Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 175–182
BBRC
www.elsevier.com/locate/ybbrc

The candidate tumor suppressor CST6 alters the gene expression profile of human breast carcinoma cells: Down-regulation of the potent mitogenic, motogenic, and angiogenic factor autotaxin

Jin Song [a,1], Chunfa Jie [b], Paula Polk [c], Ravi Shridhar [d], Timothy Clair [e], Jun Zhang [a], Lijia Yin [c], Daniel Keppler [a,f,*]

[a] Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, Shreveport, USA
[b] McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, USA
[c] Research Core Facility, Louisiana State University Health Sciences Center, Shreveport, USA
[d] Department of Pharmacology, Wayne State University School of Medicine, Detroit, USA
[e] Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, USA
[f] Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, USA

Received 22 November 2005
Available online 9 December 2005

Abstract

We recently coined CST6 as a novel candidate tumor suppressor gene for breast cancer. CST6 indeed is expressed in the normal human breast epithelium, but little or not at all in breast carcinomas and breast cancer cell lines. Moreover, ectopic expression of CST6 in human breast cancer cells suppressed cell proliferation, migration, invasion, and orthotopic tumor growth. To obtain insights into the molecular mechanism by which CST6 exhibits its pleiotropic effects on tumor cells, we compared global gene expression profiles in mock- and CST6-transfected human MDA-MB-435S cells. Out of 12,625 transcript species, 61 showed altered expression. These included genes for extracellular matrix components, cytokines, kinases, and phosphatases, as well as several key transcription factors. TaqMan PCR assays were used to confirm the microarray data for 7 out of 11 genes. One down-regulated gene product, secreted autotaxin/lyso-phospholipase D, was of particular interest because its down-regulation by CST6 could explain most of CST6's effect on the breast cancer cells. This study thus provides the first evidence that CST6 plays a role in the modulation of genes, particularly, genes that are highly relevant to breast cancer progression.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Tumor suppressors; Cytokines; Signaling; DNA microarrays; Functional genomics; Breast cancer

CST6 (or cystatin M) was initially identified by differential RNA display as a transcript that is down-regulated in metastatic when compared to primary breast tumor cells [1]. A transcript identical to the CST6 cDNA was cloned independently from embryonic lung fibroblasts and designated as cystatin E [2]. Here, we will refer to cystatin E/M as CST6. CST6 is a small protein sharing 27–32% homology to other cystatins [3]. The protein is secreted from cells as a glycosylated (17 kDa) and unglycosylated (14.4 kDa) form [1,2]. Unlike the genes of other cystatins, which are all clustered in a narrow region on chromosome 20p11.2 [3], CST6 has been assigned to chromosome 11q13 [4]. This genomic region is the site of loss of heterozygosity (LOH) in several cancer types and is believed to harbor—besides the MEN1 gene—several other tumor suppressor genes [5].

Based on the observation that CST6 is expressed in normal and premalignant breast epithelial cells but not in malignant breast cancer cell lines, its loss of expression

* Corresponding author. Fax: +1 318 675 5889.
E-mail address: dkeppl@lsuhsc.edu (D. Keppler).
[1] Present address: Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.171

was proposed to be linked to the progression of human breast cancer [1]. We recently reported that constitutive expression of CST6 in human breast cancer MDA-MB-435S cells significantly reduces cell proliferation, migration, Matrigel invasion, and tumor-endothelial cell adhesion [6]. We further established that ectopic expression of CST6 in breast cancer cells strongly delayed orthotopic tumor growth and expansion of spontaneous lung and liver metastases. Quantitative molecular and immunohistochemical studies further underscored the tumor suppressing nature of CST6. We showed indeed that microdissected normal human breast epithelial cells express high levels of this protein whereas invasive ductal carcinoma cells express little or no mRNA or protein [7].

In this study, we tested the hypothesis that CST6 expression might alter the gene expression profile of tumor cells. This hypothesis was prompted by findings showing that some cystatins could stimulate cell proliferation [8,9], increase nitric oxide (NO) production [10], and/or modulate interleukin production [11,12]. Our own previous studies suggested that CST6 suppresses cell proliferation through mechanisms that are independent of the inhibition of intracellular cysteine proteases [6]. To elucidate whether cell-secreted CST6 had autocrine function, we compared global gene expression profiles in two mock- and two CST6-transfected human MDA-MB-435S cell clones. Using oligonucleotide microarrays and various data mining tools, we analyzed 12,625 transcript species and found that ectopic expression of CST6 significantly altered expression of at least 61 transcript species: 20 were found to be up- and 41 down-regulated. Among the 61 transcript species, showing CST6-mediated changes in expression were signaling molecules, extracellular matrix components, kinases, and phosphatases, as well as several transcription factors. We further validated the microarray data for 7 out of 11 genes using independent and quantitative TaqMan PCR assays. The down-regulation of autotaxin/ecto-nucleotide pyrophosphatase 2 (ATX/ENPP-2) seemed particularly interesting to us because this enzyme has been implicated in breast cancer progression [13] and has pleiotropic but opposite effects to CST6 on tumor cells [14–16]. Taken together, our data establish for the first time that secreted CST6 might have the potential to modulate gene expression through autocrine and maybe paracrine mechanisms.

Materials and methods

Cell culture and transfection. Human breast carcinoma MDA-MB-435S cells (passage #400) were purchased from ATCC and, before transfection, cloned by limiting dilution. A single clone, representative of the parental cell 'line' with regard to morphology and cystatin expression (parental clone), was stably transfected with a pTracer-CMV2-based CST6 expression vector (CST6-clones) or the 'empty' control vector (mock-clones) as reported previously [6]. Cells were seeded into 100-mm Petri dishes and grown to sub-confluence in high-glucose DMEM (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 200 μg/ml zeocin (Invitrogen, Carlsbad, CA). Medium was replaced with fresh medium 24 h before harvesting cells for RNA extraction.

RNA extraction. Cells were trypsinized for 5 min and counted within 10 min using a hemocytometer. Two times 10^6 cells were resuspended into 100 μl RNA later to prevent RNA degradation and kept at 4 °C for storage. Cells were disrupted and homogenized using QIAshredder spin columns, and total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocols (Qiagen, Valencia, CA). The amount of RNA was determined spectrophotometrically, and the quality was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). To remove traces of contaminating genomic DNA, total RNA was treated with RNase-free DNase I (Boehringer-Roche, Indianapolis, IN) and RNA re-purified over an RNeasy spin column.

