Stanniocalcin 2 expression is regulated by hormone signalling and negatively affects breast cancer cell viability *in vitro*

Sanda Raulic^{1,3}, Yudith Ramos-Valdes¹ and Gabriel E DiMattia^{1,2,3}

¹London Regional Cancer Program, 790 Commissioners Road, Room A4-921, London, Ontario, N6A 4L6 Canada

Departments of ²Oncology and ³Biochemistry, The University of Western Ontario, London, Ontario, Canada

(Correspondence should be addressed to G E DiMattia; Email: dimattia@uwo.ca)

Abstract

Stanniocalcin 1 (STC1) and STC2 are secreted, homodimeric glycoproteins that share 30% amino acid sequence identity. Breast tumour gene profiling studies have demonstrated significantly upregulated *STC2* expression in hormone-responsive positive breast tumours; therefore, the purpose of this study was to investigate STC2 hormonal regulation and function in breast cancer cells. Here we report that *STC2* is expressed in a number of human breast cancer cell lines, regardless of their oestrogen (E₂) and progesterone (P4) receptor status, and its expression is readily detectable in human and mouse mammary gland tumours. Besides E₂, retinoic acid (RA) and P4 play an important role in the regulation of *STC2* expression, not only in MCF-7 but also in other breast cancer and non-breast cell lines. The expression of the related hormone, STC1, is not affected by

the above hormones in breast and endometrial cancer cell lines implying a fundamental difference in regulation in cancer cell lines. The induction of *STC2* expression by E₂ and RA occurs at the transcriptional level but through intermediary transcription factors. The *STC2* proximal promoter region is not responsible for hormonal induction, but exhibits a high basal transcriptional activity. Constitutive *STC2* expression in human breast cancer cell lines resulted in significant impairment of cell growth, migration and cell viability after serum withdrawal. In conclusion, STC2 is a downstream target of E₂, P4 and RA signalling pathways. In hormone receptor negative cell lines it can function in a paracrine/autocrine fashion to reduce cell proliferation.

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Introduction

Stanniocalcin 1 (STC1) and STC2 constitute a small family of secreted, homodimeric glycoproteins that have been implicated in the physiology of phosphate regulation (Wagner *et al.* 1997, Madsen *et al.* 1998, Yoshiko *et al.* 2007), metabolism (Wagner & DiMattia 2006), reproduction (Deol *et al.* 2000, Varghese *et al.* 2002, Luo *et al.* 2004, 2005), stress response (Sheikh-Hamad *et al.* 2000, Anderson 2003, Ito *et al.* 2004) and development (Stasko & Wagner 2001, Varghese *et al.* 2002, Gagliardi *et al.* 2005). Moreover, the expression of STCs has been recognised as notably altered in a variety of cancers suggesting that they play a role in tumorigenesis (Chang *et al.* 2003). This is based on gene profiling studies where *STC2* expression is significantly elevated in a specific subset of breast tumours (Gruvberger *et al.* 2001, Wilson *et al.* 2002, Kun *et al.* 2003, Amatschek *et al.* 2004, Yu *et al.* 2004, Zucchi *et al.* 2004, Esseghir *et al.* 2006, 2007) or elevated upon oestrogen (E₂) treatment of human breast cancer cell lines (Charpentier *et al.* 2000, Bouras *et al.* 2002). However, whether STC2 is responsive to other hormones that can regulate growth and what effect it might have on breast cancer cells have not been investigated.

It has been well established that oestrogens can regulate processes critical to breast tumorigenesis including cell proliferation and migration (Katzenellenbogen & Frasor 2004, Yager & Davidson 2006, Jordan 2007); therefore, those proteins regulated by E₂ may play a role in the aetiology of breast cancer. The clinical significance of breast tumour *STC2* expression was described by Iwao *et al.* (2002); they reported that the expression of 21 genes was prognostic for breast cancer and that the low expression of these genes, including *STC2*, was associated with poor prognosis. Yamamura *et al.* (2004) reported that high *STC2* mRNA steady-state levels were significantly associated with good prognosis in oestrogen (ER)- and progesterone receptor (PR)-positive breast cancer patients. More recently, a tissue microarray screen found that *STC2* expression was highly predictive for longer disease-free survival (Esseghir *et al.* 2007). Taken together, these studies open a new niche for utilising STC2 as a potentially useful breast cancer molecular marker.

The purpose of this study was twofold. Our first objective was to expand on the mechanism of STC2 regulation by E₂ and determine whether it is regulated by other growth regulatory hormones. We also asked whether STC2

regulation by specific hormones was breast cancer-specific and whether the upregulation of the *STC2* expression also extended to murine models of mammary tumorigenesis. Secondly, we addressed the function of *STC2* in human breast cancer cells by examining the phenotypic effects of *de novo* production in two human breast cancer cell lines. Presently, little is known regarding the function of *STC2*. A persistent exposure to human *STC2* in transgenic mice results in severe growth retardation and female reproductive deficits (Gagliardi *et al.* 2005). Moreover, the exogenous expression of *STC2* can produce a cytoprotective effect (Ito *et al.* 2004). These data suggest widespread effects of *STC2* and that it can have autocrine and paracrine effects in breast cancer (Gagliardi *et al.* 2005). Here, we show that *STC2* production can be regulated by hormones other than E_2 and that *STC1* expression is unaffected. We also demonstrate that *Stc2* expression is strongly upregulated in mouse mammary tumours.

To begin to address the function of *STC2* in breast cancer cells, we chose the approach described by Ito *et al.* (2004) and generated *STC2*-stable transfectants in human breast cancer cell lines. We found that *STC2* expression can result in significantly reduced cell growth, motility and viability implying that the loss of *STC2* expression in breast tumours contributes to a more aggressive phenotype.

