The *ADAMTS12* metalloprotease gene is epigenetically silenced in tumor cells and transcriptionally activated in the stroma during progression of colon cancer

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Summary

Proteases have long been associated with tumor progression, given their ability to degrade extracellular matrix components and facilitate invasion and metastasis. However, recent findings indicate that different proteases can also act as tumor-suppressor enzymes. We have recently reported that lung carcinoma cells expressing the ADAMTS-12 metalloprotease show a remarkable impairment of growth in immunodeficient mice as compared with parental cells. Here, we show that *ADAMTS12* promoter is hypermethylated in cancer cell lines and tumor tissues. Interestingly, *ADAMTS12* expression in the stromal cells surrounding epithelial malignant cells is higher than in the paired normal tissues. Moreover, the expression of this metalloprotease in colon fibroblasts co-cultured with colon cancer cell lines is higher than in those cultured alone. Furthermore, the expression of *ADAMTS-12* by these fibroblasts is linked with an anti-proliferative effect on tumor cells. Based on these findings, we hypothesize that ADAMTS-12 is a novel anti-tumor protease that can reduce the proliferative properties of tumor cells. This function is lost by epigenetic silencing in tumor cells, but concurrently induced in stromal cells, probably as part of a response of the normal tissue aimed at controlling the progression of cancer.

Key words: Metalloprotease, Thrombospondin, Methylation, Degradome

Introduction

Proteases perform fundamental processing events in multiple biological processes in all living organisms (Lopez-Otin and Bond, 2008). To date, more than 560 proteolytic enzymes and homologs have been cataloged in the human genome (Puente et al., 2003), which reflects their relevance to physiological and pathological conditions, including cancer. In fact, proteolytic activity facilitates spreading of tumor cells and formation of distant metastasis through degradation of protein components of the extracellular matrix (Overall and Lopez-Otin, 2002). However, the relationship between proteolytic enzymes and cancer is much more complex because these enzymes target a variety of substrates distinct from extracellular matrix components and influence all stages of tumor development (Egeblad and Werb, 2002; Freije et al., 2003). Recent functional studies have also revealed that some proteases exhibit potent anti-tumor functions (Lopez-Otin and Matrisian, 2007). An illustrative example is that of MMP-8, a matrix metalloprotease showing a protective role in cancer through its ability to regulate the inflammatory response induced by carcinogens (Balbin et al., 2003). Furthermore, MMP-8 expression by breast tumors correlates with a lower incidence of lymph node metastasis and confers good prognosis to breast cancer patients (Gutierrez-Fernandez et al., 2008). These findings have contributed to extend the known functions of cancer-associated proteases and emphasize the importance of targeting specific proteases for cancer treatment (Lopez-Otin and Overall, 2002; Martin and Matrisian, 2007).

The ADAMTS (a disintegrin and metalloprotease with thrombospondin domains) family comprises 19 extracellular metalloproteases closely related to MMPs and ADAMs (Cal et al., 2002; Apte, 2004; Porter et al., 2005). Gain or loss of function of different ADAMTSs are involved in serious human diseases such as osteoarthritis (Bondeson et al., 2008) and thrombotic thrombocytopenic purpura (Sadler, 2008). In relation to tumorigenesis, some ADAMTSs are overexpressed in tumors of different sources (Porter et al., 2005; Rocks et al., 2008), whereas other family members have been cataloged as tumor-protective enzymes (Lopez-Otin and Matrisian, 2007). Thus, different reports point out the angio-inhibitory properties of ADAMTS-1 (Iruela-Arispe et al., 2003; Kuno et al., 2004; Lee et al., 2006). Moreover, *ADAMTS1* expression has been found epigenetically silenced in colorectal tumors (Lind et al., 2006). Furthermore, *ADAMTS8, ADAMTS9* and *ADAMTS18* gene promoters are also hypermethylated in several carcinomas (Dunn et al., 2004; Jin et al., 2007; Lo et al., 2007). ADAMTS-12 is another member of this family and was originally identified and cloned in our laboratory (Cal et al., 2001). We have recently described that this enzyme can modulate the Ras-dependent ERK (extracellular signal-regulated kinase) signalling pathway in MDCK (Madin-Darby canine kidney)

cells, and that subcutaneous tumors induced by A549 lung carcinoma cells expressing *ADAMTS12* display a considerable growth deficiency as compared with those induced by parental cells (Llamazares et al., 2007). To further examine the relevance of ADAMTS-12 as a putative tumor-suppressor enzyme, we have analyzed the mechanisms controlling its expression in cancer cells and tissues with the finding that *ADAMTS12* is epigenetically silenced in tumor cell lines from multiple sources, whereas it is transcriptionally activated in stroma cells. Our data strongly suggest that ADAMTS-12 is part of a protective host response against tumor progression derived from the intimate crosstalk between tumor cells and the surrounding stroma.