Labeling of cRNA and hybridization of arrays. Complementary DNA for each sample was synthesized from 8 μg total RNA using a SuperScript cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) in combination with a T7-$(dT)_{24}$ primer. Following phenol–chloroform extraction of the cDNA, biotinylated cRNA was transcribed in vitro using the BioArray HighYield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). Twenty micrograms of purified cRNA was fragmented by incubation in fragmentation buffer (200-mM Tris–acetate buffer, pH 8.1, 500-mM potassium acetate, and 150-mM magnesium acetate) at 94 °C for 35 min and chilled on ice. As an additional quality control, three micrograms of fragmented biotin-labeled cRNA was hybridized (see below for conditions) to a Test3-Array comprising 5′-UTR-, CDS- and 3′-UTR-regions of several housekeeping-type genes (Affymetrix, Santa Clara, CA). This allowed us to confirm that the labeled cRNA was of the highest quality. Fifteen micrograms of fragmented, biotin-labeled cRNA was then immediately hybridized to the Human Genome U95A v.2 Array (Affymetrix, Santa Clara, CA), interrogating 12,625 transcript species (probesets). Hybridization, washing, and staining of microarrays were performed in a GeneChip Hybridization Oven 640 and an Affymetrix GeneChip Fluidics Station 400. Conditions were as follows: the arrays were hybridized for 16 h at 45 °C with constant rotation (60 rpm), washed and then stained for 10 min at 25 °C with 10 μg/ml streptavidin R-phycoerythrin (Vector Laboratories, Burlingame, CA) followed by another 10 min at 25 °C with 3 μg/ml of biotinylated goat anti-streptavidin antibody (Vector Laboratories). Arrays were then stained once again with streptavidin R-phycoerythrin for 10 min at 25 °C.

Imaging, data extraction, and analysis of microarrays. After washing and staining, arrays were scanned at 570 nm using an Agilent GeneArray Scanner (Agilent Technologies). To estimate gene expression signals, image analysis was performed with the CEL files of the Chips using the statistics' package GC-RMA (Robust Multiarray Analysis) [17]. Image processing included a normalization procedure by way of quantile normalization [18] to reduce variation between individual arrays. This variation might be introduced during the processes of sample preparation, chip manufacture, fluorescence labeling, hybridization, and/or scanning. With the signal intensities estimated above, an empirical Bayes method with gamma–gamma modeling was implemented in the R package EBarrays to estimate posterior probabilities of the differential expression of genes between CST6- and mock-clones [19]. The criterion of the posterior probability >0.5, which means the posterior probability is larger than by chance, was taken to obtain the list of genes differentially expressed in the CST6-clones. The fold changes were calculated with the geometric means of the signal intensities of CST6- and mock-clones. The geometric means have been shown previously to perform better than the arithmetic means, in terms of determining differentially expressed genes (see http://biosun01.biostat.jhsph.edu/%7Eririzarr/Talks/camda2002.pdf). All computations were performed under R environment. The Cluster & TreeView software was also used to analyze gene expressions across all transfection clones [20].

Quantitative TaqMan gene expression assays. Total RNA was, respectively, isolated from transfected mock- and CST6-clones (Mo-1, Mo-2, CM-13, and CM-17) as described above and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Briefly,

2.5 μg DNase I-treated total RNA was added into 50 μl iScript Reaction Mix with 2.5 μl iScript Reverse Transcriptase and incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. Subsequent PCRs were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Two microliters of freshly synthesized cDNAs was transferred into 25 μl TaqMan Universal PCR Master Mix containing AmpErase UNG, a 6-FAM-labeled TaqMan probe, and specific primers for each amplification. The TaqMan probe/primer sets were as follows: CST6, Hs00154599_m1; LUM, Hs00158940_m1; ATX/ENPP2, Hs00196470_m1; FOS, Hs00170630_m1; BAX (epsilon), Hs00180269_m1; ID3, Hs00171409_m1; IER3, Hs00174674_m1; TGFBI, Hs00165908_m1; ICK, Hs00248170_m1; TCF8/ZEB1/ΔEF1, Hs00232783_m1; ZNF7, Hs00171565_m1; IL8, Hs00174103_m1; and ACTB, Hs99999903_m1. At least three experiments were performed for each TaqMan assay. An experiment consisted of duplicate amplification reactions for each gene product being analyzed. The β-actin mRNA was used as an internal control for equal sampling of total RNA from one cell clone to another. The CST6 mRNA served as positive control. No cDNA template (NT) and no reverse transcriptase (RT) were used as negative controls. The cycle threshold number (C_T), at which amplification entered exponential phase, was determined for each PCR using ABI Prism's 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). This number was used as an indicator of the relative amount of initial target RNA in each sample. That is, a lower C_T indicated a higher starting amount of target mRNA. The comparative C_T method was used to calculate the relative abundance of a target transcript with regard to an internal control (β-actin). Results are expressed as relative abundance of a specific mRNA between mock- and CST6-clones (fold change ± SD, for $n = 6$–8). One-way ANOVAs were performed to compare the gene expression levels between CST6- and mock-clones.

Immunoblot analysis. Transfected mock- and CST6-clones (Mo-1, Mo-2, CM-13, and CM-17) were seeded into 100-mm Petri dishes and grown to sub-confluence in complete medium as described above. Cell monolayers were washed twice for 5 and 30 min, respectively, with serum-free medium. Medium was then replaced with 10 ml of fresh serum-free medium and cells were incubated for 24 h at 37 °C. Secreted, high-mannose-type glycoproteins were concentrated 50-fold using concanavalin A–agarose batch-affinity precipitation (Sigma) [14]. Cells were trypsinized, counted, and the cell number was used to normalize, i.e., to correct volumes of concentrated samples for each clone. Samples were electrophoresed through a 4–15% SDS–PAGE gel, and resolved proteins were electrophoretically transferred onto Protran nitrocellulose membranes (Bio-Rad, Hercules, CA) at 60 V constant voltage and 4 °C overnight. Membranes were then blocked with 3% low-fat milk and probed with rabbit polyclonal antibodies raised against human ATX or CST6 as described before [7]. Actin in the corresponding cell lysates was used as a loading control for above immunoblots. Membrane was probed with the rabbit polyclonal antibody raised against a synthesized C-terminal actin fragment (A2066, Sigma). For detection, HRP-conjugated donkey anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ) and ECL Detection Reagents (Amersham Biosciences, Piscataway, NJ) were used in conjunction with Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ).