Materials and Methods

Cell culture and hormonal treatment

The human breast cancer cell lines T-47D, MCF-7, HCC 1937, HCC 1500, MDA-MB-435, MDA-MB-231 and MDA-MB-468 (obtained from ATCC, Manassas, VA, USA) and the endometrial adenocarcinoma (Ishikawa) cell line were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St. Bruno, Canada) with 10% fetal bovine serum (FBS) (Wisent) and 50 µg/ml penicillin–streptomycin (Invitrogen). The cells were seeded at ~60% density and allowed to attach overnight. Before hormonal treatment, they were washed twice with 1× PBS and cultured in an oestrogen-depleted medium: phenol red-free DMEM-F12 medium with 10% charcoal-stripped FBS (Wisent) and 50 µg/ml penicillin–streptomycin, for 24 h. For gene regulation studies in MCF-7 cells, each hormone was applied at 10^{-6} M final concentration for 0, 1, 3 and 24 h, and Ishikawa and T-47D cell lines were treated for 0, 1, 3, 8, 24, 48 and 72 h.

Human and mouse tissues

Human breast tumour tissue samples were obtained from the London Health Sciences Tumour Bank (London, ON, Canada). Normal breast tissue was obtained by reduction mammoplasty from the Cooperative Human Tissue Network (Midwestern Division, The Ohio State University). Transgenic MMTV/activated Erb-2 (NDL) (Siegel *et al.* 1999) and MMTV/PyV MT (Guy *et al.* 1992) mouse mammary gland

tumour tissues were generously provided by Dr William Muller (McGill University, Montreal).

RNA extraction and northern blot analysis

Total RNA was isolated using TRIzol (Invitrogen). For northern blot experiments, 30–50 µg total RNA per lane was used, as described previously (Gagliardi *et al.* 2005). A Pst I/Sty I human *STC2* cDNA fragment (617 bp of coding sequence) and Pst I/Sph I human *STC1* cDNA fragment (417 bp of coding region) were used as probes. Murine *Stc2* northern blots were performed with an 896 bp coding sequence *Stc2* cDNA fragment. The whey acid protein cDNA probe was generated from the mouse IMAGE clone 5249612. To normalise for RNA loading or to demonstrate RNA integrity, blots were hybridised with an 18S rDNA fragment or with a human acidic ribosomal phosphoprotein PO (36B4) cDNA fragment. The resultant hybridisation signal was quantified using a PhosphorImager and ImageQuant software (GE Healthcare, Baie d'Urfé, Canada).

Western blot

Conditioned cell culture media from hormone-treated T-47D, Ishikawa and the pcDNA3/*STC2* stably transfected MDA-MB-231 and MDA-MB-435 cell lines were collected at the indicated time points and concentrated sixfold using Centricon YM-10 centrifugal filters according to the supplier's instructions (Fisher Scientific, Ottawa, Canada). The total protein concentration was determined using the Bradford assay (Bradford 1976). About 100 µg total protein were loaded per lane for 12% PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane (Roche). The blots were reacted with polyclonal *STC2* antisera (1:5000) raised in rabbits as described previously (Gagliardi *et al.* 2005). Immunoreactive bands were detected using the ECL Plus western blotting detection system (GE Healthcare) with subsequent exposure to Kodak Bio Max XAR film.

Generation of the pGL2-*STC2* luciferase reporter gene plasmid constructs

To generate pGL2-*STC2*-luciferase promoter constructs, an *STC2* 135A09 BAC clone from RPC11H library was used to amplify a 1.382 kb *STC2* gene promoter fragment. The PCR product was cloned into the pCR-BluntII-TOPO plasmid according to the supplier's instructions (Invitrogen). A 916 bp BamH I fragment of the *STC2* promoter was ligated to the Bgl II site of pGL2-Basic (Promega) in both orientations. The 5' truncations of the luciferase reporter were produced using convenient restriction sites.

Transient transfection and hormone treatment

Human embryonic kidney, endometrial adenocarcinoma (Ishikawa) and breast cancer T-47D cells were cultured as

described previously. The following day, the cells were transfected with 1 µg pGL2-STC2-667 plasmid and molar equivalents of pGL2-STC2-337, pGL2-STC2-172, pGL2-STC2-112, pGL2 basic and pBKSII. Co-transfection with a molar equivalent of pCMV-lacZ was done as a measure of transfection efficiency. Transfection was performed using ExGen according to the supplier's instructions (MBI Fermentas, Burlington, Canada) and the luciferase assay performed 24 h later. The hormonal responsiveness of pGL2-STC2-667 was tested by transfection of T-47D cells and compared with molar equivalents of the negative control, pGL2-basic and pERE-Luc as an oestrogen response plasmid (generously provided by Dr J Torchia, London Regional Cancer Program). One day after transfection, cells were cultured in an oestrogen-depleted medium with E₂ (10⁻⁶), RA (10⁻⁶) or an equivalent volume of solvent, for an additional 24 h before measuring the luciferase activity. β-Galactosidase assays were performed on transfected cells to normalise values for luciferase assays (Gaasenbeek *et al.* 1999).

Stable transfection of the MDA-MB-231 and MDA-MB-435 cell lines

The human breast cancer MDA-MB-231 and MDA-MB-435 cell lines were cultured and transfected with pcDNA3 or pcDNA3/STC2 using ExGen transfection agent as described previously. After transfection, they were maintained in serum and antibiotic free-Opti-MEM medium for 24 h which was subsequently replaced with complete media. The cells were allowed to recover for 24 h after transfection and a selection pressure was applied using 800 µg/ml Geneticin (Sigma-Aldrich) for 2 weeks.

