Testisin, a Glycosyl-Phosphatidylinositol–Linked Serine Protease, Promotes Malignant Transformation In vitro and In vivo

Tenny Tang, Muriel Kmet, Laura Corral, Steffan Vartanian, Andreas Tobler, and Jackie Papkoff
diaDexus, Inc., South San Francisco, California

Abstract

Human testisin, a serine protease, is highly expressed in ovarian cancer and premeiotic spermatocytes with relatively little expression in other normal tissues. We first showed that testisin was localized on the surface of cultured tumor cells as a glycosyl-phosphatidylinositol–linked protein. We next explored the biological function of testisin in malignant transformation through manipulation of testisin expression in cell culture model systems. Small interfering RNA–mediated knockdown of endogenous testisin mRNA and protein expression in tumor cell lines led to increased apoptosis and diminished growth in soft agar. Conversely, overexpression of testisin in an epithelial cell line induced colony formation in soft agar as well as s.c. tumor growth in severe combined immunodeficient mice. A catalytic domain mutant was unable to induce soft-agar growth indicating that testisin protease activity is required for transformation. Ectopic expression of testisin in a human ovarian cancer cell line without endogenous testisin expression, led to the formation of larger tumors in severe combined immunodeficient mice. Data presented here provide the first demonstration that testisin can promote cellular processes that drive malignant transformation. Our functional data coupled with the restricted normal tissue distribution of testisin and its overexpression in a majority of ovarian cancers validates this cell surface protein as a target for therapeutic intervention. (Cancer Res 2005; 65(3): 868-78)

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancy in the United States and other industrialized countries (1, 2). Because early stages of this cancer are often asymptomatic, most ovarian cancers are detected at an advanced stage when the disease has spread beyond the ovary and prognosis is very poor (3–6). Despite significant progress in chemotherapeutic development, there has been little effect on the long-term survival of women with advanced ovarian cancer (4). Consequently, identification, characterization and validation of new targets that play a role in ovarian cancer are of great importance to the diagnosis and treatment of this disease. Here we use functional validation tools in cell based model systems to show for the first time that activity of the human serine protease testisin, which is overexpressed in ovarian cancer, can play a pivotal role in promoting malignant transformation.

Serine proteases belong to a large, highly conserved protein family characterized by a catalytic motif triad of histidine, aspartate, and serine residues required for enzymatic activity (7). One serine protease subfamily is the chymotrypsin (S1) fold group which includes trypsin, chymotrypsin and numerous other secreted or cytoplasmic proteases (7, 8) as well as type I and II transmembrane or glycosyl-phosphatidylinositol (GPI)–linked proteins whose catalytic domains are exposed to the extracellular milieu (9, 10). Some membrane-associated proteases are highly expressed in epithelial cancers and have been shown to participate in tumor growth, invasion and metastasis (10–16). Others are expressed in endothelial cells during differentiation and morphogenesis and may function in physiologic as well as pathologic vasculogenesis and angiogenesis (17).

Human testisin (PRSS21 and ESP-1), encodes a predicted GPI-linked or membrane-anchored protein (18, 19). Testisin was first reported to be highly expressed in premeiotic testicular germ cells but not in a range of other normal adult tissues (18, 19). Testisin was also cloned from human eosinophils (20) and from human ovarian cancer tissue (21) where it was shown that testisin mRNA was highly expressed in ovarian carcinomas with little or no expression in normal ovaries. We independently identified testisin in a genomics project to discover up-regulated sequences in human cancers (unpublished results) and found that testisin mRNA is overexpressed in a majority of human ovarian tumors compared with normal ovarian tissues, whereas a variety of other normal human tissues showed no expression with the exception of testisin, consistent with the literature (18, 19, 21).

The specific physiologic substrates for testisin are as yet undefined and the mechanisms whereby the proteolytic activity of testisin participates in spermatocyte or tumor cell biology are unknown. Based on the significant overexpression of testisin mRNA in ovarian cancer, we explored whether testisin could play a functional role in promoting malignant transformation. We first showed that testisin localized to the surface of cultured tumor cell lines as a GPI-linked protein. We next showed that testisin promotes transformation when overexpressed in cell culture and mouse xenograft model systems and showed that the catalytic activity of testisin is required for transformation. Furthermore, small interfering RNA (siRNA)–mediated knockdown of testisin mRNA and protein in human tumor cell lines led to increased apoptosis and diminished ability to form colonies in soft agar. Our findings support a direct role for testisin in promoting malignant transformation of epithelial cells and suggest that a therapeutic strategy aimed at blocking or diminishing the activity of testisin could therefore function to inhibit the growth and progression of human ovarian cancer. The cell surface localization of testisin on tumor cells and its enzymatic activity imply that an antibody or small molecule therapeutic strategy would be useful.

