Gene expression profiling after treatment with the histone deacetylase inhibitor trichostatin A reveals altered expression of both pro- and anti-apoptotic genes in pancreatic adenocarcinoma cells

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Abstract

The histone deacetylase inhibitor trichostatin A (TSA) has been previously shown to block cellular growth in G2 and induce apoptosis in human pancreatic cancer cell lines. In order to better understand this phenomenon, we have analyzed the gene expression profiles in PaCa44 cells after treatment with TSA using microarrays containing 22,283 probesets. TSA was found to cause both the induction and repression of a large number of genes, although the number whose expression was up-regulated was greater than the number of genes that were down-regulated. When a threshold value of 3 was used as a cutoff level, a total of 306 (3.4%) of the detectable genes had altered expression. When categorized according to cellular function, the differentially expressed genes were found to be involved in a wide variety of cellular processes, including cell proliferation, signaling, regulation of transcription, and apoptosis. Moreover, Sp1/Sp3 transcription factor binding sites were significantly more abundant among TSA-induced genes. One prominent feature was the increased ratio between the levels of expression of pro-apoptotic (BIM) and anti-apoptotic (Bcl-XL and Bcl-W) genes. This result was confirmed in eight additional pancreatic cancer cell lines after treatment with TSA, suggesting that this event may be a strong determinant for the induction of apoptosis by TSA.
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1. Introduction

Although several common molecular alterations have been identified as key players in the oncogenesis and progression of primary pancreatic adenocarcinoma, including the K-*ras*, *p53*, *p16*, *DPC4*, and *FHIT* genes [1–3], current therapeutic and diagnostic strategies have gained little and prognosis remains dismal with a 5-year survival of less than 5%.

It is now well accepted that epigenetic mechanisms play a key role in the pathogenesis of cancer [4]. DNA methylation has been widely studied by a variety of experimental approaches, and molecules that interfere with DNA methylation have also been evaluated in clinical trials in both solid and hematologic malignancies [5,6]. Far less attention has been paid to histone acetylation, although multiple studies have demonstrated that histone acetylation, likewise, has an important role in the regulation of gene expression [7–9]. Histone acetylation is regulated by the activities of histone acetyl-transferase (HAT) and histone deacetylase (HDAC). Higher levels of histone acetylation have been associated with transcriptional activation due to the decreased interaction between histones and DNA, while hypoacetylated histones are generally associated with transcriptionally inactive chromatin. A role for histone acetylation and deacetylation in control of cell growth and

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apoptosis has also been demonstrated [10] and, more importantly, inhibitors of HDAC have been suggested to be applicable in the clinical treatment of human malignancies [11,12].

In recent years, a variety of compounds that inhibit HDAC have been identified, which cause growth arrest, differentiation and/or apoptotic cell death, including trichostatin A (TSA) [13,14]. Furthermore, these agents display selective toxicity to cancer cell lines [15,16] and also reduce growth of neoplastic cells in animal models at nontoxic doses [17]. Moreover, it has been reported that TSA can modulate gene expression during mouse embryogenesis with little or no apparent cytotoxic effects [18].

HDAC inhibitors may also have utility in the clinical management of pancreatic cancer. Recently, the HDAC inhibitor CI-994 was used in a randomized clinical trial in patients with advanced pancreatic cancer (cited in Ref. [19]). Disappointingly, there was no increase in survival when CI-994 was given alone or in combination with gemcitabine. However, it has been shown that CI-994 has a predominantly cytostatic effect due to its ability to arrest the cell cycle in the G1 phase [20]. From our recent studies, it is clear that TSA has a mechanism of action different from CI-994 in that it arrests cellular growth in G2 and induces apoptosis [14]. In fact, the possibility that different HDAC inhibitors may have diverse mechanisms of action has recently been reinforced by characterization of three different molecules that all induce cell death through an intrinsic apoptotic pathway as for TSA [21]. Nonetheless, a recent microarray analysis has hinted on the possibility that at least some HDAC inhibitors may have similar mechanisms of action, as hierarchical clustering of expression profiles demonstrated a greater similarity between suberoylanilide hydroxamic acid and TSA with respect to MS-27-275 [22]. At any rate, it has been suggested that the action of HDAC inhibitors on gene expression is restricted to a relatively small number of genes including *BCL2*-related pathways [21,23] and the molecular mechanisms of action of HDAC inhibitors remain poorly understood.