Chemical treatments and quantitative real-time RT-PCR (qPCR) analysis

MCF-7 or T-47D cells were cultured as described above. For antioestrogen ICI 182 780 (ICI) (Sigma-Aldrich) treatment, T-47D cells were seeded in six-well cluster plates at ~80% density and allowed to attach overnight. The cells were then washed twice with 1× PBS and incubated for 48 h in an oestrogen-depleted medium. The cells were treated overnight (18 h) with E₂ (10⁻⁸) M (Sigma-Aldrich) or following a 2 h pre-treatment with ICI. Treatments with ICI alone or solvent were used as controls. The level of STC2 mRNA was determined by qPCR using 1.5 µg total RNA and the STC2 primers described below. For actinomycin D (5 µg/ml) or cycloheximide (30 µg/ml) treatments, the cells were seeded in 60 mm dishes at ~60% density and allowed to attach overnight. Subsequently, the cells were washed twice with 1× PBS and cultured for 24 h in an oestrogen-depleted medium. The cells were pretreated for 1 h with actinomycin D (5 µg/ml) or cycloheximide (30 µg/ml) and after adding E₂ or RA (10⁻⁶) M, harvested at 0 and 3 h. The treatment with actinomycin D or cycloheximide alone was used as a control for the effects of E₂ or

RA. Total RNA was isolated and 1 µg reverse-transcribed using Superscript III RNase H reverse transcriptase kit (Invitrogen). qPCR was carried out using the Stratagene SYBER Green PCR Master Mix kit according to the manufacturer's instructions and the MxPro-3000 real-time PCR detection system. The following sets of primers were used: human STC2 sense (5'-GTTCATGACCCTGGCTTTGGGTT-3') and antisense (5'-AAACAGTGCTGGATCTCCGCTGTA-3'); human β-actin sense (5'-ACCAACTGGGACGACATGGA-GAA-3') and antisense (5'-TAGCACAGCCTGGATAG-CAACGTA-3').

Assessment of cell proliferation using the alamarBlue assay and cell counting

For the alamarBlue assay, MDA-MB-231 and MDA-MB-435 cells (stably transfected with pcDNA3 or pcDNA3/STC2) were seeded in 24-well plates at a density of 2×10⁴ cells per well in quintuplicate, and allowed to attach overnight. Subsequently, the cells were incubated with 1:20 dilution of alamarBlue (Biosource, Camarillo, CA, USA) stock solution in culture media and placed in a humidified incubator for 4 h. The fluorescence measurement was obtained at 580 nm in a Wallac Victor²-1420 microplate reader (Perkin-Elmer, Woodbridge, Canada) after 1 and 7 days of culture.

For cell counting, MDA-MB-231 cell lines expressing STC2 (lines 3 and 5) or control vector (line 6) were seeded in 24-well plates at a density of 5×10³ cells per well in quadruplicate. The cells were trypsinised, stained with Trypan blue and counted at day 3 and 7 using a haemocytometer.

Cell survival, colony-forming and scratch-wound motility assays

The breast cancer MDA-MB-231 cell lines expressing STC2 (lines 3 and 5) or control vector (line 6) were plated in 24-well plates at a density of 1.6×10⁵ cells per well in triplicate. The next day, designated as day 0, cell counts were determined to provide the starting cell number, which represented the 100% value for cell number. After washing with 1× PBS, the cells were cultured in serum-free DMEM and alamarBlue assay performed for 3, 5 and 7 days of culture. For colony-forming assay, the cells were seeded in 60 or 100 mm plates at a density of 150 or 250 cells per plate respectively in quadruplicate. After 6 days in culture, colonies were fixed and stained using the Hema 3 staining system (Fisher Scientific) according to the supplier's instructions. The colonies were counted manually using the Olympus SZ-40 microscope (Olympus, Canada). The scratch-wound assays were done in six-well plates at confluence. Twenty-four hours later, two scratches were made in the monolayer of each well using a 1000 µl plastic pipette tip. Four images were captured along each scratch using the Olympus AX-70 microscope and Olympus DP71 camera at 0, 24 and 48 h after the wound infliction. The cell migration was assessed based on the area covered by cells between 0 and 24 h using ImageJ software (<http://www.uhnresearch.ca/facilities/wcif/fdownload.html>).

Statistical analysis

Experimental data were analysed with the unpaired Student's *t*-test and by one-way ANOVA with the Dunnett's post-test (when indicated) using PRISM 3.0a software (GraphPad Software, San Diego, CA, USA). Statistically significant differences were assumed if $P < 0.05$ for all experiments.

Results

STC2 expression in various human breast cancer cell lines and breast tumour tissue

Our screen of ~40 different human cancer cell lines for *STC1* and *STC2* expression, at the protein or mRNA level, showed that *STC2* expression was highest amongst human breast cancer cell lines (unpublished results). To expand on our preliminary observations and determine whether ER signalling was essential for *STC2* gene expression in human breast cancer cells, we examined *STC2* expression in a variety of human breast cancer cell lines. Northern blot analysis detected *STC2* mRNAs in the form of ~4, ~3.6 and ~2 kb transcripts in all tested cell lines. Additionally, an ~1.8 kb transcript was detected in MCF-7, MDA-MB-468 and HCC 1500 cell lines (Fig. 1A). While others had previously indicated that the *STC2* gene expression in human breast cancer cell lines was linked to the presence of ER (Charpentier *et al.* 2000), we showed that the *STC2* gene expression was easily detectable in cell lines such as HCC 1937, MDA-MB-468 and HCC 1806 which do not contain ER or PR (Neve *et al.* 2006) (Fig. 1A). Moreover, we have detected multiple *STC2* mRNA species whereas others have reported only the presence of the ~2 kb transcript in human breast cancer cell lines (Charpentier *et al.* 2000).

To determine whether the *STC2* mRNA species seen in human breast cancer cell lines are also present *in vivo*, we investigated the *STC2* expression in a small number of normal human breast samples obtained from reduction mammoplasty and breast tumours. All three *STC2* mRNAs previously detected in human breast cancer cell lines were present (Fig. 1B), suggesting that the breast cancer cell line *STC2* gene accurately represents the *in vivo* *STC2* transcription unit.

Stc2 expression is activated in mouse mammary gland tumours

As a logical extension of the above studies, we investigated the expression of *Stc2* in the mouse mammary gland. Normal mouse mammary gland tissue was harvested from *post partum* females on different days of lactation and involution. Northern blot analysis did not detect a *Stc2* mRNA signal in these tissues (Fig. 1C).

To determine whether *Stc2* expression is altered in murine mammary gland tumours relative to its normal counterpart, we assessed the *Stc2* expression in two different types of

transgenic mouse mammary gland tumours. Northern blot analysis of transgenic MMTV/PyV MT and MMTV/activated Erb-2 (NDL) mouse mammary gland tumours revealed the presence of ~4 and ~2 kb *Stc2* mRNA species in all tumour samples (Fig. 1C). This is in sharp contrast to the normal mammary gland where *STC2* expression is undetectable by northern.

