Cell Growth Stimulation by CRASH, an Asparaginase-like Protein Overexpressed in Human Tumors and Metastatic Breast Cancers

ULRICH H. WEIDLE[1], VESNA EVTIMOVA[1], SAVERIO ALBERTI[2], EMANUELA GUERRA[2], NIKOS FERSIS[3] and SEPP KAUL[3]

[1]*Roche Diagnostics, Division Pharma, 82377 Penzberg, Germany;*
[2]*Unit of Cancer Pathology, Department of Oncology and Neurosciences, BAMS and CeSI, Fondazione "G. D' Annunzio", University of Chieti, Italy;*
[3]*Women's Hospital, University of Heidelberg, 69115 Heidelberg, Germany*

Abstract. *The gene encoding CRASH, a human asparaginase-like protein, has been cloned and its transcriptional activation has been detected in gynecologic cancers. To define the expression of CRASH in human tumors and its possible functional role, monoclonal antibodies against the CRASH protein have been generated. In non-transformed tissues CRASH was only detected in testis, brain, esophagus, prostate and proliferating endometrium. On the other hand, 36/50 ovarian carcinomas, 16/78 mammary carcinomas, 6/6 uroepithelial bladder carcinomas and 5/33 colon carcinomas scored positive for CRASH, with the absence of reactivity in the corresponding normal tissues. Strikingly, 11 out of the 16 breast cancers that expressed CRASH were metastatic, nominating CRASH to be functionally relevant in tumor progression. Twenty-eight out of 42 endometrium tumors expressed CRASH at high levels as did 5/41 prostate carcinomas, as well as ovary and breast cancers, indicating a regulation of CRASH expression by sex hormones. A bona fide estrogen responsive element was detected at bases –201/–183. This proved to be highly preserved across species, supporting an actual functional role. Asparaginase-like proteins play a role in growth regulation and signaling by p70 S6 kinase. The somatic knock-out of CRASH resulted in significant inhibition of growth of KM12L4A colon carcinoma cells, which abundantly express CRASH, whereas the proliferation of the syngeneic, weakly-expressing, slowly-growing KL12SM was not affected. These results are consistent with a selective growth advantage for aggressive cancers expressing CRASH, and nominate CRASH as a novel diagnostic and therapeutic tumor target.*

The gene encoding CRASH, a human asparaginase-like 1 protein (ALP), has been cloned and its transcriptional activation has been detected in gynecologic cancers (1). CRASH is a protein of 307 residues with a molecular weight of 31.9 kD, that has been predicted to possess L-asparaginase-1-like structure and activity [(1) and this paper]. A rat paralogue of CRASH was described as a sperm autoantigen (2), as obstruction of the male reproductive tract causes an autoimmune response that leads to the generation of anti-ALP antibodies (2). The human and rat proteins share 77% identity, suggesting strong selective pressure for a conserved function. Consistently, a broad conservation of asparaginase-like 1 protein sequences across species, *i.e.* monkey, horse, dog, cow, rodents, chicken, xenopus and zebrafish (this paper) has been detected. Asparaginases deamidate asparagine or glutamine to aspartic acid or glutamic acid, respectively, and are auto-activated by proteolysis at the catalytic site. Asparaginases are involved in key control pathways, as the selective removal of the essential Asn from the amino acid pools may severely diminish cell proliferation (3,4) and induce apoptosis (5). Notably, however, L-asparaginase also selectively inhibits the p70 S6 kinase (p70(s6k)) and the phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), that are important control pathways of the proliferation of transformed cells (6).

Northern blot analysis of rat tissues detected the highest expression of ALP in testis, with lower expression in brain, liver, kidney, heart and skeletal muscle (2). It was found that

Abbreviations: aa, amino acid(s); ALP, asparaginase-like 1 protein; IHC, immunohistochemistry; nt(s), nucleotides; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; KLM, keyhole limpet hemocyanin; MEM, minimal essential medium.

Correspondence to: Ulrich H. Weidle, Roche Diagnostics GmbH, Pharma Research, D-82377 Penzberg, Germany. Tel: +49 8856 602801, e-mail: ulrich.weidle@roche.com

Key Words: Immunohistochemistry, RNAi knock-down, asparaginase, cell growth.

CRASH mRNA levels correlate with the metastatic propensity of human colon cancer and pancreatic carcinoma cell lines. High levels of CRASH mRNA were found in endocrine-dependent uterine, mammary and ovarian tumors when compared to the corresponding normal tissues. Consistent with a regulatory role of endocrine-dependent signaling pathways, CRASH mRNA can be induced by sex hormones in BT474 breast cancer cells (1).

In this work monoclonal antibodies specific for CRASH have been generated in order to define the expression of the CRASH protein in human tumors and its possible functional role. The results demonstrate that the CRASH protein is significantly overexpressed in specific tumor types and in metastatic breast cancer. Remarkably, the knock-down of CRASH by RNAi inhibits the growth of CRASH-overexpressing metastatic colon cancer cells. These findings suggest direct relevance of CRASH expression for the growth of transformed cells and in tumor progression.

Materials and Methods

Cell lines and tissues. The breast cancer cell line KM22, isolated from metastatic bone marrow, and the ovarian cancer cell line BB, established from ascites were established at our laboratory and were cultured in DMEM/Ham's F12 supplemented with 5% fetal bovine serum. Hybridomas were grown in RPMI-1640, 8% FCS, supplemented with insulin, transferrin and selenium (Invitrogen, Karlsruhe, Germany). The colon cancer cell lines KM12SM and KM12L4A (7, 8) were cultured in RPMI-1640, 10% FCS and 2 mM L-glutamine.

Generation of monoclonal antibodies (mab) specific for CRASH. Balb/c mice were immunized *s.c.* four times with 50 μg recombinant His-tagged CRASH protein or with a CRASH-derived peptide HFGIDPPDTTITDLP coupled to KLH. Six fusions of splenocytes with non-secreting mouse myloma cell line X63-Ag8.653 were performed and the specificity of 4800 clones was evaluated by ELISA using as targets KLH-coupled synthetic CRASH peptides, recombinant CRASH and cytosolic proteins prepared from normal human fibroblasts. CRASH-positive clones were analysed by Western blotting using cytosolic proteins isolated from the breast cancer cell line KM-22. Seven CRASH-specific hybridomas were cloned and antibodies were isolated by affinity chromatography on Protein A Sepharose. All subsequent studies were performed with clone 7B6 (IgG1).

Western blotting. Cytosolic proteins from KM-22 cells (CRASH positive) were electrophoresed on a precast 15% PAA gel (BioRad, USA), which was subsequently transferred to a nitrocellulose membrane. Blocking was performed with 2% non-fat milk powder in PBS. Hybridization was performed with antibodies from the anti-CRASH hybridomas or from MOPC-21 cells for 1 h. This was followed by incubation with biotinylated horse anti-mouse IgG for 30 min at room temperature and by a subsequent incubation with streptavidin-alkaline phosphatase conjugates for 30 min. The reactivity was revealed using Fast Red as a substrate, according to the instructions of the manufacturer (BioRad, USA). A standard Kaleidoscope prestained standard (Blue 202 kD, Magenta 133 kD, Green 71 kD, Pink 41.8 kD and Blue 6.9 kD) was used as m.w. marker.

Immunohistochemistry (IHC) analysis. For histological detection of CRASH expression, tissue arrays containing human normal and tumor tissues (BioCat, Heidelberg, Germany) were analyzed. Staining was performed with mab7B6 (5 μg/mL) under standardized conditions, using an immunostainer from Ventana NexES (Ventana Medical Systems, Tucson, AZ, USA) and the Enhanced Alkaline Phosphatase Fast-Red Detection Kit, according to the instructions of the manufacturer.

Silencing of CRASH mRNA in colon cancer cell lines. Silencing of CRASH mRNA was performed with pSUPER vector-based constructs (9). Target 19-mer CRASH RNAi sequences were identified using the Whitehead Institute (jura.wi.mit.edu/bioc/siRNAext/) and the Invitrogen (rnaidesigner.invitrogen.com/rnaiexpress/) design tools, together with the Tuschl' criteria. Sixty-mer hairpin-forming oligo pairs were designed around the RNAi sequences, synthesized *in vitro* (Invitrogen, Karlsruhe, Germany), ligated into the pSUPER vector under the control of the polymerase III H1-RNA gene promoter and transformed into E. coli XL1-Blue. Transfection-grade plasmid DNA was purified from positive clones using Quiagen-tip 500 (Qiagen).