Materials and Methods

Cells and Cell Culture. Cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown according to supplied specifications.
Quantitative Real Time Reverse Transcription-PCR. RNA was prepared using Trizol RNA isolation reagent (Invitrogen Co., Carlsbad, CA) according to manufacturer's instructions and integrity was analyzed by agarose gel. Total RNA samples (2.2 μg) were treated with RNAse-free DNase I (Invitrogen). Reverse transcription was done according to manufacturer's recommendations using SuperScript II enzyme (Invitrogen) in a final volume of 33 μL. As control, 250 ng of Dnasel-treated RNA were used without the SuperScript II enzyme to check for genomic DNA contamination. Following reverse transcription the RNA/cDNA mixture was reduced to 1 ng/μL with Tris-EDTA buffer pH 7.0 (BioWhittaker, Walkersville, MD). Quantitative PCR (QPCR) was done on an ABI Prism 7900 Sequence detection system (PE Applied Biosystems, Foster City, CA) using TaqMan Universal PCR master mix according to manufacturer's directions. Amplifications were in duplicate using 10 ng template with primers and probes (Biosearch Technologies, Inc., Novato, CA) for testisin (forward primer 5′-GCAAAGCTATCTTGGAGCATG-3′, reverse primer 5′-CCAGGGTTCCACCTGAGTCA-3′, and probe 5′-CTGGCAGCAGGTCGCGG-3′) and the endogenous control ATP synthase 6 (forward primer 5′-CAGTGTGATATTGGCTTTGCCCTTCA-3′, reverse primer 5′-CGAGGATCTTTGGAATGATAGTA-3′, and probe 5′-AGCCACCTTCT-TACCACAAAGCCACA-3′). Final concentrations were 0.2 μM/liter of primer and 0.1 μM/liter of probe. PCR was done at 50 °C for 2 minutes, one cycle at 95 °C for 10 minutes, 95 °C for 15 seconds, and 40 cycles at 60 °C for 1 minute. Expression levels are represented relative to one sample named calibrator that becomes the 1× sample, and mRNA levels in all other samples are expressed as an n-fold difference relative to the calibrator (ABI Prism 7700 Sequence Detection System User Bulletin 2).

siRNA Oligonucleotides. siRNAs were designed based on the open reading frame of testisin using previously described methods (22, 23). A random "scrambled" siRNA sequence and a siRNA targeting Emerin were used (24) as negative controls and a siRNA targeting DAXX as a positive control (25). BLAST search against the human genome was done with each siRNA sequence to ensure target specificity. All siRNA molecules (HPP purified grade) were chemically synthesized by Xeragon, Inc. (Germantown, MD), dissolved in sterile buffer, heated at 90 °C for 1 minute then incubated at 37 °C for 1 hour before use. siRNA oligonucleotides were:

- Testisin: sense 5′-CACAUCCAGCCCAUCUGUC(dTdT)-3′
- Emerin: sense 5′-CGGUGUCCUCGGGGGGGGCG(dTdT)-3′
- Scrambled: sense 5′-UUCUGGUGGACUGGAC(dTdT)-3′
- DAXX: sense 5′-GGAGUUGGGAAUCCUAAG(gTdT)-3′

Transfection with siRNA Oligonucleotides. 4 × 10⁴ HeLa cells and 6 × 10⁴ CaOv3 cells were seeded in 12-well plates 18 to 24 hours before transfection carried out using Oligofectamine reagent (Invitrogen) according to the manufacturer's protocol. Final concentration (100 mmol/L) siRNA (200 mmol/L) DAXX siRNA) and 1.5 μL Oligofectamine were used per well. Experiments were done in triplicate and cells evaluated 72 hours after transfection for changes in mRNA levels by QPCR, protein levels by immunoblot and apoptosis (below). A QuantTech SYBR Green reverse transcription-PCR kit (Qiagen, Inc., Valencia, CA) was used for evaluation of mRNA knockdown. Between 20 and 40 ng of template RNA were used per reaction. QPCR was done as above. All findings were confirmed with at least two independent experiments.