Hormonal regulation of STC2 gene expression in human breast carcinoma T-47D and MCF-7 cell lines

As mentioned previously, recent gene profiling studies reported an enhanced *STC2* expression, primarily in MCF-7 cells treated with E_2 , implying that E_2 is the primary regulator of *STC2* expression in breast cancer cells (Charpentier *et al.* 2000, Gruvberger *et al.* 2001, Bouras *et al.* 2002, Frasor *et al.* 2003). To determine whether *STC2* expression was linked to other growth-modulating hormones, we investigated the role P4, RA and glucocorticoid, on *STC2* mRNA levels. We chose these hormones because MCF-7 and T-47D breast cancer cell lines express a relatively high level of E_2 , P4, RA and glucocorticoid receptors (Sutherland *et al.* 1988, Hall *et al.* 1990, Roman *et al.* 1992, Rishi *et al.* 1996). As shown in Fig. 2A, the ~2 and 4 kb *STC2* mRNA species in T-47D cells were maximally induced by E_2 (mean 17.3-fold increase), and RA (mean 4.0-fold increase) by the 24 h time point (Fig. 2A and B). An increase in *STC2* mRNA levels was discernable 3 h after hormonal treatment when compared with vehicle-treated cells. Similar results were obtained using qPCR analysis (data not shown). Experiments examining P4 regulation of *STC2* mRNA levels using northern blot showed a low level of induction and were therefore repeated using qPCR. We observed a modest twofold increase in T-47D cell *STC2* mRNA levels, and unlike E_2 and RA, P4 induction of *STC2* transcripts occurred only after a 24-h treatment (data not shown).

We also analysed *STC1* mRNA levels in the same samples because it is a highly related to *STC2* and its function may overlap with *STC2*. Moreover, others have shown the expression of *STC1* in human breast tumours and that *STC1* expression is also linked to ER expression in breast tumours and cells lines (Bouras *et al.* 2002, McCudden *et al.* 2004). Interestingly, *STC1* basal expression was low and not changed by any of the hormonal treatments of T-47D cells (data not shown).

We confirmed that *STC2* protein levels, in the conditioned culture media, also increased with time and correlated with the increase in *STC2* mRNA levels. Western blot analysis showed *STC2* protein accumulation during the late phase of the time course, reaching a maximum at 72 h for all E_2 and RA treatments (Fig. 2C). Similar experiments were carried out with MCF-7 cells to determine whether the effects we observed were cell line-specific. Treatment of MCF-7 cells with E_2 and RA induced mean *STC2* mRNA steady-state levels (mean 12- and 21-fold respectively) (Fig. 2). P4 and

Figure 1 Northern blot analysis of *STC2* expression in human and mouse tissues and cell lines. (A) *STC2* transcript sizes in human breast cancer cell lines determined by comparison with 18S (1.9 kb) and 28S (5 kb) units of rRNAs. HT1080 cell line RNA was used as a positive control. (B) *STC2* mRNA levels in normal human breast tissue and breast tumours. Fifty microgrammes of breast total RNA was loaded per lane and 20 µg T-47D total RNA was loaded in the (+) lane. (C) *STC2* mRNA levels in normal mouse mammary gland and tumour tissue. Mouse mammary gland RNAs from different days of lactation and post-lactation as indicated by lane labelling in the panel on the left (mouse pituitary GC cell line RNA was the positive control). The WAP mRNA signal confirmed that mammary glands were from lactating females. *STC2* mRNAs of ~4 and 2 kb were seen in all transgenic MMTV/activated ErbB-2 (NDL) and MMTV/PyV MT mouse mammary tumours samples. In all the blots, either 36B4 mRNA or 18S rRNA hybridisation demonstrates RNA integrity and relative lane loading.

dexamethasone treatments did not change MCF-7 cell *STC2* expression. Moreover, as we observed with T-47D cells, *STC1* mRNA steady-state levels in MCF-7 cells were not affected by the above hormones (data not shown).

To determine whether regulation of *STC2* expression by steroid hormones and RA can be extended to other steroid-responsive cancers, we tested responses in the Ishikawa

endometrial cancer cell line. Treatment with E_2 , P4 and RA resulted in a strong activation of *STC2* at the mRNA and protein level, starting at 24 h and reaching a maximum at 72 h (data not shown). Similar to human breast cancer cell lines, these treatments, including dexamethasone, did not induce *STC1* mRNA steady-state levels in the Ishikawa cells (data not shown).

Figure 2 Regulation of *STC2* gene expression by E₂ and RA in the T-47D human breast cancer cell line. (A) Representative autoradiograms of northern blots showing the effect of E₂ and RA, over a 24-h treatment period, on *STC2* mRNA levels. *STC2* mRNA levels were maximally induced by the 24-h time point for each treatment. The 18S rRNA signal shows equal RNA loading. (B) Bar graphs depicting the induction of *STC2* mRNA levels by E₂ and RA in T47-D cells. The *STC2* mRNA signal intensity was normalised to the 18S rRNA signal obtained from each blot to control for variations in RNA loading. The expression of *STC2* in vehicle-treated cells was set to 1 for comparison of different blots. Data are indicated as the mean $\pm$ s.e.m. from three independent experiments. (C) Western blot analysis of *STC2* accumulation in the conditioned culture media of hormone-treated cells. Two immunoreactive *STC2* bands (arrows) were routinely obtained as described previously (Gagliardi *et al.* 2005). Blots were stained with Ponceau S to show relative lane loading.

E₂ and RA regulation of *STC2* gene expression is a secondary response

To further examine the mechanism by which the above hormones induce *STC2* gene expression in human breast cancer cell lines, we used chemical inhibitors of transcription, translation and ER signalling. To confirm that E₂ induction of *STC2* requires ligand-activated ER, we treated cells with the antiestrogen ICI 162,780 (ICI). This compound is able to inhibit oestrogen-induced transcriptional activation by competing with E₂ for binding to ER but inhibiting homodimerisation resulting in the degradation of ER (Dauvois *et al.* 1992). The ICI treatment significantly blunted the E₂ induction of T-47D *STC2* mRNA levels from 7.5- to a threefold induction after an 18-h co-treatment indicating that ligand-bound ER mediates the E₂ induction of *STC2* transcript levels (Fig. 3A).