The three RNAi expression constructs were pooled and transiently transfected into KM12SM and KM12L4A cell lines using Lipofectamine 2000 (Invitrogen). A non-functional RNAi expression construct containing a 19-mer target sequence not present in the human genome was used as a control. Cells were routinely maintained in DMEM supplemented with 20% FBS, 100 U/ml penicillin and streptomycin (1% P/S), 1X MEM non-essential amino acids and MEM-vitamins (GIBCO, Carlsbad, CA, USA). The day before transfection, cells were trypsinized (0.25% trypsin in PBS-1 mM EDTA, 37˚C for 5 min) and seeded into 6-well plates at a density of 10^6 cells/well. Four hours before the transfection the cells were washed twice with PBS and incubated in DMEM without FBS and antibiotics. Transfection was performed using 2 μL Lipofectamine 2000 and 2 μg total plasmid DNA/well according to the manufacturers instructions.

Sequences of the oligo pairs ligated into the pSUPER vector. Details are shown in Table I.

Cell growth curves. To assess cell growth rates, cells were trypsinized 48 h after transfection and seeded at 4×10^3 cells/well into 96-well plates (six replica wells per data point). Cell numbers were quantified by staining with crystal violet (10). Briefly, adherent cells were fixed in 4% formalin in PBS for 30 min, followed by incubation with 50 mM NH_4Cl for 30 min. Cells were then stained with a solution of 0.1% crystal violet (Sigma, Taufkirchen, Germany) (diluted with water from 0.5% stock solution in 80% water, 20% methanol) for 1 h, washed extensively with water and dried. The cell-bound crystal violet was solubilized with 200 μL of acetic acid and its optical density at 550 nm was determined spectrophotometrically.

Protein sequence analysis. The accession number of the CRASH nucleotide sequence is CS000425. The RefSeq transcript for CRASH is NM_025080 (*H. sapiens* asparaginase like 1 (ASRGL1), transcript

Table I. *Sequences of the oligo pairs ligated into the pSUPER vector. Nucleotide numbering is based on the CRASH sequence as described (1). CRASH sequences are shown in bold capital letters and are underlined; the corresponding palindromic sequences are shown in bold small letters.*

CRASH 1 (position 1152-1170)
Crash1 forward
5' GATCCCC**TAAGCATCTGAATGTTTGG**TTCAAGAGA**ccaaacattcagatgctta**TTTTTA
Crash1 reverse
5' AGCT**TAAAAATAAGCATCTGAATGTTTGG**TCTCTTGAA**ccaaacattcattcagatgctta**GGG

CRASH 2 (position 2011-2029)
Crash2 forward
5' GATCCCC**GCATGGTGAAGAAAGTCAT**TTCAAGAGA**atgactttcttcaccatgc**TTTTTA
Crash2 reverse
5' AGCTTAAAAA**GCATGGTGAAGAAAGTCAT**TCTCTTGAA**atgactttcttcaccatgc**GGG

CRASH 3 (position 1648-1666)
Crash3 forward
5' GATCCCC**GCCCTTCTCTGACTTACCT**TTCAAGAGA**aggtaagtcagagaagggc**TTTTTA
Crash3 reverse
5' AGCTTAAAAA**GCCCTTCTCTGACTTACCT**TCTCTTGAA**aggtaagtcagagaagggc**GGG

Control siRNA
Control forward
5' GATCCCC**ACAAGACGACGTGGACATA**TTCAAGAGA**tatgtccacgtcgtcttgt**TTTTTA
Control reverse
5' AGCTTAAAAA**ACAAGACGACGTGGACATA**TCTCTTGAA**tatgtccacgtcgtcttgt**GGG

variant 2). BLASTp and psiBLAST analyses (www.ncbi.nlm.nih.gov /blast/) were performed using the NP_079356 CRASH protein sequence as a query. Protein sequences were analyzed using Genetics Computer Group (11), EMBnet (http:// www.ch.embnet.org) and SAPS (http://isrec.isb-sib.ch/software/SAPS_form.html) (12) programs. The asparaginase domain was analysed utilizing the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/) and MyHits MotifScan (myhits.isb-sib.ch/cgi-bin/motif_scan) (13). Consensus phospho-rylation and myristoylation sites were matched against the PROSITE database (14) (www.expasy.ch/prosite/). The consensus sequences for transcription factor binding sites were identified with the program MatInspector V2.2 (transfac.gbf.de/ TRANSFAC/) and the Eukaryotic Promoter Database (www.epd.isb-sib.ch/) (15). The Transcription Element Search System (TESS) (www.cbil.upenn.edu/cgi-bin/tess/tess) (16) and MatInspector of the Genomatix analysis package (www.genomatix.de/cgi-bin/eldorado/ main.pl?s=dc49a08a9692a9 ddf433869af4dfe273l) (17) were also utilized for these analyses. PsiPRED was used to predict protein secondary structures (bioinf.cs.ucl.ac.uk/psipred/psiform.html) (18).

Results and Discussion

Generation of anti-CRASH mabs. Transcriptional up-regulation of CRASH, a gene encoding a human asparaginase-like protein, has been demonstrated in several gynecologic cancers (1). To define the expression of CRASH in human tumors (the levels of a specific mRNA do not necessarily reflect those of the corresponding protein (19)) and its possible functional role, monoclonal antibodies against the CRASH protein were generated.

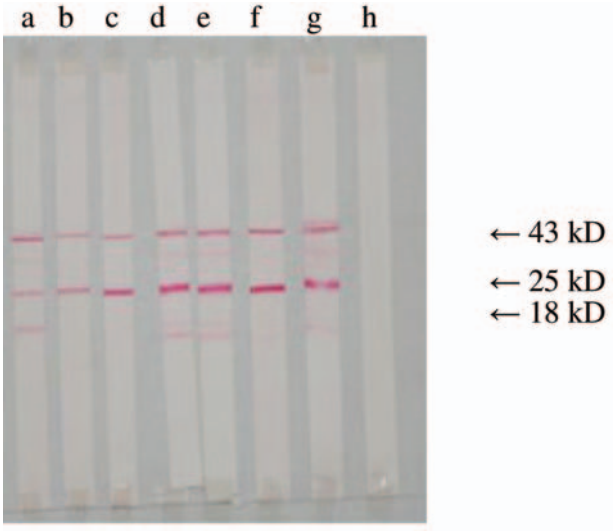

Figure 1. *Western blot analysis of mabs directed against CRASH. Cytosolic proteins from KM-22 cells (lanes a–g) and MOPC-21 cells (lane h) were electrophoresed on precast 15% PAA gels and transferred to a nitrocellulose membrane. Lanes a to g: antibodies from CRASH-specific hybridomas, lane h: MOPC-21 control antibody. Lane a: 7B6; b: 17B3; c: 9A11; d: 2A4; e: 4D11; f: 8H12; g: 5H11; h: MOPC-21.*

Figure 1 highlights the reactivity of antibodies derived from CRASH-specific hybridomas with cytosolic proteins derived from KM22 cells. According to Bush *et al*., (2), bands corresponding to unprocessed CRASH protein migrate

Table II. *Expression of CRASH in tumors and normal tissues IHC analysis was performed with mab7B6 with tissue arrays from BioCat as described in Materials and Methods.*

Organ	Tumor	Normal
Adrenal gland	0/1	0/1
Adipose	0/1	0/1
Bladder	6/6	0/4
Breast	2/4	0/4
Colon	5/33	0/33
Duodenum	1/1	0/1
Endometrium Carcinoma	28/42	0/3
Esophagus	0/5	0/5
Fallopian Tube	0/1	1/1
Gall Bladder	0/1	0/1
Kidney	0/4	1/4
Liver	0/4	0/4
Lung Squamous Cell	0/2	0/2
Lung Adenocarcinoma	0/2	1/2
Lung Alveolus Cell	1/1	0/1
Lung Small Cell Carcinoma	2/2	0/2
Lymphoma	0/2	0/2
Pancreas	0/1	0/1
Parotid gland	0/1	0/1
Prostate	5/41	3/10
Rectum	0/4	0/4
Skin	0/3	0/3
Small intestine	0/3	0/3
Soft Tissue	0/1	0/1
Stomach	0/5	0/5
Testis	-	2/2
Tongue	0/1	0/1
Throat/Pharynx	0/1	0/1
Thymus	0/3	0/3
Thyroid Adenoma	0/2	0/2
Thyroid papillary	2/2	0/2
Uterus Leiomyoma	0/3	0/3
Uterus Adenocarcinoma	0/1	0/1

Table III. *IHC analysis of breast- (A) and ovarian-related tissues (B) for expression of CRASH. A monoclonal antibody directed against CRASH (mab7B6) was used for analysis as described in Materials and Methods.*

