), and *CACNA2D2* (Hs01021049_m1). The reactions were performed in duplicate in the StepOne Real-time PCR System (Applied Biosystems), and the results were calculated using the $\Delta\Delta C_t$ method normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression levels. This endogenous control was experimentally chosen based on amplification of cDNA obtained from treated and untreated MCF7 and MDA-MD-231 cells in the TaqMan® Human Endogenous Control Plate

(Applied Biosystems). The data were evaluated using GeNorm software to detect the most stable genes under these experimental conditions.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed on breast cell lines using the MAGnify Chromatin Immunoprecipitation System (Invitrogen), according to the manufacturer's instructions. The fixed chromatin complexes were immunoprecipitated using 1 µg of either an anti-trimethyl-histone H3K9 antibody or an anti-acetyl-histone H3K9 antibody (Invitrogen). Controls were also performed using rabbit and mouse antibodies and showed negligible levels of background precipitation. Immunoprecipitated DNA was quantified by quantitative real-time PCR. Oligonucleotide primer sequences for the *TMEM115*, *TUSC2*, *NPRL2* and *ZMYND10* genes were designed using the Primer Express Software (Applied Biosystems), as indicated in Table S2. The amount of immunoprecipitated target was measured using the StepOne Real-time PCR System (Applied Biosystems). Reactions containing 10 µL volume were set up using 2x SYBR GREEN PCR Master Mix (Applied Biosystems), 200 nM of each primer and 2 µL of immunoprecipitated DNA, no-antibody control, or input chromatin for each PCR. Each experiment was set up in duplicate. The cycling conditions were as follows: 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min, repeated for 40 cycles, followed by a melt curve stage. The data were normalized using the input control and the chromatin enrichment was calculated based on the formula $\text{Input Enrichment} = 2^{(\text{Average Ct}_{\text{Input}} - \text{Average Ct}_{\text{IP}})} \times \text{dilution factor}$.

DNA isolation and sodium bisulfite treatment. Genomic DNA was prepared from untreated breast cell lines by proteinase K digestion followed by conventional extraction using a phenol/chloroform protocol. One microgram of genomic DNA was subjected to sodium bisulfite treatment using the EpiTect® Bisulphite Kit (Qiagen) according to the manufacturer's instructions. Ten nanograms of eluted DNA were used directly for the HRM assays.

High resolution melting (HRM) analysis. After DNA sodium bisulfate treatment, PCR amplification and HRM were performed on the StepOne Real-time PCR System (Applied Biosystems), according to the manufacturer's instructions. The primer sequences for *CACNA2D2*, *HYAL1*, *RASSF1*, *TMEM115*, *TUSC2*, *NPRL2* and *ZMYND10* are listed in Table S2. PCR was performed in a 20 µL volume containing 1x MeltDoctor HRM Master Mix (Applied Biosystems), 200 nM of each primer and 10 ng bisulfite-treated DNA template. A standard curve with known methylation ratios (100, 75, 50, 25 and 0%) was included in each assay to estimate the methylation ratio of each sample using CpG Methylated HeLa Genomic DNA (New England BioLabs) and EpiTect Unmethylated Human Control DNA as references. Methylation standards were performed in duplicate, and cell line samples were performed in triplicate. The cycling conditions were as follows: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min; followed by an HRM step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, and continuous acquisition from 60 to 95°C at 0.3%. HRM data were analyzed using the High Resolution Melting Software (Applied

Biosystems). The data were visualized as normalized melting curves and difference plots.

Analysis of DNA copy number by qPCR. The primer sequences for the *RASSF1*, *TMEM115*, *TUSC2*, *NPRL2* and *ZMYND10* genes are the same as used in the ChIP analysis. Reactions of 10 µL were set up using 2X SYBR GREEN PCR Master Mix (Applied Biosystems), 200 nM of each primer, and 10 ng of DNA in each PCR reaction, set up in duplicate. The cycling conditions were as follows: 10 min at 95°C, then 40 cycles at 95°C for 15 sec and 60°C for 1 min, followed by a melt curve stage, in a total volume 10 µL, in duplicate. The qPCR data were analyzed using the $2^{-\Delta\Delta\text{ct}}$ method based on the DNA copy number ratio of the target gene compared with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene in a given cell line sample relative to two standard deviations from the normal pool DNA sample.

Statistical analysis. All data are presented as the mean ± SD of independent measurements. For statistical comparisons of gene expression levels between two groups (non-tumorigenic vs. breast cancer cells), the non-parametric Mann-Whitney test was used. The Spearman correlation coefficient *r* was determined for every paired data set after each treatment. All data were analyzed using GraphPad Prism for Windows, version 5.0. *p* values of < 0.05 were regarded as statistically significant. Data Assist™ software (Applied Biosystems) was used to display graphically the results of the hierarchical clustering analysis of gene expression levels before and after each treatment, based on Euclidean distance and average linkage.

Conclusion

In summary, we have described that, among the candidate TSGs mapped to 3p21.3, the *RASSF1A* isoform was epigenetically silenced in most of the epithelial breast cell lines analyzed, whereas the *RASSF1C* isoform was overexpressed only in a subset of breast cancer cell lines. The treatment of the cell lines with the demethylating agent 5-Aza-dC, alone or in combination with TSA, led to a significant increase in the expression levels of the *HYAL1* and *CACNA2D2* genes. Genes that mapped closest to the *RASSF1* showed a more modest increase in expression levels, but pair-wise analysis showed that the *RASSF1* gene was co-regulated with *TUSC2*, *ZMYND10* and *NPRL2* genes. Furthermore, the promoter regions of these genes were unmethylated at the associated CpG islands in the untreated cells and showed low levels of trimethylation at lysine 9 of the H3 histone. These data suggest that other repressive chromatin modifications should mediate the epigenetic repression of neighboring genes in the 3p21.3 cluster.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:

www.landesbioscience.com/journals/epigenetics/article/18271

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