Next, we asked whether *STC2* was a primary E₂- and RA-responsive gene in human breast cancer cells by using a transcriptional inhibitor (actinomycin D) and a protein synthesis inhibitor (cycloheximide). As shown in Fig. 3B, a 3-h treatment

Figure 3 qPCR analysis of *STC2* expression in MCF-7 cells treated with inhibitors of transcription or translation in the presence of E₂ or RA. (A) Treatment with the type II antiestrogen, ICI, resulted in a significant reduction in E₂-induced *STC2* expression indicating that ER mediates this effect. The induction of *STC2* mRNA levels by E₂ or RA was inhibited in the presence of the transcriptional inhibitor, actinomycin D (Act. D), B and C respectively. (D) Treatment with an inhibitor of *de novo* protein synthesis, cycloheximide (CHX), showed a partial inhibition of E₂-induced *STC2* mRNA levels. Cycloheximide alone significantly increased *STC2* mRNA levels when compared with control vehicle-treated (Con.) cells. Bars represent means $\pm$ s.e.m. of three independent experiments, each done in triplicate and significant differences (indicated by asterisks) between groups were determined by Student's *t*-test and one-way ANOVA ($P < 0.0001$).

of MCF-7 cells with E₂ alone resulted in a 3.1-fold increase in *STC2* mRNA levels in comparison with vehicle-treated cells. In the presence of a transcriptional inhibitor, the E₂ induction of *STC2* mRNA levels was blocked, driving the level of its expression below that of untreated cells (Fig. 3B). We performed the same experiment with T-47D cells and obtained a similar result with a threefold induction at 3 h that was completely abrogated by actinomycin D treatment (data not shown). This mRNA synthesis inhibitor also abrogated RA induction of *STC2* mRNA levels after a 3-h treatment (Fig. 3C).

If induction of *STC2* mRNA levels in human breast cancer cells by these hormones is a primary transcriptional response, then co-treatment with an inhibitor of protein translation should not affect the upregulation of *STC2* transcript levels. Treatment with E₂ alone resulted in a significant 2.8-fold increase of *STC2* mRNA compared with untreated MCF-7 cells (Fig. 3D). Unexpectedly, we found that cycloheximide treatment in combination with E₂, partially blocked the induction of *STC2* mRNA levels by E₂ (Fig. 3D). Also, treatment with cycloheximide alone resulted in a small but significant increase in *STC2* mRNA levels in comparison with control cells, suggesting that *STC2* transcript levels can be enhanced by inhibiting the synthesis of proteins that repress *STC2* transcription or decrease the stability of *STC2* mRNA.

Functional oestrogen, RA or P4 response elements were not detected within the proximal promoter region of the STC2 gene

The above data suggest that ER, RAR and PR induction of STC2 gene expression may be mediated, in part, through binding of ligand-activated receptors to specific *cis*-active DNA elements located in and around the STC2 gene. We first followed a bioinformatics approach to search the human STC2 gene for canonical hormone response elements. Initially, we compared an ~700 nucleotide region upstream of the STC2 translation start site from several mammalian species (human, chimp, mouse, rat, dog and cow), using ClustalW multiple alignment software to identify a candidate proximal promoter region based on high DNA sequence conservation (<http://www.ebi.ac.uk/clustalw/>). The most conserved region was limited to within 200 bp of the STC2 transcription start site. Then, using Dragon oestrogen response element (ERE) finder software (<http://sdmc.lit.org.sg/ERE-V2/index>), we analysed this region for putative ERE sequences. One putative imperfect ERE sequence (5'-AT-GGTCG-AAA-GGACT-GT-3') was identified on the reverse strand at position -273 relative to the STC2 transcription start site in human and chimp STC2 sequences. However, putative P4 or RA response elements were not found within this region. We also examined the entire STC2 gene (14.8 kb) and 5 kb of the 5'- and 3'-flanking DNA region for hormone response elements using MatInspector (<http://www.genomatix.de>). Although numerous half-sites were found, sequences that show high identity with the canonical *cis*-active hormone response elements were not identified. Therefore, we focussed on testing the functional significance of the putative ERE by transient transfection. The STC2 gene promoter has not been delimited functionally; therefore, we tested the cell-specific activity of the putative promoter region and attempted to identify a minimal DNA sequence required for basal gene regulation using a series of STC2 gene promoter-luciferase reporter constructs. The basal activity of the STC2 gene promoter was readily detectable in complete cell culture media in all the three cell types regardless of endogenous STC2 expression (Fig. 4B–D). A significant change in the luciferase activity was not observed with the removal of DNA between -667 and -172 of the human STC2 proximal promoter in all three cell lines (Fig. 4A–C). However, STC2 promoter activity was lost upon deletion of DNA sequence between -172 and +127 in all three cell lines presumably due to elimination of the physiological transcriptional start sites.

To test the functional significance of the putative ERE, the largest reporter gene, pGL2-STC2-667, as well as the negative control pGL2-basic and the positive control reporter pERE-Luc were transfected into T-47D cells (Fig. 4D). The cells were treated with E₂ or an equivalent volume of control vehicle to determine whether endogenous ER would induce higher than basal luciferase activity from pGL2-STC2-667 and pERE-Luc. There was no significant difference in the relative luciferase activity between the cells transiently transfected with pGL2-STC2-667 and treated with control vehicle or E₂, whereas pERE-Luc showed a fourfold induction upon E₂

treatment. We performed the same experiment with P4 and RA to determine whether non-canonical response elements for their cognate nuclear receptors might be present within pGL2-STC2-667, but we did not observe hormonal induction of luciferase activity (Fig. 4D).