A. Breast	Tumor	Normal
Ductal Carcinoma	5/32	
Papillary Carcinoma	0/1	
Ductal In Situ Carcinoma	0/2	
Invasive Lobular Carcinoma	0/3	
Medullary Carcinoma	0/1	
Signet Ring Cell Carcinoma	0/1	
Metastatic Carcinoma	11/36	
Nipple	0/2	
Normal		0/8
B. Ovary	**Tumor**	**Normal**
Cystadenocarinoma	4/4	
Cystadenocarinoma, serous, papillary	4/4	
Cystadenocarinoma, serous	5/8	
Cystadenocarinoma, papillary	1/1	
Cystadenocarinoma, mucous	1/3	
Cystadenocarinoma, mastoid	2/2	
Adenocarcinoma	12/14	0/2
Adenocarcinoma, serous	1/1	0/4
Adenocarcinoma, papillary	0/1	
Adenocarcinoma, mucous	1/1	
Adenocarcinoma, mastoid	1/1	
Endodermal Sinus Tumor	0/2	
Transitional Cell Carcinoma	2/2	
Dysgerminoma	0/1	
Endometrium adenocarcinoma	1/1	
Granular Cell Tumor	0/2	
Cystic Teratoma & Squmous carcinoma	0/1	
Parenchymatous carcinoma	1/1	
Thecoma	0/3	
Normal		0/6

at 43 kD, *i.e.* somewhat more slowly than predicted from the sequence (307 aa; 31.9 kD m.w.) (Figure 1), whereas bands at 25 and 18 kD correspond to post-translationally processed proteins (2). Reactivity in lane (a) is consistent with the suggested processing of CRASH (2). Therefore, clone 7B6 (lane a) was used for further immunohistochemistry studies. No reactivity with the 18 kD subunit was demonstrated for hybridomas 17B3 and 9A11 (lanes b and c). Clones 2A4, 4D11, 8H12 and 5H11 displayed additional reactivity with a 10 kD band (lanes e to g).

Expression of the CRASH protein in normal and tumor tissues. The reactivity of anti-CRASH mabs in formalin-fixed, paraffin-embedded tissues was verified. IHC analysis of normal and transformed tissues was performed (Table II). Brain, testis, esophagus and prostate were the only normal tissues which scored strongly positive for CRASH expression, weaker signals being detected in the fallopian tubes, kidney and lung. Expression in testis and brain is in line with a possible role of CRASH as an evolutionarily conserved cancer-testis antigen (CTA) (20, 21). CRASH expression was not detected in placenta (Figure 2A).

Of interest, CRASH corresponds to an orthologous rat asparaginese-like protein which was identified as a sperm autoantigen (2). Anti-sperm antibodies have been observed as a response of the reproductive tract to obstruction (22, 23). Autoantigenic sperm antigens such as protamines (24), DNA polymerase (25), nuclear autoantigenic sperm protein (26) and glycoprotein FA-1 (23) have also been identified in man. Further human sperm autoantigens have been identified by Western blotting (27-29). These correlations raised interest for a potential role of CRASH as autoantigen also in man. An investigation on sera of tumor patients for the presence of anti-CRASH antibodies is ongoing.

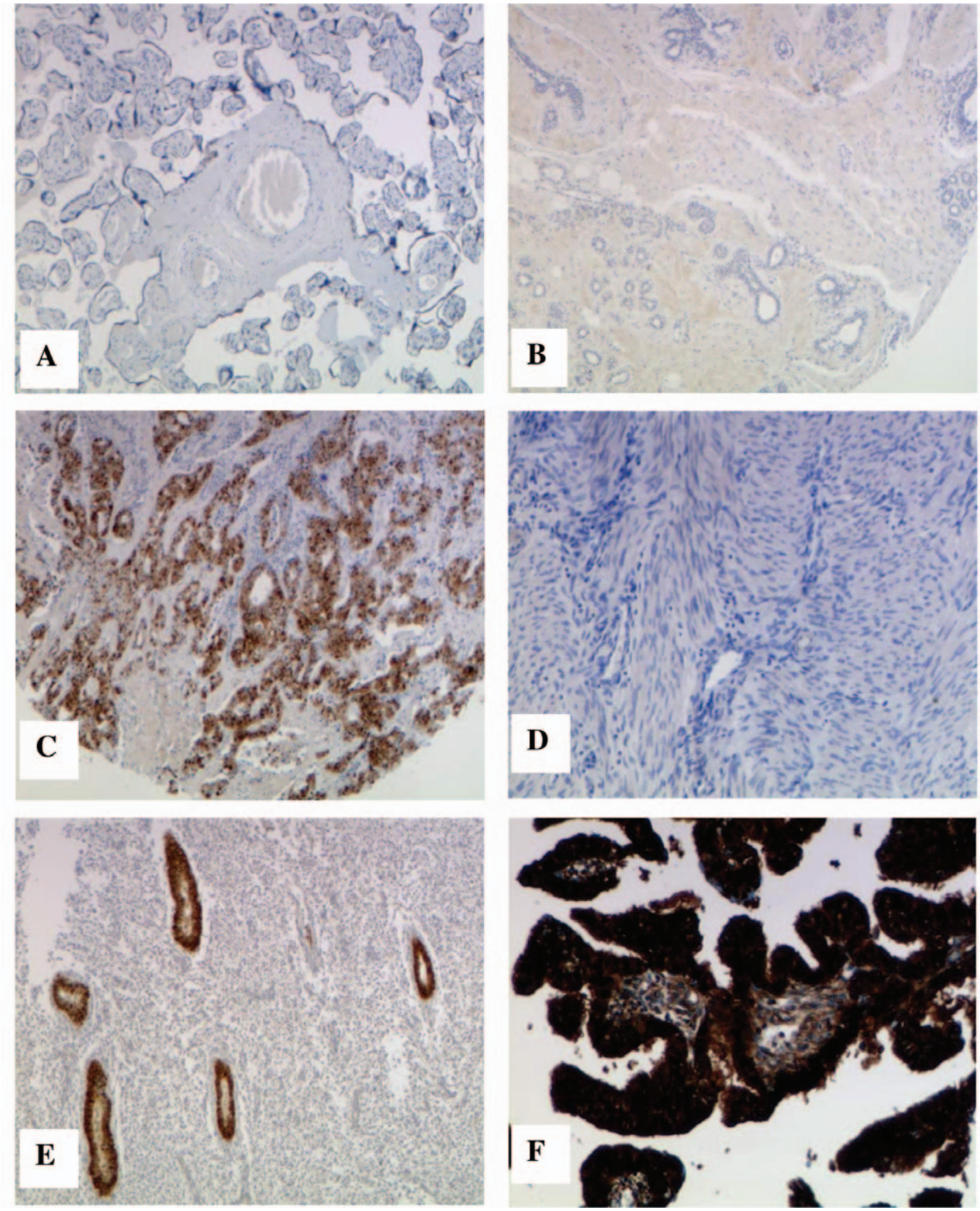

Figure 2. *IHC analysis of CRASH expression in endocrine-responsive tissues and tumors. IHC was performed with the anti-CRASH mab7B6 as described in Materials and Methods. A: placenta; B: breast (normal); C: breast carcinoma; D: uterus (normal); E: uterus (proliferating); F: uterus adenocarcinoma; Magnification: B, C, D, E=×4, A, F=×10.*

