SCCA1/SERPINC3 Promotes Oncogenesis and Epithelial–Mesenchymal Transition via the Unfolded Protein Response and IL6 Signaling

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Abstract

The serine/cysteine protease inhibitor SCCA1 (SERPINB3) is upregulated in many advanced cancers with poor prognosis, but there is limited information about whether it makes functional contributions to malignancy. Here, we show that SCCA1 expression promoted oncogenic transformation and epithelial–mesenchymal transition (EMT) in mammary epithelial cells, and that SCCA1 silencing in breast cancer cells halted their proliferation. SCCA1 overexpression in neu+ mammary tumors increased the unfolded protein response (UPR), IL6 expression, and inflammatory phenotypes. Mechanistically, SCCA1 induced a prolonged nonlethal increase in the UPR that was sufficient to activate NF-κB and expression of the protumorigenic cytokine IL6. Overall, our findings established that SCCA1 contributes to tumorigenesis by promoting EMT and a UPR-dependent induction of NF-κB and IL6 autocrine signaling that promotes a protumorigenic inflammation. Cancer Res; 74(21): 1–12. ©2014 AACR.

Introduction

Squamous cell carcinoma antigens (SCCA) belong to the clade B subset of serpins that inhibit lysosomal proteases, including cathepsins, via the irreversible interaction between its carboxyl-terminal reactive site loop (RSL) and the target proteases (1, 2). The first variant of the SCCAs, SCCA1 (SERPINB3), an inhibitor of cathepsins L, S, and K, was initially found to be elevated in squamous cell carcinoma (SCC) of the uterine cervix (3), and was later found to be highly expressed in SCCs of the lung, head and neck, and in hepatocellular carcinoma (4, 5). Its functional connection with tumorigenesis has been mainly appreciated for its anti–cell death role against lysosomal membrane permeability transition in response to various stresses such as UV, radiation, chemotherapy, TNFα, and natural killer cells (5–9). Nevertheless, accumulating evidence including that from our own group has indicated that elevated SCCA1 expression is associated with poorly differentiated and more inflammatory and aggressive human malignancies including breast cancer (10–12), pointing to additional molecular functions. We have recently reported that ectopic expression of SCCA1 leads to the inhibition of both proteasomal and lysosomal protein degradation (13), suggesting that elevated SCCA1 expression may lead to an increased unfolded protein response (UPR).

UPR is a complex signaling event that is activated by the disturbance of cellular protein homeostasis. Although it is well appreciated that excessive misfolded protein stress triggers apoptosis, UPR signaling under more physiologic conditions plays an important role in helping cells to cope with stress and to restore homeostasis. The connection between UPR and cancer has been well appreciated in light of cancer cells’ highly increased growth rate and exposure to growth-limiting conditions such as nutrient deprivation and hypoxia (14, 15). Although overactivating UPR in cancer cells can lead to cell death and has been regarded as a therapeutic opportunity using proteotoxic agents, such as the proteasome inhibitor Velcade (bortezomib; refs. 16, 17), the UPR signaling pathway has been implied in promoting tumorigenesis by increasing tumor cell survival and proliferation (15, 18). However, it remains elusive how specific cell intrinsic lesions lead to increased UPR that functions as a driving factor in tumorigenesis. In this study, we report a previously unidentified protumorigenic role of SCCA1, which is via the induction of a nonlethal level of UPR that activates NF-κB and expression of the protumorigenic cytokine IL6.
Figure 1. Ectopic expression of SCCA1 leads to EMT-like phenotype that is dependent on its protease-inhibitory activity. A–D, MCF10A cells were retrovirally infected with vector control or Flag-SCCA1 and continuously passaged. A, at passage #8, phase-contrast images were taken. B, at passage #8, total RNA was analyzed for indicated genes via qRT-PCR that was normalized against that in vector control cells. Data shown are the mean fold change SEM of two independent experiments performed in triplicates. **P < 0.05; ***P < 0.01; ****P < 0.0001. C, at passage #8, immunofluorescence was performed for E-cadherin and vimentin, and counterstained with DAPI. Images were taken by a deconvolution fluorescence microscope. Note the loss of cell surface E-cadherin and increase in vimentin expression in SCCA1-expressing cells. D, whole-cell lysates were harvested at indicated passage numbers and analyzed by immunoblotting. Right, the expression level of E-cadherin and vimentin in SCCA1-expressing cells, which were analyzed by densitometry and normalized to that of passage #1. E and F, MCF10A cells stably expressing indicated SCCA1 mutants were analyzed by immunoblotting (E), and the phase-contrast images were taken (F).
Materials and Methods

