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BMP8B Is a Tumor Suppressor Gene Regulated by Histone Acetylation in Gastric Cancer

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ABSTRACT

Different from genetic alterations, the reversible nature of epigenetic modifications provides an interesting opportunity for the development of clinically relevant therapeutics in different tumors. In this study, we aimed to screen and validate candidate genes regulated by the epigenetic marker associated with transcriptional activation, histone acetylation, in gastric cancer (GC). We first compared gene expression profile of trichostatin A-treated and control GC cell lines using microarray assay. Among the 55 differentially expressed genes identified in this analysis, we chose the up-regulated genes *BMP8B* and *BAMBI* for further analyses, that included mRNA and histone acetylation quantification in paired GC and nontumor tissue samples. *BMP8B* expression was reduced in GC compared to nontumor samples ($P < 0.01$). In addition, reduced *BMP8B* expression was associated with poorly differentiated GC ($P = 0.02$). No differences or histopathological associations were identified concerning *BAMBI* expression. Furthermore, acetylated H3K9 and H4K16 levels at *BMP8B* were increased in GC compared to nontumors ($P < 0.05$). However, reduced levels of acetylated H3K9 and H4K16 were associated with poorly differentiated GC ($P < 0.05$). Reduced levels of acetylated H3K9 was also associated with diffuse-type histological GC ($P < 0.05$). Notably, reduced *BMP8B* mRNA and acetylated H4K16 levels were positively correlated in poorly differentiated GC ($P < 0.05$). Our study demonstrated that *BMP8B* seems to be a tumor suppressor gene regulated by H4K16 acetylation in poorly differentiated GC. Therefore, *BMP8B* may be a potential target for TSA-based therapies in this GC sample subset. J. Cell. Biochem. 118: 869–877, 2017.

KEY WORDS: GENE EXPRESSION REGULATION; HISTONE ACETYLATION; TRICHOSTATIN A; GASTRIC CANCER; *BMP8B*; *BAMBI*

Conflicts of interest: None.

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Despite the declining incidence in recent years, gastric cancer (GC) is the third leading cause of cancer death worldwide [Ferlay et al., 2015]. The frequent detection at advanced stages and the limited therapeutic options in these cases contribute to the fact that GC presents a very poor prognosis and higher fatality-to-case ratio than prostate and breast cancers [Guggenheim and Shah, 2013]. Therefore, the better understanding of GC pathogenesis is essential for the identification of molecular prognosis and diagnosis markers as well as novel therapeutic methods.

Different from genetic alterations, the reversible nature of the epigenetic modifications provides an interesting opportunity for the development of clinically relevant therapeutics [Hamm and Costa, 2015]. Histone acetylation is the best characterized post-translational modification of the chromatin structure and results from a dynamic balance of the activity of two enzymatic families: histone acetyltransferases (HATs) and histone deacetylases (HDACs) [Legube and Trouche, 2003]. In general, the transfer of acetyl group to N-terminal lysine (K) residues in the histone tails by HATs results in a more relaxed chromatin and gene transcription. Conversely, the removal of acetyl group from K residues by HDACs prevents the binding of transcriptional regulatory elements to promoters, resulting in gene silencing [Yang et al., 2014]. Alterations in the balance of the activities of these enzymes lead to a disruption of cellular integrity and are frequently observed in different tumors. In particular, increased deacetylation is involved in epigenetically mediated tumor suppressor gene silencing. Thus, HDACs represent a promising class of anticancer drug targets [Seidel et al., 2012].

HDAC inhibitors (HDACis) are epigenetic agents that act by modifying gene expression to restore the normal differentiation or apoptosis of tumor cells, altering the chromatin structure, acetylating promoter regions, or disabling co-repressors [Bose et al., 2014]. As a result of a large amount data that emerged concerning the mechanisms of action of these agents, the HDACis vorinostat and romidpesin were approved for the treatment of patients with cutaneous and peripheral T-cell lymphoma, respectively [Bose et al., 2014]. Other HDACis may have the potential to be explored as antineoplastic agents in other tumors.

Trichostatin A (TSA) belongs to the hydroxamic acid chemical class HDACis, which selectively inhibit the class I and II mammalian HDAC families. Previously, our research group demonstrated that TSA treatment leads to the reduction of GC cell proliferation and increases the expression of the tumor suppressor gene, *CDKN1A* [Wisnieski et al., 2014]. These results suggest that reduced histone acetylation may play an important role in tumor suppressor gene silencing in gastric carcinogenesis and TSA may reverse this process, with potential therapeutic application.

In this study, we first aimed to identify candidate genes regulated by histone acetylation by comparing gene expression profile of TSA-treated and untreated GC cell lines. Among the candidate genes identified in our analysis, we selected two up-regulated genes belonging to the transforming growth factor-β (TGF-β) superfamily for further analysis: *bone morphogenetic 8B* (*BMP8B*) and *bone morphogenetic protein and activin membrane-bound inhibitor* (*BAMBI*). We also aimed to evaluate the mRNA and acetylated histone levels of these potential therapeutic targets in pairs of GC and the corresponding adjacent nontumor tissue samples.