Results

ADAMTS12 gene promoter is hypermethylated in colorectal carcinomas and colon cancer cell lines

Analysis of 2.0 kb upstream and 0.5 kb downstream of the first ATG codon allowed the identification of a CpG island in this region, suggesting the potential influence of CpG methylation in the transcription of the *ADAMTS12* gene. Methylation-specific PCR (MSP) amplification of a 138 bp promoter within the CpG island (Fig. 1A) was performed on a set of paired normal and tumor colon samples. As can be seen in Fig. 1B, the methylation level of *ADAMTS12* promoter was higher in 12 out of 19 (63.2%) biopsies of primary colorectal tumors than in the corresponding normal tissue. Similarly, MSP amplification using genomic DNA from the microsatellite-stable (MSS) SW620, SW480, COLO 205 and HT29 colon carcinoma cell lines, and the microsatellite-unstable (MSI) DLD-1, HCT 15, HCT-116, LoVo and RKO cell lines showed that *ADAMTS12* promoter is hypermethylated in all of them, with the exception of LoVo cells (Fig. 1B; Table 1).

We next performed high-resolution bisulfite genome sequencing of 26 CpG sites within the CpG island identified (Fig. 1A). Eleven colon carcinoma samples were selected for this assay, attending to the different intensities of PCR products in previous MSP amplifications. Seven of these samples were more methylated in the tumor than in their paired normal tissues (Fig. 1C; Table 1). Among them, tumor samples 426, 467 and 557 were highly methylated in most of the 26 CpG sites examined (70-90%). By contrast, their paired normal tissues were much less methylated (20-40%). Four additional tumor samples, 340, 401, 478 and 566, showed percentages of methylation ranging between 20 and 60%, but their paired normal tissues were scarcely methylated (below 10%). Samples 404 and 496 showed similar levels of methylation in both tumor and normal tissues, whereas samples 397 and 555 were more methylated in normal tissues. Parallel analysis carried out in colon carcinoma cell lines revealed the high frequency of methylation at these CpG sites, independently of the microsatellite instability status. Thus, percentages of 80-90% were determined for SW620, SW480 and COLO 205 (MSS cell lines), and for