Cell lines and culture

MCF10A, MDA-MB-231, MDA-MB-468, SKBR3, and HEK293T cells were obtained from the ATCC. Baby mouse kidney (BMK) cells were obtained from Dr. Eileen White’s laboratory (Rutgers University, New Brunswick, NJ), and human mammary epithelial (HMLE) cells were obtained from Dr. Robert Weinberg’s laboratory (Whitehead Institute, Cambridge, MA). All cell lines have been tested and authenticated as bacteria and Mycoplasma free following the ATCC’s instructions on a routine basis within 6 months of experiments.

Retroviral and lentiviral infection

For retroviral infections, the three plasmid system (gene of interest + helper virus + VSVG at the ratio of 4:3:1) was used to generate virus particles after transfection into HEK 293T cells using Lipofectamine 2000 (Invitrogen). Filtered...
viral supernatant along with 10 μg/mL polybrene (Sigma) was used to infect the target cells. Lentiviral infection was carried using the above-mentioned protocol by replacing the helper virus with the AR8.91 plasmid.

**IL6 ELISA, conditioned medium, and neutralization experiment**

The concentration of IL6 secreted into the media was measured using IL6 ELISA Kit (R&D Systems; D6050), as per the manufacturer's instructions.

**Subcellular fractionation**

Subcellular fractionation was carried out using the subcellular proteome extraction kit (Calbiochem). The fractions were quantified using the BCA assay, and equal amount of protein was then used for precipitation using four times the volume of ice-cold acetone. The samples were incubated for 120 minutes at −20°C and centrifuged at 13,000 × g for 15 minutes at 4°C. The pellets were air-dried for 10 minutes and resuspended in 2× SDS sample buffer and boiled at 95°C for 5 minutes. The proteins in the cytosolic and nuclear fractions were then detected by Western blotting.

**Luciferase reporter assay**

The Dual-Luciferase Activity Kit (Promega E1910) was used to examine the luciferase activity. The luciferase assay is performed following the common protocols. The NF-κB binding site of IL6 promoter was fused to a luciferase reporter gene.

**Wound-healing assay**

Equal number of vector and SCCA1-expressing cells were plated and allowed to grow to subconfluency. Two perpendicular wounds were made with the help of western loading tips across the culture dish, washed three times to remove cell debris, and imaged 6 hours later at the junction of the wound. The cell front was marked out and the area of the gap was measured using the NIS elements software (Nikon Instruments Inc.).

**Soft agar assay**

Cells (3 × 10⁴) were resuspended in 1.5-mL MCF10A complete media with 0.5% Noble agarose and overlaid onto 1.5-mL complete medium with 0.7% agarose in each well of a 6-well plate in triplicates. After 3 weeks, colonies larger than 100 μm were counted.

**Orthotopic mouse tumor experiment and SCCA1 conditional transgenic mice**

Female beige nude XID mice (HsdNIHS-Lysb+c1Fosl−1mmBtk−/−), ages 6 to 8 weeks, were obtained from the Harlan Laboratories. SCCA1 knockin transgenic mice with 129Ola/C57Bl/6 mixed background were developed by Genoway. Mice were housed and monitored at the Division of Laboratory Animal Resources at Stony Brook University (Stony Brook, NY). All experimental protocols and procedures were approved by the institutional animal care and use committee.

**Image processing and densitometry measurements**

Images captured by deconvolution microscope were viewed and processed by AxioVision LE image browser. Images were processed in Adobe Photoshop to enhance the brightness and contrast. Densitometry of immunoblot bands was determined by the ImageJ software.

**Statistical analysis**

The longitudinal data analyses were performed to assess the growth curves under different treatments. The Fisher exact test was used to test the difference between tumor incidence rates in two groups. The independent two-sample and one sample t tests were used to make comparisons between groups and to evaluate whether fold changes are different from one, respectively. The analyses were carried out using PROC MIXED, PROC FREQ, PROC MEANS, and PROC TTEST in the SAS 9.4 (SAS institute). Statistical significance level was set at P ≤ 0.05.

Additional details can be found in Supplementary Materials and Methods.