MATERIALS AND METHODS

CELL LINES AND TSA TREATMENT

The ACP02 and ACP03 cell lines were previously established by our research group from primary gastric adenocarcinomas classified as diffuse and intestinal types, respectively [Leal et al., 2009]. The culture of these cell lines and the determination of the best dosage of TSA were performed as described previously by our research group [Wisnieski et al., 2014]. The ACP02 and ACP03 cell lines were treated separately with 250 nM TSA (Sigma–Aldrich, St. Louis, MO) in three independent experiments. In each experiment, the corresponding untreated cell line was used as control. After 24 h of TSA treatment, the cells were trypsinized and washed twice with phosphate-buffered saline (GIBCO, Grand Island, NY) for RNA extraction.

CLINICAL SAMPLES

Forty-two matched pairs of GC and the corresponding adjacent nontumor tissues were obtained from patients with gastric adenocarcinoma who underwent gastric resection in João de Barros Barreto University Hospital (HUJBB) and São Paulo Hospital (HSP), Brazil, from 2009 to 2014. None of the patients had a history of exposure to either chemotherapy or radiotherapy before surgery, and there was no other co-occurrence of diagnosed cancers. Written informed consent with the approval of the ethics committees of HUJBB and HSP was obtained from all patients before sample collection (Ethics Committee number 0511/09).

All of the samples were classified according to Lauren [1965] and the tumors were staged according to the American Joint Committee on Cancer (AJCC) TNM staging criteria [Washington, 2010]. The presence of *Helicobacter pylori*, a class I carcinogen, in gastric samples was detected by PCR, as performed previously by our research group [Wisnieski et al., 2014]. Table I shows the clinicopathological characteristics of the patients diagnosed with GC.

RNA EXTRACTION

Total RNA was isolated from cell lines and tissue samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and the AllPrep DNA/RNA/Protein Kit (Qiagen), respectively, according to the manufacturer's instructions. The RNA concentration and quality were measured using the Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). The integrity of RNA was assessed by Bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technologies, Waldbronn, Germany) or gel electrophoresis. Only samples with an RNA integrity number of $\geq$7 and no DNA contamination were used in the microarray hybridization.

MICROARRAY AND BIOINFORMATICS ANALYSES

The comparison between the transcriptome of TSA-treated and control GC cell lines was performed using the Affymetrix Human Gene 1.0 ST array, following the manufacturer's instructions. Microarray data are available in the ArrayExpress database under accession number E-MTAB-4736. Gene expression values were obtained using the three-step robust multiarray average preprocessing method implemented in the Affy package from R/Bioconductor. The RankProd method was employed for the selection of

TABLE I. Associations Between Clinicopathological Features, *BMP8B*, and *BAMBI* Gene Expression

		BMP8B mRNA			*BAMBI* mRNA	
Variable	n (%)	ΔCt (mean ± SD)	*P*	n (%)	ΔCt (mean ± SD)	*P*
Sex						
Male	21 (50)	7.84 ± 1.08	0.09	23 (56)	4.45 ± 1.97	<0.01*
Female	21 (50)	8.45 ± 1.19		18 (44)	6.15 ± 1.50	
Onset (years)						
≤45	11 (26)	7.96 ± 1.36	0.56	10 (24)	4.60 ± 2.02	0.15**
>45	31 (74)	8.21 ± 1.10		31 (76)	5.39 ± 1.93	
Tumor location						
Cardia	2 (5)	7.65 ± 1.80	0.50	2 (5)	3.93 ± 1.32	0.67**
Noncardia	39 (95)	8.21 ± 1.13		39 (95)	5.26 ± 1.97	
Histopathological type[a]						
Intestinal type	28 (67)	7.96 ± 1.26	0.16	30 (73)	4.85 ± 1.91	0.09**
Diffuse type	14 (33)	8.50 ± 0.87		11 (27)	6.15 ± 1.83	
Tumor differentiation						
Poorly	18 (51)	8.53 ± 1.10	0.02*	16 (47)	5.29 ± 1.63	0.31**
Moderately	17 (49)	7.62 ± 1.16		18 (53)	4.54 ± 1.92	
Tumor invasion						
T1/T2	10 (24)	8.20 ± 1.56	0.86	10 (24)	5.37 ± 2.43	0.84**
T3/T4	32 (76)	8.13 ± 1.04		31 (76)	5.14 ± 1.83	
TNM stage[b]						
I/II	22 (52)	8.29 ± 1.13	0.41	21 (51)	5.29 ± 1.90	0.92**
III/IV	20 (48)	7.98 ± 1.21		20 (49)	5.10 ± 2.07	
Stage						
Early	3 (7)	8.70 ± 0.94	0.40	3 (7)	7.23 ± 0.94	0.26**
Advanced	39 (93)	8.10 ± 1.18		38 (93)	5.03 ± 1.93	
Lymph node metastasis						
Absent	11 (26)	8.43 ± 0.89	0.35	10 (24)	5.75 ± 1.25	0.82**
Present	31 (74)	8.04 ± 1.24		31 (76)	5.02 ± 2.12	
H. pylori infection[c]						
Absent	14 (33)	8.26 ± 1.18	0.65	13 (32)	5.39 ± 1.53	0.95**
Present	28 (67)	8.08 ± 1.17		28 (68)	5.10 ± 2.15	

n, number of samples; SD, standard deviation. Gene expression levels are inversely proportional to the ΔCt values.
*Differentially expressed between groups by univariate GLM ($P < 0.05$).
**All *P* values were adjusted for sex.
[a]According to the Lauren [1965] classification.
[b]According to the AJCC [Washington, 2010].
[c]Detected in tumoral samples as described by Wisnieski et al. [2014].

