Stanniocalcin 1 and Ovarian Tumorigenesis

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Ovarian cancer is the most lethal gynecological malignancy in women. Development of ovarian cancer involves multiple genetic and epigenetic changes that lead to the transformation of human ovarian epithelial cells to ovarian cancer cells. To facilitate the understanding of the genetic events that are involved in ovarian cancer development, we transformed human ovarian epithelial cells with the RAS oncogene, simian virus 40 (SV40) large and small tumor antigens (T and t, respectively), and the catalytic subunit of telomerase. RAS protein activates the transcription of many downstream target genes, especially those for chemokines, cytokines, and angiogenic factors (1). One gene that is activated is STC1, the gene for stanniocalcin 1 (STC1), a glycoprotein hormone. High expression of STC1 has been associated with several cancers including ovarian cancer, but its role in the development of ovarian cancer is not clear.

We used five human ovarian epithelial cancer cell lines (OVCA420, OVCA432, OVCA433, SKOV3, and HEY), immortalized human ovarian surface epithelial cells (T29 and T80), ovarian cancer tissues from 342 patients, serum from 73 ovarian cancer patients and from 58 control subjects, and 116 mice, with six or eight per group. Protein expression was assessed. Cells overexpressing STC1 protein were generated by ectopic expression of human STC1 cDNA. STC1 expression was silenced by using small interfering RNA against STC1. Cell proliferation, migration, colony formation, and apoptosis were assessed. Xenograft tumor growth in mice was studied. Neutralizing anti-STC1 antibody was used to inhibit STC1 function. All statistical tests were two-sided.

STC1 protein expression was higher in all human ovarian cancer cell lines examined than in immortalized human ovarian epithelial cell lines, higher in ovarian cancer tissue than in normal ovarian tissue (P < .001), and higher in serum from ovarian cancer patients than from control subjects (P = .021). Ovarian cancer cells with STC1 overexpression, compared with corresponding control cells, had increased cell proliferation, migration, and colony formation in cell culture and increased growth of xenograft tumors in mice. These activities in normal or malignant ovarian cells with STC1 overexpression, compared with control cells, were also accompanied by increased expression of cell cycle regulatory proteins and antiapoptotic proteins but decreased cleavage of several caspases. Within 24 hours of treatment, apoptosis in cultures of HEY ovarian cancer cells treated with neutralizing anti-STC1 monoclonal antibody was higher (17.3% apoptotic cells) than that in cultures treated with mouse IgG control cells (4.4%) (12.9% difference, 95% confidence interval = 11.6% to 14.2%).

STC1 protein may be involved in ovarian tumorigenesis.

reproduction in human STC1 transgenic mice through mineral homeostasis (15).

In mammalian tissues such as bone, skeletal muscle, brain, blood vessel, thymus, and spleen, STC1 appears to have multiple functions, including wound healing (16), mitochondria metabolism (17), angiogenesis (18), macrophage chemotaxis (19), steroidogenesis (11, 12), and muscle and bone development (20). STC1 can inhibit apoptosis (21). It is considered a molecular guard of neurons during cerebral ischemia (22) and can activate multipotent stromal cells (23). STC1 also appears to be involved in the development of other human cancers, including breast and colon cancers (24), and to be part of transcriptomes, which are responsive to hypoxia in human tumors (25). However, a direct relationship between STC1 and ovarian cancer has not been established. In this study, we investigated whether STC1 expression is associated with the development of ovarian cancer by examining STC1 protein expression in benign and malignant ovarian cancer tissue and in serum from patients with ovarian cancer and control subjects and its effect on growth of xenograft tumors in mice. We also examined whether changes in the level of STC1 expression affected the level of components that regulate the cell cycle and apoptosis by use of immortalized ovarian epithelial cells that overexpress STC1 protein or ovarian cancer cells whose STC1 protein expression was silenced.

**Patients, Materials, and Methods**

**Cell Lines and Cell Culture**

Human epithelial ovarian cancer cell lines OVCA420, OVCA432, OVCA433, SKOV3, and HEY and retroviral packaging cells (Phoenix amphotropic cells) were purchased from American Type Culture Collection (Manassas, VA). OVCA420, OVCA432, and OVCA433 cells were maintained in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, 2 mM l-glutamine, nonessential amino acids (1%), 1 mM sodium pyruvate, penicillin (100 units/mL), and streptomycin (100 µg/mL). SKOV3 and HEY cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM l-glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL). Immortalized human ovarian epithelial cells T29 and T80 were derived from the normal ovarian surface epithelial cell lines IOSE-29 and IOSE-80, which have been described elsewhere (26), and immortalized by consecutive transfections with cDNAs for SV40 T and t antigens and for the catalytic subunit of human telomerase, as previously described (1). T29H and T80H cells were derived from T29 and T80 cells that were transformed with HRAS cDNA (27). T29K and T80K cells were derived from T29 and T80 cells that were transformed with KRAS cDNA, which is similar to HRAS cDNA (27). All of these cell lines were used in our previous study (1).

**Immunohistochemical Staining and Analysis**

Tissue microarrays included core samples from seven normal human ovarian tissue specimens, 15 benign human ovarian cystadenomas, 16 borderline human ovarian tumors, and 311 human ovarian carcinomas. Use of these human specimens was approved by the Institutional Review Board. STC1 protein expression was detected by immunohistochemical staining, and an adjacent section was stained with hematoxylin–eosin and analyzed by two gynecologic pathologists (J. Liu and B. Chang). Antibodies used for immunohistochemical staining included anti-STC1 protein polyclonal antibody (product sc-14346; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution), anti-SV40 polyclonal antibody (Dako, Glostrup, Denmark; 1:200 dilution), anti-p53 monoclonal antibody (Dako; 1:100 dilution), and anti-CA 125 monoclonal antibody (Dako; 1:20 dilution). Antibody binding was detected by using avidin–biotin–peroxidase methods, as described elsewhere (1). Briefly, tissue slides were deparaffinized in xylene and rehydrated in a graded series of ethanol, and sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven for 10 minutes. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and blocking nonspecific protein binding with 1.5% normal goat serum, the sections were incubated overnight with an antibody at 4°C in a humid chamber. Antibodies were localized by incubating sections with biotinylated goat anti-rabbit IgG (28) for 30 minutes, detecting the antibody with the labeled streptavidin–biotin system (Dako), and visualizing them with the chromogen 3,3’-diaminobenzidine. Sections were lightly counterstained with hematoxylin. The primary antibody was replaced with 1x phosphate-buffered saline (PBS) as a

**CONTEXT AND CAVEATS**

**Prior knowledge**

Stanniocalcin 1 (STC1) is a secreted glycoprotein hormone that is involved in calcium and phosphate homeostasis. High STC1 expression has been associated with several cancers including ovarian cancer.

**Study design**

Human ovarian epithelial cancer cell lines, immortalized human ovarian surface epithelial cells, ovarian cancer tissues from patients, serum from ovarian cancer patients and from control subjects, and mice bearing xenograft tumors were studied. STC1 protein expression was increased by use of STC1 cDNA and was silenced by use of small interfering RNA against STC1. Cell proliferation, migration, colony formation, and apoptosis were assessed. Neutralizing anti-STC1 antibody was used to inhibit STC1 function.

**Contribution**

STC1 protein was higher in all human ovarian cancer cell lines examined than in immortalized human ovarian epithelial cell lines, higher in ovarian cancer tissue than in normal ovarian tissue, and higher in serum from ovarian cancer patients than from control subjects. STC1 overexpression increased proliferation and migration in cell culture, xenograft tumor growth, and expression of cell cycle regulatory proteins and antiapoptotic proteins but decreased cleavage of several caspases. Treatment with neutralizing anti-STC1 monoclonal antibody increased apoptosis.

**Implications**

STC1 protein may be involved in ovarian tumorigenesis.