**Figure 3.** SCCA1 promotes orthotopic breast tumor formation. MCF10A cells (7.5 × 10⁵) expressing either GFP alone (n = 5) or GFP plus SCCA1 (n = 9) were implanted into the mammary fat pad of NOD/SCID mice. A, mice were imaged for tumor formation 60 days after implantation. B, the formation of palpable tumors are summarized. Significance was judged on the basis of the Fischer exact test. ***P < 0.01. C, tumors were sectioned and stained using hematoxylin and eosin (H&E; top) or by IHC for SCCA (bottom).
SERPINB3 Induces Tumorigenesis via UPR and IL6 Signaling

A

Fold change in transcript

Vector
SCCA1

TGFβ IL6 IL8 CXCL-1 IL1β EGF TGFB HB-EGF AREG BTC EPR

B

Secreted IL6 (ng/mL)

Vector SCCA1

C

Vector + IgG SCCA1 + IgG SCCA1 + α-IL6

0 5 10 30 60 0 5 10 30 60 0 5 10 30 60 min

pSTAT3 Y705

D

Fold change in IL6 transcript

shNTC shiL6

E

Fold change in E-cadherin transcript

shNTC shiL6

F

shNTC shiL6

E-cadherin Tubulin

1.0 4.0 E-cad/Tubulin

G

shNTC shiL6

Bar, 50 μm

H

Relative growth

shNTC shiL6

I

Relative luciferase activity

Vector SCCA1

J

Vector SCCA1

C N C N

RelA/p65 65 kDa PARP 110 kDa Tubulin 55 kDa

K

Fold change in IL6 transcript

Vehicle Bay11-7082

L

DMSO Bay11-7082

Bar, 100 μm

M

BAY11-7082

0 4 8 12 16 h

C-C3 28 kDa PARP 110 kDa C-PARP 89 kDa Tubulin 55 kDa

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Results
SCCA1 expression results in EMT-associated oncogenic transformation

On the basis of our recent report that SCCA1 is associated with poorly differentiated high-grade breast carcinoma (11), we chose to study the biologic function of SCCA1 in the non-neoplastic human mammary epithelial cell line MCF10A that has a low level of endogenous SCCA1 (11). Interestingly, ectopic expression of SCCA1 in MCF10A cells led to morphologic changes from a cobblestone-like monolayer to a dispersed spindle-shaped morphology that resembles epithelial–mesenchymal transition (EMT; Fig. 1A). This is reminiscent of the EMT-like changes in HepG2, a hepatocellular carcinoma cell line, upon SCCA1 overexpression (19). Consistent with the EMT-like morphologic change, a change of transcriptional profile that included downregulation of E-cadherin (CDH1), EpCam (EPCEM), and claudin4 (CLDN4), as well as upregulation of Zeb1 (ZEB1), vimentin (VIM), and fibronectin1 (FN1), was observed in SCCA1-expressing MCF10A cells (Fig. 1B). The decreased E-cadherin and increased vimentin were also observed at the protein level by immunofluorescence (Fig. 1C) and immunoblotting (Fig. 1D). It is important to note that the morphologic changes induced by SCCA1 in MCF10A cells did not occur instantaneously, rather at approximately 2 weeks of continued passaging, accompanied by a progressive loss of E-cadherin and the reciprocal gain of vimentin (Fig. 1D). This is consistent with the notion that EMT is a multistep progressive event (20). Importantly, two catalytic-inactive mutants of SCCA1, the F352A point mutant that has decreased protease-inhibitory activity (21), and the SCCA1 Δ340–345 hinge deletion mutant that renders the RSL ineflexible, and therefore incapable of protease inhibition (22), failed to induce the EMT-like changes with regard to the expression levels of E-cadherin and vimentin (Fig. 1E) and cell morphology (Fig. 1F), indicating that the EMT-promoting function requires the antiprotease activity of SCCA1.

EMT is functionally associated with increased cell mobility and oncogenic transformation. Indeed, expression of SCCA1 in MCF10A cells conferred increased wound closure associated with individually scattered cells at the migratory front (Fig. 2A).