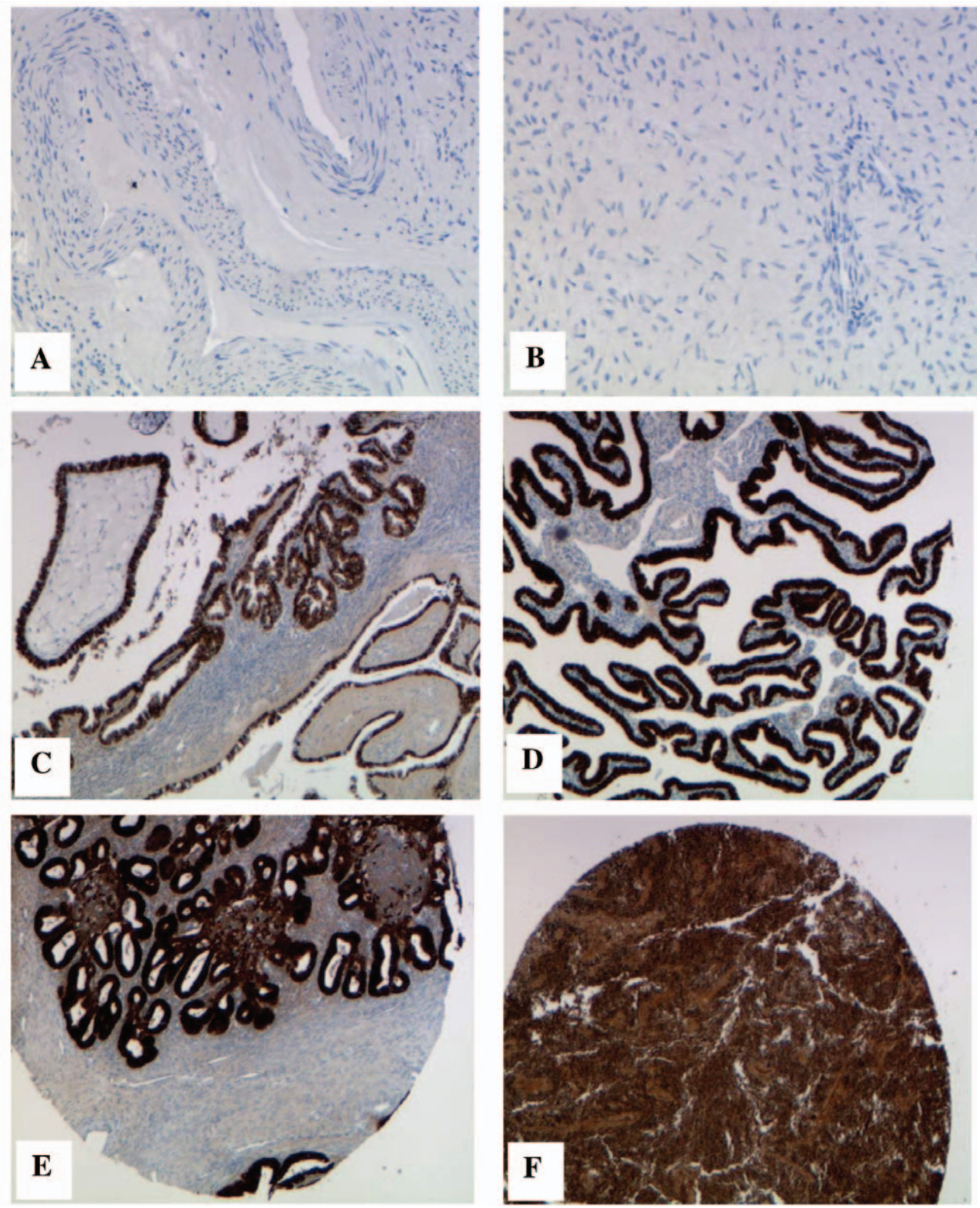

Figure 3. *IHC analysis of ovarian-related tissues. Analysis was performed with mab7B6 directed against CRASH with tumor arrays from BioCat as described in the Materials and Methods section. A: normal ovarian tissue; B: thekoma; C, D: ovary cystadenocarcinomas; E: ovary adenocarcinoma (mastoid); F: ovarian transitional carcinoma. Magnification: A, B=×10, C, D, E, F=×4.*

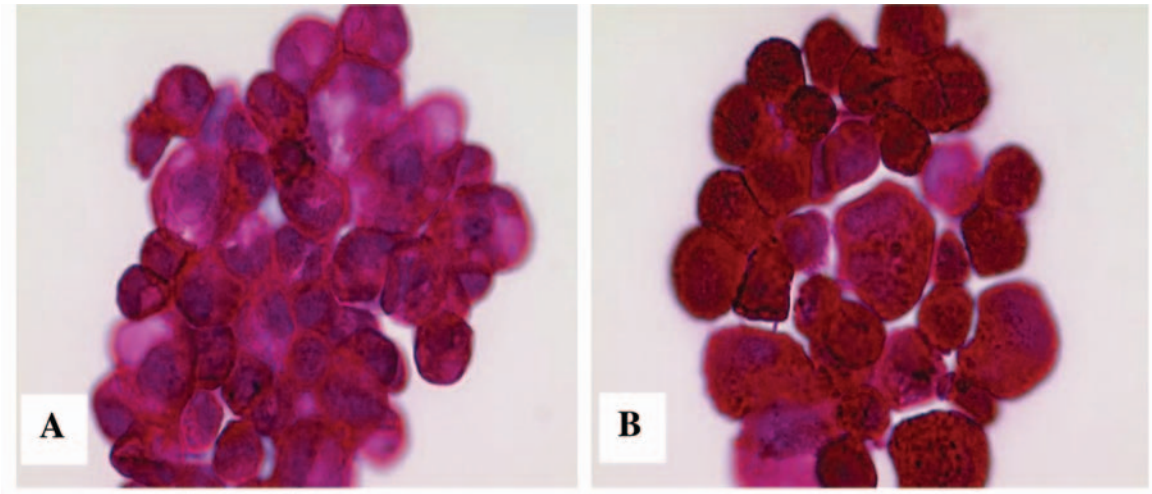

Figure 4. *EpCAM and CRASH expression in the ovarian cancer cell line BB. The BB cell line was established from the ascites of an ovarian carcinoma patient. BB cells were analyzed for the expression of EpCAM (Figure 3A) and CRASH (Figure 3B), as described in Materials and Methods. Magnification: ×40.*

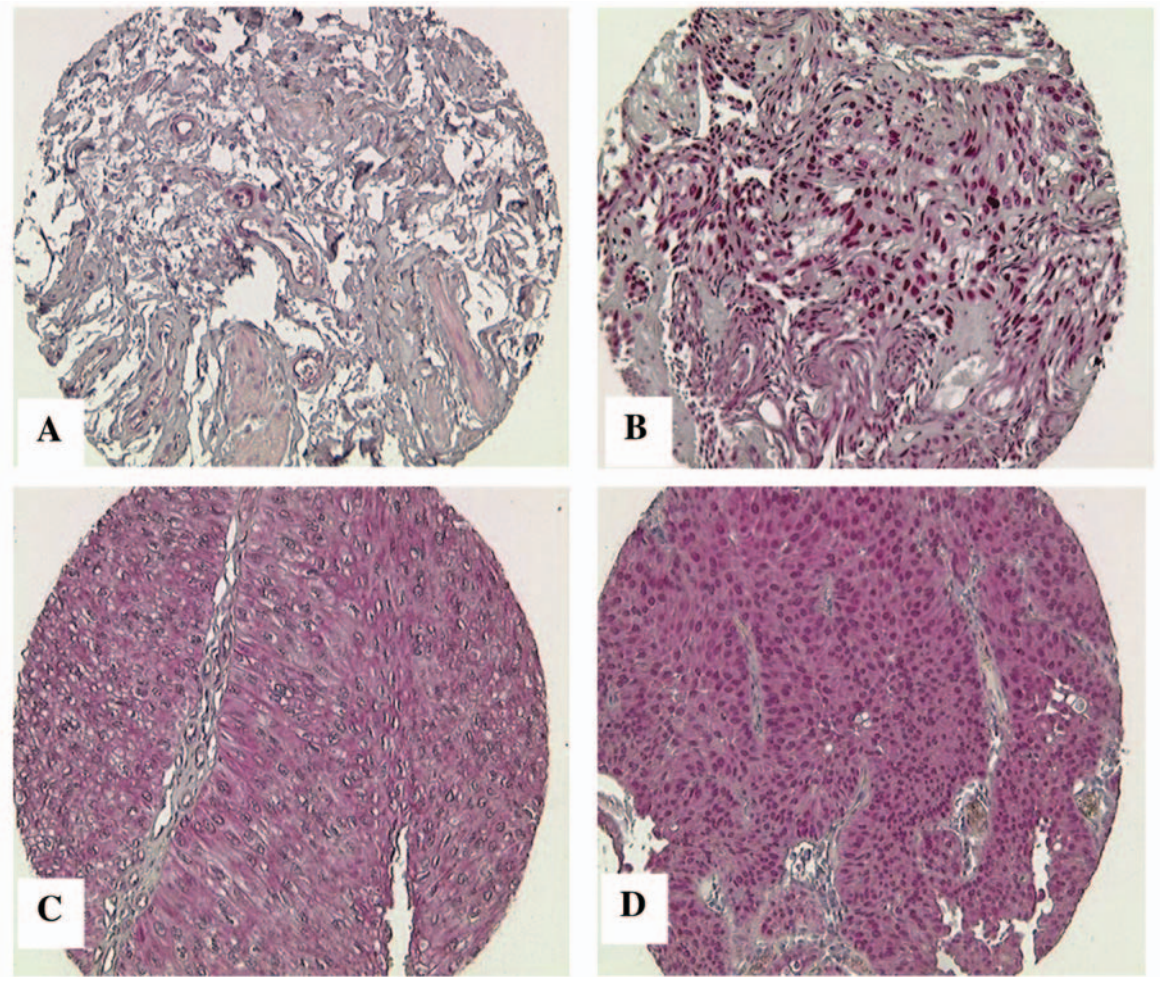

Figure 5. *IHC analysis of CRASH expression in normal bladder and uroepithelial bladder cancers. The 7B6 anti-CRASH mab was used for the analysis as described in Materials and Methods. A: normal bladder; B-D: different uroepithelial cancers of the bladder. Magnification: ×10.*