Secreted lyso-PLD assay. The lyso-phospholipase D (lyso-PLD) activity of ATX was measured as described earlier [21]. Briefly, transfected cell clones (Mo-1, Mo-2, CM-13, and CM-17) were grown and 48-h cell-conditioned media were collected as described above. Media were immediately supplemented with 10% (v/v final concentration) of ethylene glycol (Sigma, St. Louis, MO) to prevent loss of ATX activity and concentrated 40-fold using Centricon centrifugal concentrators (30-kDa cutoff). Duplicate samples (55 μl) were incubated in 100 μl final reaction volume with 1-mM lysophosphatidylcholine (LPC), for 8 h at 37 °C in Dulbecco's modified essential medium containing 0.1% (w/v) bovine serum albumin (DMEM-BSA). For determination of lyso-PLD activity on LPC, the release of choline was detected as follows: a 900 μl cocktail containing 50-mM Tris–HCl (pH 8), 5-mM $CaCl_2$, 0.3-mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS), 0.5-mM 4-aminoantipyrene (4-AAP), 5.3-U/ml HRP, and 2-U/ml choline oxidase was added to the 100 μl reaction mixture and incubated for 20 min at 37 °C. Absorbance was read at 555 nm and converted to nanomoles of choline by comparison to a choline standard curve. Means were compared using one-way ANOVA followed by Fisher's Least Significant Difference post hoc test (SPSS, Chicago, IL). LPC (18:1), choline, TOOS, 4-AAP, HRP, and choline oxidase were all from Sigma.

Results

CST6 does not alter expression of other cystatins or cathepsins

To determine the molecular mechanism(s) by which CST6 suppresses the malignant properties of tumor cells in vitro [6], we compared global gene expression profiles in two mock- and two CST6-transfected human MDA-MB-435S cell clones. For this, we used Affymetrix Hu95A v.2 oligonucleotide microarrays, which interrogate about 12,625 transcript species. First, we determined whether ectopic expression of CST6 in MDA-MB-435S cells was compensated by increased expression of a lysosomal cysteine protease or by decreased expression of another cystatin. We analyzed the expression data for the 12 presently known human lysosomal cysteine proteases (cathepsins B, C, F, H, K, L, L2, O, S, W, and Z as well as mammalian legumain) [22,23] as well as for some 11 members of the cystatin super-family (stefins A and B, cystatins C, D, F, S, SA, and SN, as well as fetuin, and high- and low-molecular mass kininogens) [24]. No compensatory effect on the expression of these genes was observed (data not shown). This is further underscored by the fact that an endogenous gene, CST3 (cystatin C), that follows a similar biosynthetic pathway as the transgene CST6, showed no changes in expression and secretion between parental, mock, and CST6 expressing cells [6,7].

CST6 alters the gene expression profile of MDA-MB-435S cells

We then compared the expression profiles of the two mock-clones (Mo-1 and Mo-2) with those of the two CST6-clones (CM-13 and CM-17) using an improved data analysis method. This allowed us to identify 61 transcript species (cDNAs, ESTs, and hypothetical ORFs) that showed significantly altered expression (posterior probability >0.5) in the two CST6-clones when compared to that of the two mock-clones (Table 1). The reader may also consult the supplementary material for the full data concerning the 61 probesets, including posterior probabilities, signal intensities, and common gene annotations. Among the 61 differentially expressed transcript species, 20 showed increased and 41 showed decreased expression upon ectopic expression of CST6 (Table 1). Owing to the fact that the transfection experiments were initially performed on a representative clone rather than the crude MDA-MB-435S cell 'line' [6], we have been able to reduce the noise due to clonal variability. Thus, we believe that the set of 61 transcript species extracted from this microarray study might truly be

Table 1
Identification of 61 transcript species showing significant altered expression upon expression of CST6