Fluorescence-Activated Cell Sorting Analysis. Either 1 million testisin-transfected 293 cells or untransfected 293 cells were dispensed in a 96-well tray in 25 μL of fluorescence-activated cell sorting buffer. Cells were incubated on ice 10 minutes with 1 μg of testisin-specific monoclonal antibody or an isotype matched control antibody and washed twice with fluorescence-activated cell sorting buffer. A biotinylated secondary antibody (Caltag, Burlingame, CA) diluted 1:50, was added for 10 minutes on ice followed by washing. Cells were incubated with a 1:50 dilution of streptavidin-R-phycocerythrin (Molecular Probes, Eugene, OR) 10 minutes on ice then washed and read on a Beckman-Coulter Epics Elite flow cytometer.

Apopptosis Assays. With the "Apo-ONE Homogeneous Caspase-3/7 Assay" kit (Promega, Inc., Madison, WI), test cells were solubilized directly on the culture plate and caspase activity measured according to supplier's instructions. With the "Guava Nexin V-PE" kit (Guava Technologies, Inc., Hayward, CA), treated cells were harvested by trypsinization. After washing, ~10⁶ cells were resuspended in 40 μL provided buffer, and 5 μL each Annexin V (+) and 7-AAD (−) were added. Cells were incubated 20 minutes on ice and analyzed using the Guava PCA machine according to manufacturer's instructions.

SDS-PAGE and Immunoblot Analysis. Cell extracts were prepared on ice using solubilization buffer (1% NP40, 10 mmol/L Na₂PO₄, and 0.15 mol/L NaCl) plus a protease inhibitor cocktail (Roche Inc., Indianapolis, IN). Snap-frozen, minced tumor tissue was homogenized in extraction buffer [50 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% IG-Pal plus protease inhibitors] followed by sonication and centrifugation to clarify extracts. Between 20 and 50 μg of protein extract were used per gel lane; protein equivalent concentrations were evaluated for comparisons on the same gel. Precast 4-12% SDS-polyacrylamide minigels (Nupro, Invitrogen) were used with MES running buffer (Nupro, Invitrogen). Gels were transferred to Immobilon-P 0.45-μm membranes (Invitrogen) followed by immunoblot analysis. A mouse monoclonal antibody against testisin was produced in in-house using recombinant testisin protein and was used at a final concentration of 1 μg/mL, a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Chemicon Inc., Temecula, CA) at 2 μg/mL, a monoclonal antibody against Na+/K+ ATPase (Santa Cruz Biotechnology, Santa Cruz, CA) at 0.2 μg/mL and a monoclonal antibody against HA (Covance, Berkeley, CA) at 1 μg/mL. Horseradish peroxidase-linked goat anti-mouse immunoglobulin (Jackson Lab, Inc., West Grove, PA) was used (1:10,000 dilution) to detect the primary monoclonal antibody with enhanced chemiluminescence reagent per manufacturer's directions (Amersham, Piscataway, NJ).

Cell Surface Biotinylation. Intact cell monolayers were washed with ice cold PBS then incubated on ice for 30 minutes with 0.5 μg/mL Sulfo-NHS-SS-Biotin ( Pierce, Rockford, IL) in PBS. Cells were washed several times with PBS including 25 mmol/L Tris then with PBS alone followed by extraction with solubilization buffer. Clarified supernatants were immunoprecipitated with streptavidin agarose (Pierce) followed by immunoblot analysis.

Phosphatidylinositol-Specific Phospholipase C Treatment. Cells were seeded in 6-well plates. At 90% confluence, the media were replaced with 1 mL fresh growth media, with or without 0.5 unit phosphatidylinositol-specific phospholipase C (PI-PLC, Sigma). After 1 hour incubation at 37 °C, media were harvested and microfuged. Unconcentrated media (15 μL) were analyzed by SDS-PAGE. Cells were solubilized for immunoblot analysis as above.

Expression Vector Construction. Human prostate cancer cDNA was prepared from polyadenylated mRNA using a BD SMART PCR cDNA synthesis kit (BD Bioscience/Clontech, Palo Alto, CA). Testisin cDNA was obtained by PCR reaction using gene-specific primers 5′-ATGGGGCGCGCGCGGGGCGCTGCTGCTG-3′ (forward) and 5′-TTATCA-GACCCGCCCAAGGAGTGAGGAAGGAGGCA-3′ (reverse). The PCR fragment was cloned into the HPV1 site of the pLXSN vector (BD Bioscience/Clontech) and sequence verified. Testisin cDNA with an in-frame COOH-terminal hemagglutinin tag was obtained using the same procedure except the reverse primer sequence was 5′-TTATCACGGGTTAGTCCGGCCAGTGG-TACGGGTGACGGCCAGGGCAGAGGAGGACCA-3′ (HA tag italized). A mutant testisin lacking protease activity was constructed using oligonucleotide directed mutagenesis to convert the catalytic triad residue serine 238 to an alanine. The mutated cDNA was sequence verified and cloned into the pLXSN vector. A retroviral vector encoding alkaline phosphatase, pLAPSN was purchased from BD Bioscience/Clontech.