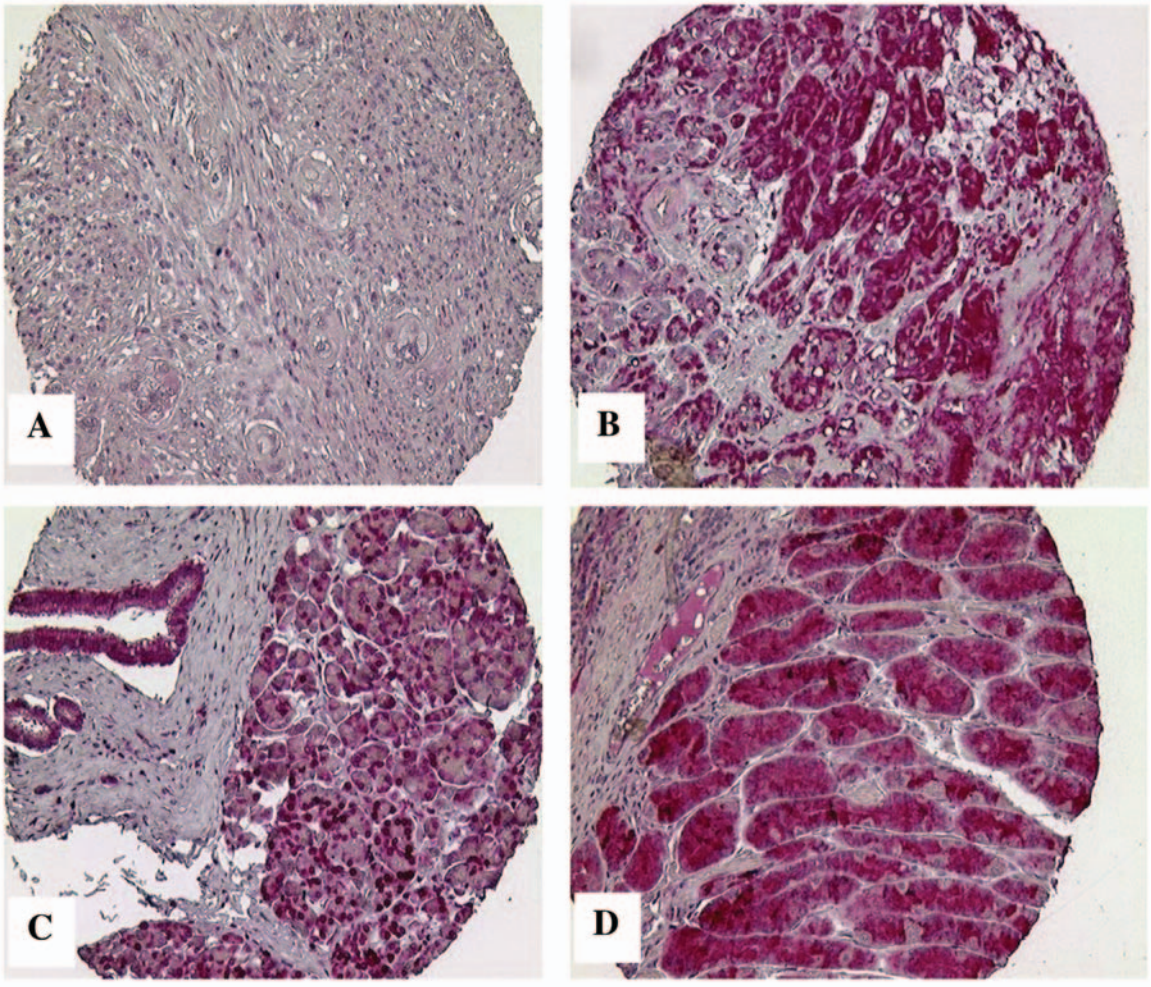

Figure 6. *IHC of CRASH expression in prostate-related tissues. IHC was performed with the 7B6 anti-CRASH mab as described in Materials and Methods. A: normal prostate; B, C and D: prostate cancer tissues derived from three patients. Magnification: A, B, C and D: ×10.*

The expression of CRASH in tumors and in the corresponding normal tissues was investigated. Five out of 33 colon tumors scored positive for CRASH expression, whereas all specimens of normal mucosa scored negative (Table II). In mammary carcinomas (Table III A) expression of CRASH was detected in 16 out of 78 cases, whereas all tissue samples derived from normal mammary gland scored negative. Strikingly, 11 of the 16 CRASH-expressing cases were those of metastatic breast cancers. Of interest, the CRASH mRNA was previously found expressed in metastatic mammary carcinoma cell lines at high frequency (1), consistently indicating that it may be specifically associated with metastasizing capacity. Representative IHC analysis of a breast cancer and of the corresponding normal tissue are shown in Figure 2 (B, C). As displayed in Table II, 28/42 endometrium carcinomas scored positive for CRASH, whereas the control tissues scored negative. CRASH was found expressed in proliferating uterus tissue; representative examples of normal uterus, proliferating uterine tissue and uterus adenocarcinoma are shown in Figure 2 (D, E, F). SDS-PAGE and Western blot analysis of proteins from placenta, normal uterus and normal ovary confirmed the negative histological results.

Prevalence of expression of CRASH was high in ovarian carcinoma, as 36/50 ovarian cancers were scoring positive for CRASH, including 12/14 ovarian adenocarcinomas (Table II). Normal ovarian tissue and a thekoma were shown not to express CRASH (Figure 3A,B). Striking homogeneity of expression of CRASH was noticed in all ovarian carcinomas analyzed by IHC (Table III B), with particularly high expression in cases of cystadenocarcinoma (Figure 3C,D), adenocarcinoma (Figure 3E) and transitional carcinoma of the ovary (Figure 3F). Strong staining of nuclei and cytoplasm was observed. An ovarian cancer cell line was

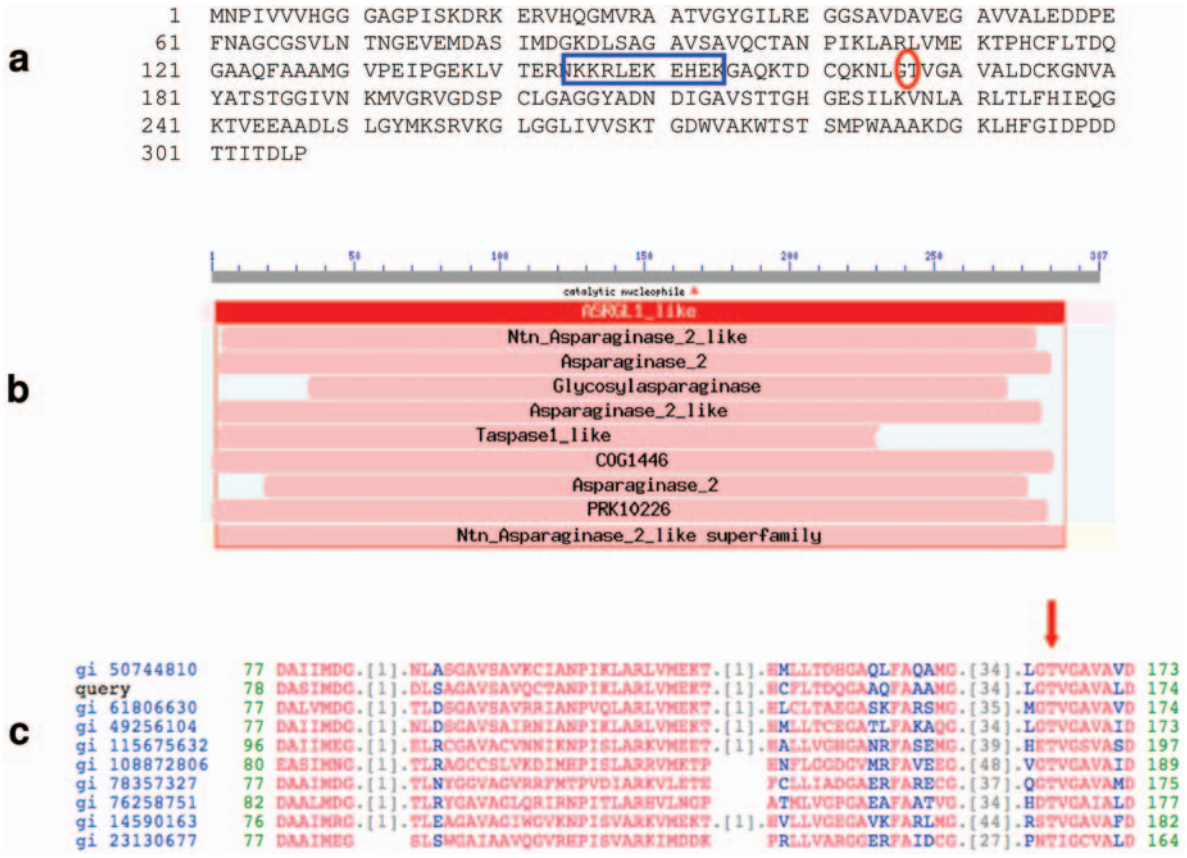

Figure 7. *CRASH sequence and asparaginase domain. (a) Amino acid sequence of CRASH. The NLS-like sequence is boxed. The conserved catalytic Thr is circled in red. (b) Overlap of the homologous regions of the Asparaginase family members. Red arrowhead: catalytic Thr. (c) Amino acid sequence alignment of Asparaginase subgroups members. Query: CRASH. The conserved catalytic Thr is indicated by a red arrow.*