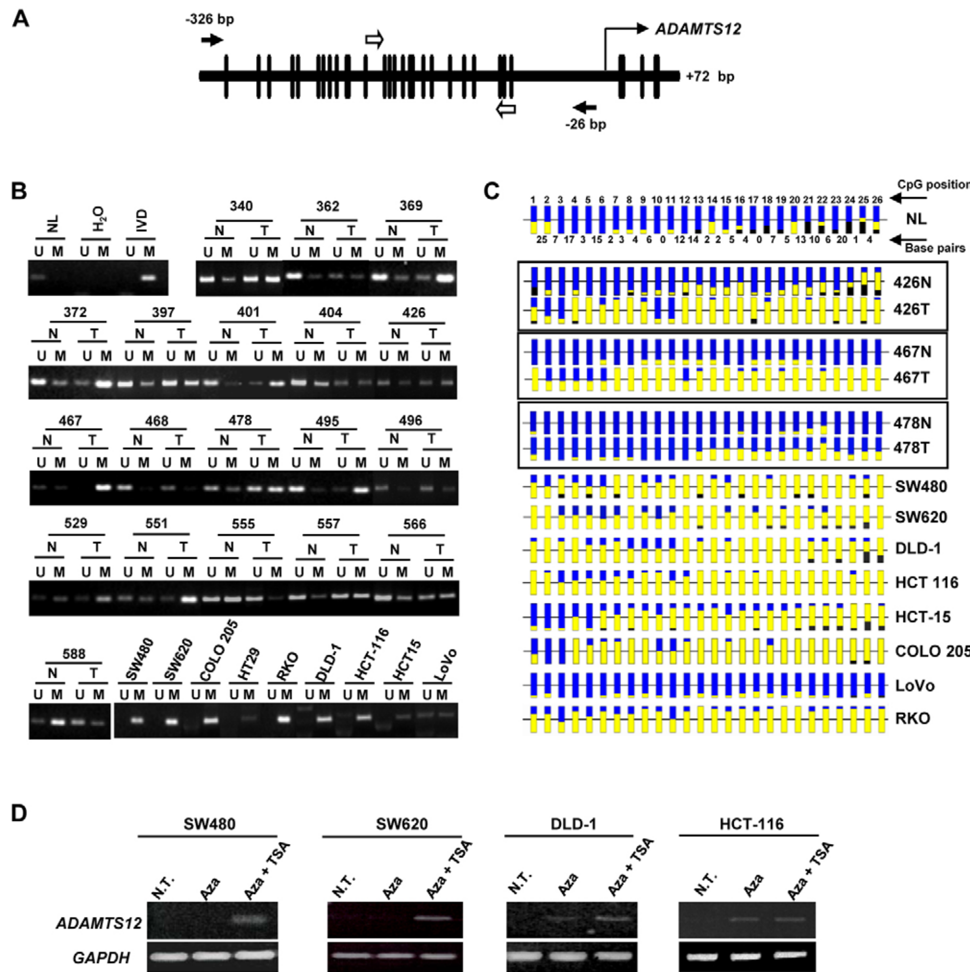

Fig. 1. Epigenetic silencing of *ADAMTS12* in colon cancer. (A) Schematic representation of the *ADAMTS12* CpG island. An interval of 2.0 kb upstream and 0.5 kb downstream from the first ATG codon of *ADAMTS12* was analyzed for CpG islands. Thin black arrow indicates the transcription start site; white arrows indicate MSP primers and thick black arrow BSP primers. (B) Methylation status of *ADAMTS12* in primary colon carcinomas and colon cancer cell lines. PCR-methylation analysis was carried out in paired normal (N) and tumor tissues (T), using the specific primers for either methylated (M) or modified unmethylated DNA (U). Genomic DNA from human lymphocytes was used as a control for unmethylated DNA (NL), and the CpGenome Universal Methylated DNA as control for methylated DNA (IVD). (C) Methylation density of *ADAMTS12* in colon cancer. Primary tumor samples and colon cancer cells were subjected to bisulfite treatment, and PCR amplification was performed using BSP primers. Relative positions of each CpG analyzed and the distance in bp are indicated in the control for unmethylated DNA (NL). Methylation percentages for the rest of the tumor samples examined are indicated in Table 1. Colors shows the methylation density of each CpG: yellow, methylated; blue, not methylated; grey, not present. (D) Activation of *ADAMTS12* expression in colon cancer cell lines. Indicated cell lines were treated with 5′-aza-2′-deoxycitidine (Aza), or Aza + TSA. *ADAMTS12* expression was assayed by RT-PCR. N.T., no treatment. *GAPDH* mRNA was amplified as an internal control.

Table 1. Hypermethylation of the *ADAMTS12* promoter

Cell lines	Patient sample number	Microsatellite instability	Mean CpG site methylation (%)		Methylation status
			Normal	Tumor	
	340	MSS	9.80	25.80	M
	362	ND	ND	ND	M
	369	MSS	ND	ND	M
	372	MSI	ND	ND	M
	397	ND	49.33	13.75	U
	401	MSI	0.64	20.33	M
	404	MSS	17.39	22.05	U/M
	426	MSS	21.42	82.25	M
	467	MSS	9.23	84.07	M
	468	MSS	ND	ND	U/M
	478	MSS	4.33	25.96	M
	495	MSS	ND	ND	M
	496	MSS	11.11	19.61	U/M
	529	ND	ND	ND	U/M
	551	MSS	ND	ND	M
	555	MSS	35.34	20.07	U
	557	MSS	31.61	70.76	M
	566	MSS	7.40	59.24	M
	588	ND	ND	ND	U
DLD-1		MSI	85.74		M
COLO 205		MSS	82.21		M
HCT-116		MSI	86.51		M
HCT 15		MSI	66.79		M
HT29		MSS	ND		M
LoVo		MSI	9.58		U
RKO		MSI	80.76		M
SW480		MSS	82.14		M
SW620		MSS	84.10		M

MSP amplification using genomic DNA from the microsatellite-stable COLO 205, HT29, SW620 and SW480 colon carcinoma cell lines, and the microsatellite-unstable DLD-1, HCT 15, HCT-116, LoVo and RKO cell lines was used to measure methylation of the *ADAMTS12* promoter. Samples were also analyzed from paired normal and primary colorectal tumor tissue from 19 patients.

U, unmethylated; M, methylated; MSS, microsatellite-stable; MSI, microsatellite-unstable; ND, not determined.

DLD-1, HCT-116 and RKO (MSI cell lines) (Fig. 1C; Table 1). The percentage of methylation for HCT 15 cells was 66.8% and, consistent with the above MSP analysis, *ADAMTS12* gene promoter was found barely methylated in LoVo cells (Table 1).

The above results strongly suggested that promoter methylation could silence *ADAMTS12* gene expression. To examine this possibility, we selected the MSS cell lines SW480 and SW620, and the MSI cell lines DLD-1 and HCT-116 to perform RT-PCR amplification. As illustrated in Fig. 1D, none of these cell lines expressed *ADAMTS12*. It is also remarkable that none of the additional cells employed in the methylation analysis showed appreciable levels of *ADAMTS12* expression (not shown). Then, we investigated whether *ADAMTS12* expression could be restored by treatment with the demethylating agent 5′-aza-2′-deoxycytidine (Aza). This treatment was carried out with or without the histone deacetylase inhibitor trichostatin A (TSA), which has also been used to examine the methylation status of *ADAMTS18* (Jin et al., 2007). As shown in Fig. 1D, *ADAMTS12* expression could be reinstated following drug treatment. Moreover, this activation was observed in DLD-1 and HCT-116 cells even in the absence of TSA. Taken together, these results provide evidence for the epigenetic silencing of *ADAMTS12* expression in colon carcinomas.

Expression of *ADAMTS12* in colon carcinomas

Next, we used RT-PCR to study expression levels of *ADAMTS12* in the set of colon cancer samples previously used to examine their methylation status (Table 1). Interestingly, and in contrast to what could be expected from the previous results, *ADAMTS12* expression levels were higher in tumor samples than in normal tissues (Fig. 2). These tumor biopsies contained at least 50% tumor cell content but no attempt at tumor-cell enrichment was made. A similar result was obtained when PCR amplification was performed using a set of five commercially available paired normal and tumor cDNA samples from colon carcinomas (Fig. 2). As a positive control for this analysis, we performed PCR amplification of *SFRP1*, a gene known to be silenced by methylation in colon cancer (Aguilera et al., 2006).

