Increasing the relative expression of endogenous non-coding Steroid Receptor RNA Activator (SRA) in human breast cancer cells using modified oligonucleotides

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ABSTRACT

Products of the Steroid Receptor RNA Activator gene (SRA1) have the unusual property to modulate the activity of steroid receptors and other transcription factors both at the RNA (SRA) and the protein (SRAP) level. Balance between these two genetically linked entities is controlled by alternative splicing of intron-1, whose retention alters SRAP reading frame. We have previously found that both fully-spliced SRAP-coding and intron-1-containing non-coding SRA RNAs co-exist in breast cancer cell lines. Herein, we report a significant (Student’s t-test, \( P < 0.003 \)) higher SRA–intron-1 relative expression in breast tumors with higher progesterone receptor contents. Using an antisense oligoribonucleotide, we have successfully reprogrammed endogenous SRA splicing and increased SRA RNA–intron-1 relative level in T5 breast cancer cells. This increase is paralleled by significant changes in the expression of genes such as plasminogen urokinase activator and estrogen receptor beta. Estrogen regulation of other genes, including the anti-metastatic NME1 gene, is also altered. Overall, our results suggest that the balance coding/non-coding SRA transcripts not only characterizes particular tumor phenotypes but might also, through regulating the expression of specific genes, be involved in breast tumorigenesis and tumor progression.

INTRODUCTION

The Steroid Receptor RNA Activator (SRA) was first identified in 1999 as a non-coding RNA specifically co-activating steroid receptors (1). Through mutation analysis, the authors unequivocally showed in their original report that the core region, corresponding to sequences encompassing exons 2 to 5 (Figure 1A), was necessary and sufficient for SRA to act as co-activator (1). Several studies have since shed light on SRA’s mechanisms of action [reviewed in (2)]. Briefly, it is believed that SRA acts embedded in ribonucleo-protein complexes recruited to the promoter of regulated genes. These complexes may contain positive regulators, such as the steroid receptor co-activator 1 (SRC-1), the DExD/H box family of RNA-helicase members p68 and p72, or the pseudouridine synthases Pus1p and Pus3p. Negative regulators, such as SMRT/HDAC1 Associated Repressor Protein (Sharp) and the recently identified SRA stem-loop interacting RNA binding protein (SLIRP), can also interact with SRA to decrease its activity (3,4).

If SRA was originally thought to specifically increase the activity of steroid receptors, further data have subsequently demonstrated that this RNA can also co-activate non-steroid nuclear receptors as well as other transcription factors such as MyoD (2). SRA has therefore a wider role than first anticipated and likely participates in signalling pathways still to be uncovered.

Additional SRA transcripts, almost identical to the original SRA and containing a full core sequence, have now been described (Figure 1B). As a result of gene polymorphism (5) they can however contain point mutations in exon-2 (SRA2) or an additional codon in exon-3 (SRA3). These transcripts can be divided in two categories: coding and non-coding SRAs. Coding isoforms have an extended exon-1, which contains two translation initiating methionine codons (Figure 1B). These coding SRAs have been proven to encode an endogenous SRA protein (SRAP) in several tissues including breast,
prostate and muscle (5–8). Even though the exact functions of SRAP remain to be fully elucidated (2), independent reports suggest that this protein also regulate steroid receptor signalling, potentially as co-activator (8,9), or as a repressor (10,11).

Non-coding SRA transcripts, generated through alternative splicing of intron-1, have also been characterized (2,12). These transcripts contain either a full (FI) or a partial (PI) intron-1 sequence (Figure 1B). The partial intron retention results from the use of an alternative 3' acceptor site located 60 bp upstream of exon-2 (12). Non-coding SRAs can also result from the concomitant use of an alternative 5' donor site (located 15 bp upstream of the end of exon-1) and the alternative 3' acceptor site (AD). All these different splicing events, which occur independently of the isoform considered (i.e. SRA1, SRA2 or SRA3), either shift SRAP open reading frame or introduce premature stop codons. The resulting alternatively spliced SRA RNAs are therefore unable to encode for SRAP.