The continuing study of the effects of histone acetylation as related to gene expression is fundamental in order to identify possible therapeutic targets, given the antiproliferative effects of TSA and other HDAC inhibitors. To date, only a limited number of studies have been performed examining variations in gene expression induced by TSA in human tumor cells [22,24–26]. To better understand its mechanism of action, we have compared the gene expression profiles of the pancreatic cancer cell line PaCa44 before and after treatment with the HDAC inhibitor TSA. Among other features, we observed that the ratio between the levels of expression of pro-apoptotic (BIM) and anti-apoptotic (BCL-XL and BCL-W) genes was significantly increased. This result was confirmed with eight additional adenocarcinoma pancreatic cell lines treated with TSA, suggesting that a strong determinant for the induction of apoptosis by TSA

may be the consistent increased ratio of expression between pro-apoptotic and antiapoptotic genes.

2. Materials and methods

2.1. Cell culture and RNA extraction

Cells were grown in RPMI 1640 supplemented with 20 mM glutamine and 10% FBS (BioWhittaker Cambrex Bioscience, Milan, Italy) and were incubated at 37 °C with 5% CO_2. TSA was obtained from Sigma Chemical Company. A 3.3 mM solution of TSA in absolute ethanol was prepared and stored at −80 °C until use. Cells were incubated with 0.2 μM TSA for 8 h before harvesting. Total cellular RNA was prepared using TRIzol Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions.

For quantitative RT-PCR on BCL2L11 (BIM), BCL2L1 (large transcript, Bcl-XL), and BCL2L2 (Bcl-W), the cell lines CFPAC1, HPAF, MiaPaCa2, Panc1, PC, PSN1, PT-45P1, PaCa44, and T3M4 were used (see Ref. [27] for molecular characterization of these cell lines).

2.2. Total cell extract and Western blot analysis

Cells were collected, washed in phosphate-buffered saline, and resuspended in 0.2 N HCl, 1% β-mercaptoethanol, 1 mM PMSF and 1× protease inhibitor cocktail. After three cycles of freeze/thaw, the cellular lysate was centrifuged at 14,000×g for 10 min at 4 °C and the supernatant was used for Western blot analysis. Protein concentration was measured using the Bradford protein assay reagent (Pierce) using bovine serum albumin as a standard. Ten micrograms of cell protein extracts was electrophoresed through a 12% SDS-polyacrylamide gel and transferred onto PVDF membranes (Millipore, Bedford, MA) by electroblotting. Membranes were then incubated overnight at 4 °C with blocking solution [5% low-fat milk in TBST (100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween-20)] and were probed for 1 h at room temperature with anti-acetyl-histone H4 polyclonal antibody (Upstate Biotechnology) or anti-human α-tubulin monoclonal antibody (Oncogene) (1:1000 in blocking solution). Horseradish peroxidase conjugated anti-mouse IgG (1:1000 in blocking solution, Upstate Biotechnology) was used to detect specific proteins. Immunodetection was carried out using the chemiluminescent substrates (Pierce) and recorded by using a Hyper-filmECL (Amersham Pharmacia Biotech).

2.3. Oligonucleotide array hybridization

First- and second-strand cDNA were synthesized from 12.5 μg of total RNA according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). After in vitro transcription, labeling and fragmentation, probes were

hybridized to the GeneChip HG-U133A containing 22,283 probesets, corresponding to about 15,000 genes. The chips were washed in a GeneChip Fluidics Station 400 (Affymetrix), and the results were visualized with a Gene Array scanner using Affymetrix software.

2.4. Data and statistical analysis

Array data were scaled to achieve a target mean of 100. The ratios and confidence intervals for each probeset were determined using the MAS5 statistical algorithm. The probesets were classified as differentially expressed when the $\log_2$ transformed expression ratio was different from zero ($P<0.0025$) and the probeset was reliably detected ($P<0.05$) in either the treated or untreated samples. Analysis was performed using Affymetrix MAS 5.0 software.

The probeset annotations were retrieved from NetAffx analysis center [28]. A set of 4784 sequences was chosen for *cis*-regulatory element analysis as they were represented by single probesets in the HG-U133A Affymetrix Chip, targeted as present in at least one hybridization and unambiguously annotated with a unique Ensembl [29] gene identifier.

The nucleotide sequences of selected loci including 0.5 kb upstream from the transcriptional start site were obtained by querying the Ensembl database. The presence of transcription factor binding sites in upstream sequences was evaluated using MatInspector 2.1 [30] and Transfac 3.1 [31] matrix V\$SP1_Q6.