Induction of *ADAMTS12* expression in stromal cells

A possible explanation for above unexpected result is that *ADAMTS12* is produced by stromal cells in the immediate vicinity of neoplastic epithelial cells and not by the tumor cells themselves. To evaluate this possibility, we performed an immunofluorescence study using 20 colon carcinoma tissue sections. As can be seen in Fig. 3A, double immunofluorescent staining for ADAMTS-12 and alpha smooth muscle actin (α-SMA) showed a close approximation of ADAMTS-12 immunoreactivity to α-SMA-positive cells. By contrast, there was no staining in epithelial cells. Moreover, detailed examination of 55 tissue areas revealed that 60% of areas of well-differentiated and moderately differentiated carcinomas showed a strong immunoreactivity for ADAMTS-12 (Table 2), and only two areas of the moderately differentiated carcinomas lacked staining for ADAMTS-12. However, only 36% of areas of advanced poorly differentiated carcinomas showed strong immunostaining for the enzyme, whereas 48% of these areas lacked staining, which is consistent with the relative low proportion of myofibroblasts in these

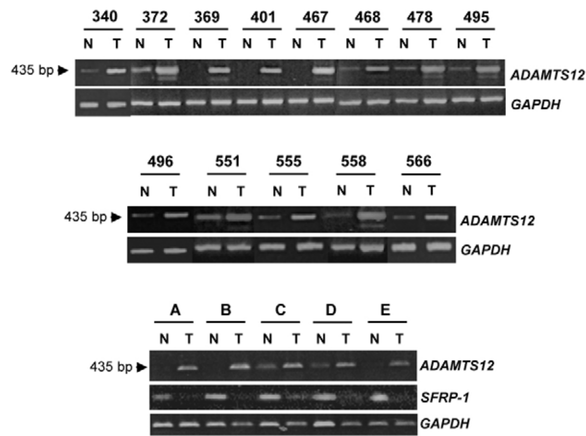

Fig. 2. *ADAMTS12* expression in human colon carcinomas. Top and middle panels*:* total RNA from the indicated colon carcinoma samples was isolated and *ADAMTS12* expression was assayed by RT-PCR (N, normal; T, tumor). The remaining colon carcinoma samples employed in this work were discarded for this analysis because of RNA quality. Bottom panel: amplification of *ADAMTS12* from a set of five (A-E) commercially available paired normal and tumor first-strand cDNA samples from colon carcinomas. Amplification of *SFRP1* was used as positive control. In all experiments, RT-PCR of *GAPDH* mRNA was used as an internal control.

undifferentiated tumours. Expression in normal mucosa or adenomatous polyps was not or hardly detectable. These data indicate that the expression of *ADAMTS12* is due to fibroblastic-like cells present in the stroma surrounding malignant cells. Moreover, these results point to a correlation between the expression of this metalloprotease and the histopathologic grade of tumors.

To examine whether colon fibroblasts could express the metalloprotease gene, we carried out co-cultures of CCD-18Co colon fibroblasts with colon cancer cells, and used quantitative RT-PCR to compare the differences in *ADAMTS12* expression levels with those detected in fibroblasts cultured alone. To discard any possible presence of tumor cells mixed with the fibroblasts following co-culture, absence of RT-PCR amplification of the carcinoma marker *EpCAM* (Trzpis et al., 2007) was also assayed (not shown). As illustrated in Fig. 3B, fibroblasts showed about twofold higher *ADAMTS12* expression when co-cultured with SW620 and DLD-1 cells, and fivefold higher levels in the case of co-culture with SW480 colon cancer cells. Additionally, we found that TGF-α (4.5-fold), TGF-β1 (3.3-fold), and IL-6 (twofold) also raised *ADAMTS12* expression in these fibroblasts. By contrast, treatment with IL-10 did not modify the expression levels of this metalloproteinase. Next, we examined whether the enhanced expression of *ADAMTS12* observed in fibroblasts co-cultured with tumor cells could be promoted by any of the analyzed cytokines. To this end, we employed different blocking antibodies and found that a considerable reduction of the metalloprotease expression was observed when blocking antibodies against TGF-β1 were added to the co-cultures, but not in the presence of blocking antibodies against TGF-α (Fig. 3C). *ADAMTS12* expression was not detected in tumor cells in the course of these experiments (not shown).

Then, we asked whether this induced expression of *ADAMTS12* in colon fibroblasts could be associated with any functional effect. To evaluate this possibility, we selected SW620 (MSS) and DLD-1 (MSI) cells because of the similar enhancement of *ADAMTS12* expression caused in CCD-18Co fibroblasts (Fig. 3B). Growth rates for the colon cancer cells were calculated during the exponential growth phases in co-culture with colon fibroblasts. Cancer cells cultured alone on the inserts were used as control. Results indicated that both cell lines had growth defects. The doubling time for DLD-1 cells in co-culture with fibroblasts was 16.9 hours, whereas it was 13.9 hours when the cell line was cultured alone. This difference was even more significant in the case of SW620 cells: the doubling times were 31.5 hours in co-culture and 25.7 hours in the absence of fibroblasts. We also determined the percentage of apoptotic cells in the assayed conditions after 96 hours of incubation (Fig. 3D). Analysis of DLD-1 revealed that the apoptotic cell population was 34% in co-culture with colon fibroblasts, and 25.5% when the tumor cell line was cultured alone. Potency of apoptosis induction by the fibroblasts was higher in case of SW620 cells: 36.5% of tumor cells were apoptotic in co-culture, but only 12.5% in the absence of fibroblasts. This study indicates the occurrence of a decrease in tumor cell proliferation concomitant with enhanced *ADAMTS12* expression.