differentially expressed genes (DEGs), considering a *P*-value cutoff of 0.05 adjusted for false discovery rate. A functional annotation analysis of DEGs was performed using the Ingenuity Pathway Analysis (IPA) tool.

cDNA SYNTHESIS AND REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

cDNA was synthesized using High-Capacity® cDNA Reverse Transcription (Life Technologies, Foster City, CA) following the manufacturer's protocol. The reaction to detect the mRNA levels of *BMP8B* (Hs01629120_s1) and *BAMBI* (Hs03044164_m1) from the cell lines and tissue samples was performed in triplicate using TaqMan® hydrolysis probes purchased as Assays-on-Demand (Life Technologies), and the Applied Biosystems 7500 fast real-time PCR system (Life Technologies). For the validation of *BMP8B* and *BAMBI* mRNA levels, we used the same RNA samples from cell lines employed in the microarray assay. The mRNA levels were analyzed using the ΔCt method, in which the target mRNA level was normalized to the geometric mean of the reference gene expression. The suitable reference genes *ACTB* + *B2M* (Hs03023943_g1 + Hs00984230_m1) and *GAPDH* + *B2M* (Hs99999905_m1 + Hs00984230_m1) were used for the analyses of cell lines and tissue samples, respectively, as determined previously by our research group [Wisnieski et al., 2013].

CHROMATIN IMMUNOPRECIPITATION (ChIP)

Briefly, 50 mg of GC and corresponding nontumor tissues were homogenized in 400 μL ice-cold douncing buffer (10 mM Tris, 4 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) and incubated with 0.4 U/mL micrococcal nuclease (Sigma–Aldrich) for 12 min at 37°C. The DNase activity was stopped by adding 0.5 M EDTA (pH 8.0). After hypotonization, 700 μL from each sample were used for input DNA and gel electrophoresis to confirm the digestion of chromatin into mononucleosomes. The remaining supernatant was divided into two 1.6 mL aliquots for ChIP. To each 1.6 mL aliquot, 160 μL of 10× incubation buffer (50 mM EDTA, 200 mM Tris, 500 mM NaCl, pH 7.5) were added together with 3.5 μL of the antibody against acetylated histone H3K9 (AcH3K9) or acetylated histone H4K16 (AcH4K16; Millipore, Temecula, CA). Negative controls were obtained using a nonantibody pull-down. After the recovery of immune complexes, samples were digested with 20 mg/mL proteinase K for 3 h at 52°C and purified using the QIAquick PCR Purification Kit (Qiagen). Input and ChIP-enriched DNA fractions were quantified in duplicate reactions using oligonucleotides for five *BMP8B* regions (Table II) in the Applied Biosystems 7500 fast real-time qPCR system (Life Technologies). SYBR Green (Qiagen) was used to detect the product amplification. The ChIP-qPCR data were analyzed in the same way as the mRNA data, except that the ChIP levels were normalized to the corresponding input DNA.

TABLE II. Oligonucleotide Sequences for the ChIP-qPCR Analysis of *BMP8B*

Amplicon[a]	Forward primer	Reverse primer
−1583	TGTACTGACCTGGCATAAGTTC	CATACCATTGGAACCTCCTCTT
−858	GCTGACTGTTACCTGCCATTA	GCAGAGGGAAGAGCTTGTG
+138	CGGCGACAGACGGATTG	TGGCTCCTGGACGAGAG
+935	TGGTCAGGAGCAGAGTGT	TTCTTACTCCTGGGTGTGTTTG
+1539	CATCTGCTTTGCCTCCTGT	CTGATCTGTGGTCCTTGGTTC

[a]The amplicon name indicates the position of the central base pair of the amplicon relative to the TSS of *BMP8B* (base pairs).

STATISTICAL ANALYSIS OF GENE EXPRESSION AND HISTONE ACETYLATION DATA

First, the Shapiro–Wilk normality test was used to evaluate the distribution of all data. Gene expression and histone acetylation data from tissues were not normally distributed and were transformed (*z*-score). Values less than −2 or more than +2 were considered outliers and excluded from the analyses. Repeated-measures general linear model (GLM) was performed to compare gene expression and histone acetylation levels between GC and corresponding adjacent nontumor samples. Univariate GLM was used to evaluate possible associations between clinicopathological features and gene expression or histone acetylation levels. This test was also used to compare the histone acetylation levels among the different studied gene regions. When more than two groups were compared simultaneously, the Bonferroni and Games–Howell post-hoc tests were applied. Gene expression data from cell lines were normally distributed and Pearson test was used to evaluate possible correlations between microarray and RT-qPCR methodologies. Spearman test was used to evaluate possible correlations between gene expression and histone acetylation levels in a sample subset. For correlations tests, values less than 0.40 were determined as a weak correlation, 0.40–0.59 as a moderate correlation, 0.6–0.79 as a strong correlation, and $\geq$0.80 as a very strong correlation. In all analyses, differences were considered significant at $P < 0.05$.