Probesets	Symbol	Title	FC	Gene ontology
Increase				
33129_at	CST6	Cystatin E/M	153	Protease inhibitor
1966_i_at	NOS2C	Nitric oxide synthase 2C (pseudogene)	2.1	NO biosynthesis
34193_at	CHL1	Cell adhesion molecule with homology to L1CAM	1.72	Cell adhesion
31665_s_at	—	—	1.71	
33440_at	**TCF-8**	TCF-8/ZEB1/ΔEF1 (represses interleukin 2 expression)	1.68	transcription
37780_at	PCLO	Piccolo (presynaptic cytomatrix protein)	1.63	Cytoskeleton organization
31954_f_at	GAGE5	G antigen 5	1.6	
34308_at	HIST1H2AC	Histone 1, H2ac	1.56	DNA binding
31498_f_at	GAGE6	G antigen 6	1.54	
1089_i_at	—	—	1.43	
37043_at	**ID3**	Inhibitor of DNA binding 3	1.4	Transcription
39827_at	DDIT4	DNA-damage-inducible transcript 4	1.4	
33149_at	HEAB	ATP/GTP-binding protein	1.39	ATP/GTP binding
32638_s_at	SMG1	PI-3-kinase-related kinase SMG-1	1.39	mRNA catabolism, kinase
38932_at	**ZNF7**	Zinc finger protein 7 (KOX 4, clone HF.16)	1.39	Nucleic acid binding
36767_at	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	1.36	Electron transport
1697_s_at	PTPRJ	Protein tyrosine phosphatase, receptor type, J	1.36	Cell–cell signaling
38825_at	FGA	Fibrinogen, A α-polypeptide	1.35	Cell proliferation
501_g_at	CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	1.35	Electron transport
35372_r_at	**IL8**	Interleukin 8	1.35	Angiogenesis, cell growth
38411_at	SORL1	Sortilin-related receptor	1.35	Transmembrane receptor
Decrease				
41544_at	PLK2	Polo-like kinase 2 (*Drosophila*)	−1.31	Cell cycle
40488_at	DMD	Dystrophin	−1.36	Cytoskeletal anchoring
33203_s_at	FOXD1	Forkhead box D1	−1.36	Transcription
219_i_at	MAP2	Microtubule-associated protein 2	−1.38	Microtubule stabilization
40913_at	ATP2B4	ATPase, Ca^{2+} transporting, plasma membrane 4	−1.39	Ca^{2+} transport/binding
35168_f_at	COL16A1	Collagen, type XVI, α1	−1.39	Cell adhesion
1005_at	DUSP1	Dual specificity phosphatase 1	−1.4	Cell cycle
32761_at	SRRM2	Serine/arginine repetitive matrix 2	−1.41	DNA binding
32242_at	CRYAB	Crystallin, αB	−1.42	Protein folding
297_g_at	—	—	−1.42	
1237_at	**IER3**	Immediate early response 3	−1.42	Apoptosis, cell growth
41431_at	**ICK**	Intestinal cell (MAK-like) kinase	−1.43	Kinase
37420_i_at	HLA-F	Major histocompatibility complex, class I, F	−1.43	Immune response
296_at	—	—	−1.43	
939_at	—	—	−1.44	
34693_at	SIAT7B	Sialyltransferase 7	−1.45	Glycosylation
40081_at	PLTP	Phospholipid transfer protein	−1.46	Lipid metabolism/transport
1152_i_at	CGB	Chorionic gonadotropin, β-polypeptide	−1.46	
1385_at	**TGFBI**	Transforming growth factor, β-induced, 68 kDa	−1.47	Cell adhesion/proliferation
39710_at	C5orf13	Chromosome 5 open reading frame 13	−1.5	
1826_at	RHOB	ras homolog gene family, member B	−1.51	Cell growth/maintenance
2067_f_at	**BAX**	BCL2-associated X protein (α, β, γ, ϵ, and σ)	−1.51	Apoptosis, cell cycle
34722_at	TIMP2	Tissue inhibitor of metalloproteinase 2	−1.51	Protease inhibitor
34478_at	RAB11B	RAB11B, member RAS oncogene family	−1.53	Signal transduction
38803_at	NCALD	Neurocalcin-δ	−1.55	Vesicle-mediated transport
40489_at	DRPLA	Dentatorubral-pallidoluysian atrophy (atrophin-1)	−1.56	Protein binding
34365_at	PPIE	Peptidylprolyl isomerase E (cyclophilin E)	−1.56	Protein folding
41124_r_at	**ENPP2**	Autotaxin (ATX)	−1.57	Cell growth/motility/angiog.
33942_s_at	STXBP1	Syntaxin-binding protein 1	−1.59	Protein transport/secretion
32243_g_at	CRYAB	Crystallin, αB	−1.6	Protein folding
2094_s_at	**FOS**	v-fos FBJ murine osteosarcoma viral oncogene homolog	−1.6	Cell growth, transcription
1916_s_at	**FOS**	v-fos FBJ murine osteosarcoma viral oncogene homolog	−1.68	Cell growth, transcription
41123_s_at	**ENPP2**	Autotaxin (ATX)	−1.69	Cell growth/motility/angiog.
2065_s_at	**BAX**	BCL2-associated X protein (α, β, ϵ, and σ)	−1.69	Apoptosis, cell cycle
34517_at	HMGCS1	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1	−1.71	Lipid metabolism
34870_at	LDB3	LIM domain binding 3	−1.72	Protein binding
434_at	H1F0	H1 histone family, member 0	−1.8	DNA binding
1915_s_at	**FOS**	v-fos FBJ murine osteosarcoma viral oncogene homolog	−1.86	Cell growth, transcription
36498_at	AP1S1	Adaptor-related protein complex 1, σ1 subunit	−1.86	Protein transport
38038_at	**LUM**	Lumican	−1.95	Cell motility
1997_s_at	**BAX**	BCL2-associated X protein (α, δ, and ϵ)	−2	Apoptosis, cell cycle

Using the Bioconductor R package, 20 transcript species showed increased and 41 showed decreased expression upon ectopic expression of CST6 (a probeset with the posterior probability >0.5 was taken as a differentially expressed transcript species). Column headings are as follows: 'Probesets' identify Affymetrix accession numbers; 'Symbol' identifies the name of the gene locus; 'Title' gives a tentative annotation of the transcript species; 'FC' gives the fold change as compared to the mock-controls. Gene symbols in bold face correspond to the transcript species that were validated using TaqMan PCR assays.

associated with expression of CST6 in human MDA-MB-435S cells. In an effort to link this set of genes to known cellular functions and pathways, we used Affymetrix' on-line Gene Ontology Mining Tool (GO). More than 24 out of 52 transcript species with useful GO-annotations appeared to be related either to cell growth/maintenance, motility, signal transduction, and/or transcriptional control (see Table 1 and the supplementary material). This is in good agreement with our previous data showing that CST6 is a pleiotropic factor with multiple effects on the malignant properties of MDA-MB-435S cells [6].

Validation of the microarray data

Before exploring how constitutive expression of CST6 might lead to altered expression of one or the other gene in human MDA-MB-435S cells, the profiling results need to be verified using alternate approaches. We have used quantitative TaqMan PCR assays to independently examine mRNA expression levels in the transfection clones. Our choice fell on a set of genes that showed little clonal variation and/or that were representative of the above gene ontology classes: BAX, ATX, FOS, and IER-3 for cell growth/maintenance; ATX and LUM for cell motility; ATX, ICK, IL-8, and TGFBI for signal transduction; and FOS, ID-3, TCF-8, and ZNF-7 for transcriptional control. Quantitative TaqMan PCR analyses showed that ectopic expression of CST6 increased expression of ID-3, IL-8, TCF-8, and ZNF-7 1.12-, 3.58-, 1.82-, and 1.35-fold, respectively (Fig. 1A, Table 2). At the same time, CST6 expression decreased mRNA expression of ATX, FOS, ICK, LUM, and TGFBI 2.44-, 1.45-, 1.82-, 3.33-, and 1.28-fold, respectively (Fig. 1A, Table 2). Statistical analyses confirmed the significance ($P < 0.05$) of the differential expression of ATX, FOS, ICK, IL-8, LUM, TCF-8, and ZNF-7. With P values of 0.2989 and 0.3147, the differential expression of TGFBI and ID-3 would require a much larger number of replicate experiments to be firmly established. Two genes out of 11, i.e., the two apoptosis-related genes BAX and IER-3, did not show consistent changes between microarray and TaqMan analyses (Figs. 1A and B, Table 2). The reason for this discrepancy remains obscure at this stage. Preliminary data from this laboratory indicate that CST6 expression did not trigger apoptosis in breast cancer cells. Overall, the TaqMan results were in good agreement with our microarray data and confirmed that expression of CST6 in MDA-MB-435S cells led to significant changes in expression of 7 genes out of 11 involved in tumor growth, invasion, and angiogenesis.