Taken together, these findings indicate that ADAMTS-12 is mainly secreted by the stromal cells surrounding cancer cells in colon carcinomas. This stromal expression of *ADAMTS12* might suggest the occurrence of a protective response, which tries to compensate for the epigenetic silencing of this gene in malignant cells.

ADAMTS12 expression is epigenetically silenced in tumors of different origin

The above results obtained in colon cancer tissues and cell lines prompted us to extend our study on *ADAMTS12* expression to tumors of different origins through methylation analysis of additional tissues and cell lines. We employed the Illumina GoldenGate methylation platform to study the DNA methylation status of two CpG positions within the *ADAMTS12* CpG island in a panel of normal tissues and cancer cell lines. As shown in Fig. 4, *ADAMTS12* was found to be highly hypermethylated in most of the analyzed tumor cell lines (21 out of 29 for probe P250, and 26 out of 29 for probe E52). By contrast, methylation was scarcely detected or not detected in the normal tissues examined. Overall, these results provide additional evidence to indicate that *ADAMTS12* is an epigenetically silenced gene in tumor cells of diverse origin and to support its potential role as a tumor suppressor gene. Interestingly, this study also showed that the promoter region of *ADAMTS12* is not methylated in LoVo cells (probe P250), which perfectly agrees with the results obtained from MSP and bisulfite-sequencing PCR (BSP) analysis (Fig. 1). However, this gene is highly methylated in these cell lines in a region contained within its first exon (E52). Thus, it could not be ruled out that methylation in positions other than those previously examined by BSP in this work could control the silencing of *ADAMTS12* expression.

Discussion

In this work, we have found that the *ADAMTS12* gene is subjected to a generalized methylation process in colorectal tumors and in a variety of tumor cells from different origins, a mechanism of growing relevance in the inactivation of tumor-suppressor genes (Esteller, 2007). We have also proven that *ADAMTS12* expression can be induced in CCD-18Co fibroblasts and is associated with a decrease in proliferation and an increase in apoptosis of the adjacent tumor epithelial cells. Finally, histopathologic analysis has revealed that ADAMTS-12 is detected in stromal cells from well-differentiated and moderately differentiated tumors rather than in