It should be stressed that both fully- and alternatively-spliced SRA transcripts contain the functional core sequence. They can therefore act as transcriptional co-activators. The additional ability of fully-spliced SRA RNAs to encode for SRAP creates a peculiar level of functional complexity to the products of the SRA1 gene. We have shown that both coding and non-coding SRA coexist in breast cancer cells (12). Interestingly, their relative expression varies between breast cancer cells lines with different phenotypes (12). In particular, we have observed that invasive breast cancer cell lines (MDA-MB-231, MDA-MB-468) expressed higher relative levels of non-coding SRA than ‘closer to normal’ MCF-10A1 breast cells. This suggests that a balance ‘tipped’ toward the production of non-coding SRA RNA in breast cells might be associated with growth and/or invasion properties. This further raises the possibility that modifying the balance between these transcripts could trigger meaningful events in breast cancer cells.

To further explore the potential relevance of the balance coding/non-coding SRAs in breast cancer, we have herein investigated SRA–intron-1 relative expression in a small cohort of invasive breast tumors. Using an antisense oligoribonucleotide designed to target SRA exon-1/intron-1 junction, we have also assessed our ability to artificially alter the balance coding/non-coding SRA transcripts in T5 breast cancer cell line.

MATERIALS AND METHODS

Cells and tumor tissues

T5 breast cancer cells were kindly provided by Murphy (13). Cells were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (CANSERA, Rexdale, ON), penicillin (100 units/ml), streptomycin (100 μg/ml) (GIBCO, Grand Island, NY) and 0.3% glucose. Cells were grown in a 37°C humidified incubator with 5% CO2.

Thirty-two tumor samples, with a wide range of estrogen receptor (ER, from 6.3 to 247 fmol/prot, median = 82.5 fmol/prot) and progesterone receptor (PR, from 12.2 to 444 fmol/prot, median = 38.5 fmol/prot) levels, were selected from the Manitoba Breast Tumor Bank (14), which operates with the approval of the Faculty of Medicine, University of Manitoba, Research Ethics Board.

Oligonucleotide treatment

2′-O-Methyl-oligoribonucleotide phosphorothioate 20-mers anti-sense to the 5′-splice site of SRA intron-1 (SRA–AS, 5′-ACCCGGCUUACGUACACGU-3′) and to the 5′-splice site of a modified β-globin intron (βgl–AS, 5′-ACCUGCCAGGCCCUCACCA-3′) were synthesized and purified by Trilink Biotechnologies, Inc. (San Diego, CA). Fluorophore (Indocarbocyanine-Cy3 and 5-Carboxyfluorescein_FAM) conjugated versions (SRA–AS–Cy3 and βgl–AS–FAM) were also obtained from Trilink Biotechnologies, Inc. Transfections were performed using DMRIE-C reagent (16 μg/ml; Invitrogen, Carlsbad, CA) according to the manufacturer’s directions using concentrations ranging from 0 to 0.5 μM. It should be noted that when no oligonucleotide was added (mock), cells were also treated with DMRIE-C reagent. Five hours post-treatment, transfection medium was replaced with fresh medium, and cells were cultured for the indicated times. For oligonucleotide and minigene co-transfections, T5 cells were plated at 3.5 × 104 cells per well in 6-well plates and co-transfected with 1.65 g of SRA minigene and 0.05, 0.1 or 0.5 μM oligoribonucleotides using DMRIE-C reagent (16 μg/ml, Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Medium containing the transfection complex was replaced by DMEM containing 5% FBS 5 h post transfection.