Virus Production and Infection. The pVpack-Eco plasmid (Stratagene, La Jolla, CA) and pVpack-Ampho plasmid (Stratagene) were used for ecotropic and amphotropic virus packaging, respectively. 293T cells seeded in a Biocat collagen coated plate (BD Bioscience) were transfected with plasmid DNA: pLXSN-testisin, pLXSN-testisin-HA, pLXSN-testisin-mut or
were implanted with Matrigel and for RK3E cells, 5 siRNA as outlined and then plated in soft agar at 10^4 cells per well. The assay was maintained by weekly feeding Iscove’s medium were applied on top of the solidified cell layer and plates were seeded in duplicate; 2 mL of 0.8% agar, 10% fetal bovine serum in 99.0% confidence level. Tumors had significantly larger volumes than SKOV3-control tumors at a 99.0% confidence level. Statistical Analysis of Tumor Xenograft Data. For the SKOV3 xenograft studies a single factor ANOVA was done to test whether on the last day of measurement the tumor volumes between control and testisins groups differed. The results indicated a >99.0% probability that the two groups do not have the same tumor volume. Furthermore, pairwise two-sample t tests assuming unequal variances with Bonferroni correction analysis were done comparing SKOV3-testisins tumors to SKOV3-control tumors. Analysis of data from the last day of measurement revealed that the SKOV3-testisins tumors had significantly larger volumes than SKOV3-control tumors at a 99.0% confidence level.

Results

Testisins Is Detected on the Surface of Tumor Cells as a GPI-Linked Protein. To identify appropriate tumor cells for testisin biochemistry and function studies, a selection of human tumor cell lines was evaluated for testisin mRNA expression by QPCR (Fig. 1A). The mRNA positive CaOv3 and HeLa cells were chosen for further analysis with Caco2 cells included as a negative control. Immunoblot of cell lysates with a monoclonal antibody against human testisin showed that HeLa cells expressed significantly more testisin protein than CaOv3 cells, consistent with the relative testisin mRNA levels, and no testisin protein was detected in Caco2 cells as expected (Fig. 1B, top). To examine whether testisin could be detected on the cell surface, live CaOv3, HeLa, and Caco2 cells were biotinylated using a protocol that specifically labels the cell surface. The cells were solubilized and biotinylated proteins selected from the mixture with avidin agarose followed by immunoblot analysis. Testisin was detected in the biotinylated fraction of both HeLa and CaOv3 cells thus demonstrating its cell surface localization (Fig. 1B, top). Na-K-ATPase, a known cell surface protein, was used as a positive control for the cell surface labeling protocol and was readily detected in the biotinylated fraction of all cells examined (Fig. 1B, middle). To ensure that internal cell proteins were not biotinylated GAPDH, an abundant cytoplasmatic protein, was evaluated. As expected, GAPDH could not be detected in the biotinylated fractions whereas it was readily detected in the total cell lysates (Fig. 1B, bottom). Fluorescence-activated cell sorting analysis using a monoclonal antibody against testisin also detected the protein on the surface of live, testisin-transfected 293 cells compared with control 293 cells which were negative (Fig. 1C).

Because human testisin was predicted to be a GPI-linked protein, this was tested by treating live HeLa cells with PI-PLC which cleaves the membrane anchor from GPI-linked proteins (26) and releases the protein. No testisin protein was shed into the medium of untreated HeLa cells however, treatment with PI-PLC released testisin into the medium where it could be detected by immunoblot (Fig. 1D). PI-PLC treatment did not release a transmembrane protease (TMPRSS4; Fig. 1D) indicating that the release of testisin was due to specific cleavage of the GPI-anchor by the PI-PLC. Together these experiments show that testisin localizes to the surface of tumor cells via a GPI-linkage.