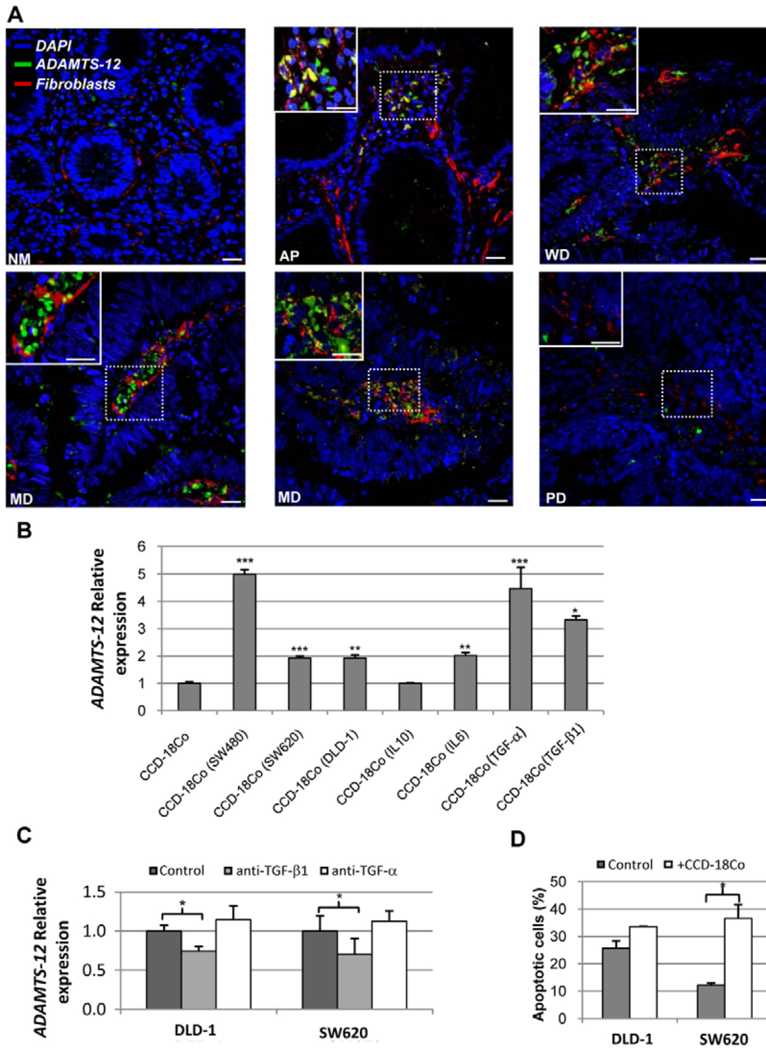

Fig. 3. Induction of *ADAMTS12* expression in stromal cells. (A) Immunofluorescence detection of ADAMTS-12 in colon carcinoma tissue sections. Detection of activated fibroblasts was performed using an anti-SM α-actin monoclonal antibody. ADAMTS-12 was detected using the H-142 antibody from Santa Cruz Biotechnology. Representative tissue sections corresponding to normal mucosa (NM), adenomatous polyp (AP), and well-differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) carcinomas are shown. Insets show higher magnifications of sections marked with a square. Scale bars: 50 μM. (B) Induction of *ADAMTS12* expression was assayed by co-culture of CCD-18Co fibroblasts with the indicated colon cancer cells, or through treatment with the indicated cytokines. After 36 hours, *ADAMTS12* expression was determined by quantitative PCR. Actin expression was simultaneously analyzed as internal control. (C) Anti-TGF-β1 blocking antibodies reduce *ADAMTS12* expression by fibroblasts co-cultured with tumor cells. A remarkable reduction of *ADAMTS12* expression was detected when anti-TGF-β1 blocking antibodies were added to the co-cultures, whereas the presence of these antibodies did not show any direct effect on the metalloprotease expression. (D), Induction of apoptosis in colon cancer cells. DLD-1 and SW620 cells were co-cultured with CCD-18Co fibroblasts or cultured alone, and the percentage of apoptotic tumor cells was determined after 96 hours. (B,C,D) Data are means ± s.e.m. Statistical significance was determined using a Student's *t*-test (****P*<0.001, ***P*<0.01, **P*<0.05).

those from advanced poorly differentiated carcinomas. Taken together, these results reinforce the proposal that ADAMTS-12 is a novel protease that can be included in the growing category of proteolytic enzymes with tumor-defying properties (Lopez-Otin and Matrisian, 2007).

ADAMTS-12 belongs to a family of metalloproteases that has been commonly associated with cancer (Rocks et al., 2008). Nevertheless, the functional relevance of these enzymes in tumor progression is still unclear and in some cases contradictory. On the one hand, ADAMTS-4 and ADAMTS-5 contribute to invasiveness of glioblastoma cells through their ability to cleave brevican (Held-Feindt et al., 2006), therefore acting as pro-tumor proteases. On the other hand, and similar to our findings on ADAMTS-12, ADAMTS-8, ADAMTS-9 and ADAMTS-18 have been proposed to act as anti-tumor enzymes because the genes encoding these proteases have been found hypermethylated in tumors of different origins (Dunn et al., 2004; Jin et al., 2007; Lo et al., 2007). Interestingly, ADAMTS-1 plays a dual role in cancer because it exhibits both pro- and anti-tumor properties (Vazquez et al., 1999; Masui et al., 2001; Iruela-Arispe et al., 2003; Kuno

Table 2. ***ADAMTS12*** **expression and histopathologic grade in colorectal carcinoma**

Sample type		Number of areas examined	Degree of staining		
			–	+/–	+
Adenomatous polyp		5	3 (60%)	2 (40%)	0 (0%)
Adenocarcinoma	Well-differentiated	5	0 (0%)	2 (40%)	3 (60%)
	Moderately differentiated	20	2 (10%)	6 (30%)	12 (60%)
	Poorly differentiated	25	12 (48%)	4 (16%)	9 (36%)
All types		55	17 (31%)	14 (25.4%)	24 (43.6%)

Twenty samples of adenomatous polyps and different grades of carcinomas were immunostained for ADAMTS-12, and 55 tissue areas examined in detail. The number and percentage of areas stained were calculated for each different group. – indicates no staining, +/– moderate staining, + intense staining. Staining resulted negative in the case of normal mucosa.

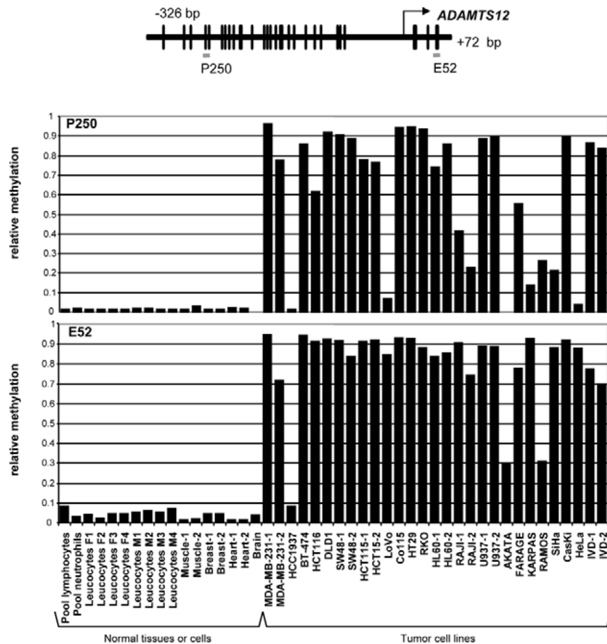

Fig. 4. DNA methylation analysis of the *ADAMTS12* promoter in a panel of normal tissues and cancer cell lines using bead arrays. Methylation was assessed at two CpG sites within the *ADAMTS12* CpG island using Illumina Goldengate Methylation Arrays. The first CpG site was located 250 bp upstream of the transcriptional start site (P250) and the second CpG site was located 52 bp downstream the transcriptional start point (E52). The amount of bisulfite-modified target DNA that hybridizes to each spot of the Illumina chip was quantified and standardized over a scale from 0.0 to 1.0 (effectively 0% and 100% likelihood of gene promoter hypermethylation, respectively).

et al., 2004; Lee et al., 2006; Lind et al., 2006). An explanation for these controversial effects might derive from the fact that this metalloprotease undergoes proteolytic processing events and the generated forms display tumor-protective or tumor-promoting properties depending on the cleavage site (Liu et al., 2006). It has been reported that the ADAMTS-1 thrombospondin domains are responsible for this protective role through their ability to sequester growth factors like vascular endothelial growth factor (Luque et al., 2003; Kuno et al., 2004). The capacity of thrombospondin domains to inhibit the function of growth factors, together with their pro-apoptotic actions (Mirochnik et al., 2008), could also help to explain the effects herein described in colon cancer cells in the presence of ADAMTS-12.