Fluorescent microscopy

Cells were cultured on coverslips and transfected with SRA–AS–Cy3 or βgl–AS–FAM at the indicated concentrations and times post-treatment. Coverslips were then briefly washed with PBS and cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Cover-slips were then rinsed with PBS and cells permeabilized with 0.2% Triton-X100 in PBS for 1–2 min prior to staining nuclei with 1 μg/ml 4′,6-diamidino-2-phenylindole-dihydrochloride (Dapi). Coverslips were mounted with FluorSave™Reagent (Calbiochem, La Jolla, CA). Fluorescent images were captured with an Eclipse E1000 epifluorescent microscope (Nikon), digitized with ACT-1 software (v.2.63; Nikon) and merged images were generated with Photoshop 6.0 (Adobe).

RT–PCR and triple-primer-PCR (TP-PCR)

Total RNA was extracted from cells or frozen tumor tissue sections using Trizol™ reagent (Gibco BRL, Grand Island, NY) and subjected to DNase treatment (Promega, Madison WI) as previously described (12,15). Half a microgram of total RNA was reverse transcribed in a final volume of 30 μl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously reported (5). One and a half microliters of reverse-transcription mixture was amplified in a final
volume of 15 µl in the presence of 60 mM Tris–HCl (pH 8.5), 15 mM [NH₄]₂SO₄, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 4 ng/µl of each primer (two or three primers for RT–PCR and TP-PCR, respectively), 1 unit of Platinum Taq DNA polymerase (Invitrogen Carlsbad, CA) and 10 nM α³²P dCTP. Each PCR consisted of a 5 min pre-incubation step at 94°C followed by 30 cycles of amplification (30 s at 94°C, 30 s at 60°C and 30 s at 72°C). The sequences of primers used are detailed in Supplementary Table S1.

Radio-labeled PCR products were then separated on denaturing poly-acrylamide/urea gels as previously described (15). Following electrophoresis, the gels were dried and exposed 30 min to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA), which allows the subsequent quantification of each observed signal.

The identity of all PCR products obtained has been confirmed following subcloning and sequencing as described previously (12,16).

Quantification and normalization of PCR signals
PCR signals were quantified using a Molecular Imager™-FX (Bio-Rad, Hercules, CA) as previously described (15). For each TP-PCR experiment (Figures 2 and 4), the relative amount of fully spliced and intron-1 retained SRA of each sample was first expressed as the percentage of the total signal measured following amplification (12). For tumor cases (Figure 2), the percentage of intron-1 retained signal was then divided by the average intron-1 retained percentage observed in T5 cells always analyzed in parallel (arbitrary unit). For Figure 2C, each dot represents the average of three independent experiments. Differences between tumor subgroups were tested using the Student’s t-test (two-tailed). For Figure 4, the average obtained in Mock transfected cells was used as reference (corresponding to 1 a.u.). For each time and experiment, differences with the signal obtained in mock cells were tested using the Student’s t-test (two-sided).

Western blot analysis
Total cell lysates were analyzed and SRAP was detected using rabbit anti-SRAP 743 (Bethyl Laboratories Inc, Montgomery, TX) antibody in conjunction with a goat anti-rabbit HRP (Sigma, St Louis, MO) antibody at dilutions of 1/1000 and 1/3000, respectively, as described (6).