**Limitations**

The mechanism of action for STC1 is not clear. Validation in animal models is required for STC1 antibody–induced apoptosis. Tumor growth was studied in immunodeficient mice.

*From the Editors*
negative control. Immunostaining for STC1 protein was independently and blindly analyzed by two gynecologic pathologists (J. Liu and B. Chang). Tissues in which more than 10% of the cytoplasm was stained for STC1 protein were considered positive, and those with less than 10% staining were considered negative. Two 1-mm cores were examined in each specimen on the tissue microarray and cells were counted in at least five high-power fields, with approximately 200 cells analyzed per high-power field.

Measurement of STC1 Protein Expression in Human Serum and in Conditioned Culture Medium by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples from 58 normal women and 73 patients with ovarian cancer and condition medium collected from T29, T29H, T29H, SKOV3, HEY, OVCA420, OVCA432, and OVCA433 cells after a 48-hour culture were analyzed for STC1 protein expression by use of an ELISA kit (Duoset ELISA development kit; R&D Systems, Inc, Minneapolis, MN) according to the manufacturer’s instructions. In brief, high-binding, flat-bottom 96-well polypropylene plates (NUNC, Naperville, IL) were coated overnight at ambient temperature with 100 µL of goat anti-human STC1 antibody (800 ng/mL). The plate was washed three times with PBS containing 0.05% Tween-20 and blocked with PBS containing 0.5% bovine serum albumin for 2 hours. Either 100 µL of a sample or 100 µL of a diluted STC1 standard (31.25–2000 pg/mL; seven dilutions) was added per well (each sample was tested in three wells). After 2 hours of incubation at room temperature and three washes with PBS containing 0.05% Tween-20, the plate was treated with a second biotinylated goat anti-human STC1 detection antibody (400 ng/mL) for 2 hours and then a solution of streptavidin conjugated to horseradish peroxidase (1:200 dilution) was added to the plates. Tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD; 10 mg/mL) and 1 M phosphoric acid were added in a volume of 50 µL, and the absorbance at 450 nm was determined for each well by use of a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA).

Cloning of STC1 cDNA and Viral Infection

Total RNA was isolated from 2 × 10⁶ T29H cells, which were found to express a high level of STC1 mRNA in our previous study (1), by using Trizol reagent (Invitrogen, Carlsbad, CA). STC1 cDNA was produced by reverse transcription–polymerase chain reaction. First, reverse transcription was performed with random hexamer primers by using the Ominoscript RT kit (Qiagen, Inc, Valencia, CA). The primers used to amplify STC1 cDNA were 5'-ATGGATCCATGCTTTCAACACTGACGATGCTTCC-3' (sense, boldface type indicates the BamHI site) and 5'-GGTGCAGGAAGAGTGCTACAGCAAGTACGCTTTTTG-3' (antisense, boldface type indicates the EcoRI site). Conditions for the polymerase chain reaction were 94°C for 2 minutes, 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 2 minutes for a total of 35 cycles, followed by 72°C for a 10-minute extension. The STC1 reverse transcription–polymerase chain reaction product was purified in a 1% agarose gel, digested with BamHI and EcoRI, and ligated to a pBabe/puromycin retroviral vector that had been digested with the same enzymes. After transformation of the plasmid in E. coli (DH5α), positive clones were selected by enzyme digestion (BamHI and EcoRI) and DNA sequencing analysis was performed at the DNA core facility at M. D. Anderson Cancer Center. The control vector used in this study was an empty pBabe/puromycin retroviral vector.

Retroviruses carrying STC1 cDNA were harvested and harvested as described previously (1). Briefly, T29, T80, and OVCA420 cells were infected twice for a total of 6 days (3 days for each infection) and the positive clones were selected with puromycin (200 ng/mL) for 10–14 days. Control cell lines were generated by infection with viruses containing the empty vector by following the same protocol. The cells were used for various analyses, including immunoblot analysis, cell cycle analysis, and apoptosis assay.

Generation and Retroviral Delivery of Small Interfering RNA (siRNA) Against STC1 mRNA

The DNA oligonucleotides used to generate siRNA against the open reading frame of STC1 mRNA (positions 369–392) were as follows: Ps1, 3'-GGGTGCAAGAAGATGCTACAGCAAGTACGCTTTTTG-3'; and Ps2, 5'-GGTGCAGGAAGAGTGCTACAGCAAGTACGCTTTTTG-3'. The DNA oligonucleotides used to generate scrambled siRNA were as follows: Pc1, 5'GGCCGCGTTTGTAGGATTGCATACGTAAACGAATCCTACAAAGCGCGCTTTTTG-3'; and Pc2, 5'-GGCCGCGTTTGTAGGATTGCATACGTAAACGAATCCTACAAAGCGCGCTTTTTG-3'. Oligonucleotides were annealed in a buffer containing 100 mM Tris–HCl (pH 7.5) and 20 mM MgCl₂ for 10 minutes at 95°C, followed by 20 minutes at 65°C. The annealed DNAs were ligated into pBabe-U6/puromycin vectors that had been cut with ApaI, blunted with the Klenow enzyme, and digested with EcoRI to generate plasmids pBabe/U6/STC1i or pBabe/U6/scrambled siRNA. Retroviruses expressing STC1 siRNA or scrambled siRNA were produced by transfection of pBabe/U6/STC1i or pBabe/U6/scrambled siRNA into Phoenix amphotropic cells and used to infect target cells (T29H, HEY, SKOV3, and OVCA432 cells) by using a method similar to that described above.

Assays for Cell Proliferation, Migration, and Anchorage-Independent Colony Formation

For analysis of cell proliferation, 5000 T29-SCT1, T80-STC1, OVCA420-STC1, T29H-STC1i, HEY-STC1i, or SKOV3-STC1i or corresponding vector or scrambled siRNA control cells in 100 µL of medium were added per well to wells of a 96-well plate or corresponding vector or scrambled siRNA control cells in 100 µL of medium were added per well to wells of a 96-well plate (three wells per each cell line), including three control wells with medium alone. Cells were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for 24 hours, and then 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; American Type Culture Collection) was added after 48 hours of incubation to measure cell proliferation by following the manufacturer’s instructions. The assay was repeated three times with triplicate samples.

For the cell migration assay, we used a high throughput screening multi-well insert 24-well two-chamber plate (BD Biosciences, San Jose, CA), with an 8-µm (pore size) polycarbonate filter between the chambers. We added 25 000 T29-SCT1, T80-STC1, OVCA420-STC1, T29H-STC1i, HEY-STC1i, or SKOV3-STC1i cells or their
corresponding vector (T29-vector, T80-vector, or OVCA420-vector, respectively) or scrambled siRNA control cells (T29H-scr, HEY-scr, or SKOV3-scr, respectively), in each upper chamber and allowed them to migrate at 37°C for 4 hours toward a lower reservoir containing medium plus fibronectin (20 µg/mL). The cells were then fixed in 100% methanol for 30 minutes and stained with Giemsa solution for 10 minutes. The migrated cells were counted as those that passed through the membrane separating the chamber. All cells were counted at ×200 magnification under a microscope. The assay was repeated three times with duplicate samples.

For anchorage-independent colony formation, we used T29-vector, T29-SC1, T80-vector, T80-SC1, OVCA420-vector, OVCA420-SC1, T29H-scr, T29H-SC1i, HEY-scr, HEY-SC1i, SKOV3-scr, and SKOV3-SC1i cells. The soft agar assay was described previously (29). Briefly, 5 × 10⁴ cells were suspended in 2 mL of medium with 0.35% agarose (Life Technologies, Rockville, MD), and the suspension was placed on top of 5 mL of solidified 0.7% agarose. Triplicate cultures of each cell type were maintained for 14 days at 37°C in an atmosphere of 5% CO2, and 95% air, with fresh medium being added at 7 days. The number of colonies that were larger than 50 µm (approximately 100 cells) in diameter in each dish was counted at 14–20 days. The assay was repeated three times with duplicate samples.

