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Sp1 Is Necessary for Gene Activation of Adamts17 by Estrogen

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ABSTRACT

Adamts17 is a member of a family of secreted metalloproteinases. In this report, we show that knockdown of Adamts17 expression induces apoptosis and inhibits breast cancer cell growth. Adamts17 expression can rapidly be induced by estrogens. siRNA knockdown of Sp1 or Myc demonstrated that Sp1 is required to induce Adamts17 gene expression in response to estrogen. Moreover, reporter assays showed that the proximal promoter and the upstream sequences were not capable of conferring estrogen responsiveness, suggesting that Sp1 elements may be located in the downstream intronic region. We further demonstrated that Sp1 and Myc binding in the proximal promoter region contributed to the Adamts17 basal expression. Furthermore, histone deacetylase (HDAC) and methylase inhibitors also induced Adamts17 expression, indicating that epigenetic alterations, such as aberrant HDAC and/or methylation are associated with dysregulated Adamts17 expression. By meta-analysis using Oncomine microarray data, we found that higher Adamts17 expression is found in several human cancer cell subtypes, especially in breast ductal carcinoma. Moreover, we found that there is an inverse correlation between higher Adamts17 expression and patients' survival. Our study suggests that Adamts17 may support breast cancer cell growth and survival. J. Cell. Biochem. 115: 1829–1839, 2014.

KEY WORDS: Adamts17; APOPTOSIS; GENE REGULATION; Sp1; BREAST CANCER

ADAMTS is a family of 19 secreted metalloproteinases that are involved in extracellular matrix degradation and turnover. The various members of this protease family are involved in a number of distinct and widespread processes, such as connective tissue maintenance, blood clotting, capillary formation, and cell migration [Le Goff Cormier-Daire, 2011; Wagstaff et al., 2011]. Previously, we showed that Adamts16 is stimulated in ovarian granulosa cells by gonadal hormones, such as follicle-stimulating hormone (FSH) or luteinizing hormone (LH), and it is capable of cleaving alpha2-macroglobulin (MG) [Gao et al., 2007]. Later, it was demonstrated that Adamts16 is upregulated in invasive ductal carcinoma of the breast (IDC) [Castellana et al., 2012] and in esophageal squamous cell carcinoma (ESCC) [Sakamoto et al., 2010]. Adamts17 is another member of this family. Mutations in the Adamts17 gene have been identified in human and several other animal species and are involved in disorders such as lenticular myopia, spherophakia and short stature [Morales et al., 2009; Farias et al., 2010; Payen et al., 2011]. Although it has been reported that Adamts17 is associated with acute promyelocytic leukemia [Lim et al., 2011], its exact role in cancer and how its gene expression is regulated have not yet been revealed.

In our previous studies, we found that *ADAMTS17* is a gene that is involved in the oxidative stress response, such as in response to the proteasome inhibitor, bortezomib, in estrogen-receptor (ER)-positive breast cancer cells. Estrogens exert profound effects on gene regulation, for example, 17-βestradiol (E2) binding to its receptor (ER) regulates human physiology through genomic and non-genomic pathways. In the genomic pathway, E2 induces ER

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translocation into the nucleus and activates the receptor transcriptional domain [Klinge, 2001]. ER binding to the estrogen response elements induces changes in downstream gene expression. Alternatively, the ER can associate with other transcriptional factors leading to gene activation via indirect promoter binding [Duan et al., 1998; Kalaitzidis and Gilmore 2005]. E2 regulation can also be mediated by non-genomic signals in which plasma membrane-associated ERs play a major role [Levin, 2005].

In the current study, we examined the expression of Adamts17 in normal human tissues and in breast cancer cells. By dissecting the Adamts17 promoter and its upstream sequences, we were able to identify Sp1 as a factor necessary for cellular response to E2 stimulation. We further demonstrated that DNA methylation and histone acetylation mediated Adamts17 expression. We also found that Adamts17 is more highly expressed in ductal, rather than in lobular, breast cancers.

RESULTS

DEPLETION OF Adamts17 SUPPRESSES CELL GROWTH AND LEADS TO APOPTOSIS

We sought to determine the effect of Adamts17 in breast cancer cells; for that purpose we used siRNA to knockdown Adamts17 expression in the breast cancer cell lines MCF7 and MDA-MB-231 (Fig. 1A). Seventy two hours after transfection with a pool of four Adamts17-siRNAs (see Materials and Methods Section), both MCF7 and MDA-MB-23 cell lines became less confluent, as determined by a CyQUANT Cell Proliferation Assay (Fig. 1B). Because the dead cells became more numerous than the controls, as evidenced by trypan blue staining (data not shown), we performed annexin V/PI staining of both control cells and Adamts17-siRNA-treated cells to investigate whether apoptosis was induced in these cells in response to Adamt17-knockdown. Fluorescence activated cell sorting analysis (FACS) indicated five- and threefold increases in the fraction of annexin V-positive MCF-7 and MDA-MB-231 cells, respectively (Fig. 1C). In contrast, the viability of the HeLa control cells was only slightly affected by Adamts17-siRNA knockdown, and HEK293 cells were not affected at all (not shown). These data imply that Adamts17 mediates cell survival by regulating an anti-apoptotic program in breast cancer cell lines, but not in all cell lines. Thus, specific down-regulation of Adamts17 significantly affected the survival and growth of breast cancer.

REGULATION OF Adamts17 EXPRESSION BY ESTROGEN

It has been shown that Adamts17 is regulated by BACH1, which directly associates with BRCA1 and is a master regulator of breast cancer bone metastasis [Cantor et al., 2001; Warnatz et al., 2011]. We were interested in investigating the Adamts17 gene expression and its regulation. Because the picture of Adamts17 expression in the published data was incomplete, we examined the Adamts17 expression by semi-quantitative RT-PCR in a number of healthy organ samples. Significantly higher expression levels of Adamts17 were detected in the heart, liver, pancreas, thymus, and ovary, but not in the brain, placenta, and leukocytes (Fig. 2A). We then examined the effect of estrogen on Adamts17 gene expression in MCF-7 cells. As shown in Figure 2B, E2 induced Adamts17 expression as early as 1 h. Puromycin and cycloheximide (CHX) did not block E2 induction, although CHX did elevate Adamts17 expression (Fig. 2C), suggesting that E2 induction does not require new protein synthesis.