Expression of CST6 reduces ATX protein level and enzyme activity

Because of ATX's role in cell proliferation, motility, and angiogenesis, we anticipated that this enzyme would be tightly regulated at both the mRNA and protein levels. Immunoblot analysis of Con A-agarose affinity precipitated ATX did

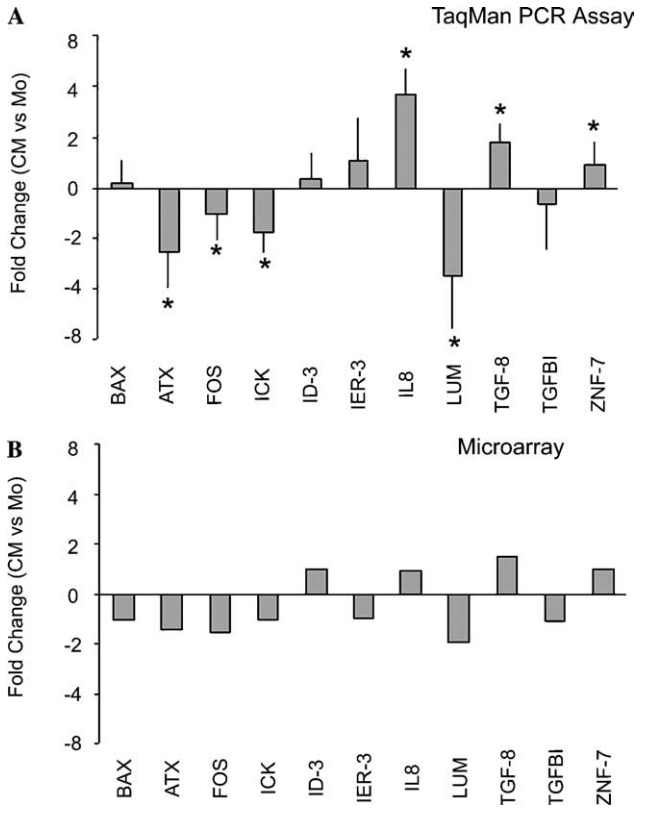

Fig. 1. Validation of microarray data by TaqMan PCR assays. TaqMan PCR assays were used to confirm the microarray data for 11 genes. (A) Mean TaqMan PCR data obtained from six to eight replicate assays. Fold changes (±SD) of CST6-clones with regard to mock-clones are shown after normalization to β-actin using the $\Delta\Delta C_t$ method. $^*P < 0.05$. (B) Microarray data obtained from duplicate mock- and CST6-transfected clones (geometric means). X-axis represents the individual genes that were probed; y-axis represents the fold changes in log-scale.

Table 2
Validation of microarray data by TaqMan PCR

Gene	Chip	TaqMan	P
BAX	−1.43	+1.06	0.5359
ATX	**−1.63**	**−2.44**	**0.0000**
FOS	**−1.71**	**−1.45**	**0.0020**
ICK	**−1.43**	**−1.82**	**0.0001**
ID-3	+1.40	+1.12	0.3147
IER-3	−1.42	+1.43	0.0532
IL-8	**+1.36**	**+3.58**	**0.0000**
LUM	**−1.95**	**−3.33**	**0.0000**
TCF-8	**+1.68**	**+1.82**	**0.0001**
TGFBI	−1.47	−1.28	0.2989
ZNF-7	**+1.39**	**+1.35**	**0.0230**

Eleven genes were validated using TaqMan PCR assays. Mean fold changes of CST6-clones with regard to mock-clones are shown after normalization to β-actin ($\Delta\Delta C_t$ method). P values are shown for the TaqMan assays. Gene symbols in bold face correspond to transcript species that exhibited $P < 0.05$ upon TaqMan validation.

indeed show greatly reduced steady-state levels of the 125-kDa enzyme in the media conditioned by CST6-clones (Fig. 2A, upper panel, CM-13 and CM-17) when compared to its level in the media conditioned by mock-clones (Fig. 2A, upper panel, Mo-1 and Mo-2). Glycosylated CST6 was used