Functional analysis was performed on the basis of Gene Ontology(GO) terms related to biological function, as retrieved from the NetAffx Analysis Center. The original GO terms enclosed with the annotations of each gene corresponded to terminal nodes of the GO functional hierarchy. Thus, we enriched the annotations by adding to each gene, every GO term representing a major class that included the original ones. Successively, for each GO term we classified the genes according to two criteria, namely: an expression change greater then threefold, either as up- or down-regulation, and the presence of the GO term in their enriched set of annotations. Thus, we obtained a series of 2×2 contingency tables representing distributions of genes with respect to altered expression and membership to GO functional classes. A Fisher exact test was performed for each contingency table to find the GO terms that were significantly over- or underrepresented among the genes showing altered expression. Only those tests with a P value less than 0.005 were considered significant. As GO terms were generally redundant in expressing biological functions, due to the hierarchical nature of GO database, and most of the genes fell in more than one category, we focused on a group of categories which were adequately generic and possibly not overlapping, in order to represent more concisely our gene set. Where not otherwise specified, all calculations were carried out using the R statistical software package.

2.5. Quantitative real-time RT-PCR

Among the highly induced genes, seven were chosen to validate the array results. These genes were selected because they were represented by unique Affymetrix probesets, have no known alternative transcripts, and suitable primers could be designed for RT-PCR with at least one primer spanning an exon–exon junction. The software Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) was used to design primers.

Quantitative, real-time RT-PCR was performed on three independent RNA preparations from PaCa44 cells (untreated or treated with 0.2 μM TSA for 8 h as for microarray analysis). RNA was extracted as described above. cDNA first strand synthesis was performed starting from 1 μg of total RNA using the AMV First Strand cDNA Synthesis Kit for RT-PCR (Roche, Basel, Switzerland) according to the manufacturer's protocol.

The following primer pairs were used: *NGFR* (forward: CCTTCAAGAGGTGGAACAGCTG, reverse: CCACTGTCGCTGTGGAGTTTTT); *PGF* (forward: TGTTCAGCCCATCCTGTGTCTC, reverse: CCCAGAACGGATCTTTAGGAGC); *TNFRSF11A* (forward: ACCCTGGACCAACTGTACCTTC, reverse: TGGTGGTTTTCTTAGCTGGCA); *MFGE8* (forward: GCTTGCATCTCTTCAGCTGGAA, reverse: ACCTGCAGCCACTGATCTTAC); *CORO1A* (forward: GCCTGGAGGTGAACAAGTGTGA, reverse: TCCTGGAACAGGTCCGACTTTC); *GAPD* (forward: ATCATCAGCAATGCCTCCT, reverse: GGACTGTGGTCATGAGTCCT); *CDKN1B* (forward: CGCTGACTTCATGGAATGGAC, reverse: CATCCAACGCTTTTAGAGGCA); *CCNA2* (forward: TCCTCGTGGACTGGTTAGTTGA, reverse: GCACTGACATGGAAGACAGGAA).

PCRs were performed in 25-μl solution containing 1% of the reverse transcription product, 10 ng of primers of each primer and 12.5-μl SYBR Green PCR Master Mix (Applied Biosystems) and were performed in triplicate. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used with the following thermal cycle protocol: 94 °C 10 min, followed by 40 cycles of 94 °C for 1 min and 60 °C for 1 min. After the run, a dissociation protocol was performed to ensure the absence of nonspecific products. The *GAPD* transcript level was used as a reference.

Quantitative RT-PCR on apoptotic genes was performed using TaqMan® Gene Expression Assays (Applied Biosystems), namely Hs00708019_s1 for BCL2L11 (BIM), Hs00236329_m1 for BCL2L1 (large transcript, Bcl-XL), Hs00187848_m1 for BCL2L2 (Bcl-W), and Hs_00355782 for CDKN1A (p21) following the manufacturer's protocol.

3. Results

We have previously shown that TSA will inhibit growth of PaCa44 cells in a dose-dependent manner with an IC_{50} of

0.09 μM at 48 h [14]. This growth arrest is caused by an increase in both apoptosis and the fraction of cells in G2 phase of the cell cycle. To better understand the mechanism of action of TSA on cellular growth and apoptosis in PaCa44 cells, we examined the differences in global gene expression profiles after inhibition of HDACs. For this purpose, RNA was isolated from cells that were treated with 0.2 μM TSA for 8 h. This time was chosen in order to give a high level of histone H4 acetylation as determined by Western blot analysis of three pancreatic cancer cell lines (Fig. 1) and to observe the most direct effects of TSA on gene expression [32]. RNA from TSA-treated and untreated cells was subjected to analysis using Affymetrix U133A gene chips, and the resulting gene expressions profiles were compared. After prefiltering to eliminate data points that were scored as absent in both treated and untreated samples, a total of 13,413 detectable spots remained, representing about 9000 genes. The complete data set is available as supplementary material.