Real-time PCR
All PCR reactions were performed in an iCycler iQ™ Real-Time PCR Detection System (Bio-rad). For each PCR run, a master-mix was prepared on ice containing 2.5 mM MgCl₂, 2.5 mM each dATP, dCTP, dGTP and dTTP, 0.1 µg each primer, 0.2x SYBR®Green1 (Molecular Probes, Inc.), 5 nM Fluorescein, 2.5% DMSO, 1x PCR Reaction Buffer (Invitrogen, Carlsbad, CA) and 1 unit of Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA) (Figure 5). Two and a half microliters of each RT sample was added to 40 µl of PCR master-mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, then 40 cycles at 95°C for 30 s, 60°C for 45 s and 72°C for 30 s. Primers used are detailed in Supplementary Table S1. Three experiments were performed. For each treatment and experiment, the number of cycles needed to reach the defined cycle threshold for fully spliced and intron-1 alternatively spliced was first corrected for the number of cycles leading to the average 36B4 threshold cycle to obtain a normalized cycle number. The average number of normalized cycles for intron-1 retained and fully spliced RNA from the three experiments performed with no oligo (Mock) was then calculated to obtain intron-1 average mock and fully spliced average mock. For each treatment and experiment, the normalized cycle number of intron-1 retained and fully spliced SRA RNA was then subtracted from intron-1 average mock and fully spliced average mock, respectively, to get the number of cycle difference between treatments (ΔCt) and establish the standard deviations. A ΔCt of +1 represents a 2¹ = 2 fold increase in expression, whereas a ΔCt of −2 represents a 2² = 4-fold decrease in expression. Differences between Mock and oligo treatments were tested using the Student’s t-test (two-sided).

Real-time PCR-array
Half a microgram of RNA was reverse transcribed using ReactionReady™ First Strand cDNA synthesis Kit (SuperArray Bioscience Corp., Frederick, MD) and applied to Breast Cancer and Estrogen Receptor RT²Profiler™ PCR arrays (SuperArray Bioscience Corp., Frederick, MD) as detailed by the manufacturer (Figures 7 and 8). For Figure 7, four experiments were performed. For each treatment (SRA–AS and βgl–AS), gene and experiment, the number of cycles needed to reach the defined threshold was normalized to the β-actin signal (ACTB) to obtain the corrected cycle numbers. For each gene the corrected cycle number per SRA–AS treatment was then subtracted from the reference gene–βgl–AS treatment to get the number of cycle difference between treatments (ΔCt) and establish the standard deviations. Differences between gene expression upon SRA–AS and βgl–AS treatments were tested using the Student’s t-test (two-sided). Levels of expression presented in Table 1 were calculated as follow. If ΔCt was higher than or equal to 0, expression corresponds to 100 x 2ΔCt whereas when ΔCt is lower than 0 (i.e. negative), expression corresponds to 100/2−ΔCt.

For Figure 8, cells were treated with SRA–AS or βgl–AS as indicated earlier. Twenty-four hours post treatment, medium was changed to medium without serum for an additional 24 h. Cells were then treated with E2 10⁻⁸ M or vehicle alone (ethanol 1/1000) for 4 h before extraction of total RNA and SuperArray analysis. Three experiments were performed. For all treatment (SRA–AS and βgl–AS with Ethanol or E2), gene and experiment, the number of cycles needed to reach the defined threshold was first normalized to the β-actin signal (ACTB) to obtain the corrected cycle numbers. For each gene, the corrected cycle number following E2 treatment was then subtracted from the Ethanol treatment to get an arbitrary measurement of E2 action: A ΔCt(Eth/E2) = 1 corresponds to
2^1 = 2-fold increase in expression, whereas a ΔCt(Eth/E2) = -1 represents a 2^1 = 2-fold decrease in expression. Differences between gene expressions upon treatments were tested using the Student’s t-test (two-sided).

**Cell proliferation assay**

Cell viability/proliferation was measured using CellTiter 96® Aqueous One Solution Reagent (Promega Co., Madison, WI) as previously described (17). Briefly, T5 cells were treated as described earlier with anti-sense oligos or not (mock). Twenty-four hours later, cells (3 × 10^5 cells) were re-seeded in 96 wells dishes in 5%FBS–DMEM. Twenty-four hours after re-seeding (48 h post oligo treatment), T0 measurements were taken. Media was changed every two days after T0. All time point measurements were taken by adding, at various times (T0, 1, 3, 5 days), 40 μl of CellTiter 96® AQuEous One Solution Reagent to each well containing 200 μl medium, followed by incubation for 3 h at 37 °C. The absorbance was then recorded at 490 nm with 96-well plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA.) All time point measurements were first blanked to the appropriate media not exposed to cells. For each experiment (performed in quadruplicate) the average of absorbance at T0 was calculated and deduced from each experimental points. For each point, the resulting mean of two independent experiments (expressed as Viability, a.u) and standard deviations were plotted using Prism 5 (GraphPad software, Inc, La Jolla, CA). Differences were tested using the Student’s t-test (two-sided).