Sp1 IS NECESSARY FOR GENE ACTIVATION OF Adamts17 BY ESTROGEN

We next investigated the molecular mechanisms leading to Adamts17 expression. Comparing the expressed sequence tags found at UniGene (National Center for Biotechnology Information

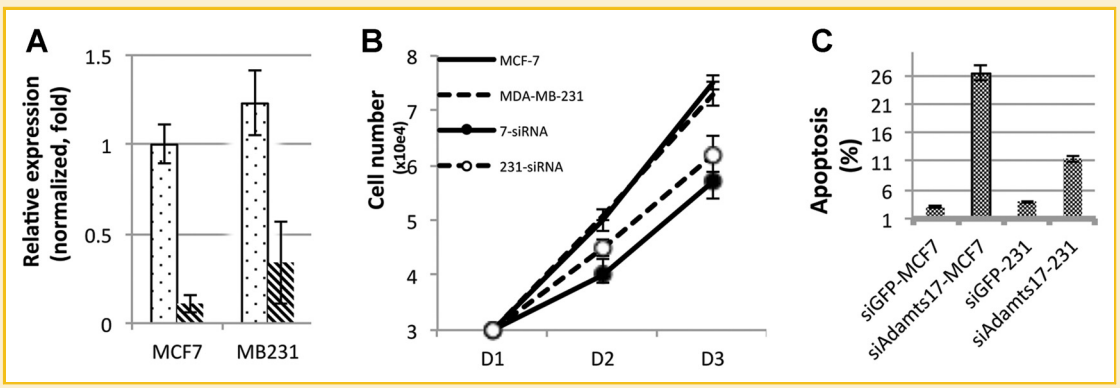

Fig. 1. Expression of Adamts17 interferes with cell growth and survival. A: Expression of Adamts17. MCF7 and MDA-MB-231 cells were transfected with either siRNA against GFP (siGFP, control, ⁞⁞) or a pool of 4 siRNAs to Adamts17 (▨). The relative level of mRNA-expression was determined by Q-PCR and indicated as fold-change normalized to POLR2J expression in three independent experiments. The expression in siGFP-transfected MCF7 cells was set as 1. B: MCF7 and MDA-MB-231 cell growth was monitored over 3 days. Cell numbers were determined in response to Adamts17-siRNA transfection compared with siGFP-transfected cells. Cell number and viability were examined by the CyQUANT Cell Proliferation Assay at various time points in triplicate. C: Adamts17 knockdown induced MCF7 and MDA-MB-231 cell apoptosis. Control MCF7 cells (siGFP-MCF7) and MDA-MB-231 cells (siGFP-231) were transfected with GFP-siRNA. MCF7 and MDA-MB-231 cells were also transfected with Adamts17-siRNA (siAdamts17-MCF7 and siAdamts17-231) and harvested 24 h later. Cells were stained with annexin V and propidium iodide (PI) and subjected to FACS. Histograms show average % (mean ± SE) of annexin V-positive (apoptotic) cells with or without Adamts17 siRNAs. Samples were run in quadruplicate.

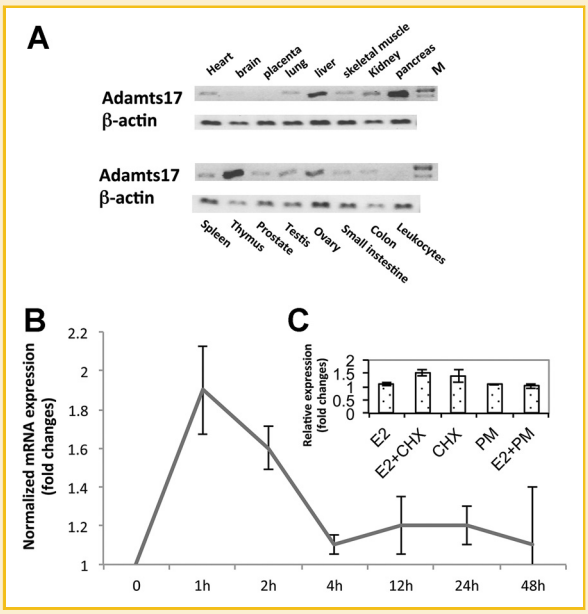

Fig. 2. Adamts17 expression in human tissues and its regulation by estrogen. A: Semi-quantitative PCR was performed on a panel of first-strand cDNAs prepared from different human tissues (Clontec). The PCR products obtained from each reaction following 28 cycles were separated on a 1.2% agarose gel and visualized by SYBR Green I. As a control of the cDNA from each tissue, the housekeeping gene β-actin was amplified in parallel. M indicates a molecular weight marker. B: Rapid induction of Adamts17 by 17β-estradiol (E2). MCF-7 cells were cultured in phenol red-free, charcoal-stripped medium for 72 h before being subjected to 50 nM E2 for 1, 2, 4, 12, 24, and 48 h. Adamts17 mRNA levels were determined relative to untreated cells (set as 1.0) by Q-PCR, as described in Materials and Methods Section. C: MCF-7 cells were treated with E2, 10 μg/ml cycloheximide (CHX), E2 plus CHX (E2 + CHX) or 15 μM puromycin (PM) or E2 plus puromycin (E2 + PM). The relative mRNA expression level was determined by real-time Q-PCR and indicated as fold-change in three independent experiments. The expression in untreated cells was normalized to 1 over POLR2J expression.

[NCBI] sequence database) to the UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg18) Assembly revealed that the transcription start sites (TSS, designated as position +1) clustered around −80 bp relative to the ATG. A TATA-like box was located at -27 bp upstream of the TSS (Fig. 3A). Moreover, when the genomic sequences were analyzed in silico using the Evolutionary Conserved Region (ECR) browser [Ovcharenko et al., 2004, http://ecrbrowser.dcode.org], the region covering the first exon and the flanking promoter (~500 bp) was found to be highly similar between humans and other primates, as well as rats and mice (*red area*, above 75% identity, Supplementary Table SI). However, further upstream sequences became divergent (green, Fig. 3B). We searched for potential regulatory regions present in this vicinity of the Adamts17 promoter (P340) using the PROMO software ([Xavier et al., 2002], Fig. 3A). Several highly conserved protein-binding sequences for transcription factors, such as Myc, Sp1, E2F, ERSE-II, AhR (CCACCTGG), lmo2, Runx2, and NF-Y, were predicted. To investigate the role of C-myc and Sp1 in mediating E2-induced Adamts17 expression, we used siRNAs that down-regulated both the mRNA and protein of C-myc and Sp1 as reported previously [Li et al., 2003; Ricci et al., 2004]. The results in Figure 3C show that transfecting Sp1 siRNA into MCF-7 cells significantly decreased the endogenous, E2-induced Adamts17 expression; however, C-myc siRNA did not cause any significant changes (data not shown). These results suggest that Sp1 is required to induce Adamts17 gene expression in response to E2.