RESULTS

IDENTIFICATION AND SELECTION OF CANDIDATE GENES REGULATED BY HISTONE ACETYLATION

Using the microarray assay, we identified a total of 55 DEGs, 28 up-regulated, and 27 down-regulated, when comparing TSA-treated and control GC cell lines (Table III). We observed four relevant functions enriched with a significant number of our DEGs (Table IV): (i) Molecular transport, RNA trafficking, and gene expression (15 genes); (ii) Cancer, cellular development, and tumor morphology (11 genes); (iii) Cell death and survival, cellular movement, cellular growth, and proliferation (3 genes); and (iv) Cell death and survival, cellular assembly and organization, and cellular development (1 gene). *BMP8B* and *BAMBI* were up-regulated DEGs identified by microarray analysis, which were included in the IPA functional categories 3 and 2, respectively.

BMP8B AND *BAMBI* ARE UP-REGULATED GENES IN TSA-TREATED GC CELLS

BMP8B mRNA levels evaluated by microarray and RT-qPCR were strong correlated (r = 0.64, $P = 0.03$; Table V). In addition, a very strong correlation between *BAMBI* mRNA levels assessed by these two methodologies was also observed (r = 0.81, $P < 0.01$; Table V).

BMP8B IS DOWN-REGULATED IN GC TISSUES

BMP8B mRNA expression was reduced in GC tissues compared to corresponding adjacent nontumor samples ($P < 0.01$; Fig. 1A). Poorly differentiated GC samples presented a significantly reduced *BMP8B* mRNA expression in relation to moderately differentiated

TABLE III. List of 55 DEGs by Comparing TSA-Treated and Control GC Cell Lines Using the RankProd Analysis

ProbeSet ID	Gene symbol	Fold change[a]	*P*
8140211	*SPDYE8P*	2.39	<0.01
8022506	–	2.29	<0.01
8147049	*FABP5*	2.25	<0.01
7904959	–	2.21	<0.01
8124645	*OR12D3*	2.20	<0.01
8167562	*GAGE12B*	2.16	<0.01
8167862	*PAGE2B*	2.15	<0.01
8016259	*LRRC37A2*	2.13	<0.01
7948420	*FABP5*	2.06	<0.01
7911283	*OR2T3*	2.05	<0.01
8091283	*PLOD2*	1.99	<0.01
8045159	–	1.92	<0.01
7914180	*SPCS2*	1.92	0.02
7965721	–	1.86	0.02
8156253	*FAM75C1*	1.85	0.01
8132830	–	1.81	0.03
8046408	*PDK1*	1.80	0.04
7986598	*GOLGA6L1*	1.78	0.03
7915252	*BMP8B*	1.77	0.04
7929256	–	1.76	0.03
7938390	*ADM*	1.75	0.04
8054800	–	1.75	0.03
8007848	*MAPK8IP1*	1.74	0.04
8035779	*ZNF253*	1.73	0.03
8136786	–	1.72	0.04
7926875	*BAMBI*	1.71	0.04
8044669	*RABL2A*	1.69	0.02
8125537	*HLA-DMA*	1.41	0.03
8108370	*EGR1*	−1.33	0.02
7975779	*FOS*	−1.35	0.03
8118548	*HLA-DRA*	−1.40	0.04
8145795	–	−1.45	0.01
7939897	*FOLH1*	−1.46	0.04
8149361	*FAM90A7*	−1.55	0.02
8180291	–	−1.56	0.04
7952750	–	−1.63	0.01
7997933	*RPL13*	−1.64	0.04
8013329	*SNORD3A*	−1.66	0.04
7953665	*DPPA3*	−1.68	0.04
8124650	*UBD*	−1.69	0.01
8139977	*STAG3L3*	−1.70	0.03
8171876	*METTL1*	−1.72	0.02
8180331	–	−1.73	0.02
8175543	*SPANXE*	−1.74	0.02
7981874	*GOLGA8E*	−1.74	0.02
7960896	*OR7E87P*	−1.76	0.02
8118028	*VARS2*	−1.79	0.01
7953812	*PHC1*	−1.80	0.01
8023154	*TCEB3CL*	−1.81	0.01
8124926	*BAT1*	−1.82	0.01
8030187	*CGB5*	−1.88	0.01
8174664	–	−1.90	0.01
8049888	*ATG4B*	−1.92	0.01
8095364	*TMPRSS11E*	−2.35	<0.01
7909146	*FAM72D*	−2.84	<0.01

[a]Estimated percentage of false-positive predictions.

TABLE IV. Functional Categories of the DEGs by IPA

Functional category (number of genes)	Score[a]	Genes
Molecular transport, RNA trafficking, and gene expression (15)	35	*ATG4B*, *DDX39B*, *FABP5*, *FAM72D*, *FOLH1*, *GOLGA6L1*, *LRRC37A2*, *METTL1*, *PHC1*, *RABL2A*, *SPCS2*, *TCEB3C*, *TMPRSS11E*, *VARS2*, *ZNF253*
Cancer, cellular development, and tumor morphology (11)	23	*ADM*, *BAMBI*, *CGB*, *EGR1*, *FOS*, *HLA-DMA*, *HLA-DRA*, *MAPK8IP1*, *PDK1*, *RPL13*, *UBD*
Cell death and survival, cellular movement, cellular growth, and proliferation (3)	5	*BMP8B*, *PLOD2*, *SNORD3A*
Cell death and survival, cellular assembly and organization, and cellular development (1)	3	*GAGE12B*

[a]The calculated score for each functional category is derived from a P-value and indicates that the probability of genes has been found in a functional category and not by chance. A score of 2 or more has at least 99% confidence to be not generated by chance.