Constitutive expression of STC2 in human breast cancer cells resulted in a significant impairment of cell proliferation

Having demonstrated that STC2 mRNA and protein abundance can be significantly upregulated by a variety of hormones that are known to have growth-promoting activity in different cell types (i.e. breast or endometrial cells), we next investigated whether STC2 could, in fact, modulate the growth of human cancer cells *in vitro*. Therefore, to begin to assess the function of STC2, we stably introduced pcDNA3/STC2 and pcDNA3 expression vectors into the MDA-MB-231 and MDA-MB-435 cell lines. These cell lines were chosen because they do not appear to express STC2 and our objective was to determine whether *de novo* exposure of endogenously produced STC2 could cause measurable changes in cell physiology. The expression of transgene-derived STC2 was confirmed by northern blot analysis and qPCR (data not shown). The level of STC2 secreted by stably transfected MDA-MB-231 cells was lower than that normally secreted by T-47D cells as determined by western blotting (data not shown).

Initially, the proliferation of MDA-MB-231 and MDA-MB-435 human breast cancer cells stably transfected with control or the STC2 expression vector was assessed using the alamarBlue assay, which is based upon metabolic activity. After 7 days of culture, we observed a significant reduction in the metabolic activity of STC2-expressing MDA-MB-231 (lines 5 and 3) and MDA-MB-435 cells relative to the control cell lines (Fig. 5A and B).

Parallel experiments were performed using cell counts to determine whether the change in alamarBlue fluorescence was due to a change in the metabolic activity or the reduction in the number of STC2-producing cells over time. As seen with the alamarBlue assay, a significant decrease in cell number was observed for STC2-expressing MDA-MB-231 cell lines, at day 7 compared with the control vector transfected cells (Fig. 5C). Furthermore, colony-forming assays showed that MDA-MB-231 cells expressing STC2 had significantly compromised the clonogenic capacity when compared with the control cell line (Fig. 5D).

Constitutive expression of STC2 in human breast cancer cells resulted in growth arrest and a significant decrease of cell viability after serum withdrawal

It has been well established that tumour cells are particularly resistant to a variety of cell stresses that gives them the growth advantage required to generate a solid mass and subsequently metastasise (Brahimi-Horn & Pouyssegur 2007, Moenner *et al.* 2007). The negative effect of STC2 on proliferation suggested that these lines were also compromised in their

Figure 4 Human *STC2* gene basal and hormone-inducible promoter activity in human cell lines. Three different human cell lines (HEK-293 embryonic kidney cells (A) T-47D breast cancer cells (B) and Ishikawa endometrial cancer cells (C)) were transiently transfected with molar equivalent quantities of different 5'-truncated *STC2*/luciferase reporter constructs. The number in bps (bp) for each construct indicates the size of the proximal promoter. The results are shown as the fold increase of normalised luciferase activity over the promoterless control vector, pGL2-Basic. The results for three independent experiments are depicted on each graph $\pm$ S.E.M.. (D) Hormonal responsiveness of pGL2-*STC2*-677 was tested in T-47D cells with pGL2-Basic as the negative control and pERE as the positive E₂-responsive promoter construct. E₂, RA, P4 or control vehicle (equivalent volume) treatment of cells transfected with the pGL2-*STC2*-677 construct did not result in significant induction of luciferase activity. These results represent mean $\pm$ S.E.M. of three independent experiments, each done in triplicate.

response to nutrient deprivation as a form of stress. Serum withdrawal is a well-documented condition that leads to decreased cell viability through induction of apoptosis (Xu *et al.* 2002). We observed two different patterns of response to serum deprivation (Fig. 6A and B). The control MDA-MB-231 cell line and the *STC2*-expressing lines exhibited an increase in alamarBlue fluorescence over the initial 3 days of the experiment indicating cell proliferation. However, this increase was significantly lower in the *STC2*-expressing lines compared with the control cell line at day 3, 5 and 7. Moreover, while the control cell line maintained a constant level of metabolic activity, both *STC2*-expressing lines showed a significant decrease from day 3 to day 7. For *STC2*-expressing line 5, this resulted in day 7 values significantly below those generated by the initial number of cells seeded (day 0) for these experiments (Fig. 6A). For line 3, the alamarBlue activity at day 7 was not significantly

different from day 0, which may be related to the lower level of *STC2* production by this line (Fig. 6B).

Ectopic expression of STC2 resulted in compromised cell motility

Certainly, one of the most lethal characteristics of an aggressive cancer phenotype is the ability of these cells to move through dense tissue, extravasate and form new tumours at distant locations (Kedrin *et al.* 2007, Sahai 2007). To test the effects of *de novo* expression of *STC2* on the motility of cancer cells, we utilised the conventional monolayer scratch-wound assay (Fig. 6C and D). The *STC2*-expressing cells (line 5 and line 3) migrated less efficiently into the wound-cleared area compared with control cells (line 6), during the first 24 h after the injury (Fig. 6C). However, this difference was not maintained after another 24 h where all cells lines had filled the gap (Fig. 6D). The results of several alamarBlue and cell-counting

Figure 5 Constitutive STC2 expression in the MDA-MB-231 and MDA-MB-435 cancer cell lines reduces cell proliferation. (A) Analysis of proliferation/viability of human breast cancer MDA-MB-231 cells stably transfected with pcDNA3/STC2 (lines 5 and 3) or pcDNA3 control vector (line 6) using the alamarBlue assay. Bars represent mean $\pm$ S.E.M. of three independent experiments done in quintuplicate. (B) The same experiment performed with MDA-MB-435 cells stably transfected with pcDNA3/STC2 showing a similar decrease in proliferation after 7 days in the cultures producing STC2. (C) An assessment of MDA-MB-231 cell growth by cell counting. Data represent means $\pm$ S.E.M. of three independent experiments done in quadruplicate confirming the alamarBlue assay results. (D) A representative quantitative analysis of clonogenic assay indicating significantly reduced colony formation by MDA-MB-231 cells expressing STC2 (lines 5 and 3) in comparison with control cell line 6. Bars represent means $\pm$ S.E.M. of three independent experiments done in quadruplicate. Significant differences (indicated by asterisks) between groups were determined by Student's *t*-test and one-way ANOVA ($P < 0.0001$).

experiments showed no significant difference in cell proliferation between MDA-MB-231 STC2-expressing and non-expressing cells during the first 2 days of culture. These data support the notion that the difference in gap closure was due to impaired migration caused by STC2 expression and not due to a difference in proliferation.