Knockdown of Testisin Expression Leads to Increased Apoptosis. To evaluate the function of testisin, we tested whether siRNA-mediated knockdown of testisin in human tumor cell lines would lead to apoptosis. HeLa cells were selected because they expressed the highest levels of testisin protein, were readily transfectable and amenable to a variety of transformation assays. Transfection of HeLa cells was optimized, four different testisin-specific siRNAs evaluated (data not shown) and the siRNA inducing the greatest decrease in testisin mRNA level was chosen for subsequent experiments to assess knockdown of testisin mRNA and protein and the effects on transformation. A siRNA representing a scrambled sequence with no homology to any mRNAs based on blast search was used as a negative control. QPCR with testisin-specific primers showed that by 72 hours maximal mRNA knockdown of ~80% was induced by testisin-specific siRNA whereas scrambled siRNA had no effect (Fig. 2A). Furthermore, neither the testisin-specific siRNA nor the scrambled siRNA induced any knockdown of GAPDH mRNA measured by QPCR with GAPDH-specific primers (Fig. 2B). Thus, testisin-siRNA specifically induced knockdown of testisin mRNA in the HeLa cell line. Immunoblot analysis of testisin siRNA treated HeLa cells revealed a corresponding decrease in testisin protein but not GAPDH protein (Fig. 2C). The effect of the siRNAs on apoptosis was evaluated with cells treated in parallel. The testisin-specific siRNA led to a significant increase in apoptosis measured with an Annexin V assay, whereas the scrambled siRNA had no effect (Fig. 2D).

Caspase activity is frequently induced during apoptosis (27, 28) and serves as a sensitive measure of induced cell death. Consequently, the activity of caspase 3 and caspase 7 was measured in siRNA-treated HeLa cells. Knockdown of testisin mRNA (Fig. 2E) led to a significant increase in caspase activity (Fig. 2F) indicating the induction of apoptosis and thus a functional role for this protein in the survival of cells. siRNA-induced knockdown of DAXX was shown to induce apoptosis in HeLa cells (25) and, therefore, was used as a positive control. Knockdown of DAXX mRNA (Fig. 2E) also induced caspase activity (Fig. 2F). In another study, emerin, a nuclear membrane protein, was knocked down with siRNA in mammalian cells and shown to be nonessential (24). As an additional specificity control for the effects of testisin siRNA on apoptosis, HeLa cells were treated with siRNA specific for emerin.
Figure 1. Testisin mRNA expression and cell surface detection. A, relative testisin mRNA expression in human tumor cell lines. B, immunoblots of avidin agarose-selected proteins from CaOv3, HeLa, and Caco2 cells which were surface biotinylated (+) or mock treated (−) along with samples of total cell lysate. Antibodies against testisin (top), NaK-ATPase (middle), or GAPDH (bottom) were used. C, fluorescence-activated cell sorting profiles of testisin-transfected or control 293 cells with a testisin-specific antibody (white area) or a control antibody (grey area). D, immunoblots of media and cell extracts from live cells treated either with (+) or without (−) PI-PLC. HeLa cells with a testisin-specific antibody (top). 293T cells transfected with HA-tagged TMPRSS4 with a HA-specific antibody (bottom).
Figure 2. Knockdown of testisin mRNA and protein expression in HeLa cells and apoptosis measurement. QPCR evaluation of testisin mRNA levels (A) or GAPDH mRNA levels (B) in HeLa cells treated with either scrambled- or testisin-specific siRNA. C, immunoblots of siRNA-treated HeLa cells with antibodies against testisin or GAPDH. D, apoptosis induction in siRNA-treated cells measured with Annexin V assay and Guava detection system. E, QPCR evaluation of testisin, DAXX, or emerin mRNA levels after treatment of HeLa cells with target-specific or scrambled siRNA. F, measurement of caspase 3 and/or caspase 7 activity in siRNA-treated cells from E. G, QPCR evaluation of testisin, DAXX, or emerin mRNA levels after treatment of SKBR3 cells with target-specific or scrambled siRNA. H, measurement of caspase 3 and/or caspase 7 activity in siRNA-treated cells from G.
which led to a 50% decrease in emerin mRNA levels yet had no effect on caspase activity (Fig. 2E and F). To further exclude nonspecific effects on apoptosis from testisin siRNA, a similar experiment was done using SKBR3 breast tumor cells that do not express testisin mRNA. In this case, treatment with the testisin siRNA did not induce apoptosis whereas DAXX and emerin controls performed as expected (Fig. 2G and H).