GC ($P = 0.02$; Table I; Fig. 1B). Moreover, poorly differentiated GC also showed reduced *BMP8B* mRNA expression compared to nontumor samples ($P = 0.03$; Fig. 1B).

BAMBI mRNA expression did not differ between GC tissue and corresponding adjacent nontumor samples ($P = 0.63$). Reduced *BAMBI* expression (fold change < 0.5) was observed in 30% of GC samples in relation to nontumor samples. On the other hand, increased *BAMBI* expression (fold change > 2.0) was observed in 37% of GC samples in relation to nontumor samples. Increased *BAMBI* expression was associated with male individuals compared to females ($P < 0.01$; Table I).

HISTONE ACETYLATION LEVEL AT *BMP8B* IS ALTERED IN GC TISSUES

In GC tissues, an enrichment of AcH3K9 was observed in *BMP8B* regions nearest to transcription start site (TSS) compared to the other evaluated regions ($P < 0.05$ for all comparisons; Fig. 1C). A very similar pattern was observed in nontumor samples (Fig. 1C). However, GC tissues presented significantly increased AcH3K9 levels in relation to nontumor samples at all evaluated regions ($P < 0.05$ for all comparisons; Fig. 1C), with exception of the +935 ($P = 0.14$; Fig. 1C).

The distribution of AcH4K16 in both GC and adjacent nontumor samples was similar to H3K9 results. However, only adjacent nontumor samples presented statistical differences among the studied BMP8B regions ($P < 0.05$ for all comparisons; Fig. 1D). In addition, GC tissues presented significantly increased AcH4K16 levels compared to nontumor samples at all studied regions ($P < 0.05$ for all comparisons; Fig. 1D), with exception of the +935 ($P = 0.13$; Fig. 1D).

We also observed that reduced AcH3K9 at −1583 ($P = 0.03$), −858 ($P = 0.02$), and +138 ($P = 0.01$) regions was associated with diffuse-type GC compared to intestinal-type GC (Table SI). A significantly reduced AcH3K9 at +138 ($P = 0.02$), as well as AcH4K16 at −858 ($P = 0.03$), +935 ($P = 0.03$), and +1539 ($P = 0.01$) regions, was also associated with poorly differentiated GC compared to moderately differentiated tumors (Table SI).

BMP8B mRNA AND ACETYLATED HISTONES LEVELS ARE CORRELATED IN GC TISSUES

A strong positive correlation between *BMP8B* mRNA expression and AcH4K16 levels was observed at −858 ($r = 0.69$, $P = 0.01$; Table VI) and +138 ($r = 0.65$, $P = 0.04$; Table VI) regions in GC samples. A moderate positive correlation between these data were also detected at +1539 ($r = 0.59$, $P = 0.03$; Table VI). Additionally, we detected a strong to very strong correlation of specifically AcH3K9 and AcH4K16 levels between the different combinations of the studied *BMP8B* regions (Table VI).

DISCUSSION

The use of microarray assay has been considered an important tool for the screening of genes regulated by DNA and histone methylation in GC [Liu et al., 2014a; Zhu et al., 2015]. However, to our knowledge, the large-scale screening of genes regulated by histone acetylation was performed only in GC cell lines established from tumors of East Asian patients [Yasui et al., 2003; Lee et al., 2004]. Our results demonstrated 55 DEGs after TSA treatment, confirming a previous study that demonstrated a selective action of this HDACi in cancer cell lines [Van Lint et al., 1996]. Most of the DEGs identified in our study were associated with important biological functions, including gene expression regulation, carcinogenesis, cell death, and proliferation. Because increased expression of HDACs is known to be involved in tumor suppressor silencing in carcinogenesis and increased acetylation via TSA treatment may restore the activity of these genes, we chose and validated two up-regulated DEGs belonging to the TGF-β superfamily, *BMP8B* and *BAMBI*.

TABLE V. Correlation Between Data From Microarray Assay and RT-qPCR

	BMP8B			*BAMBI*		
GC cells	Microarray[a] (mean ± SD)	RT-qPCR[b] (mean ± SD)	r (*P*)	Microarray[a] (mean ± SD)	RT-qPCR[b] (mean ± SD)	r (*P*)
Control	3.44 ± 0.26	0.0004 ± 0.0003	0.64	7.14 ± 0.51	0.38 ± 0.17	0.81
TSA	4.26 ± 0.49	0.0024 ± 0.005	(0.03*)	7.91 ± 0.46	0.74 ± 0.33	(<0.01*)

SD, standard deviation; r, Pearson co-efficient of correlation.
*Significant correlation ($P < 0.05$).
[a]Fluorescence signal values.
[b]$2^{-\Delta Ct}$ values, in which the expression of the target gene was normalized by the geometric mean of *ACTB* + *B2M* expression.