To verify the results of the microarray analysis, quantitative real-time RT-PCR was performed on seven randomly chosen genes among those scored as differentially expressed. As shown in Fig. 2, the fold change in these seven genes was similar to that found by microarray analysis, indicating the validity of the results. Fig. 3A shows a distribution of mRNA expression on the basis of the ratio of expression in the TSA-treated and untreated samples, while the data are presented numerically in Fig. 3B. TSA induced both the induction and repression of a large number of genes. However, the number of probesets whose expression was up-regulated was greater than the number of probesets that were down-regulated, especially when higher threshold values were considered (Fig. 3A and B). When a threshold value of 3 was used as a cutoff level for differential expression, a total of 306 genes (corresponding to 346 probesets) had altered expression, representing 3.4% of detectable genes. Of these, 59 were down-regulated while 247 were up-regulated. The 60 most interesting genes, selected on the basis of those playing a known or deduced

Fig. 2. Comparison of changes in the expression of selected genes by microarray and quantitative RT-PCR analysis after treatment of PaCa44 cells with TSA. Differences in expression by either technique are as shown. Gray bars indicate the average of three independent determinations by quantitative RT-PCR and the standard deviations are also indicated. Black bars represent the results observed in microarray analysis.

role in cellular proliferation/apoptosis/cell-cycle control, are shown in Table 1.

The genes with altered expression after treatment with TSA were subsequently classified according to cellular function. The Gene Ontology categories that most summar-

A

B

		Fold-change (range)			
		>5	3-5	2-3	1-2
Number of probesets	up	88	193	583	1975
	down	5	60	214	2116
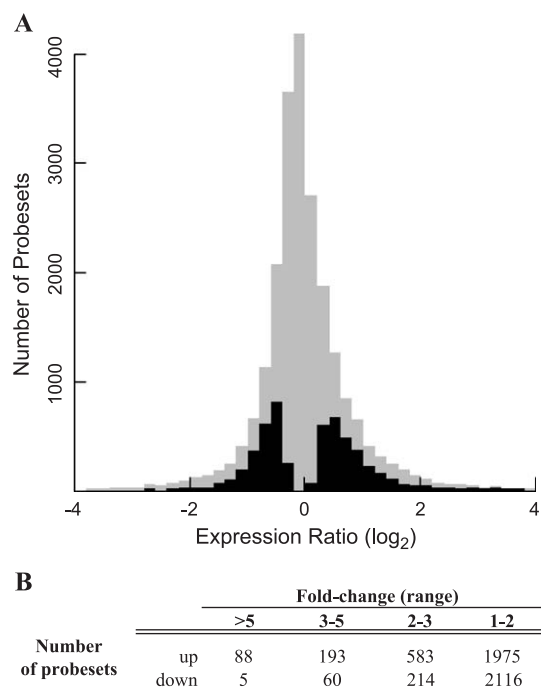
Fig. 3. Distribution of differentially expressed genes after treatment of PaCa44 cells with TSA. (A) Histogram representation. The number of probesets showing altered expression is plotted against the difference in the ratio of expression. The black area shows probesets scored as differentially expressed genes by MAS5 software. The gray area represents the distribution of all probesets. (B) Number of differentially expressed probesets at selected cutoff values.
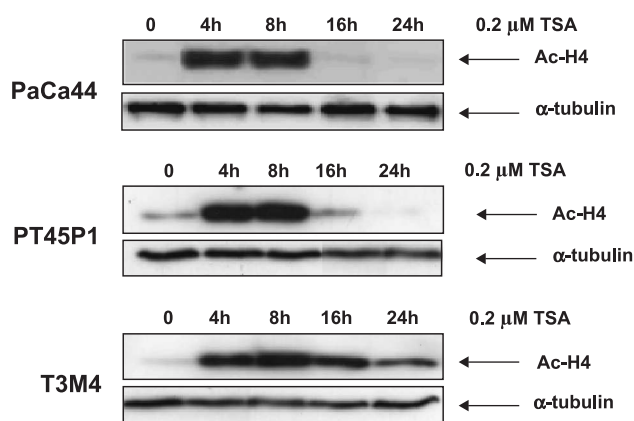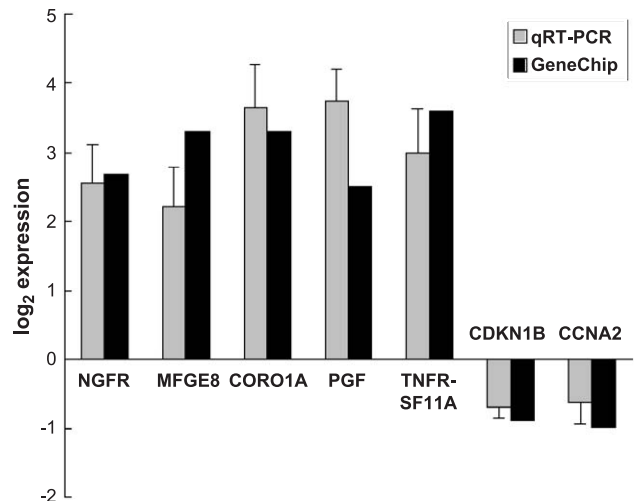
Fig. 1. Western blot analysis of histone acetylation following treatment of various cell lines with TSA at different times. Cells were treated as described in the Materials and methods.