These cells were also resistant to cell death induced by loss of adherence (anoikis; Fig. 2B), and displayed colony formation in soft agar (Fig. 2C). Furthermore, while MCF10A cells are dependent on growth factors and hormones [epidermal growth factor (EGF), insulin, hydrocortisone, and cholera toxin], SCCA1 expression rendered them independent of EGF (Fig. 2D) and enabled them to proliferate even when deprived of all four hormonal factors (Supplementary Fig. S1). The independence of EGF was accompanied by increased steady-state levels of phospho-ERK and phospho-Akt, which did not decrease upon EGF deprivation (Fig. 2E). We then examined the tumor-promoting function of SCCA1 in an orthotopic mouse tumor setting by injecting SCCA1-expressing MCF10A cells into the mammary fat pad of the immunocompromised NOD-XID mice. Although MCF10A cells are non-tumorigenic, 9 out of 9 mice implanted with SCCA1-expressing cells developed tumors as shown by GFP imaging (Fig. 3A and B) where the ectopic expression of SCCA1 was confirmed by immunohistochemistry (Fig. 3C). Taken together, our results indicate that SCCA1 can promote an EMT-like phenotype and oncogenic transformation in a manner that is dependent on its antiprotease activity.

SCCA1 expression induces IL6 expression

Because the SCCA1-expressing cells displayed an EMT-like phenotype, EGF independence, and oncogenic transformation (Figs. 1 and 2), we speculated that SCCA1 might promote the expression of autocrine factors that are implicated in EMT and/or EGFR signaling and oncogenic transformation. We performed quantitative real-time PCR (qRT-PCR) to determine the expression of well-characterized EMT-associated factors including transforming growth factor-β (TGFβ), interleukin-6 (IL6), IL8, IL1β, and (C-X-C motif) ligand 1 (CXCL1; refs. 23–26), as well as the EGF family members EGF, TGFA, heparin-binding EGF-like growth factor (HBEGF), amphiregulin (AREG), betacellulin (BTC), and epiregulin (EPR; ref. 27). Among these, IL6 transcription was significantly upregulated in SCCA1-expressing cells (Fig. 4A). Correlating with the increased IL6 transcript level, a dramatically increased amount of IL6 was detected in the culture medium of SCCA1-expressing cells.

Figure 4. SCCA1 promotes IL6 expression by activating NF-κB. A, total RNA isolated from vector control or SCCA1-expressing MCF10A cells were analyzed for the expression of indicated hormonal ligands by qRT-PCR. Data shown are the mean transcript level normalized to that of vector control cells ± SD of triplicate experiment. *, EGF was below detectable level, B, cell culture medium of vector control and SCCA1-expressing cells from overnight cultures was collected and measured for the level of secreted IL6 by ELISA. †, the absolute concentration of IL6 in the control cells was below the sensitivity of the kit (3.25 pg/mL). Data shown are the mean ± SD of two independent experiments performed in triplicates. C, vector control or SCCA1-expressing cells were cultured in EGF-free medium for 24 hours. The medium from vector control cells was incubated with control IgG and that from SCCA1 cells was incubated with IgG or an IL6 neutralizing antibody. These media were added to EGF-starved MCF10A cells. Whole-cell lysates were collected at indicated times and probed for phospho-STAT3 (Y705) and total STAT3. D, relative cell proliferation was determined by crystal violet staining. Data shown are the mean ± SD of three independent experiments performed in triplicates. EGF was below detectable level. F, whole-cell lysates were analyzed by immunoblotting. G, phase-contrast images of the cell culture was taken. H, relative cell proliferation was determined by crystal violet staining. Data shown are the mean ± SD of three independent experiments performed in duplicates. ***, P < 0.001. I, indicated cells were transfected with the NF-κB luciferase reporter and a Renilla luciferase construct. Twenty-four hours after transfection, NF-κB luciferase activity was calculated by normalizing against the Renilla luciferase activity. Data shown are a representative graph of three independent experiments performed in triplicates, showing the mean ± SD. ***, P < 0.0001. J, indicated cells were subjected to subcellular fractionation. The cytosol (C) and nuclear (N) fractions were analyzed by immunoblotting. K, left, cells were treated with either DMSO or 5 μM/L BAY-117082 for 4 hours. Right, SCCA1-expressing cells were retrovirally infected with vector control or IκBxM (S32A, S36A) for 4 days. Expression of IκBxM was verified by immunoblotting. IL6 transcript level was analyzed by qRT-PCR. Data shown are the mean ± SD of three independent experiments performed in triplicate. ***, P < 0.0001. L and M, SCCA1-expressing MCF10A cells cultured in nonadherent culture dish, in the presence of DMSO or 5 μM/L BAY-117082, for 4 days. phase-contrast image of the cell culture was taken (L) and indicated proteins were probed by immunoblotting (M).
SERPINB3 Induces Tumorigenesis via UPR and IL6 Signaling