Histopathologic analysis has revealed that ADAMTS-12 is detected in the stroma of well-differentiated tumors rather than in advanced carcinomas. It is noteworthy that immunofluorescent double-staining analysis for ADAMTS-12 and α-SMA resembles that shown for periostin, which is expressed by stromal cells in colon carcinoma (Kikuchi et al., 2008). Periostin is a secreted extracellular matrix protein found in close proximity to the cells responsible for its expression, the α-SMA-positive cancer-associated fibroblasts. The roles of periostin in cancer appear to be quite diverse, but studies with periostin-deficient mice have shown that this protein negatively regulates tumor growth by promoting capsule formation. This suggests that, similar to our proposal for ADAMTS-12, periostin can also function as an anti-tumor protein (Shimazaki and Kudo, 2008).

In this work, we also found that *ADAMTS12* expression is enhanced in colon fibroblasts co-cultured with colon cancer, which is linked to an anti-tumor effect on cancer cells. On this basis, we speculate that the expression of this metalloprotease could form part of a stromal response promoted by cancer-associated fibroblasts to control the pro-angiogenic properties induced by different soluble growth factors. These fibroblasts, also termed myofibroblasts, are the predominant cell type in the stroma of most carcinomas (Ronnov-Jessen et al., 1996) and constitute a heterogeneous population of cells that cannot always be identified as α-SMA-positive (Sugimoto et al., 2006). These cells are phenotypically different from fibroblasts present in normal tissue, but similar to those found in wounds during the healing process (Gabbiani, 2003). Myofibroblasts are intricately interwoven with the epithelial tumor cells (Micke and Ostman, 2004) and, therefore, different pro-tumor or anti-tumor responses can be elicited by the stroma as a consequence of its interaction with the malignant cells (Mueller and Fusenig, 2004).

Among the cytokines assayed in this study, TGF-α and TGF-β1 induced the highest levels of *ADAMTS12* expression when the human colon fibroblasts were cultured alone. However, the use of blocking antibodies in co-cultured assays suggests that TGF-β1 could be one of the factors responsible for expression of the metalloprotease as a consequence of the crosstalk between tumor and stromal cells. This finding perfectly agrees with previous studies showing that TGF-β1 is one of the main inducers of *ADAMTS* gene expression, including that of *ADAMTS12*, in different conditions (Cal et al., 2001; Wang et al., 2003). Moreover, Porter et al. have also reported that *ADAMTS12* mRNA levels are higher in stromal fibroblasts than in epithelial tumor cells from breast cancer (Porter et al., 2004), a type of tumor that commonly expresses high levels of TGF-β1 (Chang et al., 2007). Altogether, these findings indicate that, following its induction in stromal fibroblasts through the action of factors such as TGF-β1, ADAMTS-12 might act as a tumor-protective protease in colon cancer and also in tumors of different origin such as breast cancer. Finally, the fact that other members of this metalloprotease family have also been found in stromal cells but not in the corresponding cancer cells (Cross et al., 2005), leads us to propose that stromal expression of different *ADAMTSs* could be a common feature in tumor development or progression.

In summary, we provide new data indicating a dual regulation of *ADAMTS-12* expression in colon carcinoma: epigenetic inactivation in epithelial malignant cells and induction in myofibroblasts, suggesting that this metalloprotease could form part of a protective stromal response aimed at limiting tumor progression. Our data also suggest that ADAMTS-12 might have potential clinical applications because this enzyme could be a prognosis marker not only in colon cancer but also in other tumors in which ADAMTS-12 is detected (Cal et al., 2002). However, further studies will be necessary to determine the precise role of *ADAMTS12* expression during progression of colon cancer. Likewise, analysis of susceptibility to colon cancer in *Adamts12*-null mice, currently ongoing in our laboratory, will help to clarify the precise role of this enzyme in the context of the growing number of tumor-defying proteases that are known to be produced by human cells.

Materials and Methods

DNA samples

Genomic DNAs from paired normal and tumor colon samples were obtained from 19 patients undergoing surgery at the Catalan Institute of Oncology (Barcelona, Spain). Genomic DNA from human lymphocytes was used as a control for unmethylated DNA, and the CpGenome Universal Methylated DNA (Chemicon), for methylated

DNA. A set of five paired normal and tumor colon cDNA samples to evaluate *ADAMTS12* expression was purchased from Clontech.

Cell culture

Colon cancer cell lines and CCD-18Co fibroblasts were from the American Type Culture Collection. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 50 μg/ml streptomycin.