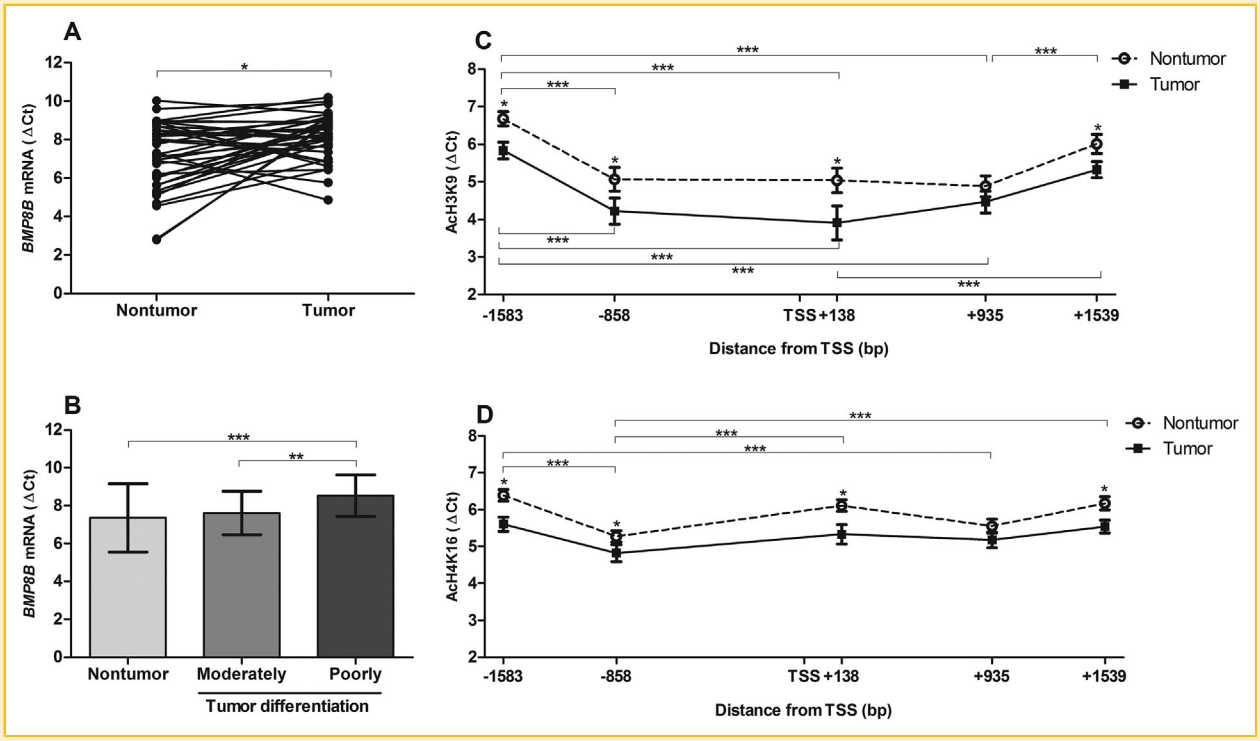

Fig. 1. *BMP8B* mRNA and acetylated histone levels in gastric tissues. (A) *BMP8B* mRNA in pairs of GC and the corresponding adjacent nontumor samples, (B) association between *BMP8B* mRNA and tumor differentiation, (C) AcH3K9, and (D) AcH4K16 levels at the five *BMP8B* regions assessed by Chromatin Immunoprecipitation. Data are expressed as the mean ± standard deviation. * Significant difference between groups by repeated-measures GLM, ** by univariate GLM, *** or by univariate GLM followed by Bonferroni and Games–Howell post-hoc tests ($P < 0.05$). Ac: acetylated. Gene expression and histone acetylation levels are inversely proportional to the ΔCt values.

The TGF-β superfamily consists of more than 30 different members, including the TGF-βs, activins, NODAL, BMPs, growth and differentiation factors, and anti-Müllerian hormone [Wakefield and Hill, 2013]. Although the TGF-β ligands themselves have been widely studied in cancer, the other members belonging to the TGF-β superfamily have also crucial roles in cancer development and metastasis. The role of these less studied ligands in tumorigenesis frequently reflects their function in embryonic development or in adult tissue homeostasis, and their influence extends from the tumor microenvironment to cancer complications [Wakefield and Hill, 2013]. Therefore, they are plausive therapeutic targets to be studied.

BMPs were originally identified as molecules that induce ectopic bone and cartilage formation in rodents [Hogan, 1996]. Currently, it is well known that these secreted signaling molecules are involved in several biological processes, including organogenesis, cell proliferation, differentiation, migration, immune response, angiogenesis, and apoptosis [Khin et al., 2009]. Although BMPs gained increasing attention in cancer research in the last years, complex and controversial results were reported involving tumor suppression and tumor promotion aspects that depend on the tumor type and stage. In gastric carcinogenesis, BMP2, BMP4, and BMP7 were described to enhance cell migration, invasion, and metastasis [Katoh and Terada, 1996; Park et al., 2010; Aoki et al., 2011]. Conversely, BMP2, BMP4, BMP9, and BMP10 have been also reported to inhibit cell proliferation and migration as well as to induce cell cycle arrest and apoptosis [Shirai et al., 2011; Zhang et al., 2012; Duan et al., 2015; Lei et al., 2016]. In addition, BMP signaling has been described to involve targets previously studied by our research group. A study described that BMPs inhibit MYC transcriptional activity and expression in colorectal cancer [Lee et al., 2010]. MYC mRNA and protein overexpression and its gene amplification is a common finding in GC samples, GC cell lines, and some preneoplastic gastric lesions as well as in a nonhuman primate model of gastric carcinogenesis [Leal et al., 2016]. Furthermore, BMP2 was reported to demonstrate antiproliferative activity in GC via the activation of p21 [Wen et al., 2004], which is a known tumor suppressor with reduced expression in gastric tumors [Mitani et al., 2005; Do Nascimento Borges et al., 2010; Wisnieski et al., 2014, 2015].