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Fold change in transcript

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shNCT, shPERK

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shNCT, shATF6α

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shNCT, shXBP1

Bar, 100 μm
OF8 Cancer Res; 74(21) November 1, 2014

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(Fig. 4B). The secreted IL6 is functionally active because the culture medium collected from SCCA1-expressing, but not vector control cells, induced STAT3 phosphorylation, a known downstream consequence of IL6 signaling, and this effect was abrogated when the conditioned medium was preincubated with an IL6-blocking antibody (Fig. 4C). It is important to note that a slight, yet apparent, upregulation of IL6 transcript was detected in the early passages post-SCCA1 expression, which progressively increased in subsequent passages, resulting in over a 100-fold elevation (Supplementary Fig. S2). Silencing IL6 at the early-passage post-SCCA1 expression led to a drastic increase in E-cadherin expression at both the transcript and protein levels (Fig. 4D–F), delayed the change to spindle-like morphology (Fig. 4G), and decreased proliferation (Fig. 4H). These results, while they do not rule out the possible involvement of other cytokines, indicate that expression of SCCA1 leads to increased IL6 production, which is likely a causative factor rather than the consequence of the SCCA-induced EMT-like phenotype and cell transformation.

SCCA1-induced IL6 expression is mediated by the activation of NF-κB

Expression of IL6 is largely driven by the NF-κB transcription factors, which have been shown to be essential for EMT in breast cancer (28, 29). Because we detected increased IL6 expression in SCCA1-expressing cells, we determined whether SCCA1 could promote the activation of NF-κB. Indeed, a significant increase of NF-κB activity was detected in SCCA1-expressing cells using an NF-κB luciferase reporter assay (Fig. 4I), which was accompanied by a marked increase of nuclear RelA/p65 (Fig. 4J). The increased expression of IL6 in SCCA1-expressing cells was abrogated by a pharmacologic inhibitor of NF-κB, BAY11-7082, at a concentration tolerated by the parental MCF10A cells (24), and by the ectopic expression of the 1kBΔ2M (S32A, S36A) super-repressor mutant (Fig. 4K). Accordingly, treatment with BAY11-7082 resulted in the abrogation of anchorage-independent sphere formation (Fig. 4L) and increased apoptosis (Fig. 4M) in SCCA1-transformed cells. These results indicate that activation of NF-κB plays an essential role in SCCA1-mediated IL6 production and cell transformation.

SCCA1 induces proinflammatory signaling by activating a low-level chronic UPR

We have recently reported that expression of SCCA1, through its protease-inhibitory activity, leads to impaired lysosomal and proteasomal protein turnover (13). This impaired protein degradation caused by elevated SCCA1 expression is nonlethal and may lead to UPR that has been implicated in proinflammatory response in numerous human diseases including cancer (30). Hence, we speculated that SCCA1 could promote NF-κB activation and subsequently IL6 production by inducing a nonlethal, yet prolonged, UPR.

There are three arms of the UPR signaling pathway that are mediated by PERK, ATF6α, and IRE1α/β (31). It is generally accepted that while the three arms are closely interconnected, each of them has predominant molecular signatures: activation of PERK leads to increased translation of ATF4 transcriptional factor rather than the consequence of the SCCA-induced EMT-like phenotype and cell transformation.

Figure 5. SCCA1 induces chronic UPR that is essential for IL6 production and transformation. A, vector control or SCCA1-expressing MCF10A cells were treated with 5 μmol/L tunicamycin and analyzed by immunoblotting. B, cells were subjected to subcellular fractionation. The cytosol (C) and nuclear (N) fractions were analyzed by immunoblotting. C, cells were probed for ATF6α by immunofluorescence and counterstained with DAPI. Images were taken by a deconvolution fluorescence microscope. D, primers across the splice junction of XBP1 were used to amplify XBP1 by semi-quantitative RT-PCR. The PCR product was divided into two halves. One was subjected to PstI digestion and the other was not, and then resolved on an agarose gel. PCR of GAPDH was used as a control for equal amplification. Note that SCCA1 cells had increased amount of XBP1u and decreased XBP1 splicing. E, vector and SCCA1-expressing cells were treated with 5 μg/mL tunicamycin for indicated times. XBP1 transcript was detected by semi-qPCR. F–N, SCCA1-expressing MCF10A cells were lentivirally infected with short hairpins of control (shNTC) and PERK (F–H), ATF6α (I–K), or XBP1 (L–N). Cells were harvested 4 days later and qRT-PCR was performed for the transcript level PERK, ATF6α, IL6, and immunoblotting for XBP1. Data shown are the mean ± SEM of three independent experiments performed in triplicate. Relative cell growth was determined by crystal violet staining normalized to the reading of cells at day 1 (G, J, and M). Significance judged by longitudinal data analysis was *, P < 0.05; **, P < 0.01; ***, P < 0.001 for G and J, and nonsignificant for M. Cells were cultured in suspension for 10 days. Phase-contrast images of cell spheres were taken (H, K, and N).
UPR-promoting function of SCCA1 is dependent on its protease-inhibitory activity as the SCCA1 F352A and SCCA1Δ340–345 inactive mutants did not alter the levels of ATF6α-p50 (Supplementary Fig. S3). These data indicate that increased SCCA1 expression leads to a chronic UPR with the activation of the PERK and ATF6 arms and slight inhibition of the IRE1 arm.