**Knockdown of Testisin Expression Leads to Diminished Colony Formation in Soft Agar.** As shown above, specific knockdown of testisin mRNA and protein in HeLa cells led to increased apoptosis, measured by two different methods. We next examined whether knockdown of testisin affected the ability of HeLa cells to form colonies in soft agar. HeLa cells were treated with scrambled, testisin- or DAXX-specific siRNA and plated in soft agar. HeLa cells form numerous large colonies in agar and this was not affected by the scrambled siRNA (Fig. 3A). In contrast, both testisin- and DAXX-specific siRNAs inhibited the number of colonies formed by ~88% and 80%, respectively and the colonies formed were smaller (Fig. 3A). Testisin and DAXX mRNA levels decreased by 50% and 80% respectively, 72 hours after siRNA transfection (Fig. 3B) and again induced caspase activity whereas the scrambled siRNA did not (Fig. 3C).

We next evaluated knockdown of testisin in the CaOv3 tumor cell line which also expresses testisin mRNA and cell surface testisin protein (Fig. 1A and B). Treatment with testisin-specific siRNA decreased testisin mRNA levels by 55% (Fig. 4A) with a corresponding decrease in testisin protein (Fig. 4B), whereas there was no effect on GAPDH mRNA levels (data not shown) or protein (Fig. 4B). Testisin mRNA knockdown increased apoptosis, measured by the Annexin V assay, whereas scrambled siRNA had no effect (Fig. 4C). In a subsequent experiment, treatment of CaOv3 cells with either testisin or DAXX siRNA induced caspase activity (Fig. 4D) in conjunction with specific knockdown of the respective targeted mRNAs (Fig. 4E). Emerin siRNA treatment reduced emerin mRNA levels by 50% (Fig. 4E) with no effect on caspase activity (Fig. 4D). CaOv3 cells formed only tiny colonies in soft agar but the number was diminished by ~60% to 70% upon treatment with either testisin or DAXX but not scrambled siRNA (data not shown).

Together, data obtained with HeLa and CaOv3 cells showed that specific reduction of testisin mRNA and protein levels led to increased apoptosis and diminished growth in soft agar thus suggesting that testisin participates in the malignant transformation of these cells.

**Overexpression of Testisin Promotes Colony Formation in Soft Agar.** To test whether testisin is capable of promoting transformation we overexpressed testisin in cell lines and evaluated soft agar growth. We used the RK3E model system which has previously been used successfully to evaluate transformation induced by different genes and signaling pathways including Ras, c-Myc, and β-catenin (29–31). Wild-type testisin protein, with and without a COOH-terminal hemagglutinin tag, and testisin protein with a point mutation in the catalytic triad thus lacking enzymatic activity (8, 32) were expressed from a retroviral expression vector, which included a neomycin resistance gene. A retroviral vector expressing alkaline phosphatase (AP) was used as a control. To avoid clonal effects, G418-selected, polyclonal
pools of infected RK3E cells were used for all experiments. Expression of testisin proteins in the G418-selected cell pools was verified by immunoblot (Fig. 5A). RK3E cells do not express endogenous testisin mRNA (data not shown) nor was testisin protein detected in the AP-expressing RK3E cells (Fig. 5A). Expression of AP in the G418-selected cells was evaluated by staining cell monolayers for AP activity showing that essentially all of the cells were positive (Fig. 5B) and, therefore, most of the G418-selected cells expressed the gene of interest. Virus-infected, selected cells were plated in soft agar and monitored for colony formation. Neither parental RK3E cells nor AP-expressing cells formed any colonies under these assay conditions (Fig. 5C and D), similar to previous results (30). However, cells expressing either HA-tagged or untagged testisin protein formed colonies demonstrating that ectopic expression of the protein promotes transformation in this assay system (Fig. 5C and D). The mutant testisin protein did not induce soft agar growth of RK3E cells (Fig. 5C and D) indicating that the catalytic function of testisin is required for transformation.

**Overexpression of Testisin Protein Promotes Xenograft Tumor Formation in Severe Combined Immunodeficient Mice.**

To further evaluate the transforming ability of testisin, RK3E cells expressing testisin or AP were implanted s.c. into SCID/Beige mice and tumor formation was monitored. All nine mice implanted with
testisin-expressing RK3E cells developed large tumors whereas none of the mice implanted with AP-expressing cells formed tumors (Fig. 6A). At the conclusion of the xenograft study tumors were harvested and evaluated by immunoblot for the presence of testisin protein. The tumors maintained expression of testisin protein at a level similar to that observed in the infected RK3E cells before implantation (Fig. 6B).