Table 1

60 Most interesting differentially expressed genes after treatment with TSA

Gene description	Gene symbol	Fold change (log$_2$)
A disintegrin and metalloproteinase domain 19 (meltrin beta)	ADAM19	−2.1
BCL2-like 1	BCL2L1	−1.8
BCL2-like 11 (apoptosis facilitator)	BCL2L11	3.4
BCL2-like 2	BCL2L2	−0.6
BTB and CNC homology 1, basic leucine zipper transcription factor 2	BACH2	1.8
Ca^{2+}-promoted Ras inactivator	CAPRI	3.7
Colon cancer-associated protein Mic1	MIC1	1.7
Cyclin A2	CCNA2	−1
Cyclin-dependent kinase (CDC2-like) 10	CDK10	−1.2
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	1.6
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	−0.9
Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	1
Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	CDKN2D	1.8
DNA (cytosine-5-)-methyltransferase 3 beta	DNMT3B	1.9
Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	DYRK3	1.6
Fas apoptotic inhibitory molecule	FAIM	−1.6
G protein-coupled receptor 39	GPR39	−1.7
G protein-coupled receptor 87	GPR87	−1.8
G protein-coupled receptor kinase 5	GPRK5	3.8
GATA binding protein 3	GATA3	−5.5
GTP binding protein overexpressed in skeletal muscle	GEM	1.8
Hairy/enhancer-of-split related with YRPW motif 1	HEY1	1.6
Histone deacetylase 7A	HDAC7A	−2.2
Histone deacetylase 9	HDAC9	−1.8
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2	1.6
Katanin p80 (WD40-containing) subunit B1	KATNB1	2.1
MAD, mothers against decapentaplegic homolog 1 (*Drosophila*)	MADH1	−1.6
MAP kinase-interacting serine/threonine kinase 2	MKNK2	1.6
MAX interacting protein 1	MXI1	2.1
msh homeo box homolog 2 (*Drosophila*)	MSX2	1.9
myc-induced nuclear antigen, 53 kDa	MINA53	−1.6
Notch homolog 1, translocation-associated (*Drosophila*)	NOTCH1	2

Table1 (*continued*)

Gene description	Gene symbol	Fold change (log$_2$)
Nuclear receptor coactivator 2	NCOA2	−1.9
Nuclear receptor coactivator 3	NCOA3	1.6
Nuclear receptor interacting protein 1	NRIP1	−1.6
Phosphatidylinositol 4-kinase type II	PI4KII	1.8
Phosphoinositide-3-kinase, catalytic, delta polypeptide	PIK3CD	1.8
Protein kinase C binding protein 1	PRKCBP1	−3.9
Protein kinase, cAMP-dependent, regulatory, type II, beta	PRKAR2B	2.3
Protein phosphatase 2, regulatory subunit B (B56), beta isoform	PPP2R5B	1.6
RAB3A, member RAS oncogene family	RAB3A	3
RAB40B, member RAS oncogene family	RAB40B	3.3
RalGDS-like gene	RGL	2.6
Ras and Rab interactor 2	RIN2	−1.8
RAS guanyl releasing protein 2 (calcium and DAG-regulated)	RASGRP2	2.9
Ras-induced senescence 1	RIS1	3.3
Ras-like protein TC10	TC10	2.2
Signal transducer and activator of transcription 1, 91 kDa	STAT1	−0.5
Single-stranded DNA binding protein 2	SSBP2	2.5
Snail homolog 2 (*Drosophila*)	SNAI2	1.7
SWI/SNF related, matrix associated, subfamily a, member 2	SMARCA2	4.5
Transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	0.7
Tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	2.2
Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	0.9
Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	0.9
Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	3.9
Tumor necrosis factor receptor superfamily, member 25	TNFRSF25	−1.7
Tumor necrosis factor receptor superfamily, member 9	TNFRSF9	0.7
zinc finger protein 161 homolog (mouse)	ZFP161	−1.8
zinc finger protein, X-linked	ZFX	−2.1

ized the set of differentially expressed genes are shown along with their gene abundance in Table 2.