Next, to determine whether activation of PERK and ATF6α are critical for SCCA1-mediated IL6 expression and cell transformation, we silenced PERK or ATF6α in SCCA1 cells. Silencing either PERK or ATF6α led to downregulation of IL6, impaired cell proliferation, and decreased anchorage-independent sphere formation in SCCA1-expressing MCF10A cells (Fig. 5F–K). In contrast, silencing XBP1 led to an increase of IL6 and no decrease in cell proliferation or sphere formation (Fig. 5L–N). Similarly, silencing PERK and ATF6α in MDA-MB-231 cells with the endogenous level of SCCA1 also led to decreased IL6 expression and reduced cell proliferation (Supplementary Fig. S4A–S4D). The specific role of SCCA1 in mediating UPR, IL6 production, and transformation was further tested by silencing the overexpressed SCCA1 in MCF10A cells (Supplementary Fig. S5A–S5C), as well as endogenous SCCA1 in MDA-MB-231 cells (Supplementary Fig. S5D–S5F) and MDA-MB-468 cells (Supplementary Fig. S5G–S5I), which resulted in decreased expression of ATF4, IL6, and cell proliferation.

Taken together, the above data indicate that elevated SCCA1 expression leads to IL6 production and oncogenic transformation by upregulating a nonlethal level of UPR in mammary epithelial cells. The generality of this notion is strengthened by further observations that overexpression of SCCA1 led to increased IL6 production and UPR response in the BMK epithelial cells (Supplementary Fig. S6A–S6C; ref. 39), in HMLE cell line (Supplementary Fig. S6D and S6E), and in human breast cancer cell line SKBR3 (Supplementary Fig. S6F and S6G).

**SCCA1 alters UPR and inflammatory conditions in vivo**

To further study the role of SCCA1 in affecting UPR and tumorigenesis in vivo, we generated a conditional SCCA1 transgenic mouse strain with a 129Ola/C57Bl/6 mixed background. The human SCCA1 open reading frame cDNA was cloned into the housekeeping Hprt gene located on the X-chromosome. A stop cassette flanked by LoxP sites was placed upstream of SCCA1 to allow for Cre-specific tissue expression of SCCA1 (LSL-SCCA1; Fig. 6A). The LSL-SCCA1 mice were bred to the MMTV-Cre mice to induce SCCA1 expression in mammary glands, which was confirmed by immunoblotting and IHC (Fig. 6B and C). The SCCA1-expressing mice were fertile, sustained pregnancies to term, produced viable offspring, and displayed no apparent defects in ductal morphogenesis, indicating that elevated expression of SCCA1 is not sufficient to drive mammary tumorigenesis by itself. Because our earlier results showed that SCCA1 can promote orthotopic tumor development in MCF10A cell line, which harbors loss of p19Arf tumor-suppressor gene, we are currently investigating the role of SCCA1 in tumorigenesis in spontaneous mouse tumor models with loss of tumor-suppressor genes including the p19Arf flox/flox model (40). Separately, as our above results also showed that SCCA1 can promote chronic UPR and IL6 production, we bred the LSL-SCCA1 mice to the MMTV-neu mice (with FVB background; ref 41) to determine whether expression of SCCA1 can modulate tumor microenvironment and accelerate tumorigenesis. We chose the MMTV-neu model because it has a relatively long tumor latency of 7 to 14 months with 50% incidence. A total of 8 neu+SCCA1+ mice and 10 neu+ littermate control female mice were generated. Four mice from each group developed palpable tumors. The average latency of tumor formation was 407 days and 551 days for neu+SCCA1+ and neu+ only mice, respectively (P = 0.24). The prolonged tumor latency is reminiscent of that of the F1 virgin mice of the FVB and B6 crossing (42). Metastatic tumors were not observed in these mice at endpoint (tumor size, 300–500 mm3). We were able to collect eight tumors for histology and four tumors for Western blot analysis (two tumors from each group). Strikingly, when compared with neu+only tumors, all neu+SCCA1+ tumor cells invaded the basement membrane into the stromal tissue (Fig. 6D). The SCCA1+ tumors also displayed elevated UPR indicated by increased expression of ATF4 and ATF6α-p50 (Fig. 6E), as well as increased intratumoral IL6 expression and the infiltration of F4/80-positive cells (Fig. 6F). These results indicate that while SCCA1 is not able to accelerate tumor development in the MMTV-neu background, it can clearly alter the tumor microenvironment and increase the potential of tumor invasiveness. Further characterization of the role of SCCA1 in tumorigenesis in tumor models with other genetic lesions is under way.