**Immunoblot Analysis of STC1, Cell Cycle Regulatory, and Apoptosis-Associated Proteins**

The expression level of secreted STC1 protein was examined by immunoblot analysis. Cells that overexpressed STC1 protein (T29-SC1, T80-SC1, and OVCA420-SC1 cells), cells whose STC1 expression was silenced with STC1 siRNA (T29H-scr, HEY-scr, SKOV3-scr, or OVCA420-scr) or scrambled siRNA, respectively, were cultured in six-well plates in serum-free medium for 2 days, and 2 mL of supernatant was collected and centrifuged at 2000g for 5 minutes to pellet the cell debris. The supernatant was filtered through a 0.22-µm (pore size) membrane to remove additional cell debris and used for analysis. To analyze STC1 expression in cells, cell lysates were prepared at 75% of confluence by using 500 µL of radioimmunoprecipitation assay buffer (25 mM Tris·HCl at pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) in 10-cm culture dish, with a 20-minute incubation on ice. Protein concentrations of the lysates were measured with a Bio-Rad protein assay kit (Hercules, CA). Immunoblot analyses were performed as previously described (29). Samples (50 µg each) were prepared with 6× sodium dodecyl sulfate loading buffer (125 mM Tris·HCl at pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol, and 0.2% bromophenol blue) and boiled in a water bath at 100°C for 5 minutes. Proteins in lysates or medium were separated in 10%–12% Tris–glycine gels under denaturing conditions. Proteins were transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and the blot was blocked in 10% nonfat milk (Bio-Rad) overnight at 4°C. Antibodies against the following proteins were obtained from Santa Cruz Biotechnology: STC1 (goat anti-STC1 polyclonal antibody, product sc-14346; 1:1000 dilution), BAX polyclonal antibody (product sc-6236; 1:2000 dilution), BCL-2 monoclonal antibody (product sc-7382; 1:500 dilution), cyclin-dependent kinase (CDK) 2 polyclonal antibody (product sc-70829; 1:1000 dilution), CDK4 polyclonal antibody (product sc-260; 1:1000 dilution), cyclin A monoclonal antibody (product sc-239; 1:1000 dilution), cyclin B1 (V152) polyclonal antibody (product sc-53236; 1:1000 dilution), cyclin D1 polyclonal antibody (product sc-246; 1:500 dilution), and cyclin E monoclonal antibody (product sc-247; 1:500 dilution). Monoclonal antibodies against the following proteins were from Cell Signaling Technology (Danvers, MA): β-actin (product mAb 3700; 1:2000 dilution), BAD (product mAb 9239; 1:1000 dilution), pBAD (product mAb 5284, Ser-112; 1:1000 dilution), caspase-3 (mAb 9665; 1:1000 dilution), caspase-7 (mAb 9494; 1:1000 dilution), and caspase-10 (product mAb 9752; 1:1000 dilution). Antibodies against the following proteins were obtained from Biologend (San Diego, CA): caspase-1 polyclonal antibody (product 645101; 1:1000 dilution), caspase-8 polyclonal antibody (product 622001; 1:1000 dilution), and caspase-9 polyclonal antibody (product 621901; 1:2000 dilution). The polyclonal antibody against BCL-XL (product AM05; 1:1000 dilutions) was from Calbiochem (San Diego, CA). The secondary antibodies were F(ab), fragment of donkey anti-mouse immunoglobulin (product NA931) or of donkey anti-rabbit immunoglobulin (product NA9340) linked to horseradish peroxidase from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Immunoblot reagents were from an electrochemiluminescence kit (Amersham Biosciences). The intensity of protein bands was quantified from the originally exposed x-ray films with FluorChem Q imaging analysis software from Alpha Innotech Corporation (San Leandro, CA).

**Xenograft Tumors in Nude Mice**

All mouse experiments followed institutional guidelines and were approved by the Institutional Animal Care and Use Committee. Subcutaneous injection of tumor cells allows visual monitoring of tumor growth, and intraperitoneal injection of tumor cells allows tumor cells to grow in a peritoneal microenvironment that mimics the environment of human ovarian cancer. Mice were kept in a pathogen-free environment. For the subcutaneous group, we investigated the ability of T29-SC1, T80-SC1, OVCA420-SC1, T29H-SC1i, HEY-SC1i, and SKOV3-SC1i cells or corresponding control cells injected subcutaneously to generate xenograft tumors. Briefly, we harvested 2 × 10⁶ cells by incubation in trypsin–EDTA, washed the cells twice with PBS, resuspended the cells in 0.15 mL of PBS, and injected all cells subcutaneously into 4- to 6-week-old BALB/c athymic nude mice (National Cancer Institute-Frederick, National Institutes of Health). For the subcutaneous injection group, six mice (for OVCA420-SC1, T29H-SC1i, and HEY-SC1i and their control cells OVCA420-vector, T29H-scr, and HEY-scr) or eight mice (for T29-SC1, T80-SC1, and SKOV3-SC1i and their control cells T29-vector, T80-vector, and SKOV3-scr) were used per cell line and each mouse received two injections, each of 2 × 10⁶ cells, in bilateral flank to form two tumors per mouse. The tumor growth of modified and control cell lines was monitored until the day that mice were killed. The date on which the first grossly visible tumor appeared for subcutaneous injection was recorded, and the tumor size was measured every 3 days. Two-dimensional measurements were taken with an electronic caliper after injection, and tumor volume was calculated
with the use of the following formula: tumor volume (in mm$^3$) = $a \times b \times 0.52$, where $a$ is the longest diameter, $b$ is the shortest diameter, and 0.52 is a constant to calculate the volume of an ellipsoid. When a tumor reached 1.5 cm in diameter, the mouse was killed by exposure to 5% carbon monoxide. The two tumors were excised, fixed in 10% formalin overnight, and subjected to routine histological examination by investigators who were blinded to the tumor status. For the intraperitoneal injection group, eight mice were used for each group and each mouse received one injection of 2×10$^6$ T29-STC1 or HEY-STC1i cells or their corresponding control cells. Mice were observed for lethargy, poor appetite, and abdominal enlargement. Mice were killed at the end of 42 days for HEY-STC1i or HEY vector control and 6 months for T29-STC1 or T29-vector control cells. All tumor nodules were counted and dissected; each tumor nodule was also weighed and its volume was determined as described above for subcutaneous tumors. Thus, a total of 116 mice were used in these experiments.

**Immunofluorescence Staining for CA 125 Protein**

T29-STC1 and T80-STC1 cells and their corresponding control cells were used for analysis of epithelial ovarian cancer marker CA 125 by immunofluorescence staining as described previously (30). Briefly, cells were cultured in chambered slides for 24 hours (50% confluence) and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 4 minutes at room temperature and then incubated with 0.2% Triton X-100 for 5 minutes at room temperature. Primary antibodies against CA 125 (Dako; monoclonal antibody, 1:100 dilution) and actin (Santa Cruz Biotechnology; polyclonal antibody, 1:500 dilution) were applied to the slides and incubated at room temperature for 1 hour. After washing, secondary antibodies (donkey anti-mouse IgG conjugated with fluorescein isothiocyanate [product 715-095-150; 1:1000 dilution] or goat IgG [product 705-075-003; 1:1000 dilution] conjugated with Texas red [Jackson ImmunoResearch Laboratory, West Grove, CA]) were used. We used 4′,6-diamidino-2-phenylindole as the nuclear dye. Cells were visualized and photographed under fluorescence microscopy.