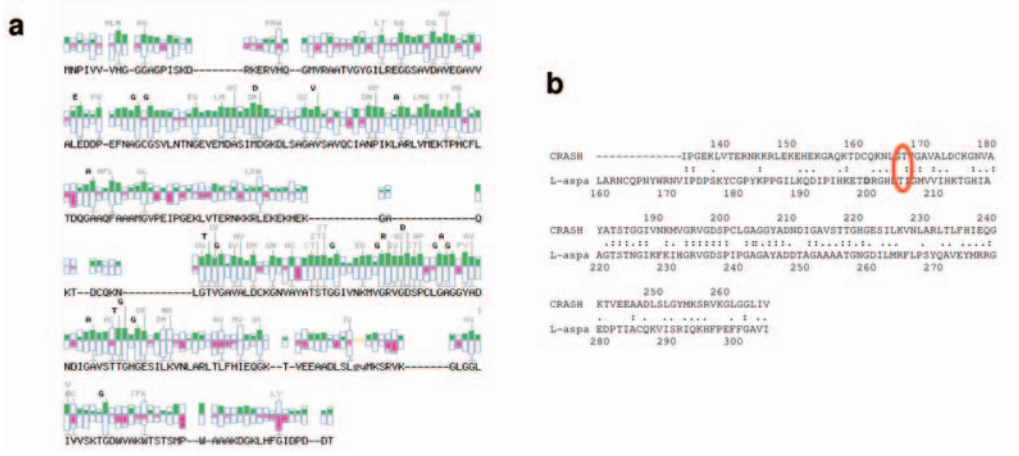

Figure 8. *Sequence comparison of CRASH and Asparaginase sequences. (a) Myhits PFAM analysis of CRASH versus asparaginase 2; quantitative profiling. The gray boxes indicate the maximal/minimal possible scores at each position of the asparaginase 2 profile while the green/red bars represent the actual profile score of CRASH. Positions with three or less different amino acids showing a positive profile score at this position are explicitly labeled as such irrespective of the actual CRASH residue. In case only one amino acid has a positive score at a defined position its label is set in bold (note that CRASH fulfills all these key profile positions). Gaps of CRASH against the profile are marked by dashes in the CRASH sequence, while gaps of the profile against CRASH are marked by orange dashes in the profile and lowercase CRASH sequence. (b) LALIGN alignment of CRASH with L-asparaginase. Conserved, similar and dissimilar residues are indicated by colons, dots and spaces, respectively. The conserved catalytic Thr are circled in red.*

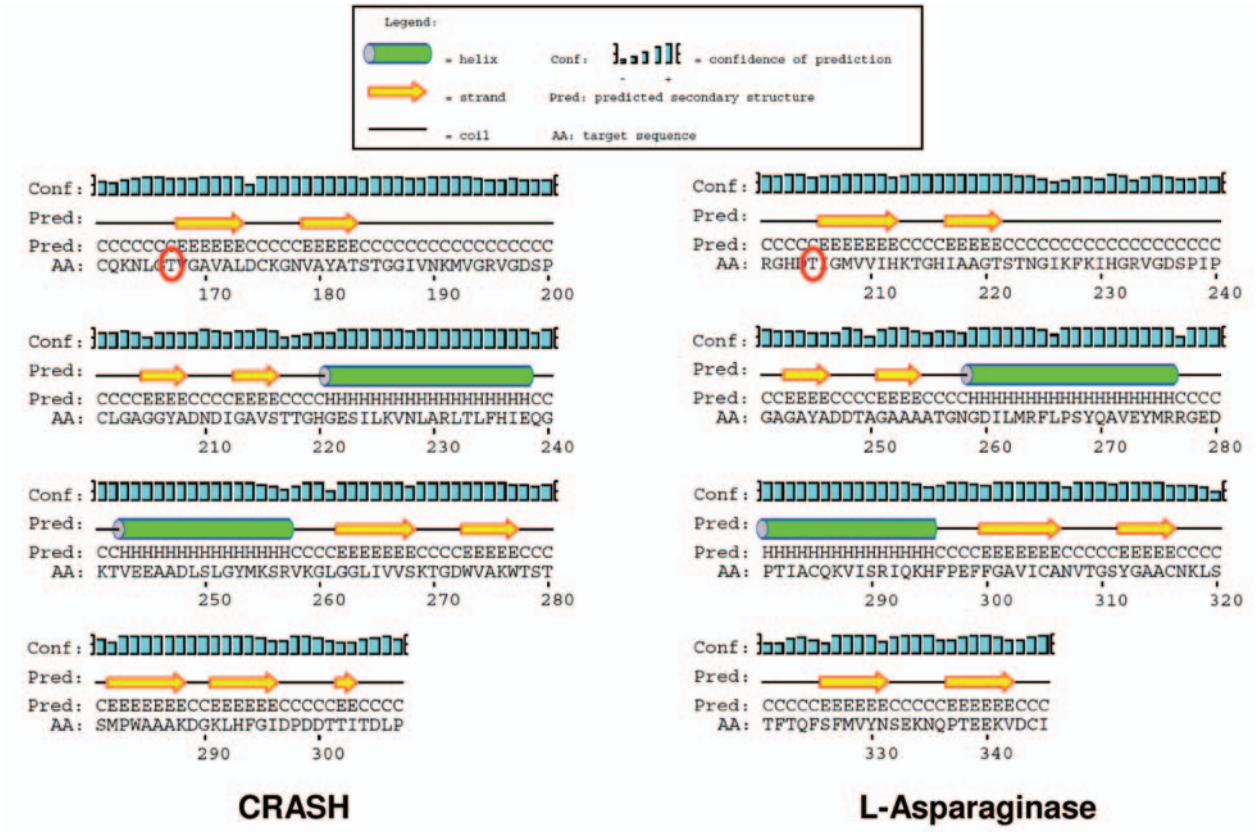

Figure 9. *Structure of CRASH. The secondary structure of CRASH and L-Asparaginase were analysed with JPRED / psiPRED. β-strands (E underscores) are indicated as yellow arrows; α-helices (H underscores) are depicted as green cylinders. The confidence of the prediction for each amino acid is indicated by the vertical cyan bars. The conserved Thr are circled in red.*

generated from the ascitic fluid of a patient with a primary adenocarcinoma. As expected, EpCAM expression was associated with the plasma membrane and with intracellular deposits (Figure 4A). Surprisingly, in addition to its expected patterns of intracellular expression, CRASH was also found associated with the plasma membrane and at cell–cell junctions (Figure 4B), raising the possibility that CRASH may play an unexpected role in cell-cell adhesion. These findings also raise the possibility that specific adapter(s) or other CRASH-interacting signaling molecules may operate to bring CRASH to specific cell membrane regions.

Expression of CRASH in bladder-related tissues is shown in Figure 5. Only very weak staining of scattered areas was revealed within normal bladders (Figure 5A). CRASH expression in uroepithelial bladder cancer specimens is shown in Figure 5B,C and D. Figure 6 displays examples of expression of CRASH in prostate-related tissues. In prostate cancer, only a subgroup (5/41) scored positive for CRASH expression (Table II), with expression of CRASH confined to epithelial cells, smooth-muscle cells proving negative. However, when compared to normal prostate tissue (Figure 6A, Table I), CRASH-expressing cases demonstrated a

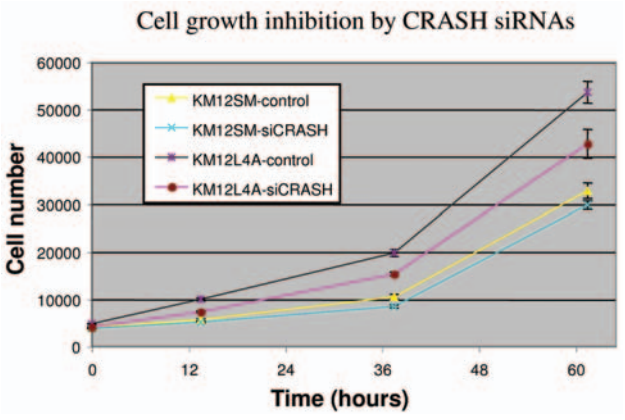

Figure 10. *Cell growth inhibition by CRASH siRNA. Three RNAi expression constructs for CRASH were pooled and transfected in KM12 cells. A non-functional RNAi expression construct was used as a negative control. Cells were seeded for evaluation of proliferation 48 hours after transfection as described in Materials and Methods; time 0 was 4 hours after seeding. Growth was assessed by crystal violet staining as described (10). Cells transfected with the ineffective control siRNA are in black (KM12L4A) and yellow (KM12SM), respectively. Cells transfected with the pooled CRASH siRNAs are in magenta (KM12L4A) and cyan (KM12SM). Standard deviation bars of cell number measurements are shown.*