We next evaluated the effect of ectopic testisin expression on tumor formation by the human SKOV3 ovarian cancer cell line, chosen for this purpose since it does not express endogenous testisin mRNA (Fig. 1A), nor testisin protein (Fig. 6C). SKOV3 cells were infected with either a retrovirus expressing testisin or the AP control followed by G418 selection. Testisin protein expression in the selected cells was verified by immunoblot (Fig. 6C). AP control and testisin-expressing SKOV3 cells were implanted s.c. into SCID/Beige mice and monitored for tumor formation. SKOV3 cancer cells form tumors as xenografts in mice (33, 34). As expected, the AP control-expressing SKOV3 cells were also capable of growth as xenografts where all 10 mice implanted formed tumors (Fig. 6D). However, cells expressing ectopic testisin protein formed larger tumors throughout the time course when compared with the AP-control cells (Fig. 6D). Statistical analysis of the data showed that the increased size of testisin-SKOV3 tumors compared with AP control-SKOV3 tumors was significant.

**Discussion**

Human testisin is a predicted GPI-linked serine protease overexpressed in ovarian epithelial cancers with little or no expression in other tissue types except premeiotic testicular germ cells where it is abundantly expressed (18, 19, 21). We showed that testisin is localized to the cell surface and explored potential functions of testisin in malignant transformation through overexpression and siRNA-mediated knockout of testisin expression in cell culture and mouse xenograft model systems. Our data provide the first demonstration that the catalytic activity of testisin can promote cellular processes that drive malignant transformation. These functional data coupled with the restricted normal tissue distribution of testisin and its overexpression in a majority of ovarian cancers validate this cell surface protein as an exciting new cancer therapeutic target. Testisin would be particularly amenable to targeting with an antibody or a small molecule inhibitor.

Interestingly, whereas testisin is overexpressed in ovarian cancer, expression is lost in testicular germ cell tumors and consequently

![Figure 5](#)

**Figure 5.** Soft agar assays with RK3E cells overexpressing testisin proteins. A, immunoblot detection of testisin proteins in G418-selected cell pools of RK3E cells infected with retrovirus vectors expressing wild-type testisin protein (testisin), with and without a COOH-terminal hemagglutinin tag (HA), testisin protein with a point mutation within the catalytic triad (mut), or AP (control). B, staining for AP activity in monolayers of G418-selected cells. C, photographs of agar assay results for each cell type. D, average number of colonies counted within multiple representative microscopic fields for each test cell type.
testisin was initially postulated to serve a tumor suppressor function in the testis (18). Whereas the function of testisin could be different depending on cell and tissue context it is possible that loss of expression in testicular tumors is rather indicative of the tumor cell differentiation state such that they no longer express testisin whose expression is normally restricted to premeiotic germ cells during human spermatogenesis. Alternatively, the testisin gene localizes to a region of chromosome 16 (16p13.3) that exhibits loss of heterozygosity in testicular cancers (35) and is associated with significant genetic instability due to many repetitive elements (36), which could indicate that testisin expression is lost by virtue of colocalization with a tumor suppressor gene.

Our data show conclusively that native human testisin protein is displayed on the surface of tumor cells, attached via a GPI linkage. We have not detected testisin shed into the medium of cells cultured under a variety of conditions (unpublished results). Mouse testisin, which shares 64% amino acid identity with human testisin, was also proven to be a GPI-linked protein in transfected cells and localized into lipid rafts of the sperm membrane (37). GPI-linked proteins are known to be targeted to lipid rafts, plasma membrane domains enriched in cholesterol and glycolipids (38, 39). Other proteins, including transmembrane receptors and membrane-associated proteins involved in signal transduction also localize to lipid rafts (38, 39). The Src family kinase, Lck, is fatty acylated and concentrated in lipid rafts where it interacts with GPI-linked proteins and the T-cell receptor to participate in stimulatory T-cell signaling (40). Membrane localization and activation of the receptor tyrosine kinase Ret is accomplished by interaction with a GPI-linked coreceptor localized to rafts and this is required for signal transduction, differentiation and neuronal survival (41). By analogy, testisin could function in transformation by localizing to lipid rafts and participating in signal transduction together with other signaling proteins. It is also conceivable that a non–cell surface population of testisin could play a biologically relevant role in transformation.