Discussion

Our studies of STC2 regulation and function in human tumour cell lines are based on the fact that it is an extracellular messenger produced by cancer cells and upregulated by hormones that can regulate proliferation, suggesting that STC2 can indirectly influence tumorigenesis. The objective of this study was to determine whether STC2 gene expression

was linked to hormones that can positively regulate cancer cell growth and whether *de novo* STC2 expression could alter the growth properties of breast cancer cells *in vitro*.

STC2 expression in breast cancer

Our northern blot analysis revealed STC2 expression in ER-positive as well as ER-negative human breast cancer cell lines in contrast to Charpentier *et al.* (2000). Clearly, STC2 expression is not exclusively dependent on ER signalling in breast cancer cells and may have different effects in ER-positive and -negative cells. We also analysed human breast RNA for STC2 expression. Previously, STC2 expression data were derived from commercially available northern blots that utilised polyA⁺ enriched mRNA and did not include human breast tissue (Chang & Reddel 1998, DiMattia *et al.* 1998, Moore *et al.* 1999). Our data agree with previously published data showing several STC2 transcripts in human tissue and cell line RNAs (Chang & Reddel 1998, DiMattia *et al.* 1998, Moore *et al.* 1999) likely arising from the use of alternative polyadenylation signals in the > 3 kb 3'-untranslated region of the mRNA as predicted by Aceview (Thierry-Mieg & Thierry-Mieg 2006). Those earlier experiments indicated that STC2 expression in human tissues is low; therefore, it was surprising to find that all three species of STC2 mRNA were detectable by northern blot in normal human breast and breast tumour tissue RNAs. Therefore, it would appear that breast is a major site of STC2 expression implying that it plays a physiological role in the mammary gland.

Given the high level of STC2 expression in human breast tissue and breast cancer cell lines, we investigated its expression in normal and tumorigenic mouse mammary gland. This was important because of the potential utility of mutant mouse mammary tumour models to study the role of STC2 in cancer. Unlike human breast tissue, *Stc2* expression was not detectable in normal mouse mammary gland RNA using northern analysis. However, we readily detected the expression of *Stc2* in MMTV/ERBB2/Neu and MMTV/PyVMT transgenic mammary tumours. This suggests that signalling mechanisms involved in the tumorigenic pathways initiated by the above transgenes regulate *Stc2* expression. Whether or not STC2 plays a positive or negative role in this process requires further investigation with *Stc2* null and STC2-overexpressing mouse strains (Varghese *et al.* 2002, Chang *et al.* 2005, Gagliardi *et al.* 2005). A model of familial adenomatous polyposis is the only other mouse tumour model that showed elevated *Stc2* expression specifically in adenomatous lesions, which correlated with the STC2 expression in human colorectal cancers with *Apc* gene mutations (Andreu *et al.* 2006). Our data imply that specific transgenic murine models of mammary gland tumour formation may be helpful in understanding the role of STC2 in cancer and that the molecular mechanisms controlling tumour cell STC2 expression may be conserved between mouse and human cells.

Figure 6 Constitutive expression of STC2 in MDA-MB-231 cells significantly decreased the cell viability after serum withdrawal and negatively affected cell movement. STC2-expressing and control vector-transfected MDA-MB-231 cell line viability was determined by alamarBlue assay at the indicated time points. Over the 7 days in culture, a significant decrease in cell viability was observed for STC2-expressing line 5 (A), and line 3 (B) compared with the control cell line. The viability of the control cell line (line 6) was not significantly affected by serum deprivation over the assay period. Asterisks indicate statistically significant differences between the control cells and the STC2 expressing by one-way ANOVA ($P < 0.0001$). (C) The scratch-wound assay was used to assess the effect of STC2 expression on random cell movement. The MDA-MB-231 lines expressing STC2 closed the gap less efficiently than the control cell line at the 24-h time point. Data are presented as the percentage of area populated by cells between 0 and 24 h $\pm$ S.E.M. from four independent experiments done in octuplicate. (D) Representative images of scratch-wound area at different time points showing that by 48 h all three lines had completely filled the gap.

Hormonal regulation of STC2 and STC1 expression in human cancer cell lines

Others have reported the induction of *STC2* expression in response to E_2 treatment in human breast cancer cells (Charpentier *et al.* 2000, Bouras *et al.* 2002). Our results revealed that *STC2* represents a downstream target of E_2 , P4 and RA signalling pathways in human breast cancer cell lines (MCF-7, T-47D) and a human endometrial cancer cell line (Ishikawa). We observed some differences in the level of *STC2* induction

and in the temporal pattern of expression amongst the different cell lines. For example, P4 treatment of MCF-7 or T-47D cells did not result in strong upregulation of *STC2* steady-state mRNA levels, whereas a significant induction was seen in the Ishikawa cells. This could be due to the fact that nuclear receptor-mediated gene expression can be differentially modulated in the different types of human cancer cells under identical hormone treatments due to cell-specific differences in the level of nuclear receptor co-regulators (Hyder *et al.* 1998, Magklara *et al.* 2000, Liang *et al.* 2005). Additionally, we found

that dexamethasone had no effect on the STC2 mRNA levels suggesting that STC2 is not under the control of this hormone, at least not in these cell types. This is not the case for *Stc1*, which showed potent downregulation when exposed to glucocorticoids in a murine pituitary cell line (Groves *et al.* 2001). Interestingly, none of the hormonal treatments applied in this study influenced *STC1* expression in Ishikawa, MCF-7 or T-47D cells. This agrees with Frasor *et al.* (2003) and Katzenellenbogen & Frasor (2004) where they showed that *STC1* was actually downregulated by E₂ in MCF-7 cells.

Collectively, our results indicate that STC2 is a hormonally responsive gene in breast cancer and non-breast cancer cell lines implying that it plays a general function in cell homeostasis that is associated with a variety of nuclear receptor signalling pathways. In addition, the lack of STC1 and STC2 co-regulation in our studies may indicate that STC1 and STC2 play distinct roles in human tumour cells with STC2 function linked to nuclear receptor signalling.