**RESULTS**

**Higher SRA–intron-1 relative expression in breast tumors with higher progesterone receptor contents**

Thirty-two different human breast tumors were selected from the Manitoba Breast Tumor Bank. These tumors spanned a wide range of estrogen receptor alpha (ER) and progesterone receptor (PR) levels, as assessed by ligand binding assay. Total RNA was extracted from frozen tumor tissue sections, reverse-transcribed and the relative expression of non-coding SRA transcripts containing intron-1 analyzed by radioactive TP-PCR, as described in the ‘Materials and Methods’ section. TP-PCR, which relies on the use of three primers during the PCR reaction, has been previously validated to assess the relative proportion of transcripts sharing a common extremity but differing in the other (12,16,18). As illustrated Figure 2A, the lower primer (primer E) recognizes the common exon 3 sequence, whereas the upper primers (C and D) are designed to recognize intron-1 and exon-1 sequence, specific of non-coding and coding SRA transcripts, respectively. Two PCR products (377–380 and 360–363 bp), corresponding respectively, to SRA transcripts containing intron-1 sequences (FI, PI and AD in Figure 1B) or fully spliced (FS), are expected. The potential 3 bp size difference corresponds to the additional codon specific for SRA3 isoform and located in the amplified part of exon-3 analyzed (Figure 1B). PCR products corresponding to both coding and non-coding transcripts were detected in all samples analyzed (Figure 2B). For each sample, signals were quantified and the relative proportion of SRA intron-1 containing RNA was expressed in arbitrary unit (a.u, Figure 2C). Relative level of this transcript varies from one case to another (min: 0.71 a.u, max: 1.61 a.u, median 1.10 a.u). No difference was found in the relative expression of non-coding SRA when comparing cases with low ER (n = 16, ER < 82.5 fmol/mg prot., intron-1 median: 1.04 a.u) and high ER (n = 16, ER > 82.5 fmol/mg prot., intron-1 median: 1.14 a.u).

In contrast, a significant (Student’s t-test, two-sided, P < 0.003) higher intron-1 retention was observed in samples with high PR (n = 16, PR > 38.5 fmol/mg prot., intron-1 median: 1.27 a.u.), than with low PR (n = 16, PR < 38.5 fmol/mg prot., intron-1 median: 1.02 a.u).

**Altering intron-1 splicing events using a modified anti-sense oligoribonucleotide**

Reprogramming splicing events through transfection of modified antisense oligoribonucleotides targeting exon–intron junctions to interfer with donor or acceptor splicing sites has been successfully achieved in different systems (19,20). We hypothesized that an oligonucleotide complementary to the junction exon-1–intron-1 could reprogram the fate of pre-messenger RNA and lead to the production of more alternatively spliced non-coding SRA transcripts Figure 3.
To establish proof of principle that such an approach might be suitable to block SRA intron-1 donor site and ultimately alter SRA intron-1 splicing events, we initially used a previously described artificial SRA–β-globin minigene model (12). Two different 2′-O-methyl-modified antisense oligoribonucleotide phosphorothioate 20-mers were designed to recognize the splice donor sites of β-globin (βgl–AS) and SRA intron-1 (SRA–AS), present on this minigene (Supplementary Figure S1). We first established that both SRA–AS and βgl–AS similarly entered and remained in T5 cells (Supplementary Figure S2). We then demonstrated that SRA–AS oligoribonucleotide, as opposed to βgl–AS, had the ability to interfere with SRA intron-1 donor site and promoted SRA intron-1 retention within transcripts originating from our artificial SRA minigene (Supplementary Figure S3).