To further study the effects of E2 induction on the Adamts17 gene, human genomic fragments (−1,129, F900) were cloned into the pGL3-basic Luc reporter plasmid. Promoter activities were assessed by measuring the luciferase activity after transiently transfecting the constructs into MCF-7 cells. However, we found that the elements within the luciferase reporter (F900) did not show any response to E2 in MCF-7 cells, although they were required for maximal basal promoter activity (Fig. 3D). Additionally, the bio-informatic analysis did not predict the presence of a potential estrogen receptor binding site in the Adamts17 proximal promoter and upstream sequences (Fig. 3A). These results indicate that although Sp1 is required to induce Adamts17 gene expression in response to E2, Sp1 elements may be located in the downstream intronic regions rather than in the proximal promoter.

Sp1 REGULATION OF BASAL Adamts17 EXPRESSION

We next studied the Adamts17 basal proximal promoter activity, although it did not respond to E2 as shown above. As shown in Figure 4A, a series of 5′-end deletion luciferase reporters containing different Adamts17 promoters were also constructed. The activities of the full-length promoter (F900) and truncated forms with deleted sequences between −1,129 and −590 bp (B660, S550, P340) were tested and found to be constitutively expressed. Conversely, a significant loss of activity at −213 bp (A100) was found, which was similar to the pGL3-Basic control (Fig. 4B). These results indicated that a functional promoter existed in the −1,129/+90 bp region relative to the TSS of the Adamts17 gene.

There are four Sp1-like boxes clustered within the basal promoter region (P340). We next analyzed the contribution of these sequences by investigating if the Sp1 transcriptional factor could bind to the individual sites by EMSA. Four oligonucleotides containing each Sp1 box were incubated with recombinant human Sp1 produced in Schneider SL2 cells. As shown in Figure 4C, all oligonucleotides yielded DNA–protein complexes with Sp1 proteins, although two of them bound Sp1 more strongly (lanes 1–4). Consistent with these findings, the luciferase assays showed that the reporter carried internal substitution mutations of these two Sp1 sites (CC→AA) and reduced the activity by 40%, compared to that of the wild type P340 (Figs. 4D and E). We further confirmed their roles by introducing an internal deletion of the other two Sp1 boxes. As depicted in Figure 4E, deletions between −50 and −75 bp also caused decreases in the reporter activities by approximately 40%. These results indicate that Sp1 elements may play pivotal roles in maintaining the Adamts17 basal transcriptional activity.

Myc REGULATION OF BASAL PROMOTER ACTIVITY

There are two consensus sequences (CANNTG) for Myc at position −27 to −20 (Fig. 3A). Therefore, we focused on the function of these binding sites. We carried out chromatin immunoprecipitation (ChIP) analyses to measure the potential interaction between Myc and the

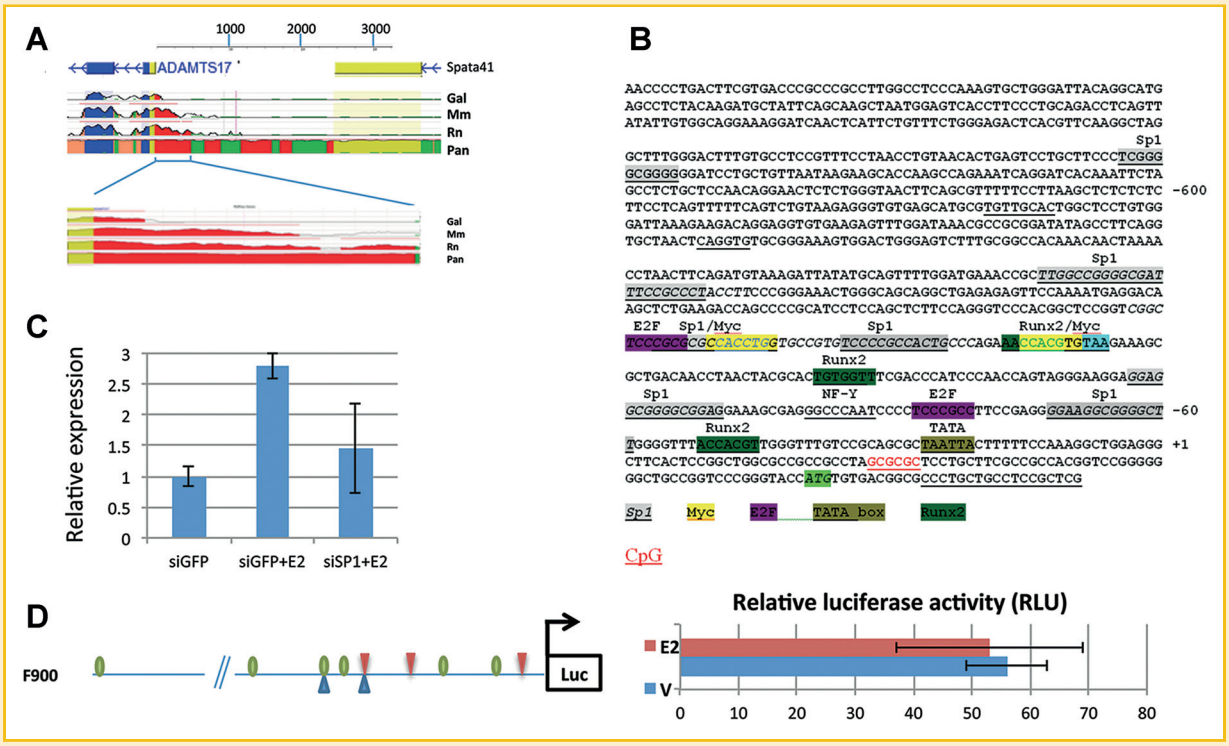

Fig. 3. E2-responsiveness of the Adamts17 gene promoter and its basal promoter activity. A: The ECR browser shows the sequence alignment of two exons of human Adamts17 and its 3 kb sequence immediately upstream with chicken, mouse, rat, and chimpanzee. Red areas denote sequence identity above 70% and green bars are transposons and repeats. The coordinate is shown in base pairs. B: Promoter sequence of human Adamts17. C: Effects of Sp1 siRNA on Adamts17 transcriptional activation. MCF7 cells were transfected with either control siRNA (siGFP) or Sp1 siRNA (siSP1). Two hours after E2 treatment, samples were analyzed for Adamts17 mRNA levels by quantitative RT-PCR. Relative expression levels were obtained after normalization to POLR2J levels. E2 responsiveness of the −1,129 to +90 region of the Adamts17 gene (F900) in MCF-7 cells is shown. Transient transfection of Adamts17 promoter-luciferase reporter plasmids (1 mg each) and luciferase assays were carried out as described in the Materials and Methods Section. Luciferase activities were normalized to that for b-gal transfection (the reference plasmid).