Transcriptional regulation of STC2 expression by oestrogen and RA

We used chemical inhibitors of transcription or translation to determine whether *STC2* was a direct E₂ or RAR target gene in breast cancer cells. The transcriptional block eliminated the upregulation of STC2 mRNA abundance at the 3-h time point; however, we also observed that blocking protein translation also diminished E₂-induced STC2 expression in two different breast cancer cell lines. Based on Dean & Sanders' (1996) proposal, steroid-responsive genes can be classified into three main categories: primary response genes, delayed primary response genes and secondary response genes. The primary responsive genes interact with steroid receptors directly and their response usually occurs within minutes after exposure to steroids. The secondary responsive genes generally represent downstream targets of regulatory proteins synthesised by primary responsive genes. They exhibit a time lag in response to steroid treatment usually measured in hours. Our time-course experiments showed that multi-fold upregulation of STC2 mRNA levels, upon E₂, RA or P4 treatment, occurred with a time lag between 3 and 24 h. Based on the above discussion, and taking into consideration the findings of Carroll *et al.* (2006), it is likely that *STC2* represents an E₂ secondary responsive gene and that E₂ regulates *STC2* expression at the transcriptional level through ER, but this also requires intermediary proteins (i.e. newly synthesised co-factors).

To further investigate whether *STC2* was a direct target for the E₂, P4 and RA receptors, we examined the *STC2* gene for the presence of canonical DNA-binding sites for these nuclear receptors. One putative ERE was identified; however, this sequence (5'-AT-GGTCG-AAA-GGACT-GT-3') differs from the consensus core ERE (5'-CA-GGTCA-nnn-TGACC-CT-3') at three nucleotide positions in both arms of the consensus sequence. Several studies have shown that nucleotide alterations from the core sequence on each side of an ERE palindrome, results in a significant decrease in

ER-binding affinity and transcriptional activation (Klinge 2001). This lack of sequence identity with a canonical ERE does not preclude direct ligand-activated ER regulation as demonstrated for the imperfect ERE present in the proximal promoter of the human *WISP2* gene (5'-GGTCA-CAC-CCACC-3') (Fritah *et al.* 2006). Therefore, we directly tested the hormone inducibility of the *STC2* proximal promoter region; however, E₂, RA or P4 treatments did not induce the *STC2* promoter. These data suggest that the putative ERE, we identified in the proximal promoter region is not responsive to E₂ and that putative *dis*-active regulatory elements responsive to RA and P4 are not present within the *STC2* proximal promoter region, lending support to the hypothesis that regulation of STC2 by these hormones is indirect. Moreover, we did not uncover perfect or imperfect response elements for the E₂, P4 and RA receptors in other regions of the *STC2* gene suggesting that these hormones function indirectly to regulate *STC2* expression. This is not surprising, given that Kwon *et al.* (2007) found that only 6% of E₂-regulated genes were bound by ER in the proximal promoter region. In addition, a genome-wide screen for ER-binding sites in MCF-7 cells found that the majority of the high-confidence sites are located outside of proximal promoter regions and they did not identify a functional ERE within 100 kb of the *STC2* gene (Lin *et al.* 2007). Therefore, it seems likely that for E₂ regulation of the *STC2* gene, other transcription factors may be mediating the effect as has been documented for the Sp1, AP-1 and nuclear factor-κB (Chambliss & Shaul 2002, Wintermantel *et al.* 2005, McDevitt *et al.* 2007). Therefore, functional identification of hormone-responsive regions of the *STC2* gene will require a systematic testing of the entire locus through a combination of transfection assays coupled with ChIP-chip assays.

Constitutive expression of STC2 in human breast cancer cells alters cell growth

Given that *STC2* expression can be significantly increased in cancer cells by a variety of growth-promoting hormones, we asked fundamental questions regarding the role of STC2 in breast cancer cell biology. As demonstrated by alamarBlue assay, cell-counting and colony-forming assay, *de novo* production of STC2 by MDA-MB-231 and MDA-MB-435 breast cancer cells resulted in a significant impairment of cell growth. It is important to note that the level of STC2 produced by the stably transfected cells was substantially lower than produced by T-47D cells indicating that overexpression of the protein is not likely responsible for the growth impairment effect of STC2. This is the first study to show that STC2 can exert a growth-suppressive effect on human breast cancer cells or any cell type *in vitro*. Recently, Gagliardi *et al.* (2005) demonstrated that constitutive expression of human STC2 in transgenic mice, results in a significant reduction of intramembranous and endochondral bone development, as well as high neonatal morbidity, suggesting that STC2 can act as a potent growth inhibitor *in vivo*. The mechanism responsible for the STC2 growth inhibitory effect is not known, although it seems to be

independent of GH/IGF I axis and may be linked to a metabolic disturbance (Gagliardi *et al.* 2005). Whether STC2 is having a negative effect on MDA-MB-231 cell metabolism and thus reducing cell proliferation will require further study to evaluate ATP production and mitochondrial function.

Our results are in contrast to those of Ito *et al.* (2004) since they reported that STC2 overexpression resulted in selective protection of HeLa cells against endoplasmic reticulum stress-induced cell death. A significant decrease in cell death was observed in STC2-overexpressing HeLa cells treated with thapsigargin, while STC2 overexpression did not affect tunicamycin-treated HeLa cell viability (Ito *et al.* 2004). These findings suggest that STC2 expression is differentially responsive to stress stimuli and that the activation of distinct signalling pathways could implicate STC2 in a cytoprotective or pro-apoptotic role. Collectively, our data suggest that STC2 expression could be detrimental to cell viability *in vitro* and that human breast cancer MDA-MB-231 cells may be a good model to study its mechanism of action.

In summary, our data indicate that STC2 expression can be induced through a variety of nuclear receptor signalling pathways in different cell types through an indirect mechanism. Functionally, *de novo* STC2 production in aggressive human cancer cell lines decreases cell viability. This coupled with clinical data indicating that loss of STC2 expression positively correlates with a more aggressive breast tumour phenotype suggests that STC2 may act as an autocrine or paracrine brake on cell growth and its expression is associated with a more differentiated phenotype (Iwao *et al.* 2002, Yamamura *et al.* 2004, Essegir *et al.* 2007).

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