Since at least a major part of the effects of TSA can be ascribed to induction of apoptosis, we chose to further analyze the expression of key apoptotic genes with altered expression by microarray analysis in eight additional pancreatic cancer cell lines by quantitative RT-PCR that we have previously characterized for growth inhibition by TSA

Table 2
Functional classification of genes showing altered expression after treatment with TSA

Functional category	Number of genes[b]	OR	P[a]	Total number of genes
Metabolism	94	0.61	0.001	2995
Cell–cell signaling	17	2.63	0.001	187
Cell cycle	7	0.41	0.015	413
Development	33	1.57	0.030	589
Ion transport	12	1.57	0.139	206
Regulation of transcription	21	0.73	0.206	704
Cell proliferation	9	1.11	0.710	210
Signal transduction	45	1.20	0.315	1016
Vesicle-mediated transport	7	1.13	0.678	161
Chromosome organization and biogenesis	5	1.08	0.809	120
Cell motility	5	0.86	1.000	148

[a] Fisher's exact test.
[b] Based on a cutoff value of 3.0.

[14]. Fig. 4 shows that the proapoptotic gene BIM was significantly up-regulated by TSA in all cell lines tested, while Bcl-XL and Bcl-W showed significant down-regulation. To obtain more information on how the levels of histone acetylation influence gene expression, a time course experiment was performed in which the expression of selected apoptosis and cell cycle related genes was monitored at various times after treatment with TSA. These results demonstrate that the expression of the genes analyzed generally shows maximal alteration within 8 h (Fig. 5), a time that coincides with the maximal effects on histone acetylation observed in Western blot analysis (Fig. 1).

It has been previously suggested that the transcriptional factors belonging to the Sp1 family may play a role in alteration of gene expression after inhibition of HDAC [33–36]. To further explore this possibility, we determined the presence and number of Sp1 (GC-box) binding sites within 500 bp of the transcriptional initiation site of genes that showed altered expression after treatment with TSA (Table 3). It was found that Sp1 sites were significantly more abundant among induced genes (14%) with respect to those that were not differentially expressed (7%) or down-regulated (6%) ($P<0.001$). The distribution of the binding sites for the transcription factor NF-κB was not significantly different among promoters of genes showing differential expression after TSA treatment (data not shown).

4. Discussion

The interest in the use of inhibitors of HDAC stems from the fact that they induce apoptosis as well as chromatin remodeling, which may be responsible for the observed antitumor effects. Clinically, HDAC inhibitors have shown promise only in hematologic malignancies, although their possible application to solid tumors, including pancreatic cancer, has nonetheless been demonstrated in vitro. The development of additional molecules that alter the equili-

brium of histone acetylation would benefit from a greater understanding of the molecular mechanism of action and the pathways involved. The present work addresses this issue by examining the global gene expression patterns induced in pancreatic cancer cells by microarray analysis after treatment with TSA, a well-known HDAC inhibitor.

Our data add support to the concept that TSA does not have global effects on gene expression, but rather alters the expression of a relatively small number of specific genes. Among those with known or deduced cellular function, the differentially expressed genes were found to be involved in a wide variety of cellular processes, including cell proliferation, signaling, regulation of transcription, and apoptosis. Lastly, on a genomic scale, we showed that there was a significant correlation between the presence of a binding site for the transcription factor Sp1 and the probability of induction by TSA.

Previous investigations into the gene alterations caused by TSA have reported widely varying percentages (from 1–7%) with regards to the number of total genes that are differentially expressed after treatment by this inhibitor of HDAC [37]. While in part these apparent discrepancies can be explained by the different methodologies used, the reported differences may also be explicable by the different treatment times with TSA. In the present study, considering a threshold value of 3.0 as significant for differentially expressed genes, a total of 3.4% of genes showed altered expression after treatment with TSA, a value consistent with the range found from previous studies.

To our knowledge, this is the first functional classification of differentially expressed genes following TSA treatment. Not unsurprisingly, TSA caused the up- and down-regulation of numerous genes involved in a wide variety of cellular processes. The categories of metabolism and cell–cell signaling were underrepresented, although the significance of this is unclear at present.

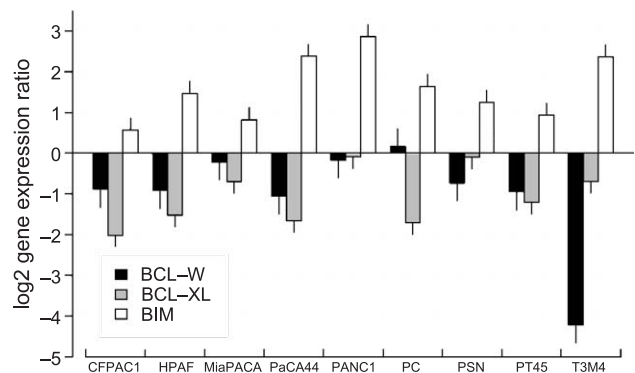Only a limited number of studies have analyzed the effects of TSA on gene expression by microarray analysis