**Cell Cycle Analysis**

For cell cycle analysis, 1–2×10$^6$ T29-STC1, T80-STC1, OVCA420-STC1, T29H-STC1i, HEY-STC1i, or SKOV3-STC1i cells and their corresponding control cells were harvested, washed twice with 1x PBS, and resuspended in 200 µL of 1x PBS. The cells were fixed in 4 mL of ice-cold 75% ethanol at 4°C for a minimum of 4 hours and then washed twice with 1x PBS. The cells were then resuspended in 500 µL of 1x PBS and stained with 200 µL of propidium iodide (50 µg/mL; Sigma-Aldrich, St Louis, MO) and 20 µL of RNase (1 mg/mL; Sigma-Aldrich) to remove RNA in a 37°C water bath for 15–20 minutes. The percentage of cells in each phase of the cell cycle was determined by flow cytometry (FACStation; BD Biosciences), and data were analyzed by using CellQuest software as described previously (31). The assay was repeated three times.

**Terminal Deoxynucleotidyltransferase–Mediated Deoxyuridine Triphosphate–Biotin Nick End Labeling (TUNEL) Assay for Apoptosis**

Samples of tumors from nude mice that had been established by injecting T29H-scr, T29H-STC1i, HEY-scr, or HEY-STC1i cells were fixed in formalin and embedded in paraffin by use of an Apo-BrdU In Situ DNA Fragmentation Assay Kit (Medical & Biological Laboratories Co, Ltd, Woburn, MA) according to the manufacturer’s instructions. Tumor tissues from two mice for each group were analyzed. Frozen tissue sections were fixed in fresh 4% formaldehyde in PBS, 50 µL of DNA Labeling Solution was applied in a dark, humidified 37°C incubator for 60 minutes. The slides were viewed and photographed immediately afterward. Apoptotic cells have strong green nuclear fluorescence. Cells stained with propidium iodide have strong red counterstaining. The total number of apoptotic cells with strong green nuclear fluorescence staining was counted under a fluorescence microscope. For quantification, the total number of cells and the number of apoptotic cells in each field were counted, and the percentage of apoptotic cells was calculated. At least 1000 cells per slide were counted in 10 randomly selected fields. The assays were repeated twice.

**Apoptosis and Anti-STC1 Antibody Treatment**

We used cells that stably expressed STC1 or cells in which STC1 expression was silenced, and their corresponding control cells to investigate the relationship between STC1 expression and apoptosis. Specifically, we used T29-STC1 (control = T29-vector), T80-STC1 (control = T80-vector), and OVCA420-STC1 (control = OVCA420-vector) cells, which stably expressed STC1; and we used T29H-STC1i (control = T29H-scr), HEY-STC1i (control = HEY-scr), and SKOV3-STC1i cells (control = SKOV3-scr), in which STC1 expression was silenced. For all cells, we stained 1×10$^6$ cells with annexin V and propidium iodide at room temperature for 30 minutes, according to the annexin V fluorescence apoptosis detection kit I (BD Biosciences PharMingen), and then subjected them to flow cytometry analysis to determine the proportion of apoptotic cells. Each assay was repeated three times. For treatment of cells with anti-STC1 antibody, HEY and T29H cells that overexpressed STC1 at 75% confluence in serum-free medium were first treated with anti-STC1 monoclonal antibody (R&D Systems, Inc; 2 µg/mL for HEY cells and 0.5 µg/mL for T29H cells). Mouse IgG at 2 µg/mL (R&D Systems Inc) was used as the negative control. After 5 hours of incubation in a cell culture incubator at 37°C, various concentrations of STC1 protein (100–1000 ng/mL for HEY cells and 20–500 ng/mL for T29H cells) were added and cells were harvested 2, 12, or 24 hours later and stained with annexin V and propidium iodide according to the annexin V fluorescence apoptosis detection kit I (BD Biosciences PharMingen). Apoptosis was assessed by flow cytometry as described above. The percentage of apoptotic cells was calculated on the basis of the amount of the M2 peaks in the histogram data, which represents an early apoptotic population (ie, annexin V-positive and propidium iodide-negative cells) (32). All samples were assessed in duplicate, and the experiment was repeated twice.

**Treatment of Cells With Carboplatin**

We used the following cells that stably expressed STC1 or whose STC1 expression was silenced and their corresponding controls for this experiment: T29-STC1 (control = T29-vector), T80-STC1 (control = T80-vector), T29H-STC1i (control = T29H-scr), and
HEY-STC1i (control = HEY-scr). We added 5000 cells to each well in a 96-well plate and cultured them for 24 hours. Cells were then treated with carboplatin at 0.5–500 µM to determine half-maximal inhibitory concentration for each cell line, by incubating cells with carboplatin for 12 hours and then replacing the medium with fresh medium without drug for an additional 48 hours. Cell viability was measured by an MTT assay at 570 nm as described above. The survival of each cell line was compared with that of its corresponding control cell line.

Statistical Analysis
For mice in the subcutaneous group, the number of mice (ie, sample size) required to reach statistical significance was determined with preliminary pilot studies that used the following formula (33): \( n = 16 \times (SD/difference \text{ in mean tumor volume})^2 + 1 \). Results of that pilot study indicated that six mice would be required to detect tumor size differences with 80% power at a P value of less than .05. Each mouse had two bilateral flank injections, from which the mean volume of tumor in each mouse generated from \( 2 \times 10^6 \) cells was computed for growth curve (the mean tumor volume in each group = total mean volume from each mouse divided by number of mice). Statistical analysis was performed by \( t \) test at different time points between the mean tumor sizes of each group.

The statistical significance of the differences in the percentage of cells with STC1-positive cytoplasm among normal, benign, and malignant ovarian tissues was calculated by using the \( \chi^2 \) test. The statistical significance of differences in serum levels of STC1 protein between women with ovarian cancer and healthy women and in cell growth, migration, colony formation, tumor volume, and apoptotic levels between cells that overexpressed STC1 protein or lacked STC1 protein expression and their parental control cells was calculated by the Student’s \( t \) test. A \( P \) value of less than .05 was considered to be statistically significant. Statistical significance associated with ovarian carcinomas of different histotypes (serous, endometrioid, mucinous, and other histotypes) and with borderline tumors, both as compared with normal ovarian epithelial cells and control cystadenoma, was computed. The difference in STC1 expression in ovarian carcinoma by age (<55 years or ≥55 years) or by stage (stages I, II, III, or IV) was also examined. Data were analyzed with Statistica version 6 software (StatSoft, Inc, Tulsa, OK). All statistical tests were two-sided.

Results

STC1 Protein Expression in RAS-Transformed Ovarian Epithelial Cell Lines and Human Ovarian Cancer Cell Lines
A cDNA expression array study has shown previously that the expression of STC1 mRNA was increased 5- to 13-fold in different RAS-transformed ovarian lines compared with vector-transfected control cells (1). So, we first used immunoblot analysis to evaluate STC1 protein expression in RAS-transformed ovarian surface epithelial cell lines (T80H, T80K, T29H, and T29K cells) and their corresponding parental cells (T80 and T29 cells) and in a panel of ovarian cancer cell lines (SKOV3, HEY, OVCA420, OVCA432, and OVCA433). Higher STC1 protein expression was detected in all four RAS-transformed cell lines than in their corresponding parental cells (Figure 1, A). Higher STC1 expression was also detected in HEY ovarian cancer cells, which contain two KRAS mutations at codons 12 (TGT to GAT) and 13 (GGC to AGC), which resulted in alteration of amino acids 12 (Cys to Val) and 13 (Gly to Ser), respectively (Liu J, unpublished data, The University of Texas MD Anderson Cancer Center). Higher STC1 expression was also detected in SKOV3, OVCA432, and OVCA433 cells, which have no known RAS mutation, but not in OVCA420 cells, probably because of heterogeneous genetic background (Figure 1, A) compared with immortalized ovarian surface epithelial T29 cells. Thus, STC1 may be activated by both RAS-dependent and -independent mechanisms.