Adamts17 promoter. As shown in Figure 5A, the ChIP analysis revealed that Myc was bound to the promoter in MCF7, even in the absence of E2. These results were confirmed by CHIP-QPCR (Fig. 5B), suggesting that Myc binding to the promoter is independent of estrogen stimulation. We then assayed the activity of the P340 construct that was transiently transfected into MCF-7 cells in response to C-myc overexpression. As shown in Figure 5C, the luciferase activity was induced over fivefold by Myc proteins, but the E2 treatment did not further increase the level. However, when we examined how the endogenous Adamts17 expression was regulated by overexpressing C-myc or by knocking down endogenous Myc through siRNA, we found surprisingly that neither triggered any change in Adamts17 expression (Fig. 5D). The discrepancy between the endogenous Adamst17 regulation and the in vitro luciferase reporters suggests that the Myc binding to the Adamts17 promoter in vivo did not ensure Adamts17 activation.

GENE TRANSCRIPTION IS COUPLED TO HISTONE DEACETYLATION/ DNA DEMETHYLATION

Hypermethylation and histone acetylation both modify the chromatin structure and regulate gene activation. The search of genomic sequences that cover the first exon and its flanking regulatory region revealed that this region contains a CpG island with over 50 CpG dinucleotides (Supplementary Fig. S1). To explore any epigenetic link to Adamts17 gene activation, we examined Adamts17 expression by using 5-Aza-2′-deoxycytidine (5-Aza) to inhibit DNA methyltransferases (DNMTs) and trichostatin A (TSA) to inhibit histone deacetylases (HDACs). As shown in Figure 6A, the mRNA level was significantly changed in 5-Aza-treated MCF7 cells (1 μM 5-aza-dC) compared to vehicle-treated cells, although not in mb-231 cells. For the controls, other cell types, such as HT-29 colon cancer cells, had increased Adamts17 levels following 5-Aza treatment, but not in LNCaP and HepG2 cells (Fig. 6A). We next determined whether deacetylase activity was associated with Adamts17 expression by using TSA. Following treatment with TSA, Adamts17 mRNA levels increased in MCF7 and LNCaP cells compared to vehicle-treated cells. In contrast, the expression remained unaffected in MB231 and HepG2 cells (Fig. 6). These results suggested that histone acetylation and DNA methylation affects Adamts17 transcription in breast cancer cells.

ASSOCIATION OF ELEVATED *ADAMTS17* EXPRESSION WITH HIGHER HISTOLOGICAL GRADE AND WORSE CLINICAL OUTCOME IN BREAST CANCERS

Previously we evaluated the involvement of Adamts17 in various cancer cell types by carrying out a meta-analysis to examine

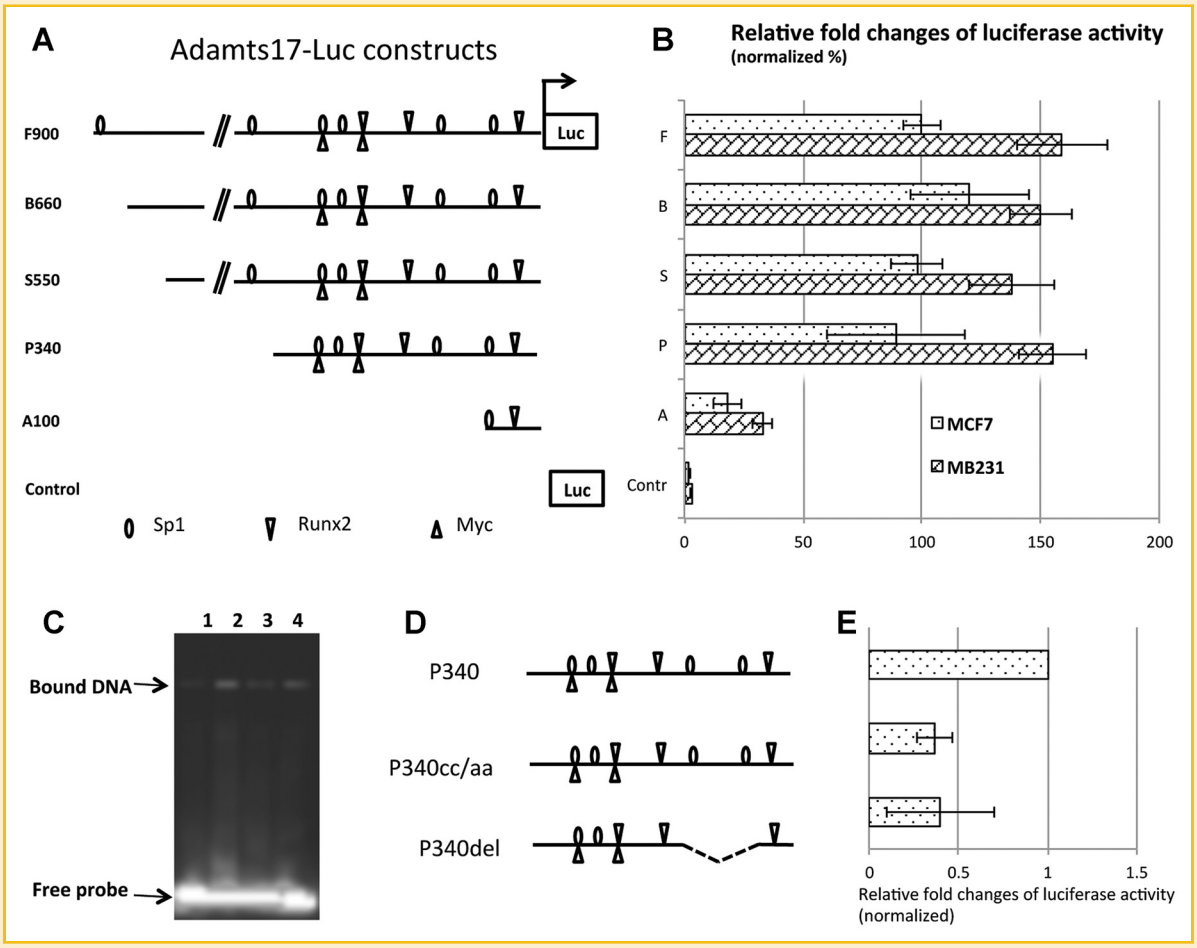

Fig. 4. Sp1 regulates the promoter activity of Adamts17. A: Schematic representations of the human Adamts17 promoter and the construction of deletions, which were derived from F900 (−1,129 to +90). B: Deletion analysis of F900. The F900 luciferase activity was arbitrarily set at 100% in MCF-7 cells. Measurements were expressed with error bars for three independent experiments for every construct. C: Binding of SP1 proteins to four oligonucleotides derived from putative Sp1 binding sequences in the Adamts17 proximal promoter by gel mobility shift assays described in the Materials and Methods Section. Sp1 proteins were incubated with 5′ Dyomics 781-labeled sp1–520 (1), sp1–420 (2), sp1–425 (3), and sp1–100 (4). D: Deletion analysis of P340. E: Luciferase activities were normalized to the reference plasmid (b-gal). The differences were compared to P340, where 1 was arbitrarily set in MCF7 cells. Measurements were expressed with error bars for three independent experiments for every construct.