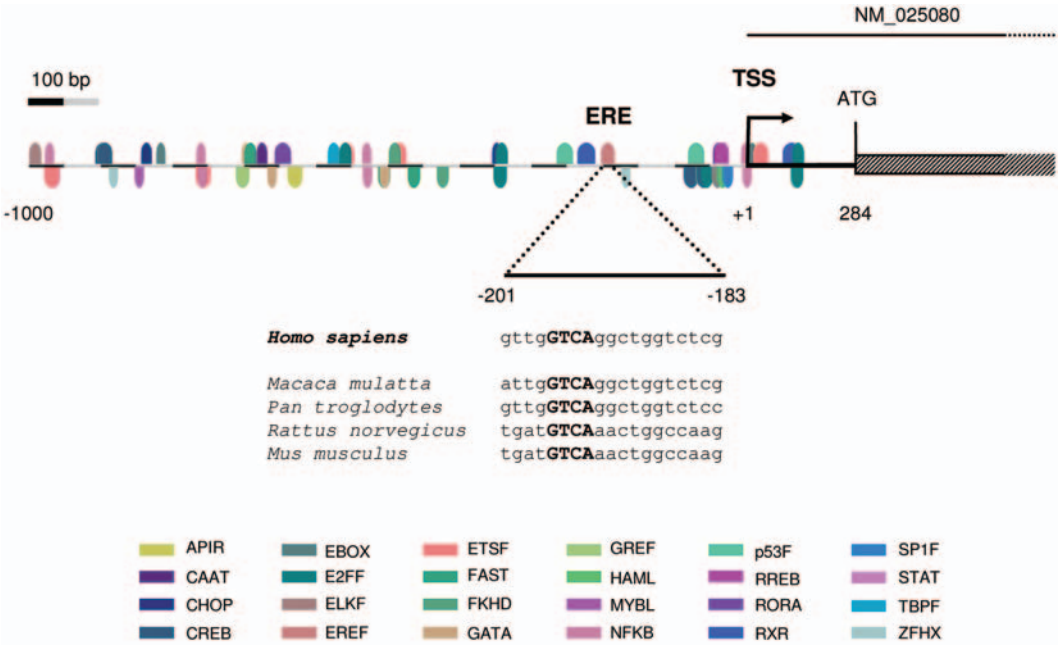

Figure 11. *The CRASH gene promoter elements. The analysis of the promoter region of CRASH was performed as described in Materials and Methods. The binding sites for the most frequent transcription factors are shown in color along the promoter cartoon. The human ERE sequence in the CRASH promoter is shown, together with the corresponding ERE sequences in the orthologous genes of rhesus monkey, chimpanzee, rat and mouse NM_025080: RefSeq CRASH sequence; ERE Estrogen Response Element: TSS Transcription Start Site; ATG translation start site. The scale bar for 100 bp is shown. The transcription factor color code is detailed at the bottom. APIR: MAF and AP1-related; CAAT: CCAAT binding factor; CHOP: C/EBP homologous protein; CREB: cAMP-responsive element binding protein; EBOX: E-box binding factor; E2FF: E2F-myc activator/cell cycle regulator; ELKF: basic and erythroid krueppel like factors; EREF: estrogen response element binding factor; ETSF: human and murine ETS1 factor; FAST: FAST-1 / SMAD interacting protein; FKHD: forkhead domain factor; GATA: GATA binding factor; GREF: glucocorticoid-responsive and related factor; HAML: human acute myelogenous leukemia factor; MYBL: cellular and viral myb-like transcriptional regulator; NFKB: Nuclear factor kappa B/c-rel; p53F: p53 tumor suppressor; RREB: Ras-responsive element binding protein; RORA: v-ERB and RAR-related orphan receptor alpha; RXR: retinoid X receptor; SP1F: GC-box factor; STAT: signal transducer and activator of transcription; TBPF: TATA-binding protein factor; ZFHX two-handed zinc finger homeodomain.*

dramatic increase in staining intensity (Figure 6B, C, D). A possible correlation with patient and disease characteristics is presently being investigated.

Of interest, in several cases of tumors *in vivo* and of cell lines *in vitro* CRASH was also detected in the nucleus. The size of CRASH is below the cut-off for passive diffusion into the nucleus (30). However, both the selectivity of nuclear localization and the occurrence of cases with nuclear localization-only speak against passive diffusion alone. Both active transport into the nucleus and nuclear retention require specific association with regulatory / transporter molecules and / or nuclear retention sequences or nuclear localization sequences (NLS) (30). Of interest, a candidate NLS-like sequence (145 **KKRLEKEHEK** 154), that is similar to that found in PKCδ (31), was identified in the CRASH peptide sequence (Figure 7A). Taken together, the findings above indicate a positive selective pressure for CRASH expression, particularly in specific tumor histotypes. As in several instances the corresponding normal tissues do not express this molecule, these findings suggested a selective advantage for CRASH-expressing tumor cells and a possible relevance of CRASH expression for tumor growth.

Sequence analysis, domain identification and function of the CRASH protein. Asparaginases hydrolyse asparagine and glutamine, yielding aspartic/glutamic acid and ammonia. Glycosylasparaginases in mammalian cells play the additional role of removing carbohydrate side chains from asparagine, as a final step in the degradation of cell surface glycoproteins after asparagine has been released from the peptide chains in the lysosome (32). Hence, asparaginases may exert a control role in signaling from the cell surface, in the metabolism of tumor cells and in cell growth. Consistently, amino acid sufficiency has been demonstrated to selectively regulate p70 S6 kinase (p70(s6k)) and the activity of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (6). Addition of L-asparaginase to human leukemic cells inhibits the activity of p70(s6k) and the

phosphorylation of 4E-BP1, and L-asparaginase, as well as rapamycin, selectively suppress the synthesis of ribosomal proteins at the level of mRNA translation (6). Consistent with a metabolic role of the enzyme, ALP colocalizes with a mitochondrial marker at the midpiece of spermatozoa (2).

Asparaginase-like 1 protein sequences were found to be highly conserved across species (monkey, horse, dog, cow, rodents, chicken, xenopus and zebrafish), and CRASH was found to be highly similar to both L-asparaginases and glycosylasparaginases (33, 34) (Figure 7B, C). In particular, conservation of the catalytic nucleophile threonine was observed (Figures 7 and 8), which suggests conservation of catalytic activity, as the threonine provides the nucleophile for both autoproteolysis and hydrolase activities. Consistently, Western blot analysis detected actual cleavage of a sizable fraction of the CRASH proteins.

Conservation of function in homologous sequences implies conservation of folding and of overall structure. Hence, the CRASH potential structure was analysed to detect conserved elements across the asparaginase family. Programs that predict secondary structure possess the highest accuracy (80.7%) (18) among the structure-predicting tools, and were used to compare CRASH with a *bona fide* L-asparaginase. Strikingly high conservation of 2D structural elements was demonstrated in the catalytic domain (Figure 9). Both β-strand and α-helix elements, position and length were shown to be conserved. Little conservation was present in the N-terminal region (not shown). Largely conserved phosphorylation (Casein Kinase II at amino acids 43, 71, 80, 243 and 303; PKC at amino acid 141) and myristoylation sites consensus sequences were also identified.

CRASH regulates cell proliferation in an endocrine-dependent manner. In order to investigate a functional involvement of CRASH in tumor cell proliferation, CRASH expression was knocked-down by RNAi in a syngeneic colon cancer cell system: KM12LSM, a line with moderate metastatic potential that expresses low levels of CRASH, and KM12L4A, a line with strong metastatic potential that expresses high levels of CRASH (7, 8, 35). Notably, KM12L4A proliferates considerably faster than KM12LSM, and were significantly inhibited by the CRASH siRNA (close to the basal levels of KM12SM cells). On the other hand, the CRASH siRNA had essentially no effect on the proliferation of the KM12SM cells (Figure 10). Hence, CRASH can play a stimulatory role in cell growth.

