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

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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 testis, 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.

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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'-GCAAGGTTCACTGTGAGC-3', reverse primer 5'-CCAGGTTCACTGTGAGC-3', and probe 5'-CTTGCAATGCCAACAGGCGG-3') and the endogenous control ATP synthase (forward primer 5'-CAGTGATTATAGGCTTTCGCTCTAA-3', reverse primer 5'-CCGUGCUCCUGGGGCUGGG(dTdT)-3', and probe 5'-AGCGCATTCT-TACCACAAAGCCA-3'). Final concentrations were 0.2 μmol/L for primer and 0.1 μmol/L for 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. A mouse monoclonal antibody against testisin was produced 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 Sulfon-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 (PF-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'-ATGGGCGGCGGCGGGGCGGTGCTGCTG-3' (forward) and 5'-TTATCA-GAGCCGCCCCAGGATGGGGAGGCCCA-3' (reverse). The PCR fragment was cloned into the HpaI 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'-TTATCAAGCGGTTATGAGGCGCGGTGCTGCTG-3'. The PCR fragment was cloned into the HpaI 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'-TTATCAAGCGGTTATGAGGCGCGGTGCTGCTG-3'. The PCR fragment was cloned into the HpaI site of the plXSN vector (BD Bioscience/Clontech) and sequence verified.

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 Biocoat 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 with growth medium. For knockdown assays the cells were transfected with
were incubated at 37
Iscove’s medium were applied on top of the solidified cell layer and plates
well, 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
comparing SKOV3-testisin tumors to SKOV3-control tumors. Analysis of
tests
not have the same tumor volume. Furthermore, pairwise two-sample
test
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
data from the last day of measurement revealed that the SKOV3-testisin
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
transfection assays. A scrambled sequence with no homology to any mRNAs based on
blast search was used as a negative control. PCR 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

Results

Testisin 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
cytosplasmic 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).

pLXSN-AP plus pVpack-Eco/Ampho and pVpackGP (Stratogene) using
Lipofectamine with PLUS reagent (Invitrogen) according to manufacturers
recommendations. Virus-containing media were harvested 24 hours later.
Filtered virus-containing medium with 4 μg/mL hexadimethrine bromide
(Sigma) was used to infect RK3E or SKOV3 cells. Infected cells were grown
for 60 to 72 hours when 350 μg/mL G418 sulfate (Cellgro, Herndon, CA) was
added. Following G418 selection, pools of cells were used for all subsequent
experiments. Expression of ectopic proteins in the virus-infected, selected
cells was verified by immunoblot and expression of alkaline phosphatase
(AP) was monitored by staining.

Soft Agar Assays. Assays, repeated 2 to 4 times, were conducted using
6-well plates (Corning, Inc., Big Flats, NY). The 2-mL bottom agar consisted
of 0.8% agar, 10% fetal bovine serum in Iscove’s medium (Invitrogen).
Trypsinized cells were suspended in 0.4% agar, 10% fetal bovine serum in
Iscove’s medium and applied in a 5-mL final volume on top of the solidified
base layer. Three different viable cell numbers, 105, 104, and 5 × 103 cells per
well, were seeded in duplicate; 2 mL of 0.8% agar, 10% fetal bovine serum in
Iscove’s medium were applied on top of the solidified cell layer and plates
were incubated at 37°C. Colonies appeared after ~10 to 14 days and were
counted between 2 and 4 weeks. The assay was maintained by weekly
feeding with growth medium. For knockdown assays the cells were transfected with
siRNA as outlined and then plated in soft agar at 105 cells per well.

Tumor Xenografts. Retrovirus-infected, G418-selected pools of SKOV3 or
RK3E cells expressing either AP or testisin were injected s.c. into SCID/Beige
mice (Charles River Laboratories, Wilmington, MA). Nine or 10 mice were
used per group as indicated. For SKOV3 cells, 105 cells in 100 μL PBS were
implanted with matrigel and for RK3E cells, 5 × 105 cells in 100 μL PBS
were implanted without matrigel. Tumor formation was monitored by
palpation and caliper measurement and tumor volume was calculated using
the formula (length × width2)/2. All animal experiments were done in
complete compliance with institutional guidelines.

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 testisin 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-testisin tumors to SKOV3-control tumors. Analysis of
data from the last day of measurement revealed that the SKOV3-testisin
tumors had significantly larger volumes than SKOV3-control tumors at a
99.0% confidence level.
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).
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 non-specific 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

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**Figure 3.** Knockdown of testisin expression in HeLa cells and measurement of colony formation in soft agar. A, photographs of HeLa cells plated in soft agar after treatment with scrambled, testisin-specific, or DAXX-specific siRNAs. Colonies within multiple representative microscopic fields for each test cell type were counted. B, QPCR evaluation of testisin and DAXX mRNA levels 72 hours after siRNA treatment. C, measurement of caspase 3 and/or caspase 7 activity in siRNA-treated cells.
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

Figure 4. Knockdown of testisin mRNA and protein expression in CaOv3 cells and measurement of apoptosis. A, QPCR evaluation of testisin mRNA levels in CaOv3 cells treated with either scrambled or testisin-specific siRNA. B, immunoblot of testisin and GAPDH protein levels in scrambled or testisin siRNA-treated cells. C, apoptosis induction in siRNA treated cells from A/B measured with Annexin V assay and Guava detection system. D, measurement of caspase 3 and/or caspase 7 activity in CaOv3 cells treated with scrambled, DAXX-, emerin-, or testisin-specific siRNAs. E, QPCR evaluation of DAXX, testisin, and emerin mRNA levels from the experiment in D.
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](https://www.aacrjournals.org/cancerres/2005/65/3/875/figure5.png)

**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/prostatin, 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 including urokinase plasminogen activator, tissue-type plasminogen activator, coagulation factors (X, XI, and XII) and matritapese, 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 cleavage of the growth factor proMMP-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.

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