Adamts17 expression using Oncomine microarray data of 14,182 samples across 68 human cancer datasets [Rhodes et al., 2004]. We found significantly higher Adamts17 expression levels in some, but not all, subtypes of various cancer specimens compared to the corresponding healthy tissue (Figs. 7A–C and Supplementary Fig. S2).

In breast cancer, Adamts17 expression levels were also significantly higher compared to normal mammary tissue (Figs. 7A and B). As a control, we also examined the expression of an array of biomarkers currently used for breast cancer staging, such as MUC1, PLAU, and SERPINE1 (Fig. 7A). More specifically, Adamts17 expression was higher in ductal breast carcinoma than in lobular breast carcinoma in five out of six datasets. For instance, there was a 3.2-fold difference between ductal and lobular breast carcinomas (Fig. 7B). However, the expression level of Adamts17 was not clearly correlated with the ER, progesterone receptor or ERBB2 status (Supplementary Fig. S3A–C) and the BRCA1/2 status (that could only be analyzed in one dataset, Supplementary Fig. S3D). We also analyzed the Adamts17 expression in Grades 1, 2, and 3 breast cancer subtypes, and the results demonstrated that Adamts17 was significantly overexpressed with increasing tumor grading from 1 to 3 ($P = 0.0001$) (Fig. 7D and Supplementary Fig. S3E).

E-cadherin (CDH1)is one of the best-studied discriminators for differentiating ductal and lobular breast cancer specimens [Sarrió et al., 2003]. As a control, we evaluated the co-expression patterns of Adamts17 and CDH1. As shown in Figure 7E, both genes were expressed at higher levels in ductal breast cancer samples, although the expression levels varied among individual patients.

Furthermore, we analyzed the association between Adamts17 expression and patients' survival. As shown in Figures 2F and G, high expression was significantly associated with patients' poor survival, indicating that elevated Adamts17 expression influenced clinical outcomes. Taken together, these data suggest that Adamts17 can be a potential biomarker of ductal breast cancer.

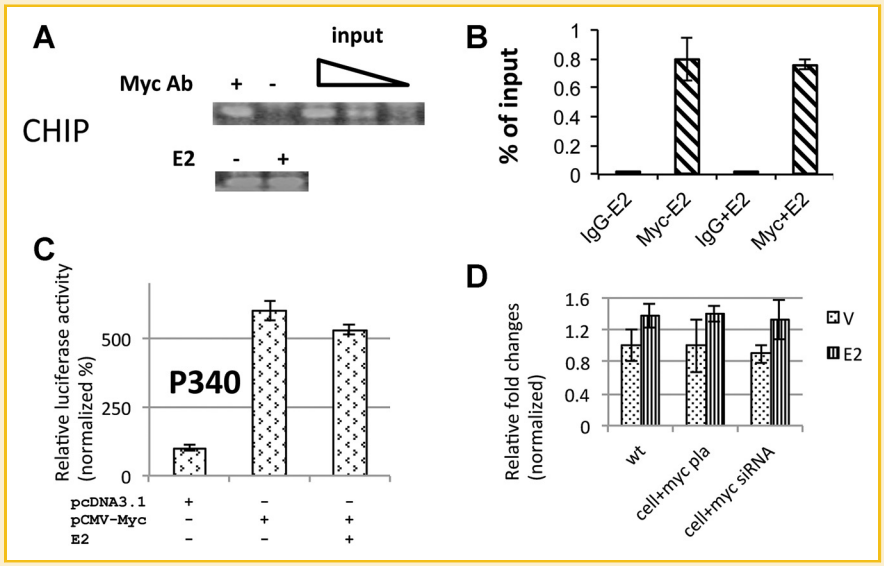

Fig. 5. Interaction of C-myc with the Adamts17 promoter. A: Binding sites and amplicons used in chromatin immunoprecipitation (ChIP) analyses are shown in the promoter and the first exon of Adamts17. ChIP assays were performed with anti-C-myc antibody (N-262, +) or IgG (−) from MCF-7 cells synchronized by serum starvation and then stimulated with 50 nM E2. Cells were formaldehyde crosslinked and processed for ChIP analyses by semi-quantitative PCR. 1×, 1/10×, and 1/100× titration of input chromatin were included as additional controls. B: ChIP assays were performed with anti-C-myc antibody (N-262) or IgG. DNA was analyzed by ChIP-QPCR. C: Constructs of P340 were transfected with pcDNA3.1 or Myc expression plasmids. Six hours after transfection, cells were treated with E2 for 2 h and assayed for luciferase activity. D: Cells were either transfected with Myc expression plasmids (+ cell pla) or with Myc siRNAs (+ myc siRNA) for 6 h. (V) indicates DMSO treatment.

DISCUSSION

Many proteins in the human ADAMTS family have been shown to be involved in tumorigenesis and metastasis [Wei et al., 2010]. As our results demonstrated, Adamts17 knockdown inhibited cell growth and induced apoptosis; therefore, it is conceivable that Adamts17, along with other members of this protease family, is a degrading protease for certain components of the extracellular matrix, thereby promoting tumor cell growth. Conversely, factors or substrates other than the extracellular matrix that inhibit cell survival may be targeted by Adamts17. Therefore, it is important to understand the gene regulation involved with Adamts17 expression.

It has been shown that Adamts17 is regulated by BACH1, which is directly associated with BRCA1 and is a master regulator of bone metastasis in breast cancer [Cantor et al., 2001; Warnatz et al., 2011]. We found that Adamts17 expression is induced by E2 and that epigenetic regulation also plays a role in its gene expression.