Because STC1 is a secreted glycoprotein hormone, we investigated whether it could be identified in cell culture medium and serum from patients with ovarian cancer by immunoblot analysis by ELISA. We found moderate to high levels of STC1 protein in conditioned culture medium from T29H, T29K, HEY, SKOV3, OVCA432, and OVCA433 cells but not from T29 or OVCA420 cells (Figure 1, B and C). In addition, the level of STC1 protein was statistically significantly higher in serum from patients with ovarian cancer (\( n = 73 \), mean = 1516 pg/mL) than in serum from control women (\( n = 58 \), mean = 865 pg/mL) (difference = 651 pg/mL, 95% confidence interval [CI] of the difference = 419.1 to 882.9 pg/mL; \( P = .021 \)) (Figure 1, D).

To further investigate the association between STC1 protein expression and ovarian cancer, we used a tissue microarray containing specimens from seven normal ovarian cystadenomas, 342 ovarian cancers (15 benign ovarian cystadenomas, 16 borderline ovarian tumors, and 311 ovarian carcinomas) for immunohistochemical detection of STC1 protein. STC1 protein was localized predominantly to the cytoplasm of epithelial cells as indicated by arrows (Figure 1, E, high-grade serous carcinoma, high power). Low levels of STC1 protein were detected in two (28.6%) of the seven normal ovarian surface epithelial tissue specimens and in two (13.3%) of the 15 benign ovarian cystadenoma tissue specimens. High levels of STC1 expression were detected in 13 (81.3%) of the 16 borderline tumor tissue specimens and in 280 (90.0%) of the 311 ovarian carcinoma tissue specimens (Table 1 and Figure 1, E).

We found that STC1 expression was statistically significantly associated with ovarian carcinomas of different histotypes (serous, endometrioid, mucinous, and other histotypes; \( P < .001 \)) and with borderline tumors (\( P < .001 \)), both as compared with normal ovarian epithelial cells and cystadenoma control. Expression of STC1 in ovarian carcinoma did not reach statistical significance in analysis by age (<55 years or ≥55 years; \( P = .311 \)) and stage (stages I, II, III, or IV; \( P = .056 \)). Thus, increased expression of STC1 appears to be associated with human ovarian cancer but not normal ovarian epithelial cells or benign cystadenomas.

Overexpression of STC1 Protein in Ovarian Epithelial Cell Lines and Cell Proliferation, Migration, and Anchorage-Independent Colony Formation
We investigated the effects of altering the expression of STC1 protein on ovarian cancer cells. To increase the expression of STC1 protein, we used retrovirus infection to introduce an STC1 cDNA expression vector into the immortalized ovarian epithelial cell lines T29 and T80 and into the ovarian cancer cell line OVCA420. The cell lines that overexpressed STC1 protein were T29-STC1,
T80-SC1, and OVCA420-SC1 cell lines. To silence SC1 expression, we introduced retroviruses carrying siRNA against SC1 mRNA into T29H, HEY, SKOV3, and OVCA432 cell lines, which have high expression of SC1 protein (Figure 1, A–C). We found that SC1 protein expression was increased after the insertion of SC1 cDNA and decreased after the insertion of SC1 siRNA (Figure 2, A). To investigate whether silencing the expression of wild-type HRAS reduces SC1 protein expression, we examined the level of SC1 protein in SKOV3-HRASi cells whose HRAS had been silenced with HRAS siRNA, as described in our previous study (29) and found that these cells had a lower level of SC1 protein than control cells (Figure 2, A). In addition, we detected secreted SC1 protein in the conditioned culture medium of T29 cells that overexpress SC1 protein but not in T29 parental cells (Figure 1, B).

We next evaluated cell proliferation, migration, and anchorage-independent colony formation of paired cell lines whose expression of SC1 protein was increased or decreased, compared with corresponding control cells containing empty vector or control siRNA. Cells that overexpressed SC1 protein (T29-SC1, T80-SC1, and OVCA420-SC1 cells) had higher levels of cell proliferation, migration, and anchorage-independent colony formation than the corresponding vector control cells; however, cells whose SC1 expression was silenced with siRNA (T29H-SC1i, HEY-SC1i, SKOV3-SC1i) had lower levels of cell proliferation, migration, and anchorage-independent colony formation than the corresponding vector control cells (Figure 2, B–D). For these quantitative analyses, we used T29-SC1, which overexpressed SC1; T29H-SC1i, whose SC1 expression was silenced; and their control cells, T29-vector and T29H-scr cells, for examples.

Cell proliferation, as shown by optical density at 570 nm, was higher in T29-SC1 cells (0.22) than in T29-vector control cells (0.17) (difference = 0.05, 95% CI = 0.04 to 0.06) but was lower in T29H-SC1i cells (0.19) than in T29H-scr control cells (0.29) (difference = 0.1, 95% CI of the difference = 0.09 to 0.11). In the cell migration assay, more T29-SC1 cells (257 cells) migrated...
through the membrane in the migration chamber than T29-vector control cells (178 cells) (difference = 79 cells, 95% CI = 61 to 97 cells); but fewer T29H-STC1i cells (228 cells) migrated than T29H-scr control cells (374) (difference = 146, 95% CI of the difference = 124 to 168). In the anchorage-independent colony formation assay, T29-STC1 cells formed more colonies (1356 colonies) than T29-vector control cells (12 colonies) (difference = 1344, 95% CI = 1307 to 1381 colonies); but T29H-STC1i cells formed fewer colonies (523 colonies) than T29H-scr control cells (941 colonies) (difference = 418 colonies, 95% CI = 339 to 497 colonies). Thus, overexpression of STC1 protein appears to increase cell proliferation, migration, and anchorage-independent growth of ovarian cancer cell lines, and silencing of STC1 expression appears to decrease these activities.

**STC1 Expression and Transformation of Ovarian Epithelial Cells and Growth of Ovarian Tumors in Nude Mice**

To test the effect of altering the STC1 expression on tumor growth, cells that carried the ectopic STC1 expression vector (T29-STC1, T80-STC1, and OVCA420-STC1) and their corresponding control cells (T29-vector, T80-vector, and OVCA420-vector, respectively) or cells in which the expression of STC1 mRNA was silenced (T29H-STC1i, HEY-STC1i, and SKOV3-STC1i) and their corresponding control cells (T29H-scr, HEY-scr, and SKOV3-scr, respectively) were subcutaneously or intraperitoneally injected into nude mice. Subcutaneous injection generates tumors whose growth is easy to monitor, and intraperitoneal injection allows tumor cells to grow in a peritoneal microenvironment that mimics the microenvironment of human ovarian cancer growth. As shown in Figure 3, A and B, after 19 weeks of observation, no tumors were found in all mice that were injected subcutaneously with T29 or T80 control cells (eight mice per cell line) and no tumors were found in all mice that were injected intraperitoneally with T29 or T80 control cells. T29 and T80 cells express very low levels of STC1 protein. However, tumors were detected in six of the eight mice injected subcutaneously with T29-STC1 cells and in five of eight mice injected subcutaneously with T80-STC1 cells (both of these cell lines express STC1 protein). The average tumor volume per mouse (from mean of two injections) reached 33 mm³ (95% CI = 31.4 to 34.6 mm³) at 19 weeks in six of eight mice injected with T29-STC1 cells. In mice injected with T80-STC1 cells, the mean volume of tumor from five of eight mice was 118 mm³ (95% CI = 111.5 to 124.5 mm³) after 19 weeks postinjection. In addition, six of eight mice injected intraperitoneally with T29-STC1 cells developed peritoneal tumors but not in control T29 cells. Histopathological analysis found that subcutaneous T29-STC1 and T80-STC1 tumors were poorly differentiated carcinomas (data not shown) and that intraperitoneal T29-STC1 tumors were papillary carcinomas (Figure 3, C) with morphological features similar to those of human ovarian carcinoma. Immunohistochemical analysis found that the intraperitoneal T29-STC1 tumors had strong staining for STC1 protein in the cytoplasm of tumor cells. These tumors were also positive for SV40 T antigen and p53, as expected because T29-STC1 cells were derived from SV40-immortalized parental cells with stabilized p53 expression (Figure 3, C). The cytoplasm and cell membranes in T29-STC1 tumors or in T29-STC1 and T80-STC1 cells, compared with control cells, had strong staining for CA 125 protein by immunohistochemistry (Figure 3, C) and by immunofluorescence (Figure 3, D). In addition, overexpression of STC1 protein increased the growth of OVCA420 human ovarian tumors in nude mice compared with that of control tumors (Figure 3, E). The average tumor volume per mouse reached 11 mm³ at 89 days in mice injected with OVCA420-STC1 cells compared with control mice injected with vector control cells (mean tumor volume = 1 mm³) (difference = 10 mm³, 95% CI = 9.6 to 10.4 mm³, P < .001). Thus, overexpression of STC1 protein appeared to induce transformation of immortalized ovarian surface epithelial cells (T29 and T80) and to enhance tumorigenesis of human ovarian cancer cells (OVCA420), but overexpression of STC1 protein did not induce transformation of immortalized human mammary epithelial cells that had been similarly immortalized with SV40 T and/or t antigen and human telomerase catalytic subunit (data not shown) (26). Thus, the transforming activity of STC1 protein may differ across epithelial cell lines.