CpG island identification and DNA methylation analysis

An interval of 2.0 kb upstream and 0.5 kb downstream from the first *ADAMTS12* ATG codon was analyzed for CpG island identification using the Methyl Primer Express Software v1.0, (https://produtcs.appliedbiosystems.com). This program was also used to design MSP primers. Primers for the unmethylated reaction were 5′-GAGTTTGGGAGGAAGATGTATT-3′ and 5′-CTAACAATATCCACTTTCA-ACAAAA-3′, and for the methylated reaction 5′-GAGTTCGGGAGGAAGAT-GTATC-3′ and 5′-ACAATATCCGCTTTCGACG-3′. To analyze methylation density of genomic DNA, two BSP primers, 5′-TGGTTTGGGGGTTTTTTATT-3′ and 5′-AACTAAACACCTTTTTCCCCTC-3′, were designed using the indicated program. Bisulfite-modified genomic DNA from paired normal and tumor tissues and cell lines was used as template.

RT-PCR amplification

Total RNA from tissue samples and cell lines was isolated by guanidium thiocyanate-phenol-chloroform extraction and reverse-transcription reactions were carried out with 300 ng of RNA, using the Thermoscript RT-PCR system with Platinum Taq polymerase (Invitrogen). The *ADAMTS12*-specific primers used were 5′-CAG-AAAGGACATCTTGCTGG-3′ and 5′-TCCTGGCAGAAGGTGCATTC-3′. PCR amplifications were performed under the following conditions: one cycle at 95°C for 1 minute, and 35 cycles at 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. *GAPDH* was amplified as an internal control.

Treatment of colon cancer cell lines with demethylating agents

Colon cancer cells were seeded in 10 mm dishes and incubated in culture media with 10 μM Aza for 3 days. For the combined treatment, cells were treated for 3 days with Aza and subsequently with 10 ng/ml TSA for 24 hours. *ADAMTS12* expression before and after treatment was determined by RT-PCR.

Co-culture assays and cytokine treatment

Induction of *ADAMTS12* expression in colon fibroblasts was examined through their co-culture with colon cancer cell lines or by cytokine treatment. For co-cultures, subconfluent CCD-18Co fibroblasts in six-well dishes were cultured with cell-culture inserts (8.0 μm pore size; BD Biosciences) overlaid with the indicated colon cancer cell lines and incubated for 36 hours in DMEM medium containing 0.25% FBS. Cytokines were added to CCD-18Co fibroblasts cultured in six-well dishes at 2 ng/ml final concentration (except TGF-α, which was used at 25 ng/ml). Then, 9 μl of a 1:5 dilution of cDNA was employed in quantitative PCR using TaqMan probe HS00917112_m1, TaqMan Master Mix and AbiPrism 7900HT (Applied Biosystems). When indicated, anti-TGF-α or anti-TGF-β1 blocking antibodies (Calbiochem) were added to the co-cultures at 1.5 μg/μl. Actin expression was analyzed as an internal control. Statistical significance was determined using a Student's *t*-test. For determination of cell growth, overlaid colon cancer cell lines (SW620 and DLD-1) were trypsinized and counted directly in a hemocytometer. After plotting total cell number against time, data were adjusted to exponential curves from where doubling-time and regression values were calculated. For apoptosis analysis, 5×10^5 cells were evaluated using the Anexin V-FITC/IP kit from Sigma-Aldrich. Each cell population was identified in a Cytomics FC500 (Beckman-Coulter).

Tissue immunofluorescence analysis

Paraffin-embedded colon tissues from 20 different tumors were cut into 5-micron sections and mounted onto glass slides. The sections were deparaffinized in xylene followed by ethanol hydration. Samples were permeabilized for 30 minutes with 0.1% Triton X-100 and incubated for 15 minutes in PBS with 0.1 M glycine. Nonspecific labeling was blocked with 5% BSA in PBS for 30 minutes. Sections were incubated overnight at 4°C with an anti alpha smooth muscle primary antibody (α-SMA, dilution 1:50; DakoCytomation) to detect activated fibroblasts. After incubation, sections were washed with PBS and PBS-Tween 20 (0.5% v/v), and then incubated with Alexa Fluor 546-conjugated goat anti-mouse antibody (dilution 1:500; Invitrogen) for 45 minutes at room temperature. After washing with PBS and PBS-Tween, sections were then incubated with a second primary antibody against ADAMTS-12 (H-142, dilution 1:25; Santa Cruz Biotechnology) overnight at 4°C and washed. H-142 is a polyclonal antibody raised against amino acids 1441-1582 of the human ADAMTS-12. Alexa Fluor 488-conjugated goat anti-rabbit antibody (dilution 1:200; Invitrogen) was added and incubated for 45 minutes at room temperature. The corresponding negative controls were set by omitting the anti-ADAMTS-12 antibody. We then washed the slides and stained nuclei with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 1 μg/ml in Vectashield mounting medium (Vector Laboratories). Images were obtained using a confocal microscope, Leica TCS-SP2-AOBS.

Analysis of DNA methylation status using bead arrays

Methylation analysis of *ADAMTS12* promoter in a panel of normal tissues and cancer cell lines was assessed using Illumina Goldengate Methylation Arrays (Bibikova et al., 2006). The two CpG sites analyzed were located 250 bp upstream and 52 bp downstream of the *ADAMTS12* transcriptional start point (E52). Thge amount of bisulfite-modified target DNA that hybridizes to each spot of the Illumina chip was quantified and standardized over a scale from 0.0 (0% likehood of gene promoter methylation) to 1.0 (100% likelihood of gene promoter hypermethylation).

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