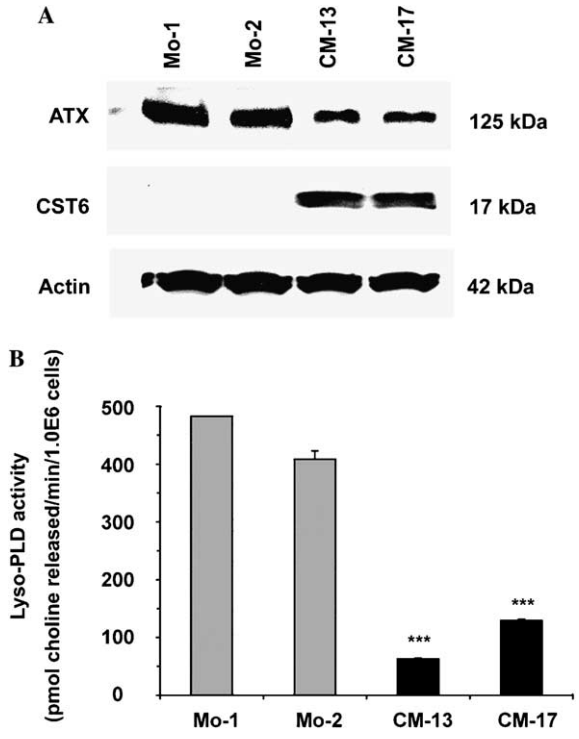

Fig. 2. Ectopic expression of CST6 reduced ATX protein level and enzyme activity. Transfected cell clones (Mo-1, Mo-2, CM-13, and CM-17) were grown and cell-conditioned media collected, and concentrated as described in Materials and methods. Cells were trypsinized, counted, and cell number used to normalize concentrated secretions of each clone. (A) Fiftyfold concentrated samples were analyzed by 4–15% SDS–PAGE, resolved proteins transferred onto nitrocellulose, and probed with rabbit polyclonal antibodies raised against human ATX or human CST6. Ectopic expression of CST6 (A, middle panel, CST6) led to decreased expression of ATX in CM-13 and CM-17 when compared to Mo-1 and Mo-2 (A, upper panel, ATX). Actin in the corresponding cell lysates was used as a loading control for above immunoblots (A, lower panel, actin). (B) In a separate experiment, 40-fold concentrated media were assayed for ATX's lyso-phospholipase D (lyso-PLD) activity using LPC as substrate. Ectopic expression of CST6 reduced lyso-PLD activity 3- to 8-fold. Enzyme activity is expressed as picomole choline released/min/10^6 cells. Results are the average of duplicate assays. One-way ANOVA followed by Fisher's LSD post hoc test was used to compare CST6- to mock-clones; ***$P < 0.001$.

as a positive control for Con A-binding (Fig. 2A, middle panel, CM-13 and CM-17).

Like other members of the NPP-family, ATX has multiple enzymatic activities [25]. Enzyme activity appears to be essential for many of its biological functions, since a single point mutation at amino acid T_{210} (rat enzyme: T_{206}) abolishes the enzyme's (1) nucleotide-phosphodiesterase activity using pNP-TMP as substrate as well as melanoma cell migration in response to autotaxin [26]; (2) lyso-PLD activity using LPC as substrate [27]; and (3) lyso-PLD activity using sphingosylphosphorylcholine (SPC) as substrate [25]. To further confirm the immunoblotting data, we examined lyso-PLD activity of autotaxin in whole media concentrated 40-fold using Centricon centrifugal concentrators (30-kDa cut-off). Ectopic expression of CST6 resulted in a 3- to 8-fold reduction in lyso-PLD activity on LPC (Fig. 2B). This reduction in lyso-PLD activity of the two CST6-clones was statistically highly significant ($p < 0.001$) when compared to the enzyme activity of each mock-clone. In conclusion, our results strongly suggest that secreted CST6 was able to modulate expression of autotaxin. Further studies are now in progress to understand the molecular mechanism of this modulation.

Discussion

Normal epithelial cells undergo multiple genetic and epigenetic changes to acquire the highly proliferative, invasive, vasculogenic/angiogenic, immunosuppressive, and metastatic properties that are characteristic of malignant tumors [28]. These genetic changes lead in turn to global changes in gene expression [29], and more specifically, in overexpression of tumor-promoting genes and loss of expression of tumor-suppressing genes. Close to 100 genes have so far been identified as tumor-promoting or tumor-suppressing, and the products of these genes belong to various protein superfamilies, such as cytokines, adhesion molecules, cell surface receptors, signal transducers, and transcription factors [30]. Data from several laboratories now show that protease inhibitors have to be included as a novel superfamily of proteins with tumor-promoting and/or tumor-suppressing properties [31,32].

Previously, we have shown that ectopic expression of the lysosomal cysteine protease inhibitor CST6 in a breast cancer cell line leads to suppression of several cellular properties that are characteristic of malignant tumor cells. More specifically, CST6 was found to inhibit cell proliferation, migration, invasion of a reconstituted extracellular matrix, as well as adhesion to an experimental endothelium [6]. These multiple effects of CST6 on tumor cells suggested that this inhibitor might have 'autocrine' properties. The literature already provides several intriguing leads in this direction: brain CST3 (cystatin C or γ-trace) was initially believed to be a neuroendocrine hormone with biochemical properties similar to those of glucagon [33]. More recently, CST3 or its chicken and rat orthologues were found to stimulate DNA synthesis and proliferation of a number of cells, including fibroblasts, mesangial cells, and neuronal stem cells [8,9,34,35]. In particular, this inhibitor seems to act in synergy with FGF-2 to sustain survival and proliferation of neuronal stem cells [9]. However, CST3 is also a potent antagonist of TGFβ signaling [36]. Others have shown that cystatins can increase nitric oxide production in interferon-primed macrophages [11,37]. The stimulation of cell proliferation and production of nitric oxide are both independent of the inhibition of lysosomal cysteine proteases [9,11]. Moreover, cystatins of filarial nematodes are able to down-regulate T-cell proliferation in hosts and induce anti-inflammatory interleukin responses [12,38,39].

Our observations that CST6 was able to reduce cell proliferation, migration/invasion as well as cell adhesion in vitro suggested that this protein might behave like an autocrine factor regulating cellular properties. To further

explore this lead, we have used DNA microarray analysis to profile gene expression changes induced by the putative 'CST6' signal. This approach, combined to a powerful statistical analysis, identified 20 up- and 41 down-regulated genes in human MDA-MB-435S cells overexpressing CST6. More than 24 out of 45 genes with useful gene ontology annotations were either related to cell growth/maintenance, motility, signal transduction, and/or regulation of transcription. To our knowledge, this is one of the very first studies to show that a cystatin has the capacity to alter expression of genes. This is of particular interest since secreted CST6 has hitherto been considered to play a role only in the regulation of intracellular protein degradation [2,40]. In contrast, our studies suggest that CST6 might suppress malignant properties of MDA-MB-435S cells partly through modulation of expression of two major secreted signaling molecules, ATX and IL-8. Both, ATX and IL-8, have been described in the literature as mitogenic, motogenic, and angiogenic cytokines [14–16,41,42]. Our microarray and TaqMan data show that CST6 down-regulates ATX but up-regulates IL-8. This suggests that CST6 might behave like a tumor suppressor for some types of cancers [6,7] while acting perhaps like a tumor promoter for other types of cancers [43,44]. We have shown previously [6] that most breast cancer and melanoma cells have lost expression of CST6 while two of three prostate cancer cell lines were found to express high levels of transcript. Hence, as for many cytokines there is no doubt that the cellular context, the level, and timing of the expression of CST6 are going to play an important role in its biological activity.