To further explore the association of STC1 protein expression with RAS-mediated transformation and with tumor growth, we examined the effect of decreased STC1 protein expression on tumor growth. Tumor size was measured every 3 days after the tumor appeared in all mice. The average tumor volume per mouse injected with T29H-STC1i cells was 156 mm³ at 30 days compared with tumor volume formed by T29-scr (mean volume = 904 mm³) (difference = 748 mm³, 95% CI = 721.7 to 774.3 mm³).

**Table 1.** Patient clinicopathological characteristics associated with level of stanniocalcin 1 (STC1) expression in ovarian cancers on tissue microarrays*

| Patient characteristics | STC1 protein expression, No. of patients (% positive) | P  
|-------------------------|------------------------------------------------------|---
| Age at diagnosis         |                                                      |  
| <55 y                   | 11 126 137 (92)                                       | P<.001†  
| ≥55 y                   | 20 154 174 (88.5)                                     |  
| Stage                   |                                                      |  
| I                       | 4 21 25 (84.0)                                        | P<.001†  
| II                      | 5 14 19 (73.7)                                        |  
| III                     | 19 209 228 (91.7)                                     |  
| IV                      | 3 36 39 (92.3)                                        |  
| Histotype (diagnosis)   |                                                      |  
| Ovarian carcinoma       | 31 280 311 (90.0)                                     | P<.001†  
| Serous                  | 15 160 175 (91.4)                                     |  
| Endometrioid            | 3 22 25 (88.0)                                        |  
| Mucinous                | 0 7 7 (100.0)                                         |  
| Others                  | 13 91 104 (89.4)                                      |  
| Borderline tumor        | 3 13 16 (81.3)                                        | P<.001†  
| Cystadenoma             | 13 2 15 (13.3)                                        |  
| Normal ovarian epithelium | 5 2 7 (28.6)                                         |  

* The STC1 expression difference by age or by stage was analyzed in ovarian carcinomas, and STC1 expression in ovarian carcinomas (including serous, endometrioid, mucinous, and others; n = 311) or in borderline tumor (n = 16) by histotype was analyzed in comparison with normal ovarian epithelia and cystadenomas (n = 32).

† Statistical significance (P value) was computed by two-sided χ² test.

‡ Statistical significance (P value) was computed by two-sided Fisher’s 2 × 5 table.
cells was 243 mm³ at 35 days compared with tumor volume formed by HEY-scr control cells (mean volume = 485 mm³) (difference = 242 mm³, 95% CI = 207.3 to 276.7 mm³). The average tumor volume per mouse injected with SKOV3-STC1i cells was 204 mm³ at 35 days compared with tumor size formed by SKOV3-scr (mean volume = 443 mm³) (difference = 239 mm³, 95% CI = 208.3 to 269.7 mm³). Lower expression of STC1 protein was detected in xenograft mouse tumors derived from HEY-STC1i or SKOV3-STC1i cells, whose STC1 expression had been silenced, than in tumors derived from the corresponding control cells, as expected (Figure 3, I and J). Thus, STC1 expression appears to be associated with transformation of ovarian epithelial cells (Figure 3, A and B) and sustained tumor growth of ovarian cancer cells (Figure 3, E–H).

Similar numbers of tumor nodules were found in the peritoneal cavity of mice after intraperitoneal injection with HEY-scr control cells (mean nodule number = 67) and with HEY-STC1i cells (mean nodule number = 62) (Supplementary Figure 1, A, available online) (Supplementary Figure 1, A, available online) and mean tumor nodule weight per mouse (mean weight = 0.04 g) (Supplementary Figure 1, B, available online) were lower in intraperitoneal tumors produced by cells whose STC1 expression had been silenced than in the corresponding control cells (mean size = 347 mm³, difference = 298 mm³, 95% CI = 275.8 to 320.2 mm³; and mean weight = 0.24 g, difference = 0.2 g, 95% CI = 0.1 to 0.3 g), demonstrating that STC1 expression is also required for optimal tumor growth in peritoneal cavity of nude mice, a natural microenvironment for ovarian cancer development.

**STC1 Expression and Cell Cycle Progression**

To elucidate the mechanism of tumorigenesis mediated by STC1 protein, we used flow cytometry to determine whether the level of STC1 expression affects cell cycle progression (Figure 4, A and B). When STC1 was overexpressed as in T29-STC1, T80-STC1, or OVCA420-STC1 cells, the number of cells in G1 and G0 phases was lower than that of the corresponding control cells, but the number of cells in S phase was higher (Figure 4, A). For example, there were 24.6% of T29-STC1 cells and 44.2% of T29-vector cells in G0 and G1 phases (difference = 19.6%, 95% CI = 16.1% to 23.1%), but there were 35.4% of T29-STC1 cells and only 25.4% of T29-vector cells in S phase (difference = 10%, 95% CI = 7.6% to 12.4%), as shown by quantitative analysis in Figure 4, B. Silencing STC1 expression appeared to increase the number of cells in G0 and G1 phases compared with that in control cells but appeared to decrease the number of cells in S phase. For example, there were 21.5% of T29H-STC1i cells and 38.2% of T29H-scr cells in G0 and G1 phases (difference = 16.7%, 95% CI = 9.7% to 23.7%), but there were 27.1% of T29H-STC1i cells and 35.1% of T29H-scr cells in S phase (difference = 8%, 95% CI = 5.6% to 10.4%), as indicated by quantitative analysis in Figure 4, B.