Adamts17 expression can be stimulated rapidly by E2, but its expression then remains at a lower level. The molecular regulation of many estrogen-regulated genes, such as TFF1/pS2 in breast cancer, has been elucidated as direct ER binding to the consensus ER elements (ERE, [Pearce and Jordan 2004]). In the present study, we found that Sp1 proteins were able to bind to the Sp1 consensus elements. Substitutions and a deletion on Sp1 sites located within the proximal promoter demonstrated that these binding sequences serve as critical elements for basal expression. Furthermore, estrogen induction of the Adamts17 gene was suppressed by Sp1 knockdown. These results suggest that Sp1 was required for Adamts17 expression induced by estrogen. Our results also demonstrated that C-myc overexpression stimulated Adamts17 reporter activities, but it was not able to induce endogenous expression. Consistent with these observations, Adamts17 was not considered as a Myc-responsive gene in previous studies (http://www.myccancergene.org/). Moreover, E2 could not increase Myc recruitment to its binding region, although Myc has been shown to be a regulator for E2 stimulation. Thus, Myc is not responsible for E2 induction of Adamts17 expression. The expression of Adamts17 induced by estrogens would be regulated by the cooperative effects of Sp1 in the proximal promoter with factors binding to downstream sequences and/or the chromatin structure.

Several acetylases/deacetylases and methylases/demethylases directly or indirectly interact with ERα and facilitate the decondensation of chromatin at the transcription starting site [for review, see Green and Carroll, 2007; Mann et al., 2011]. Previously, it was shown that TSA mimics the estrogen-induced activation of BRCA1 by increasing p300-dependent histone acetylation at the BRCA1 promoter [Jang et al., 2010]. Moreover, the association of estrogen receptors with histone deacetylases 2, 4, and 6 has been demonstrated in a ligand-dependent manner [Leong et al., 2005; Saji et al., 2005; Itoh et al., 2007]. Indeed, both TSA and Aza can induce expression, indicating that histone deacetylases and methylases are key regulatory mediators of Adamts17 gene expression. We found that the E2 stimulation did not require protein synthesis. As shown in the results, CHX may regulate Adamts17 expression via chromatin

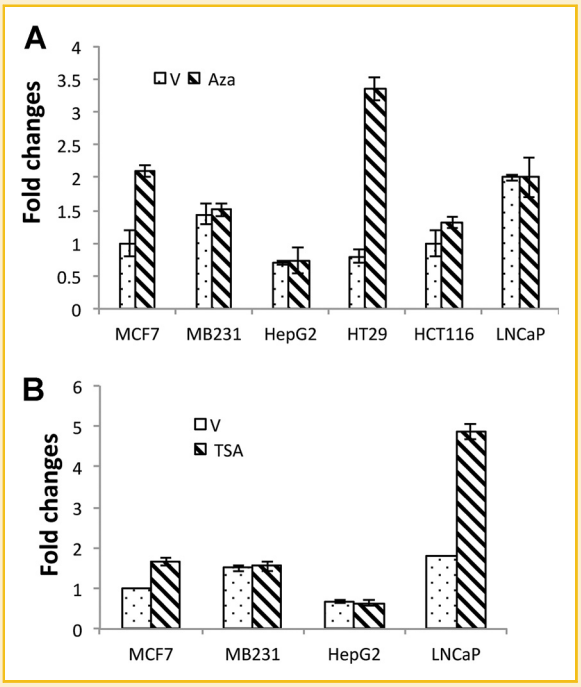

Fig. 6. Expression of Adamts17 in cells following treatments with Aza or TSA. A: Cells were treated with 10 μM Aza for 24 h. B: Cells were treated with 100 nM TSA for 24 h. Adamts17 expression was determined by Q-PCR. Bars show the mean of relative Adamts17 mRNA levels (n = 3) with s.d. (error bars) after treatments. Adamts17 mRNA levels were first normalized to POLR2J expression, and then the Adamts17 expression in control MCF-7 cells with DMSO treatment (the vehicle treated control, V) was normalized to 1.

modifications or mRNA degradation inhibition as suggested in some previous studies [Jacobson and Peltz, 1996; Cesari et al., 1998]. Therefore, it is possible that 17β-estradiol affects the rates of histone acetylation and deacetylation in MCF-7 cells, meaning that estrogens affect the level of histone acetylation. Thus, rapid E2 induction of Adamts17 expression can be due to the change of the chromatin structure.

Mutations in Adamts17 are the genetic cause of Weill–Marchesani-like syndrome, which is characterized by lenticular myopia, ectopia lentis, glaucoma, spherophakia, and short stature [Morales et al., 2009]. However, the role of Adamts17 in cancer has not been demonstrated before, although a novel PML-Adamts17-RARA gene rearrangement has recently been found in a patient with pregnancy-related acute pro-myelocytic leukemia [Lim et al., 2011]. Our studies showed an important "in silico" finding that Adamts17 was expressed at low levels in invasive lobular carcinoma of the breast, but was highly expressed in both forms of breast ductal carcinoma. Our results are consistent with a previous report of a proteomic study, which demonstrated that Adamts17 is a protease abundantly present in invasive ductal cell carcinoma [Overall et al., 2004]. Moreover, we found that there is an inverse correlation between higher Adamts17 expression and patients' survival. Therefore, further studies are underway to confirm these findings with primary tissue samples.

MATERIALS AND METHODS

MATERIALS

The expression plasmid for Myc was obtained from imaGenes (Berlin, cDNA clone MGC: 64962 IMAGE: 6012670, in pCMV-SPORT6). FlexiTube Adamts17 siRNAs (SI00151655, SI00151676, SI03043887, and SI03101672) and Sp1 siRNA were from Qiagen (Hilden, Germany). One microgram of each siRNA or a pool of all four Adamts17 siRNAs was transfected with RNAiFect (Qiagen). Anti-Adamts17 (Q-12)-antibody was purchased from Santa Cruz (USA, sc-100480). Anti-C-myc [9E11] antibody was from Abcam. Recombinant human Sp1 proteins were obtained from ALX-201-106-R050 (http://www.enzolifesciences.com). 17β-Estradiol (E2) was from Calbiochem (cat. 3301).

CELL CULTURE

MCF-7, MDA-MB-231, HeLa, and HEK293 cells were cultured as previously described in detail [Gao et al., 2007]. MCF7 cells were cultured in 10% FBS, charcoal-stripped serum, endogenous steroids, and phenol red-free DMEM. Cells were then treated with 17β-estradiol (E2, at 50 nM) for the indicated times. Each experiment was performed in triplicate. Cells were seeded at 3×10^4 cell density in 6-cm dishes. Cells in three dishes were harvested at days 1, 2, and 3 and the number of cells in each dish was measured by the CyQUANT Cell Proliferation Assay Kit (C7026) from Invitrogen, following the manufacturer's instructions.