Testisin does not seem to have additional amino acid sequences or domains that would enable nonproteolytic functions, nor does it have a transmembrane or cytoplasmic domain that could directly function in signal transduction (10). Our data conclusively showed that the ability of ectopic testisin protein to induce cell growth in soft agar is abrogated by mutation of a single critical residue in the catalytic domain thus implicating the enzymatic activity in testisin function. Based on the structural information and our functional data we conclude that the catalytic activity of testisin at the cell membrane or extracellular space, and the resulting cleaved substrates, are pivotal to the downstream consequences of cellular transformation.

Testisin and other serine proteases of the S1 subfamily are predicted to have trypsin-like substrate specificity (8, 10, 32).

---

Figure 6. Xenograft tumor formation in SCID/Beige mice with cells overexpressing testisin. A, graph of mean group tumor volume over time for subcutaneous implants of RK3E cells expressing testisin or AP into nine SCID/Beige mice per cell type. B, immunoblot of testisin in harvested RK3E-testisin tumors compared with the RK3E cell line used for implantation. C, immunoblot of testisin protein in G418-selected SKOV3 ovarian cancer cells infected with either a retrovirus expressing testisin or an AP-control retrovirus. D, graph of mean group tumor volume over time for subcutaneous implants of SKOV3 cells expressing testisin or AP into 10 SCID/Beige mice per cell type.
Differences in the otherwise highly conserved catalytic domains of the membrane-associated serine proteases suggest unique substrate specificities (8, 32). However, the specific substrates and cellular functions of testisin in cancer or in spermatogenesis have yet to be determined. During germ cell maturation, testisin is postulated to participate via its proteolytic activity in modulation of cell-ECM interactions and/or regulation of growth factor activity (18, 42). Similar functions are ascribed to acrosin and the murine serine proteases TESP1 and TESP2 which are present in the sperm acrosome and activated during the acrosome reaction (43, 44).

Another membrane-associated protease, CAP1/prostasin, is thought to modulate the activity of an amiloride sensitive sodium channel (45).

We favor the hypothesis that substrates for testisin in a cancer context will be growth factors, receptors, or extracellular matrix molecules, whereby their proteolytic cleavage will facilitate growth-promoting or antiapoptotic signals that contribute to malignant transformation of ovarian epithelial cells. In one related scenario, aberrant expression of testisin in ovarian cancer could expose the testisin catalytic activity to growth promoting substrates usually not accessible to the enzyme in its normal expression locale of the spermatocyte. In these contexts, the proteolytic activity of testisin could serve to activate a specific ligand for a colocalized receptor. A protease activation mechanism of this sort has been described for hepatocyte growth factor/scatter factor. This cytokine, together with its receptor tyrosine kinase Met, plays a pivotal role in development and cancer through control of cell growth, migration, invasion and metastasis (46). The inactive precursor, pro-hepatocyte growth factor/scatter factor, is activated via cleavage by several serine proteases that play a role in cancer and tissue repair with urokinase plasminogen activator, tissue-type plasminogen activator, coagulation factors (X, XI, and XII) and matritapase, a type II transmembrane serine protease (46–49). A related mechanism has been described whereby epidermal growth factor receptor is transactivated upon G-protein coupled receptor activation and mitogenic signaling (50). The epidermal growth factor receptor activation resulted from a G-protein coupled receptor–induced metalloproteinase/cysteine proteinase activity of the growth factor proHB-epidermal growth factor (50). Thus, the proteolytic activity of testisin could function in a similar fashion to activate growth factors that serve to promote tumor cell growth, migration, angiogenesis, or metastasis.

Discovering physiologic substrates for testisin will enable understanding of the molecular actions of testisin in normal as well as pathologic settings. Experiments are under way to identify potential substrates for testisin in cancer as well as to uncover signal transduction pathways that may be activated through the functions of testisin.

References


Testisin, a Glycosyl-Phosphatidylinositol–Linked Serine Protease, Promotes Malignant Transformation *In vitro* and *In vivo*

Tenny Tang, Muriel Kmet, Laura Corral, et al.


Updated version: Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/65/3/868](http://cancerres.aacrjournals.org/content/65/3/868)

Cited articles: This article cites 47 articles, 18 of which you can access for free at: [http://cancerres.aacrjournals.org/content/65/3/868.full.html#ref-list-1](http://cancerres.aacrjournals.org/content/65/3/868.full.html#ref-list-1)

Citing articles: This article has been cited by 5 HighWire-hosted articles. Access the articles at: [http://cancerres.aacrjournals.org/content/65/3/868.full.html#related-urls](http://cancerres.aacrjournals.org/content/65/3/868.full.html#related-urls)

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.