We used immunoblot analysis to investigate the associations between altered STC1 expression and the corresponding changes...
Figure 3. Xenograft ovarian tumor burden in mice and stanniocalcin 1 (STC1) expression. Cells with overexpression of STC1 (T29-STC1, T80-STC1, and OVC420-STC1) and with silenced expression of STC1 (T29H-STC1i, HEY-STC1i, and SKOV3-STC1i) and their corresponding control cells were used to establish tumors. A and B) Volume of tumors produced by subcutaneous injection for T29-STC1 cells or T29-vector control cells (A) and for T80-STC1 cells or T80-vector control cells (B). Each cell line was injected into the bilateral flanks of eight mice, which produced 16 subcutaneous tumors (two tumors per mouse). Error bars = 95% confidence intervals (CIs). C) Hematoxylin–eosin staining or immunohistochemical staining for STC1 protein, SV40 large T antigen, p53, and CA 125 in xenograft tumors produced by intraperitoneal injection of T29-STC1 or T80-STC1 cells. Antibodies against STC1, SV40 large T antigen, p53, or CA 125 were used. Antibody binding was detected by using avidin–biotin–peroxidase and visualized with the chromogen 3,3′-diaminobenzidine. Scale bars = 50 µm. D) Immunofluorescence staining for CA 125 in T29-STC1 and T29-vector control cells and in T80-STC1 and T80-vector control cells. Anti-CA 125 monoclonal antibody was used, and the secondary antibody was donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (green). β-Actin was detected with a polyclonal primary antibody and donkey anti-goat secondary antibody conjugated with Texas red to show the actin cytoskeleton. Nuclei were stained with 4,6-diamidino-2-phenylindole. Overlaid images were merged to show CA 125 (green), cytoskeletal marker actin (red), and nuclei (blue). Scale bars = 50 µm. E) Growth of tumors produced by subcutaneous injection of mice with OVC420-STC1 cells or vector control cells. A total of six mice were used for each cell line, with each mouse receiving injections at two sites to form two tumors per mouse. Error bars = 95% CI. F–H) Growth of tumors produced by subcutaneous injection of mice with cells transfected with small interfering RNA (siRNA) against STC1 (eg, T29H-STC1i) or corresponding control scrambled siRNA (eg, T29H-scr). A total of six mice for T29H-STC1i, HEY-STC1i, T29H-scr, and HEY-scr cells, or eight mice for SKOV3-STC1i and SKOV3-scr cells were used and with each mouse receiving injections as two sites to form two tumors per mouse. Error bars = 95% CI. F) T29H-STC1i cells and T29H-scr control cells. G) HEY-STC1i cells and HEY-scr control cells. H) SKOV3-STC1i cells and SKOV3-scr control cells. I) STC1 expression in xenograft tumors generated by the following cells: HEY-STC1i and control HEY-scr cells and by SKOV3-STC1i and control SKOV3-scr cells. IHC = immunohistochemistry.
Figure 4. Cell cycle distribution of ovarian cancer cells and stanniocalcin 1 (STC1) expression. Cells with overexpression of STC1 (T29-STC1, T80-STC1, and OVCA420-STC1) and their corresponding controls (T29-vector, T80-vector, and OVCA420-vector) or with silenced expression of STC1 (T29H-STC1i, HEY-STC1i, and SKOV3-STC1i) and their corresponding controls (T29H-scrambled small interfering RNA [scr], HEY-scr, and SKOV3-scr) were tested. A) Cell cycle distribution of cells that overexpress STC1 or cells in which STC1 expression was silenced and their control cells. The cell cycle distribution of cells stained with propidium iodide was determined by flow cytometry. B) Quantitative analysis of cell cycle distribution. Data from three independent experiments were used in this analysis. Error bars = 95% confidence intervals. C and D) Immunoblot analysis of cell cycle regulatory proteins (cyclin E, cyclin-dependent kinase 2 [CDK2], cyclin A, cyclin B1, cyclin D1, and CDK4). Expression of these proteins was assessed by immunoblot analysis with the corresponding antibodies. Bands were visualized by electrochemiluminescence chemiluminescence. β-Actin was used as the loading control. C) Cells with STC1 overexpression (STC1+) or their control cells (STC1−). For each protein, the ratio of expression in each pair of cells (ie, STC1 cDNA−/STC1 cDNA+) is shown under the corresponding immunoblot bands. D) Cells whose STC1 expression was silenced (STC1i−) or their corresponding control cells (STC1i+). For each protein, the ratio of expression in each pair of cells (ie, STC1i−/STC1i+) is shown under the corresponding immunoblot bands. F = full length form of cyclin E; L or * = low molecular weight of cyclin E; PI = propidium iodide.

in the expression of various proteins that are involved in cell cycle regulation, including cyclin E, CDK2, cyclin A, cyclin B1, cyclin D1, and CDK4. T29-STC1, T80-STC1, or OVCA420-STC1 cells, which overexpressed STC1 protein, had higher levels of the short isoform of cyclin E that usually increases cell proliferation than the corresponding control cells (Figure 4, C). T29H-STC1i, HEY-STC1i, SKOV3-STC1i, and OVCA432-STC1i cells, in which the expression of STC1 mRNA was silenced, had lower levels of the short isoform of cyclin E than the corresponding control cells (Figure 4, D). In addition, the level of CDK2, which binds with cyclin E and cyclin A and promotes cell cycle progression in S phase, was also higher in T29-STC1, T80-STC1, or OVCA420-STC1 cells, which overexpressed STC1, than in the corresponding control cells; whereas the level of CDK2 was
lower in T29H-STC1i, HEY-STC1i, SKOV3-STC1i, or OVCA432-STC1i cells whose STC1 expression had been silenced. Finally, the pattern for the expression of cyclin B1, which promotes cell cycle progression in M phase, was similar to that of CDK2, but the levels of cyclin D1 and its binding protein CDK4 remained constant as the expression of STC1 was altered (Figure 4, C and D). Thus, STC1 expression appears to be associated with the entry of quiescent cells into S phase of the cell cycle and with the expression of the cell cycle regulatory proteins CDK2, cyclin A, cyclin E, and cyclin B.

**STC1 Expression and Apoptosis in Ovarian Cancer Cells**

We investigated the association between STC1 expression and apoptosis in ovarian cancer cells by use of an annexin V fluorescence apoptosis assay (Figure 5, A). In T29-SCC1, T80-SCC1, or OVCA420-SCC1 cultures, which overexpressed STC1, the percentage of apoptotic cells was lower than that in the corresponding control cells (in T29-SCC1 by 9.0%, 95% CI = 8.3% to 9.7%; in T80-SCC1 by 5.7%, 95% CI = 4.5% to 6.9%; and in OVCA420-SCC1 cells by 4.1%, 95% CI = 3.6% to 4.6%). In contrast, in T29H-STC1i, HEY-STC1i, or SKOV3-STC1i cultures, in which STC1 expression is silenced, the percentage of apoptotic cells was higher than that in the corresponding control cells (in T29H-STC1i cells, 3.5% higher, 95% CI = 3.1% to 3.9%; in HEY-STC1i cells, 2.7% higher, 95% CI = 2.2% to 3.2%; and in SKOV3-STC1i cells, 4.7% higher, 95% CI = 4.5% to 4.9%). Thus, STC1 protein appears to have antiapoptotic activity in ovarian cancer cells.

We next investigated the antiapoptotic activity of STC1 in ovarian xenograft tumors that were produced in nude mice by subcutaneous injection with T29H-STC1i and HEY-STC1i cells, in which the expression of STC1 is silenced, by use of an in situ TUNEL assay (Figure 5, B and C). Nearly twice the percentage of apoptotic cells was found in tumors derived from HEY-STC1i and T29H-STC1i cells than in tumors derived from the corresponding control cells. Quantitatively, the percentage of apoptotic cells was 10.5% in HEY-STC1i tumors but was 4.8% in HEY-scr tumors (difference 5.7%, 95% CI = 4.2% to 7.2%). The percentage of apoptotic cells was 10.2% in T29H-STC1i tumors but was 4.2% in T29H-scr tumors (difference 6%, 95% CI = 4.2% to 7.8%) (Figure 5, B). Thus, STC1 also appears to have antiapoptotic activity in tumors.

To determine whether secretion of the STC1 protein is required for its antiapoptotic activity, we treated T29H and HEY cells, which express high levels of STC1 protein as shown in Figure 1, A and C, with a monoclonal antibody against STC1 that neutralizes
secreted STC1 protein in medium and measured the number of apoptotic cells in such cultures before and after antibody treatment. As shown in Figure 5, D and E, the number of apoptotic cells after treatment with anti-STC1 antibody was higher than that after treatment with control mouse IgG. An increased level of apoptosis was observed as early as 2 hours after incubation with anti-STC1 monoclonal antibody, and the maximum effect of apoptosis—a more than fourfold increase in the percentage of apoptotic cells in both T29H and HEY cultures—was observed at 24 hours. Quantitatively, the percentage of apoptotic cells was 26.3% and 6.7% in T29H cells treated, respectively, with STC1Ab and mouse IgG at 24 hours (difference = 19.6%, 95% CI = 17.5% to 21.7%). The percentage of apoptotic cells was 17.3% in HEY cells treated with a neutralizing antibody against STC1 and 4.4% in HEY cells treated with control mouse IgG at 24 hours (difference = 12.9%, 95% CI = 11.6% to 14.2%). Thus, secreted STC1 protein appears to have antiapoptotic activity in ovarian cancer cells and so antibody-based therapy against STC1 should be explored further as a potential treatment for ovarian cancer.