The proportion of endogenous SRA transcripts retaining intron-1 is increased following treatment of T5 cells with SRA–AS

To further determine if the introduction of SRA–AS could also alter the balance of endogenous coding and non-coding SRA RNAs, dose and time dependence experiments were performed and the relative proportion of SRA intron-1 retention assessed by triple-primer PCR (TP-PCR). T5 cells were transfected with increasing concentrations of either SRA–AS or βgl–AS oligoribonucleotides and total RNA analyzed by TP-PCR as described earlier. Co-amplification of two products, migrating at an apparent size of 377 and 360 bp, occurred (Figure 4A). These products were found, following sequencing, to correspond to the expected non-coding (containing intron-1) and coding (fully spliced) SRA transcripts. No products were amplified when RNA was not reverse-transcribed, confirming that no DNA could possibly be amplified in this assay. Signals were quantified and SRA intron-1 relative levels expressed in arbitrary unit as described in the ‘Materials and Methods’ section. A dose-dependent shift towards more intron-1 retention was observed in T5 cells when treated with SRA–AS. At the highest concentration tested of 0.5 μM, which corresponded to a 100% transfection efficiency (Supplementary Figure S2), SRA–AS led to an approximate 4-fold increase (2.5 ± 0.6 SD) in relative intron-1 retention at t: 24h upon SRA–AS

Figure 2. TP-PCR amplification of coding and non-coding SRA mRNAs in breast cancers. (A) Principle: three primers are used during the PCR amplification. The lower primer (E) is common to the two sequences whereas, the upper D and C primers are specific for exon-1 (gray) and intron-1 (black), respectively (12). Triple-primer PCR will lead to an intron-1 or Fully spliced (FS) products, corresponding to non-coding and coding SRA RNAs, respectively. (B) PCR products, run on an acrylamide gel, are visualized using a Biorad Phosphorimager. (C) Signals from three experiments have been quantified and the average relative proportion of non-coding SRA RNA expressed in arbitrary unit (intron-1 a.u) as detailed in the ‘Materials and Methods’ section has been graphed for each tumor. For analysis purposes, tumors have been grouped according to their ER levels: lower and higher than the median; or according to their PR levels: lower and higher than the median. The horizontal bar indicates the median of intron-1 values for each subgroup. Differences have been tested using the Student’s t-test two-tailed.

Figure 3. Principle: an oligonucleotide complementary to the junction exon-1/intron-1 is designed to interfere with the proper splicing of SRA pre-messenger. As a result, a higher proportion of alternatively spliced transcripts (containing Full—FI- or partial—PI-intron-1 retention, or resulting from the use of an alternative donor site (AD) as defined Figure 1) relative to the fully spliced (FS) should be observed.

Figure 4A: 0.5 μM SRA–AS, as compared to mock transfected control (Figure 4A, 0). As expected, no effect was observed on the proportion of endogenous intron-1 retaining SRA RNAs when cells were transfected with equivalent amounts of βgl–AS oligoribonucleotides (Figure 4A, 0.05 μM βgl–AS). βgl–AS oligonucleotides have been used as negative controls in subsequent experiments.

Time course experiments similarly performed with no oligoribonucleotides (Mock), 0.5 μM SRA–AS or 0.5 μM βgl–AS oligoribonucleotides, revealed a significant (n = 5, p < 0.05, Student’s t-test) increase (2.5 ± 0.6 SD fold) in relative intron-1 retention at t: 24h upon SRA–AS
A maximal 3.5-fold average increase in the relative level of intron-1 retention was observed at \( t \): 48 h (Figure 4B, 48 h SRA–AS) over that observed for time-matched mock transfected cells. This effect is maintained, albeit decreased at \( t \): 72 h when levels of intron-1 retention have returned to that of samples at \( t \): 24 h. As expected, \( \beta \text{gl–AS} \) oligoribonucleotide transfection had no effect in promoting endogenous SRA intron-1 retention in T5 cells.