APOPTOSIS ASSAY

Viability was determined at various time points by trypan blue exclusion, counting at least 100 cells in each individual culture. The percentage of cell survival was defined as the number of surviving cells per total cell count. Significant differences ($P < 0.05$) between mean values were analyzed using the Kruskal–Wallis test and growth curves were calculated. Annexin V and propidium iodide (PI) staining: 24 h after transfection cells were harvested, stained with annexin V–Alexa Fluor 488 and PI (Molecular Probes, Eugene, OR) and subjected to Fluorescence Activated Cell Sorting (FACS). A total of 10,000 cells were counted per sample and the data were processed using the FlowJo software (Tree Star, Ashland, OR).

EXPRESSION ANALYSIS BY SEMI-QUANTITATIVE RT-PCR AND Q-PCR

The expression pattern of Adamts17 mRNA in a number of different human tissues was examined by semi-quantitative RT-PCR using the primers listed in Table I and panels of first-strand cDNAs from various human tissues (Clontech USA. Human MTC™ Panel I&II, Catalog No. K1420-1, K1421-1). The PCR conditions were as described previously with 28 cycles and β-actin as a control [Gao et al., 2007].

For Q-PCR studies, total RNA was extracted from cells using a commercially available RNeasy Total RNA kit from Qiagen. Primers for real-time PCR were synthesized by Microsynth, Balgath, Switzerland (Table I). Power SYBR Green PCR Master Mix (AB Applied Biosystems) for real-time PCR and POLR2J Control Reagent as an internal control were employed. cDNA was

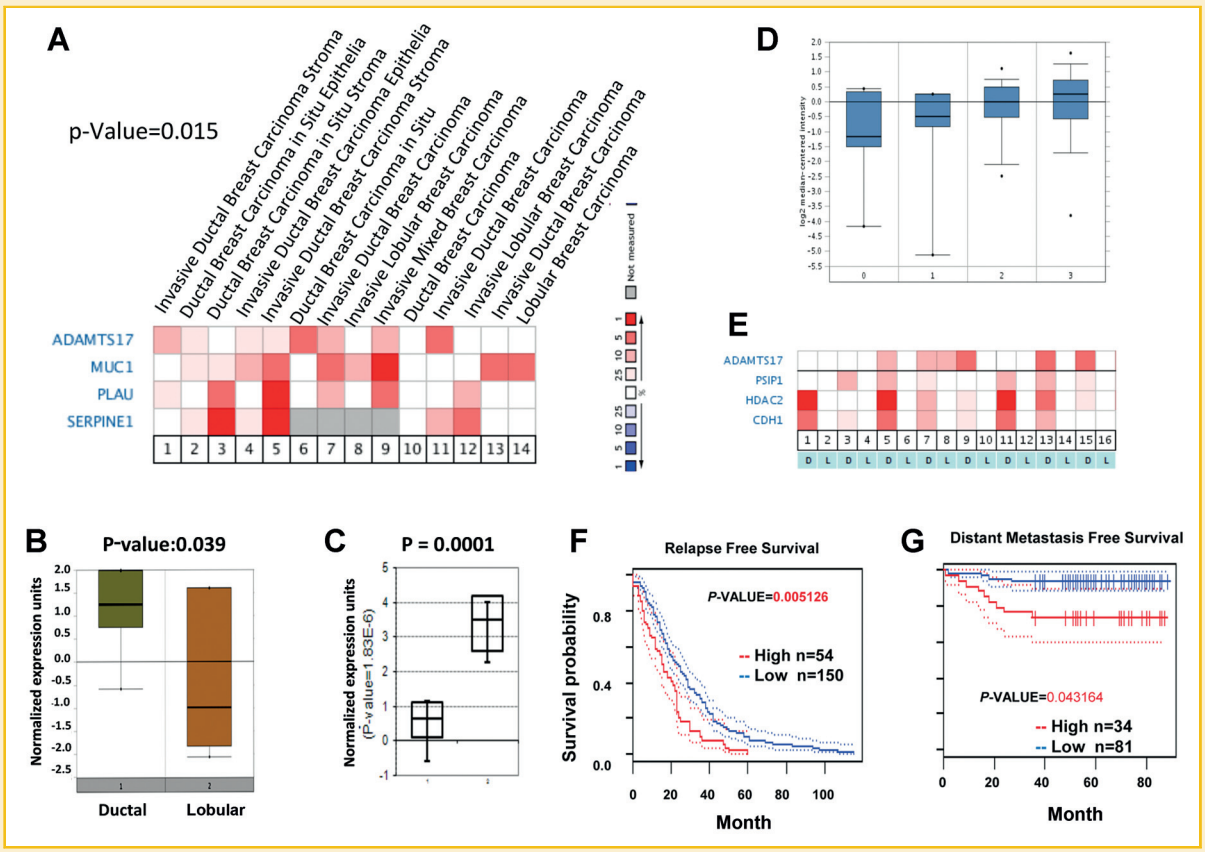

Fig. 7. Expression of Adamts17 in cancer. A: Oncomine was used to analyze microarray data published previously. Expression of Adamts17 with genes commonly used for the staging of breast cancer in clinical practice was analyzed. All data, including *P* values, were calculated from Oncomine. Expression level (normalized expression units) was expressed as the log 2 median-centered intensity. Samples 1–14 were from six datasets as described in the Materials and Methods Section. B: Elevated expression levels were revealed in ductal breast carcinoma (1) when compared with breast lobular carcinoma (2). Data were extracted from the Turashvili Breast dataset. Normalized expression units were calculated as the log 2 median-centered intensity. C: 1 indicates normal skin tissue, 2 indicates skin basal cell carcinoma. Data were extracted from the Riker Melanoma dataset [Riker et al., 2008]. D: 0 indicates normal tissues, 1 as Grade 1, 2 as Grade 2, and 3 as Grade 3. E: Expression of Adamts17 with CDH1 and HDAC2, molecular markers of ductal breast cancer specimens. D indicates ductal breast carcinoma and L indicates lobular breast carcinoma (the data were extracted from six datasets). F: Probability of patients' survival was analyzed by a Kaplan–Meier plot for tumors stratified according to the Adamts17 expression level (high in red and low in blue) [Bos et al., 2009]. G: Kaplan–Meier analysis for Distant Metastasis Free Survival according to the Adamts17 expression level in the Li dataset [Li et al., 2010].

subjected to 40 cycles of real-time PCR amplification carried out using an ABI PRISM 7500 Sequence Detector System (AB Applied Biosystems). Q-PCR was performed in triplicate using the SYBR Green SuperMix (Quanta Biosciences). The results from the Q-PCR were calculated as differences in mRNA levels with delta CT and normalized to the level of POLR2J according to the manufacturer's instructions (SA Biosciences). Primer pairs used for Q-PCR are described in the Supplementary Table SI. The difference between the results was assessed statistically with the Mann–Whitney *U*-test.