Current studies are aimed at determining whether the 'CST6 signal' is generated via inhibition of some proteolytic event at the cell surface or, rather via direct interaction of CST6 with a specific cell surface receptor. In addition, we would like to determine whether CST6 alters the transcription rate of the respective genes or, whether it acts at a post-transcriptional level such as the stability of the pre-mRNAs or mRNAs. Remarkably, a number of genes such as FOS and TCF-8/ZEB-1/ΔEF-1, which have been identified in this study as modulated by CST6, represent highly cancer-relevant transcription factors [45,46]. These results suggest that CST6 expression may impact on transcription of target genes. The studies presented here further underscore the importance of CST6 - and maybe cystatins in general—as novel signaling molecules during cancer progression. Future investigations will determine whether and how the 'cystatin' signal integrates into other signaling pathways and whether this ultimately leads to altered tumor growth, invasion, neovascularization, and/or metastasis.

Acknowledgments

This study was in part supported by a Research Grant CA91785 (DK) and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research (TC). JS received a Takeda Scholar-in-Training Award from the American Association for Cancer Research to present part of this work at the Special Conference on Cancer Research: 'New Directions in Angiogenesis Research,' Chicago, October 15–19, 2003. RS was partly supported by Research Grant CA36481 (Bonnie F. Sloane, Wayne State University) from the National Cancer Institute. Our sincere thanks go to Drs. Omar Skalli and Athena W. Lin for critical reading of the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.171.

References

[1] G. Sotiropoulou, A. Anisowicz, R. Sager, Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer, J. Biol. Chem. 272 (1997) 903–910.
[2] J. Ni, M. Abrahamson, M. Zhang, M.A. Fernandez, A. Grubb, J. Su, G.L. Yu, Y. Li, D. Parmelee, L. Xing, T.A. Coleman, S. Gentz, R. Thotakura, N. Nguyen, M. Hesselberg, R. Gentz, Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins, J. Biol. Chem. 272 (1997) 10853–10858.
[3] M. Abrahamson, Cystatins, Methods Enzymol. 244 (1994) 685–700.
[4] G. Stenman, A.K. Astrom, E. Roijer, G. Sotiropoulou, M. Zhang, R. Sager, Assignment of a novel cysteine proteinase inhibitor (CST6) to 11q13 by fluorescence in situ hybridization, Cytogenet. Cell Genet. 76 (1997) 45–46.
[5] E.S. Srivatsan, R. Chakrabarti, K. Zainabadi, S.D. Pack, P. Benyamini, M.S. Mendonca, P.K. Yang, K. Kang, D. Motamedi, M.P. Sawicki, Z. Zhuang, R.A. Jesudasan, U. Bengtsson, C. Sun, B.A. Roe, E.J. Stanbridge, S.P. Wilczynski, J.L. Redpath, Localization of deletion to a 300 kb interval of chromosome 11q13 in cervical cancer, Oncogene 21 (2002) 5631–5642.
[6] R. Shridhar, J. Zhang, J. Song, B.A. Booth, C.G. Kevil, G. Sotiropoulou, B.F. Sloane, D. Keppler, Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells, Oncogene 23 (2004) 2206–2215.
[7] J. Zhang, R. Shridhar, Q. Dai, J. Song, S.C. Barlow, L. Yin, B.F. Sloane, F.R. Miller, C. Meschonat, B.D. Li, F. Abreo, D. Keppler, Cystatin m: a novel candidate tumor suppressor gene for breast cancer, Cancer Res. 64 (2004) 6957–6964.
[8] Q. Sun, Growth stimulation of 3T3 fibroblasts by cystatin, Exp. Cell Res. 180 (1989) 150–160.
[9] P. Taupin, J. Ray, W.H. Fischer, S.T. Suhr, K. Hakansson, A. Grubb, F.H. Gage, FGF-2-responsive neural stem cell proliferation requires CCg, a novel autocrine/paracrine cofactor, Neuron 28 (2000) 385–397.
[10] L. Verdot, G. Lalmanach, V. Vercruysse, S. Hartmann, R. Lucius, J. Hoebeke, F. Gauthier, B. Vray, Cystatins up-regulate nitric oxide release from interferon-gamma-activated mouse peritoneal macrophages, J. Biol. Chem. 271 (1996) 28077–28081.
[11] L. Verdot, G. Lalmanach, V. Vercruysse, J. Hoebeke, F. Gauthier, B. Vray, Chicken cystatin stimulates nitric oxide release from interferon-gamma-activated mouse peritoneal macrophages via cytokine synthesis, Eur. J. Biochem. 266 (1999) 1111–1117.
[12] A. Schonemeyer, R. Lucius, B. Sonnenburg, N. Brattig, R. Sabat, K. Schilling, J. Bradley, S. Hartmann, Modulation of human T cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode *Onchocerca volvulus*, J. Immunol. 167 (2001) 3207–3215.