Finally, we investigated the association between STC1 expression and chemoresistance to carboplatin in ovarian cancer cells, because resistance to carboplatin is the most common problem in the treatment of patients with ovarian cancer. The above-established T29-STC1 and T80-STC1 cells (which overexpress STC1) and T29H-STC1i and HEY-STC1i cells (in which STC1 expression is silenced) and their corresponding control cells were treated with carboplatin at various concentrations from 0.5 to 500 µM for 12 hours. We found that STC1 overexpression was associated with increased resistance to carboplatin treatment, whereas the lack of STC1 expression was associated with increased sensitivity to carboplatin treatment (Figure 6, A and B). Specifically, the half-maximal inhibitory concentration of carboplatin was higher in T29-STC1 cells (5.5 µM) than in control cells (1.5 µM) (difference = 4 µM, 95% CI = 3.7 to 4.3 µM) and higher in T80-STC1 cells (7.5 µM) than in control cells (2.5 µM) (difference = 5 µM, 95% CI = 4.5 to 5.5 µM), but the half-maximal inhibitory concentration was lower in T29H-STC1i cells (4.5 µM) than in control cells (13.5 µM) (difference = 9 µM, 95% CI = 8.7 to 9.3 µM) and lower in HEY-STC1i cells (7.5 µM) than in control cells (32.5 µM) (difference = 25 µM, 95% CI = 22.6 to 27.4 µM) (Figure 6, C). Thus, the level of STC1 expression appears to be associated with chemoresistance to carboplatin in ovarian cancer.

To investigate which apoptotic signaling pathway is involved in STC1-associated apoptosis, we used cells that overexpress STC1 shown under the corresponding immunoblot bands. * = cleaved caspase-8; ** = cleaved caspase-9; *** = cleaved caspase-3; arrow = procaspase or cleaved caspase.
(T29-SC1, T80-SCT1, and OVCA420-STC1 cells) and cells in which STC1 expression is silenced (T29-STC1i, HEY-STC1i, SKOV3-STC1i, and OVCA432-STC1i cells) as well as their corresponding control cells. Expression of the antiapoptotic proteins BCL-2 and BCL-XL was increased in all cell lines that overexpress STC1, but expression was decreased in all cell lines in which STC1 expression was silenced (Figure 6, D). Expression of the proapoptotic proteins BAD, pBAD, and BAX was not affected by changes in the level of STC1 expression. Overexpression of STC1 was associated with increased accumulation of procaspase-8 and -9 in STC1-overexpressing cells and with a concomitant decrease of the cleaved products from procaspase-3. In contrast, silencing expression of STC1 was associated with the accumulation of procaspase-8 and procaspase-9 and with concomitant increase in cleaved products of procaspase-8 in HEY-STC1i cells, the accumulation of procaspase-9 in T29H-STC1i cells, and the accumulation of procaspase-3 in T29H-STC1i cells. Expression of caspase-1, caspase-7, or caspase-10 was not affected by changes in the level of STC1 expression. Thus, STC1 protein appears to inhibit apoptosis primarily by promoting expression of the antiapoptotic proteins Bcl-2 and Bcl-XL, which leads to the inhibition of cleavage of procaspases-3, -8, and -9.

**Discussion**

In this study, we found that the expression of human STC1 was associated with the development of ovarian cancer. We found that increased expression of STC1 transformed immortalized human ovarian epithelial cells to ovarian cancer cells and promoted growth of xenograft ovarian tumors in mice. Further, we found that STC1 expression promoted cell proliferation by controlling entry of quiescent cell into S phase of the cell cycle and thus enhanced growth of tumors generated with transformed ovarian epithelial cells. We also found that STC1 overexpression increased the expression of cyclin A, cyclin B1, CDK2, and a short cyclin E isoform; the elevated expression of these proteins is possibly required for regulation of the transition from G1 to S phase. Finally, we showed that the expression of Bcl-2 and Bcl-XL, well-characterized antiapoptotic proteins, was increased when STC1 was overexpressed. The antiapoptotic effect was likely associated with the extracellular (secreted) function of STC1 because the effect was blocked by anti-STC1 monoclonal antibody. Our data support the hypothesis that STC1 may be a novel secreted oncoprotein in ovarian cancer development.

**STC1** was initially identified as a target gene after the introduction of the RAS oncogene in three immortalized ovarian epithelial cells. RAS mutations are commonly detected in low-grade serous ovarian cancers (34,35), endometrioid ovarian cancers (36), and mucinous ovarian cancers (37,38). In this study, we found that **STC1** is a downstream target of the RAS oncogene as shown by increased expression of STC1 protein in RAS-transformed cells. However, we also found that expression of STC1 protein was elevated in ovarian cancer cell lines without known RAS mutation and in high-grade serous carcinoma in which RAS mutations are rare but p53 mutations are very common. Therefore, STC1 expression can be activated by either a RAS-dependent or a RAS-independent pathway (possibly mutation in p53) and may be a common downstream target of epithelial ovarian cancer.

On the basis of these results, we propose the following model for STC1-mediated oncogenesis (Figure 7): After activation of the RAS oncogene or other possible oncogenic event such as loss of p53, STC1 expression and secretion increase. Secreted STC1 protein then binds to its undefined receptors on the membrane of tumor cells, which activates this receptor. Activation of the STC1 receptor shifts the balance between antiapoptotic and proapoptotic signals to decrease apoptosis and cell cycle progression, which in turn leads to increased tumor growth. Because elevated STC1 protein expression decreased apoptosis, the sensitivity of the ovarian cancer cells with elevated STC1 expression to the most commonly used chemotherapy drug, cisplatin, was also decreased.

This study had several limitations. The detailed molecular mechanism involved in STC1-mediated cell proliferation and apoptosis and in cisplatin-mediated resistance is also not clear. Furthermore, STC1-mediated cisplatin resistance was only tested in ovarian cancer cell lines and it will require further validation in animal models. Similarly, antibody-induced apoptosis of ovarian cancer cells also needs to be validated in animal models. In addition, the tumor growth was studied in immunodeficient mice, but it remains to be determined whether those conclusions will also apply to human patients with ovarian cancer. Finally, it remains to be determined whether STC1 is specific to ovarian cancer or whether it plays a similar functional role in other epithelial cancer.

Results of this study have several potential clinical implications. First, **STC1** expression appeared to promote ovarian tumor growth through an antiapoptotic effect; therefore, it may be a target for therapeutic intervention. Small-molecule compounds that mimic the effect of STC1 siRNA should be investigated for their therapeutic value against ovarian cancer. Second, the antiapoptotic
effect of STC1 was blocked by neutralizing anti-STC1 monoclonal antibodies; therefore, antibodies against the secreted form of STC1 or its undefined receptor should be investigated further for their therapeutic value against ovarian cancer. Third, because STC1 is a secreted protein in ovarian cancer, it should be investigated as a marker for early detection, diagnosis, or therapeutic response of ovarian cancer. It has been proposed that STC1 protein might be a novel blood and bone marrow marker for breast cancer and for the detection of minimal residual breast cancer (39,40). Thus, further studies are warranted to elucidate the mechanisms involved in STC1-mediated ovarian tumorigenesis and to further explore the effect of anti-STC monoclonal antibody on ovarian cancer growth in animal models and the diagnostic value of STC1 protein in serum in a large cohort of ovarian cancer patients.

References


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