Although different BMP members have been studied in gastric carcinogenesis, there is little information about the role of *BMP8B* in cancer. All these aforementioned factors make the study of *BMP8B* interesting in GC samples.

Our results demonstrated that *BMP8B* mRNA is significantly reduced in GC compared to the corresponding adjacent nontumor samples. To our knowledge, only two studies have reported the role of this gene in carcinogenesis. In pancreatic cancer tissue, *BMP8B* was found down-regulated compared to the adjacent normal tissue [Cheng et al., 2014]. The authors also reported that the induction of *BMP8B* overexpression inhibited cell growth, whereas its silencing exerted antiapoptotic effects in human pancreatic cancer cell lines.

TABLE VI. Correlation Between *BMP8B* mRNA and Histone Acetylation Levels in GC Tissues

		BMP8B mRNA	AcH3K9 −1583	AcH3K9 −858	AcH3K9 +138	AcH3K9 +935	AcH3K9 +539	AcH4K16 −1583	AcH4K16 −858	AcH4K16 +138	AcH4K16 +935	AcH4K16 +1539
BMP8B mRNA	ρ	1	−0.03	0.13	0.01	0.14	0.24	0.29	0.69*	0.65*	0.55	0.59*
	P	-	0.93	0.68	0.98	0.64	0.43	0.34	<0.01	0.04	0.05	0.03
AcH3K9 −1583	ρ	−0.03	1	0.66*	0.68*	0.71*	0.85*	0.08	0.19	−0.06	0.10	0.05
	P	0.93	-	0.01	0.02	<0.01	<0.01	0.79	0.53	0.88	0.75	0.87
AcH3K9 −858	ρ	0.13	0.66*	1	0.98*	0.96*	0.86*	−0.10	0.25	−0.08	0.01	−0.12
	P	0.68	0.01	-	<0.01	<0.01	<0.01	0.80	0.41	0.83	0.98	0.69
AcH3K9 +138	ρ	0.01	0.68*	0.98*	1	0.98*	0.86*	−0.08	0.27	−0.07	−0.13	−0.16
	P	0.98	0.02	<0.01	-	<0.01	<0.01	0.81	0.42	0.86	0.71	0.65
AcH3K9 +935	ρ	0.14	0.71*	0.96*	0.98*	1	0.89*	−0.06	0.23	−0.08	−0.01	-0.09
	P	0.64	<0.01	<0.01	<0.01	-	<0.01	0.85	0.46	0.83	0.99	0.76
AcH3K9 +1539	ρ	0.24	0.85*	0.86*	0.86*	0.89*	1	0.10	0.33	0.14	0.22	0.07
	P	0.43	<0.01	<0.01	<0.01	<0.01	-	0.75	0.27	0.70	0.48	0.82
AcH4K16 −1583	ρ	0.29	0.08	−0.10	−0.08	−0.06	0.10	1	0.72*	0.67*	0.70*	0.79*
	P	0.34	0.79	0.80	0.81	0.85	0.75	-	<0.01	0.03	<0.01	<0.01
AcH4K16 −858	ρ	0.69*	0.19	0.25	0.27	0.23	0.33	0.72*	1	0.76*	0.82*	0.90*
	P	<0.01	0.53	0.41	0.42	0.46	0.27	<0.01	-	0.01	<0.01	<0.01
AcH4K16 +138	ρ	0.65*	−0.06	−0.08	−0.07	−0.08	0.14	0.67*	0.76*	1	0.89*	0.76*
	P	0.04	0.88	0.83	0.86	0.83	0.70	0.03	0.01	-	<0.01	0.01
AcH4K16 +935	ρ	0.55	0.10	0.01	−0.13	−0.01	0.22	0.70*	0.82*	0.89*	1	0.87*
	P	0.05	0.75	0.98	0.71	0.99	0.48	<0.01	<0.01	<0.01	-	<0.01
AcH4K16 +1539	ρ	0.59*	0.05	−0.12	−0.16	−0.09	0.07	0.79*	0.90*	0.76*	0.87*	1
	P	0.03	0.87	0.69	0.65	0.76	0.82	<0.01	<0.01	0.01	<0.01	-

ρ, Spearman ρ co-efficient of correlation; Ac, acetylated.
*$P < 0.05$.

On the contrary, Mima et al. [2013] reported that high *BMP8B* mRNA expression was associated with a shorter GC patient survival time following a curative resection. Furthermore, the authors revealed that high *BMP8B* mRNA expression had an independent prognostic power compared to other standard prognostic markers, such as tumor size and the presence of the histological diffuse-type GC. The fact that Mima et al. evaluated mRNA expression in GC tissues and did not compare the mRNA levels of these samples to nontumor samples may explain the contradictory results. Other factors may also interfere in the results, such as ethnicity and experimental conditions for mRNA quantification.