**Discussion**

In this study, we report that an intracellular serine/cysteine protease inhibitor, SCCA1, promotes the production of IL6 by inducing chronic UPR and subsequent activation of NF-κB. This leads to an EMT-like phenotype and oncogenic transformation in the nontumorigenic mammary epithelial cell line MCF10A. Silencing of the ectopically expressed SCCA1 in MCF10A cells and the endogenous SCCA1 in MDA-MB-231 and MDA-MB-468 cells leads to decreased UPR, IL6 signaling, and halted cell proliferation. We also show that overexpression of SCCA1 in the neu-induced mammary tumors resulted in increased UPR, IL6 expression, and proinflammatory condition.

Our study uncovers a novel protumorigenic role of SCCA1 and helps to explain the association between elevated SCCA1 expression and poorly differentiated and more aggressive human malignancies, namely by inducing IL6 autocrine signaling and EMT-like phenotype. Although we cannot rule out the possibility that other cytokines/growth factors may also be involved, our data strongly indicate that IL6 autocrine signaling plays a critical role. This is in line with recent literature showing that IL6 acts to sustain the positive feedback loop to achieve chronic inflammation (24, 25), and that cell autonomous inflammation mediated by IL6 is a key component in driving EMT that have been shown to account for oncogenic transformation and tumor cell de-differentiation (43, 44).

Our work also provides SCCA1 as a molecular signature that connects protein homeostasis, UPR, NF-κB activation, and tumorigenesis. We have previously reported that expression
of SCCA1 leads to a suppression of both lysosomal and proteasomal protein turnover, in a manner that requires its protease-inhibitory activity (13). Although the precise mechanism underlying this SCCA1-mediated blockade of protein turnover remains to be determined, our finding is in line with a recent report that the cathepsin L-deficient cells display impaired lysosomal turnover, accumulation of ubiquitinated proteins and protein aggregates, and disrupted ER homeostasis (45). It is conceivable that the blockade of protein turnover can lead to UPR, which we show here promotes protumorigenic IL6 production. Our work hence establishes a case in which cell intrinsic blockade of protein turnover promotes EMT and tumorigenesis via UPR, which has been implicated in cell de-differentiation and EMT (46, 47), as well as for tumorigenesis in animal models of breast cancer (48, 49).

The UPR-inducing and protumorigenic function of SCCA1 suggests it may be a predictor and target for therapy. We previously reported that while SCCA1 protects cells from lysosomal disruption resulting from DNA-alkylating damage and hypotonic shock, it sensitizes cells to ER stressors (13), suggesting a therapeutic opportunity of using proteotoxic agents, such as proteasome inhibitors, to treat cancers with elevated SCCA1. In addition, as we show here...
that the antiprotease activity of SCCA1 is essential for it protumorigenic function, targeting the protease-inhibitory function of SCCA1 may be another vital therapeutic approach.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Other (contributing animal work including characterization of the phenotype of animal, endpoint tumor collecting, fixation, processing, embedding, and the H&E and IHC staining for tumor sections): Y. Sun

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SCCA1/SERPINC3 Promotes Oncogenesis and Epithelial–Mesenchymal Transition via the Unfolded Protein Response and IL6 Signaling

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