This regulatory role was suggested to be mechanistically linked to endocrine-dependent pathways of growth control. Indeed, CRASH can be induced by androgens and progesterone in BT474 breast cancer cells (1), and is overexpressed in a large fraction of hormone-sensitive cancers. Hence, to investigate a hormone-responsive regulation of transcription, we looked for the presence of bona fide endocrine-responsive elements in the promoter of the CRASH gene. Transcription factor binding site searching was performed on the promoter region of the transcript, as retrieved from the human genome sequence assembly, from 1000 bp upstream the transcription start site (TSS) to 100 bp downstream the TSS. A first layer of analysis was performed with TESS. TESS uses binding site or consensus strings and positional weight matrices from the TRANSFAC, IMD, and CBIL-GibbsMat databases. This allows to identify all potential core binding sites, including those too short to be distinguishable from random occurrences. Seven hundred and fifty candidate estrogen receptor α binding sites were identified as predicted by TESS with the core consensus sequence GGTCA (data not shown). This analysis was refined with MatInspector, a program that utilizes a large library of matrix descriptions for factor binding sites, to locate matches along regions of DNA sequences. MatInspector assigns a quality rating to matches on the basis of individually optimized matrix similarity thresholds, thus permitting sensitive quality-based filtering and selection of matches. This allowed to refine the binding site search (188 confirmed binding sites) (data not shown). Based on the palindromic consensus 5' GGTCAnnnTGACC 3', utilizing searching for canonical half palindromes (36,37), an estrogen response element (ERE) IR3 was identified at position -201/-183 (gcccaggctgGAGTgcaatggcgcg) with a core similarity of 1 (in capital) and a matrix similarity of 0.843. This element was also found conserved in the promoters of the CRASH orthologous genes from *Macaca mulatta, Pan troglodytes, Rattus norvegicus* and *Mus musculus* consistent with its predicted functional role (Figure 11).

In summary, the results suggest a direct relevance of CRASH expression for the growth of transformed cells and in the progression of endocrine-responsive tumors, candidating CRASH as a novel diagnostic and therapeutic target in cancer.

Acknowledgements

We thank Alessandra Madeo for help during the course of this work. We are also grateful for the support of the Fondazione of the Cassa di Risparmio della Provincia di Chieti.

References

1 Evtimova V, Zeillinger R, Kaul S and Weidle UH: Identification of CRASH, a gene deregulated in gynecological tumors. Int J Oncol *24*: 33-41, 2004.

2 Bush LA, Herr JC, Wolkowicz M, Sherman NE, Shore A and Flickinger CJ: A novel asparaginase-like protein is a sperm autoantigen in rats. Mol Reprod Dev *62*: 233-247, 2002.

3 Chuang JC, Yu CL and Wang SR: Modulation of lymphocyte proliferation by enzymes that degrade amino acids. Clin Ex Immunol *82*: 469-472, 1990.

4 Becker FF and Broome JD: L-asparaginase: inhibition of early mitosis in regenerating rat liver. Science *156*: 1602-1603, 1967.

5 Bussolati O, Belletti S, Uggeri J, Gatti R, Orlandini G, Dall'Asta V and Gazzola GC: Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. Exp Cell Res *220*: 283-291, 1995.

6 Iiboshi Y, Papst PJ, Hunger SP and Terada N: L-Asparaginase inhibits the rapamycin-targeted signaling pathway. Biochem Biophys Res Commun *260*: 534-539, 1999.
7 Morikawa K, Walker SM, Jessup JM and Fidler IJ: *In vivo* selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res *48*: 1943-1948, 1988.
8 Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM and Fidler IJ: Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res *48*: 6863-6871, 1988.
9 Brummelkamp TR, Bernards R and Agami R: A system for stable expression of short interfering RNAs in mammalian cells. Science *296*: 550-553, 2002.
10 Orsulic S, Li Y, Soslow RA, VitaleCross LA, Gutkind JS and Varmus HE: Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell *1*: 53-62, 2002.
11 Devereux J, Haeberli P and Smithies O: A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res *12*: 387-395, 1984
12 Brendel V, Bucher P, Nourbakhsh IR, Blaisdell BE and Karlin S: Methods and algorithms for statistical analysis of protein sequences. Proc Natl Acad Sci USA *89*: 2002-2006, 1992.
13 Pagni M, Ioannidis V, Cerutti L, Zahn-Zabal M, Jongeneel CV and Falquet L: MyHits: a new interactive resource for protein annotation and domain identification. Nucleic Acids Res *32*: W332-335, 2004.
14 Falquet L, Pagni M, Bucher P, Hulo N, Sigrist CJ, Hofmann K, Falquet L, Pagni M, Buche P, Hulo N, Sigrist CJ, Hofmann K and Bairoch A: The PROSITE database, its status in 2002. Nucleic Acids Res *30*: 235-238, 2002.
15 Schmid CD, Perier R, Praz V and Bucher P: EPD in its twentieth year: towards complete promoter coverage of selected model organisms. Nucleic Acids Res *34*: D82-85, 2006.
16 Schug J: Using TESS to predict transcription factor binding sites in DNA sequence. Curr Protoc Bioinformatics Chapter *2*: Unit 2 6, 2008.
17 Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A. Cartharins K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M and Werner T: MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics *21*: 2933-2942, 2005.
18 Jones DT: Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol *292*: 195-202, 1999.
19 Varambally S, Yu J, Laxman B, Rhodes DR, Mehra R, Tomlins SA, Shah RB, Chandran U, Monzon FA, Becish MJ, Wei JT, Pienta KJ, Gosh D, Rubin MA and Chinnaiyan AM: Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell *8*: 393-406, 2005.
20 Suri A: Cancer testis antigens – their importance in immunotherapy and in the early detection of cancer. Expert Opin Biol Ther *6*: 379-389, 2006.
21 Zendman AJ, Ruiter DJ and Van Muijen GN: Cancer/testis-associated genes: identification, expression profile, and putative function. J Cell Physiol *194*: 272-288, 2003.
22 Naz RK, Deutsch J, Phillips TM, Menge AC and Fisch H: Sperm antibodies in vasectomized men and their effects on fertilization. Biol Reprod *41*: 163-173, 1989.
23 Alexander NJ and Anderson DJ: Vasectomy: consequences of autoimmunity to sperm antigens. Fertil Steril *32*: 253-260, 1979.
24 Samuel T, Linnet L and Rumke P: Post-vasectomy autoimmunity to protamines in relation to the formation of granulomas and sperm agglutinating antibodies. Clin Exp Immunol *33*: 261-269, 1978.
25 Witkin SS, Higgins PJ and Bendich A: Inhibition of viral reverse transcriptase and human sperm DNA polymerase by anti-sperm antibodies. Clin Exp Immunol *33*: 244-251, 1978.
26 Batova IN, Richardson RT, Widgren EE and O'Rand MG: Analysis of the autoimmune epitopes on human testicular NASP using recombinant and synthetic peptides. Clin Exp Immunol *121*: 201-209, 2000.
27 Snow K and Ball GD: Characterization of human sperm antigens and antisperm antibodies in infertile patients. Fertil Steril *58*: 1011-1019, 1992.
28 Shetty J, Naaby-Hansen S, Shibahara H, Bronson R, Flickinger CJ and Herr JC: Human sperm proteome: immunodominant sperm surface antigens identified with sera from infertile men and women. Biol Reprod *61*: 61-69, 1999.
29 Lee CY, Lum V, Wong E, Menge AC and Huang YS: Identification of human sperm antigens to antisperm antibodies. Am J Reprod Immunol *3*: 183-187, 1983.
30 Kininis M and Kraus WL: A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. Nucl Recept Signal *6*: e005, 2008.
31 DeVries TA, Neville MC and Reyland ME: Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence. Embo J *21*: 6050-6060, 2002.
32 Saarela J, Laine M, Tikkanen R, Oinonen C, Jalanko A, Rouvinen J and Peltonen L: Activation and oligomerization of aspartylglucosaminidase. J Biol Chem *273*: 25320-25328, 1998.
33 Lough TJ, Reddington BD, Grant MR, Hill DF, Reynolds PH and Farnden KJ: The isolation and characterisation of a cDNA clone encoding L-asparaginase from developing seeds of lupin Lupinus arboreus. Plant Mol Biol *19*: 391-399, 1992.
34 Noronkoski T, Stoineva IB, Petkov DD and Mononen I: Recombinant human glycosylasparaginase catalyzes hydrolysis of L-asparagine. FEBS Lett *412*: 149-152, 1997.
35 De Lange R, Burtscher H, Jarsch M and Weidle UH: Identification of metastasis-associated genes by transcriptional profiling of metastatic *versus* non-metastatic colon cancer cell lines. Anticancer Res *21*: 2329-2339, 2001.
36 Tora L, Gaub MP, Mader S, Dierich A, Bellard M and Chambon P: Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. Embo J *7*: 3771-3778, 1988.
37 Kato S, Tora L, Yamauchi J, Masushige S, Bellard M and Chambon P: A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically. Cell *68*: 731-742, 1992.

Received October 13, 2008
Revised December 29, 2008
Accepted February 11, 2009