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

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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 promotor 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.


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 E2 and that STC1 expression is unaffected. We also demonstrate that Stc 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 1X 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 10−12 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 northerns 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’Urfe, 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 1382 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-SC2-667 plasmid and molar equivalents of pGL2-SC2-337, pGL2-SC2-172, pGL2-SC2-112, pGL2 basic and pBKSI. Co-transfection with a molar equivalent of CMV-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 responsivity of pGL2-SC2-667 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 STC2 performed 24 h later. The hormonal responsivity of pGL2-SC2-667 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 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. The fluorescence measurement was obtained at 580 nm in a Wallac Victor^{2–1420} 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 x 10^3 cells per well in quadruplicate. The cells were trypsinised, stained with trypan blue and counted at day 3 and 7 using a haemocytometer.

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 x 10^5 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.uhrresearch.ca/facilities/wcif/idownload.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 E2, implying that E2 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 E2, 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 E2 (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 E2 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 E2 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 E2 and RA induced mean STC2 mRNA steady-state levels (mean 12- and 21-fold respectively) (Fig. 2). P4 and
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 E2, 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).
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 antioestrogen ICI 182 780 (ICI). This compound is able to inhibit oestrogen-induced transcriptional activation by competing with E2 for binding to ER but inhibiting homodimerisation and 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 a 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 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 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).

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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://sdiml.org.sg/ERE-V2/index), we analysed this region for putative ERE sequences. One putative imperfect ERE sequence (5’-AT-GGTGC-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 give 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
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 experiments.
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′ 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, St2 expression was not detectable in normal mouse mammary gland RNA using northern analysis. However, we readily detected the expression of St2 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 St2 expression. Whether or not STC2 plays a positive or negative role in this process requires further investigation with St2 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 St2 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.
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$_2$, P$_4$, 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

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 octoplicate. (D) Representative images of scratch-wound area at different time points showing that by 48 h all three lines had completely filled the gap.
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 Stt1, 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 RA 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 ER response was identified; however, this sequence (5'-AT-GGTC-GAA-GGACT-GT-3') differs from the consensus core ER response sequence (5'-CA-GGTC-nnnTGACC-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-CACCCACC-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, which we identified in the proximal promoter region is not responsive to E2 and that putative cis-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, Wintertmant 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, Esseghir et al. 2007).

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