Fig. 4. The plot represents expression changes of apoptosis related genes (BCL-XL, BCL-W and BIM), in various pancreatic cell lines, following treatment with TSA 0.2 μM after 8 h. Each bar corresponds to the base-two logarithm of gene expression ratio between treated and untreated samples, normalized using GAPD transcript levels. The means and S.E. are obtained using three replicates.
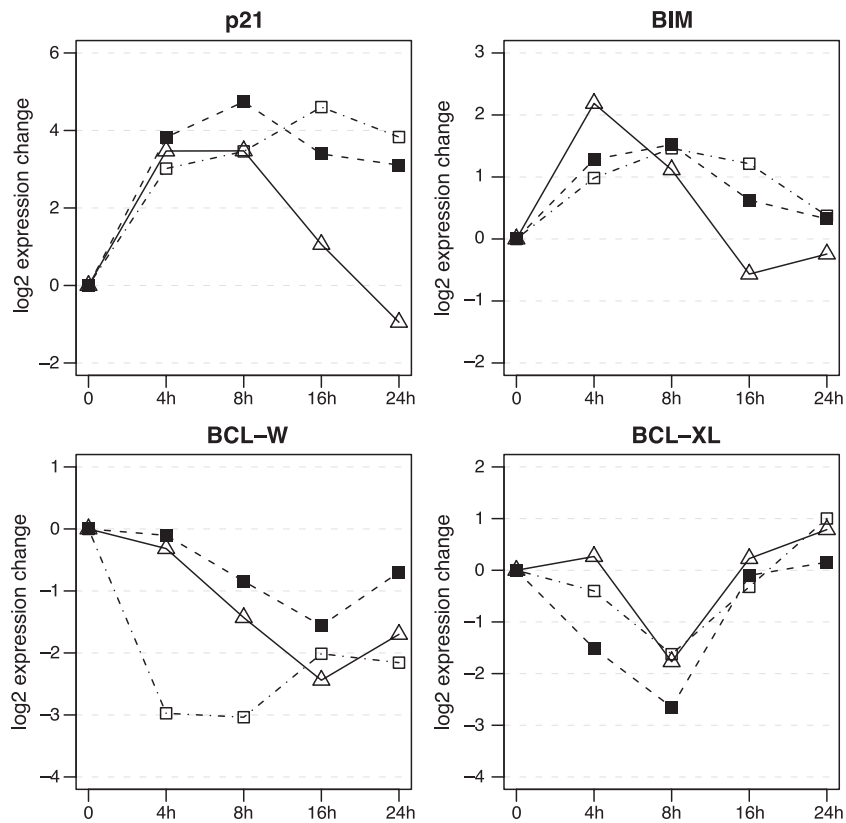
Fig. 5. Time-dependent effects of TSA on the expression of selected genes as determined by quantitative RT-PCR. The base-two logarithm of gene expression ratio between untreated and samples treated for various times is shown. Samples were normalized using GAPD transcript levels as a reference. Empty triangles, PT45; solid squares, PaCa44; empty squares, T3M4.

often in combination with demethylating or other agents [22,24–26]. Regarding pancreatic cancer, only a single study has been reported on the gene expression profile after treatment with TSA [25]. However, in that report, TSA was used as an agent to allow for gene reexpression, before treatment with a demethylating agent, in order to screen for targets of aberrant methylation; the most direct effects of TSA on gene expression after a relatively short treatment period, i.e., about 8 h, were not examined. We observed that maximal levels of gene expression occurred at 8 h following TSA treatment, while its effects were less evident at earlier or later time points, depending on the gene under examination (Fig. 5). These results correlate well with the levels of histone acetylation observed by Western blot analysis (Fig. 1). Interestingly, a recent study has attempted

to define a common gene set, consisting of eight up-regulated and five down-regulated genes, which were all altered by HDAC inhibition from analysis of bladder and breast cancer cell lines [22]. Considering this set of 13 genes, all were up- or down-regulated, although to different extents, in our present analysis of a pancreatic cancer cell line (data not shown). This group of commonly altered genes included p21, histone H2B, TRPM-2, and the transformation-related protein TRP [22].

Additionally, we found that Sp1/Sp3 sites were significantly more abundant among TSA-induced genes with respect to either uninduced or down-regulated genes. This provides further confirmation of the activating role on transcription that these *cis*-acting elements play upon treatment with TSA. In fact, Sp1/Sp3 transcription factor binding sites have been frequently implicated in changes in gene expression following alterations in chromatin acetylation [38,39]. In particular, Sp1/Sp3 binding sites situated in the proximity of the transcription start site have been reported as necessary for the TSA induction of the p21 promoter [40]. A repressor function has also been attributed to the Sp1/Sp3 family of transcription factors due to their ability to form repressor complexes with HDACs [41,42]. Moreover, it has been suggested that an increase in the acetylation status of Sp3 induced by TSA may cause a switch from a repressor to an activator function, leading to

Table 3
Distribution of GC-boxes in the proximal promoter region

	Number/presence of GC-boxes[a]				
	Absent	Present	1	2	>2
Up-regulated	682	113	94	14	5
Down-regulated	699	44	42	2	0
No change	3090	219	184	31	4

Differential expression tag determined by the MAS5 algorithm.