Our results also showed that reduced *BMP8B* mRNA level was associated with poorly differentiated GC. Gastric adenocarcinomas may be classified as poorly, moderately, and well-differentiated types depending on its histological, biological, and genetic characteristics [Katoh and Terada, 1996]. Studies have demonstrated that cell differentiation is important for estimating the tumor progression and outcomes of patients with GC [Adachi et al., 2000; Zu et al., 2014]. The degree of differentiation of GC cells has been shown to correlate with tumor aggressiveness [Dicken et al., 2005]. Poorly differentiated tumors tend to grow quickly and be more invasive than well or moderately differentiated GC. In addition, bone metastasis is more frequently detected in poorly differentiated GC than in the well-differentiated tumors [Katoh and Terada, 1996]. Furthermore, a study has demonstrated that a BMP member modulates the differentiation of gastric cells by increasing the pepsinogen II, a differentiation marker of the stomach [Wen et al., 2004]. Therefore, our results suggest that *BMP8B* is a tumor suppressor gene down-regulated in GC and plays an important role in gastric epithelial cell differentiation.

BAMBI is a transmembrane glycoprotein that is homologous to the TGF-β type I receptors, except that it lacks an intracellular kinase domain required for signal transduction [Onichtchouk et al., 1999]. As a pseudoreceptor, this molecule was described to inhibit the TGF-β signaling by forming a heterodimer with TGF-β type II receptors [Onichtchouk et al., 1999]. Increased BAMBI expression has been reported in colorectal [Fritzmann et al., 2009], bladder [Khin et al., 2009], ovarian [Pils et al., 2010], and lung [Miao et al., 2009] tumors. However, little is known about the role of *BAMBI* in gastric carcinogenesis as well as the interrelationship between *BAMBI* and *BMP8B* in this neoplasia. To our knowledge, only two studies have reported increased *BAMBI* expression in GC from a Chinese population and it was associated with poor prognosis [Liu et al., 2014b; Zhang et al., 2014]. In our study, we detected a heterogeneous *BAMBI* mRNA expression in GC and corresponding adjacent nontumor samples. We believe that the observed heterogeneous pattern hindered us to determine the role of *BAMBI* in our sample size.

To understand the regulation of *BMP8B* as a tumor suppressor gene in gastric tumors, we quantified the histone acetylation levels in these samples. Our results first demonstrated that gastric samples present an enrichment of both AcH3K9 and AcH4K16 within the studied *BMP8B* region. This enrichment showed the same pattern of distribution in both GC and nontumor samples. Increased AcH3K9 and AcH4K16 levels were observed near to TSS compared to the

other evaluated *BMP8B* regions. This finding is in agreement with previous genome-wide studies that had stated a predominant distribution of AcH3K9 [Karmodiya et al., 2012] and AcH4K16 [Hayashi-Takanaka et al., 2015] in regions surrounding the TSSs. It is known that the acetylation of nucleosomal histones in TSS region facilitates the transcription by stabilizing the binding of chromatin remodeling factors to promoter regions and/or decreasing nucleosome occupancy at TSS [Horikoshi et al., 2013].

Although GC and nontumor samples demonstrated the same pattern of H3K9 and H4K16 acetylation distribution, GC samples showed an increased acetylation in these histone markers compared to adjacent nontumor samples. Similar results were reported recently by our research group, which demonstrated H3K9 and H4K16 hyperacetylation associated with promoter and coding *CDKN1A* regions in GC compared to adjacent nontumor samples [Wisnieski et al., 2015]. We hypothesize that the deregulation of HATs and HDACs in GC, as reported by our group [Wisnieski et al., 2014] may contribute to the *BMP8B* hyperacetylation observed in our tumor samples.

When histone acetylation levels in tumor samples were evaluated considering the clinicopathological features, reduced AcH3K9 and AcH4K16 at *BMP8B* promoter and coding regions was associated with poorly differentiated GC compared to moderately differentiated tumors. Reduced AcH3K9 at *BMP8B* promoter and coding regions was also associated with diffuse-type histological GC compared to intestinal-type GC. Diffuse-type GC presents poor prognosis and rapid progression than intestinal-type tumors. In general, this histological type is poorly differentiated with infiltrating, noncohesive tumor cells [Tokuhara et al., 2015]. Therefore, our results suggest that reduced levels of AcH3K9 and AcH4K16 within the studied *BMP8B* region may be involved in the development of poorly differentiated gastric tumors.

Because we detected significantly reduced *BMP8B* mRNA expression and histone acetylation levels associated with poorly differentiated GC, we performed correlation analyses to determine a possible relationship between mRNA and histone acetylation levels in this sample subset. Our results showed a positive correlation between the reduced levels of *BMP8B* mRNA and AcH4K16 in poorly differentiated GC. AcH4K16 has been identified as a marker of actively transcribed genes by creating or securing binding platforms for transcriptional factors as well as other chromatin-modifying enzymes [Zippo et al., 2009; Ruthenburg et al., 2011]. Moreover, a previous study had identified transcription factor motifs overlapping AcH4K16 sites localized near promoters and suggested that AcH4K16 directly regulates gene expression through effects on transcription factors [Horikoshi et al., 2013]. Therefore, we believe that reduced AcH4K16 levels may play a crucial role in the down-regulation of *BMP8B* gene expression in poorly differentiated GC.

In conclusion, *BMP8B* seems to be a tumor suppressor gene in GC, especially in poorly differentiated tumors. Although *BMP8B* expression was decreased in GC, increased AcH3K9 and AcH4K16 levels were detected in most of this gene studied regions. Conversely, reduced AcH4K16 levels may be a mechanism that explains the down-regulation of *BMP8B* in poorly differentiated GC. Therefore, *BMP8B* is a potential target for TSA-based therapies in this GC subset.

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