SEQUENCE ANALYSIS OF Adamts17 PROMOTER FRAGMENTS

DNA sequence screening for putative transcription factor-binding sites was performed using the web-based prediction programs PROMO3.0 using version 8.3 of TRANSFAC (transfac.gbf.de/) and TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html). Putative Runx-binding sites were identified by searching for the published consensus sequence motif ACCPuCPu [Kamachi et al., 1990].

pGL3 LUCIFERASE ASSAY

DNA fragments containing various lengths of the Adamts17 promoter region were subcloned into a pGL3-basic vector. Substitutions were performed by in vitro site-directed mutagenesis using the QuikChange kit (Stratagene). The primers for the constructs are listed in Table I. In brief, for transient transfections, either charcoal-stripped MCF7 or MB231 cells were transfected with 1 μg of each luciferase reporter plasmid together with 0.25 μg b-galactosidase vector (Amaxa Nucleofector kit) according to the manual. For each plasmid reporter, cells at 2.0×10^5 cells/mL were cultured in 12-well plates. Eighteen hours after transfection, cells were treated with or without 50 nM E2 for 2 h. The vehicle control was 0.01% DMSO. Then, cells were lysed with 50 μl 1× reporter lysis buffer (Promega) and both the luciferase and b-galactosidase enzyme activities were measured from 20 μl of lysate. The luciferase activity was normalized to the transfection efficiency according to the b-galactosidase enzyme activity. Luciferase activity is represented as either relative light units (RLU) or fold-change relative to

TABLE I. Sequences for Primers and siRNAs

Gene name	Primer name	Sequence
ADAMTS17 (RT-PCR)	Adamts17-f299	CAGCTGTGGTAAAGGGGTGT
	Adamts17-r299	TGGCGTTGATCCTGTCGT
ADAMTS17 (Q-PCR)	Adamts17-s5	GGTCTCAATTTGGCCTTTACCAT
	Adamts17-s3	GACCTGCCAGCGCAAGAT
Sp1 (siRNA)	siSp1	AATGAGAACAGCAACAACTCC
ADAMTS17 (siRNA)	SI00151655	AACGACAGTGACTGCCCTCAA
	SI00151676	CAGGGACTTCTATGCAAACAA
	SI03043887	AGCGCGATACCTCGGCAATAA
	SI03101672	GACCGTCATGAACATGGTATA
Sp1	sp1–520	TTG GCC GGG GCG ATT TCC GCC CTA CCT T
5′ Mod.: Dyomics 781	520-c	AAG GTA GGG CGG AAA TCG CCC CGG CCA A
	sp1–420	CGG CTC CCG CGC GCC ACC TGG TGC CG
	420-c	CGG CAC CAG GTG GCG CGC GGG AGC CG
	sp1–425	CCC GCG CGC CAC CTG GTG CCG TGT CCC CGC CAC TGC CCA
	425-c	TGG GCA GTG GCG GGG ACA CGG CAC CAG GTG GCG CGC GGG
	sp1–100	AGG AGG AGG CGG GGC GGA GGA
	100-c	TCC TCC GCC CCG CCT CCT CCT
Myc (Chip)	Chip-5	TGTAAAGATTATATGCAGTTTTGGATG
	Chip-3	TACCATGTGTGACGGCGC
Reporters	*F900*	5′ACCG AGCT CGAGTGCAGTGGCACAATCTC
		5′AAGTA AGCT T CTCCAGCCTTTGGAAAAA
	B660	5′ACCG AGCT CGCCTCTGCTCCAACAGGAACT
		5′AAGTA AGCT T CTCCAGCCTTTGGAAAAA
	S550	5′ACCG AGCT CGATAAACGCCGCGGATATAG
		5′AAGTA AGCT T CTCCAGCCTTTGGAAAAA
	P340	5′ACCG AGCT CAGCAGGCTGAGAGAGTTCC
		5′AAGTA AGCT T CTCCAGCCTTTGGAAAAA
	A100	5′ACCG AGCT CGAAAGCGAGGGCCCAATC
		5′AAGTA AGCT T CTCCAGCCTTTGGAAAAA
	P340cc/aa	*CGCGCCACCTGGTGCCGTGTCCCCGCCACTG→*
		CGCGAAACCTGGTGCCGTGTCCaaGCCACTG
	P340del	5′ACCG AGCT CCAGCAGGCTGAGAGAGTTCC
		5′AAGTA AGCT T CCTCCAGCCTTTGGAAAAAGTAATTAGCG-CTCCTCCTTCCCTACT
Adamts17 (Chip-QPCR)	Promoter-chip-5	ATCCCAACCAGTAGGGAAGG
	Promoter-chip-3	GGACAAACCCAACGTGGTAA

the appropriate DMSO vehicle control. Three independent experiments were performed.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

5′-labeled Dyomics 781 single-stranded oligonucleotides were synthesized by Microsynth (CH). The double probes were generated by mixing the complementary oligonucleotides at a 1:1 ratio. The sequences are listed in Table I. For binding reactions, 50 ng recombinant human Sp1 proteins were incubated with 25 nM probes for 30 min at room temperature. The sample mixtures were then separated by 4% non-denaturing PAGE. The gel was scanned with a LICOR odyssey scanner.

DNA CHIP ASSAY

MCF-7 cells were synchronized by serum starvation and then stimulated with 50 nM E2. After 24 h, cells were formaldehyde crosslinked and processed for ChIP analyses by using the Chip-it Express kit (active motif). Immunoprecipitations were performed using anti-cMyc (9E11) antibodies. Semi-quantitative PCR was performed with primers specific for the gene promoters. Products were analyzed on a 6% acrylamide gel.

DNA MICROARRAY META-ANALYSIS FOR HUMAN CANCER GENE EXPRESSION

The Oncomine gene expression signature database was used to identify the association with human cancer [Rhodes et al., 2004]. An association was considered significant with an odds ratio >2. We also searched for overlap using different filtering criteria, such as (i) "cancer versus normal" and (ii) "clinical outcome," to study the origin and clinical characteristics. Six breast cancer mRNA expression datasets that were available were analyzed, including the Karnoub Breast dataset [Karnoub et al., 2007]; Ma Breast [Ma et al., 2009]; Radvanyi Breast [Radvanyi et al., 2005]; Richardson Breast [Richardson et al., 2006]; Turashvili [Turashvili et al., 2007], and Zhao Breast data set [Zhao et al., 2004]. Normalized expression units were calculated as the log 2 median-centered intensity.

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