[a] Occurrence of GC-box within 500 bp of the transcriptional initiation site.

an increased expression of genes with receptive binding sites [43]. Thus, the greater abundance observed of Sp1 motifs in the genes up-regulated by TSA may be explained by either changes in the posttranslational acetylation status of GC-box binding transcription factors or the impairing of a repressor complex activity due to inactivation of HDACs.

We have previously shown that TSA arrests cellular growth in G2 and induces apoptosis in pancreatic cancer cell lines [14]. With regard to the former phenomenon, the cyclin-dependent kinase inhibitors p21 (CDKN1A), p19 (CDKN2D), and p57 (CDKN1C) have been previously associated with cell growth arrest in G2 phase [44–47]. All three genes were up-regulated after treatment with TSA. In addition, cyclin A (CCNA2) and cdk10 (CDK10), modulators of progression from G2 to M [48,49], were down-regulated. Thus, the change in expression of these genes is highly consistent with the observed growth arrest in G2 caused by TSA in pancreatic cancer cell lines [14].

Consistent with the apoptotic effects of TSA on pancreatic cancer cells [14], we observed the up- and down-regulation of genes involved in apoptotic cell death, in both the mitochondrial and the death receptor pathways. Interestingly, BIM (BCL2L11), a proapoptotic BH3-only BCL-2 family member [50,51], showed significant induction by TSA, in both microarray analysis and RT-PCR of pancreatic cancer cell lines. The BH3 domain is a nine-amino-acid region required in the binding of Bcl-2-like pro-survival proteins, and participates in the initiation of apoptosis through the mitochondrial pathway. It has been recently demonstrated that transient transfection of BIM in NIH 3T3 mouse fibroblasts determines apoptotic cell death, which can be blocked by overexpression of Bcl-2 [51]. Moreover, we demonstrated that TSA represses the expression of the antiapoptotic genes BCL-XL (BCL2L2) and BCL-W (BCL2L1). It has been previously reported that treatment with antisense oligonucleotide for these antiapoptotic genes induced apoptotic cell death [52–54]. Thus, our data on the consistent alteration of expression of BIM, BCL-XL, and BCL-W in all nine pancreatic cancer cell lines tested suggest that the increased ratio between the levels of expression of pro-apoptotic and anti-apoptotic genes may be a strong determinant for the induction of apoptosis by TSA. In pancreatic cancer, the enhanced expression of BCL-XL may be associated with decreased survival [55], and resistance to chemotherapy may be at least partly mediated by this gene [56]. Furthermore, in pancreatic cancer cells, the ratio between the levels of expression of pro-apoptotic and anti-apoptotic genes is predictive of sensitivity towards cytotoxic drugs [57,58]. Thus, our results suggest that pancreatic cancer treatment with TSA may enhance its sensitivity towards chemotherapy.

A second group of related genes involved in apoptotic signaling by TNF were also induced by TSA, namely the TNF ligand TNFSF9 and the TNF receptor members TNFRSF10D, TNFRSF10B and TNFRSF9. It has been amply demonstrated that TNF is able to kill cells through apoptosis by a mechanism that involves signaling by the death receptor family to which the TNF receptor belongs [59,60]. This is in agreement with the observation that HDAC inhibitors sensitize some cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [61]. More recent studies have shown that NF-κB, a transcription factor activated upon TNF signaling, is partly responsible for masking the apoptotic effect of TNF [59], in agreement with the observation that NF-κB prevents cells from undergoing apoptosis. In contrast, the induction of another transcription factor, STAT-1, has been found to play a key role in TNF-mediated apoptotic cell death [62]. Since our data show that TSA represses the gene for the p105 precursor of p50 NF-κB and activates the STAT1 gene, it may be that the up-regulation of TNF and its receptor by TSA may represent additional factors that contribute to the apoptotic cell death induced by TSA.

In summary, with specific regards to the biological effects of TSA in pancreatic cancer cells, namely the block of cell growth in G2 and induction of apoptosis, expression profiling after treatment with TSA identified several genes that may be related to this phenomena. These include several cyclin-dependent kinase inhibitors, members of the BCL family, and genes involved in apoptotic signaling by TNF that at least in part may be responsible for the observed effects of TSA on cellular growth.

Acknowledgements

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