[13] S.Y. Yang, J. Lee, C.G. Park, S. Kim, S. Hong, H.C. Chung, S.K. Min, J.W. Han, H.W. Lee, H.Y. Lee, Expression of autotaxin (NPP-2) is closely linked to invasiveness of breast cancer cells, Clin. Exp. Metastasis 19 (2002) 603–608.
[14] M.L. Stracke, H.C. Krutzsch, E.J. Unsworth, A. Arestad, V. Cioce, E. Schiffmann, L.A. Liotta, Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein, J. Biol. Chem. 267 (1992) 2524–2529.
[15] S.W. Nam, T. Clair, C.K. Campo, H.Y. Lee, L.A. Liotta, M.L. Stracke, Autotaxin (ATX), a potent tumor motogen, augments invasive and metastatic potential of ras-transformed cells, Oncogene 19 (2000) 241–247.
[16] S.W. Nam, T. Clair, Y.S. Kim, A. McMarlin, E. Schiffmann, L.A. Liotta, M.L. Stracke, Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor, Cancer Res. 61 (2001) 6938–6944.
[17] R.A. Irizarry, B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, T.P. Speed, Exploration, normalization, and summaries of high density oligonucleotide array probe level data, Biostatistics 4 (2003) 249–264.
[18] B.M. Bolstad, R.A. Irizarry, M. Astrand, T.P. Speed, A comparison of normalization methods for high density oligonucleotide array data based on variance and bias, Bioinformatics 19 (2003) 185–193.
[19] C.M. Kendziorski, M.A. Newton, H. Lan, M.N. Gould, On parametric empirical Bayes methods for comparing multiple groups using replicated gene expression profiles, Stat. Med. 22 (2003) 3899–3914.
[20] M.B. Eisen, P.T. Spellman, P.O. Brown, D. Botstein, Cluster analysis and display of genome-wide expression patterns, Proc. Natl. Acad. Sci. USA 95 (1998) 14863–14868.
[21] E. Koh, T. Clair, E.C. Woodhouse, E. Schiffmann, L. Liotta, M. Stracke, Site-directed mutations in the tumor-associated cytokine, autotaxin, eliminate nucleotide phosphodiesterase, lysophospholipase D, and motogenic activities, Cancer Res. 63 (2003) 2042–2045.
[22] D. Bromme, J. Kaleta, Thiol-dependent cathepsins: pathophysiological implications and recent advances in inhibitor design, Curr. Pharm. Des. 8 (2002) 1639–1658.
[23] J.M. Chen, P.M. Dando, N.D. Rawlings, M.A. Brown, N.E. Young, R.A. Stevens, E. Hewitt, C. Watts, A.J. Barrett, Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase, J. Biol. Chem. 272 (1997) 8090–8098.
[24] D. Keppler, Towards novel anti-cancer strategies based on cystatin function, Cancer Lett. (2005) [Epub ahead of print].
[25] T. Clair, J. Aoki, E. Koh, R.W. Bandle, S.W. Nam, M.M. Ptaszynska, G.B. Mills, E. Schiffmann, L.A. Liotta, M.L. Stracke, Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate, Cancer Res. 63 (2003) 5446–5453.
[26] H.Y. Lee, T. Clair, P.T. Mulvaney, E.C. Woodhouse, S. Aznavoorian, L.A. Liotta, M.L. Stracke, Stimulation of tumor cell motility linked to phosphodiesterase catalytic site of autotaxin, J. Biol. Chem. 271 (1996) 24408–24412.
[27] R. Gijsbers, J. Aoki, H. Arai, M. Bollen, The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site, FEBS Lett. 538 (2003) 60–64.
[28] B. Vogelstein, K.W. Kinzler, Cancer genes and the pathways they control, Nat. Med. 10 (2004) 789–799.
[29] J.R. Pollack, T. Sorlie, C.M. Perou, C.A. Rees, S.S. Jeffrey, P.E. Lonning, R. Tibshirani, D. Botstein, A.L. Borresen-Dale, P.O. Brown, Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors, Proc. Natl. Acad. Sci. USA 99 (2002) 12963–12968.
[30] F. Radtke, K. Raj, The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat. Rev. Cancer. 3 (2003) 756–767.
[31] S. Sheng, J. Carey, E.A. Seftor, L. Dias, M.J. Hendrix, R. Sager, Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells, Proc. Natl. Acad. Sci. USA 93 (1996) 11669–11674.
[32] C.G. Huh, K. Hakansson, C.M. Nathanson, U.P. Thorgeirsson, N. Jonsson, A. Grubb, M. Abrahamson, S. Karlsson, Decreased metastatic spread in mice homozygous for a null allele of the cystatin C protease inhibitor gene, Mol. Pathol. 52 (1999) 332–340.
[33] A. Grubb, H. Lofberg, Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis, Proc. Natl. Acad. Sci. USA 79 (1982) 3024–3027.
[34] J. Leung-Tack, C. Tavera, M.C. Gensac, J. Martinez, A. Colle, Modulation of phagocytosis-associated respiratory burst by human cystatin C: role of the N-terminal tetrapeptide Lys-Pro-Pro-Arg, Exp. Cell Res. 188 (1990) 16–22.
[35] C. Tavera, J. Leung-Tack, D. Prevot, M.C. Gensac, J. Martinez, P. Fulcrand, A. Colle, Cystatin C secretion by rat glomerular mesangial cells: autocrine loop for in vitro growth-promoting activity, Biochem. Biophys. Res. Commun. 182 (1992) 1082–1088.
[36] J.P. Sokol, W.P. Schiemann, Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells, Mol. Cancer. Res. 2 (2004) 183–195.
[37] S. Hartmann, A. Schonemeyer, B. Sonnenburg, B. Vray, R. Lucius, Cystatins of filarial nematodes up-regulate the nitric oxide production of interferon-gamma-activated murine macrophages, Parasite Immunol. 24 (2002) 253–262.
[38] B. Vray, S. Hartmann, J. Hoebeke, Immunomodulatory properties of cystatins, Cell Mol. Life Sci. 59 (2002) 1503–1512.
[39] P. Schierack, R. Lucius, B. Sonnenburg, K. Schilling, S. Hartmann, Parasite-specific immunomodulatory functions of filarial cystatin, Infect. Immun. 71 (2003) 2422–2429.
[40] P.L. Zeeuwen, I.M. van Vlijmen-Willems, D. Olthuis, H.T. Johansen, K. Hitomi, I. Hara-Nishimura, J.C. Powers, K.E. James, H.J. op den Camp, R. Lemmens, J. Schalkwijk, Evidence that unrestricted legumain activity is involved in disturbed epidermal cornification in cystatin M/E deficient mice, Hum. Mol. Genet. 13 (2004) 1069–1079.
[41] R.M. Strieter, P.J. Polverini, D.A. Arenberg, A. Walz, G. Opdenakker, J. Van Damme, S.L. Kunkel, Role of C–X–C chemokines as regulators of angiogenesis in lung cancer, J. Leukoc. Biol. 57 (1995) 752–762.
[42] K. Xie, Interleukin-8 and human cancer biology, Cytokine Growth Factor Rev. 12 (2001) 375–391.
[43] N. Vigneswaran, J. Wu, S. Muller, W. Zacharias, S. Narendran, L. Middleton, Expression analysis of cystatin C and M in laser-capture microdissectioned human breast cancer cells—a preliminary study, Pathol. Res. Pract. 200 (2005) 753–762.
[44] N. Vigneswaran, J. Wu, W. Zacharias, Upregulation of cystatin M during the progression of oropharyngeal squamous cell carcinoma from primary tumor to metastasis, Oral Oncol. 39 (2003) 559–568.
[45] K. Belguise, N. Kersual, F. Galtier, D. Chalbos, FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells, Oncogene 24 (2005) 1434–1444.
[46] V. Bolos, H. Peinado, M.A. Perez-Moreno, M.F. Fraga, M. Esteller, A. Cano, The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors, J. Cell Sci. 116 (2003) 499–511.