Increased relative expression of non-coding SRA transcripts corresponds to both an increase in intron-1 containing SRA RNAs and a decrease in the levels of fully spliced SRA RNA

To clarify whether the relative increase in non-coding SRA RNAs expression observed by TP-PCR resulted from an increase in intron-1 retaining RNA, a decrease in fully spliced RNA or both, we assessed the expression of these SRA RNAs by real-time PCR. T5 cells were treated with no oligoribonucleotide (Mock), 0.5\( \mu \)M SRA–AS (orange circle) or 0.5\( \mu \)M \( \beta \text{gl–AS} \) (blue circle). At \( t \): 24 h, total RNA was extracted, reverse-transcribed and analyzed by real-time PCR as described in ‘Materials and Methods’ section. Following amplification with a lower primer annealing to exon-3 and upper primers annealing to either intron-1 or exon-1, the percentage change in the level of intron-1 retained or fully spliced was quantified in three independent experiments, as described in the ‘Materials and Methods’ section. Dots represent the average \( \Delta C_t \) for each treatment whereas bars correspond to standard deviations. Asterisk indicate a significant difference (Student’s \( t \)-test, two-sided) in the corresponding SRA (intron-1 alternatively spliced or fully spliced) expression between mock and oligo-transfected cells.
Modulation of the balance coding/non-coding endogenous SRA RNAs alters gene expression in T5 breast cancer cells

To establish whether this artificial alteration of the balance between coding/non-coding SRA1 RNAs impacts on gene expression, total RNA from T5 cells transfected with SRA–AS or βgl–AS oligoribonucleotides was extracted and analyzed 24 h post-transfection by real-time quantitative PCR using a Breast Cancer and Estrogen Receptor (ER) RT 2 Profiler™ PCR Array (SuperArray Biosciences USA) as described in the ‘Materials and Methods’ section. This technology consists of ready-to-use PCR plates with each well containing an optimized pair of primers corresponding to a series of known genes. It provides the fastest way to interrogate the effect of a given treatment on genes historically linked to different pathways or pathologies (21). The expression of 57 genes was evaluated in cells treated with SRA–AS and βgl–AS oligoribonucleotides and differences in expression assessed using the Student’s t-test (see Table 1 for a full description of the genes assessed and the results obtained). The expression of 51 genes remained constant upon SRA–AS treatment (Figure 7). The expression of five genes was however significantly increased. A strong increase (∆Ct = 2.87, corresponding to a 729% increase in the expression compared to the control) in the expression of the urokinase plasminogen activator (PLAU) gene intimately linked to invasion mechanisms (22), was observed. Similarly, enhanced levels (∆Ct = 1.73, corresponding to a 331% increase in the expression compared to the control) of estrogen receptor beta mRNA (ESR2) was also found. Expression of Stanniocalcin 2 (STC2), vascular endothelial growth factor (VEGF) and Thrombospondin 1 (THBS1) was significantly increased, although to a lesser extent.

Modulation of the balance coding/non-coding endogenous SRA RNAs alters the response to estrogen of T5 breast cancer cells

The modification of the expression of estrogen receptor beta together with the known importance of estrogen signaling in breast cancer led us to investigate the effect of altering the balance coding/non-coding SRA RNAs on the response of T5 breast cancer cells to estrogen. T5 cells were transfected with βgl–AS or SRA–AS oligoribonucleotides and treated 48 h later with vehicle (ethanol) or E2 (10−8M) for a period of 4 h, as described in the ‘Materials and Methods’ section. The expression of the 57 genes listed in Table 1 has been assessed as described earlier. Estradiol treatment of control cells pre-treated with βgl–AS oligoribonucleotide significantly altered the expression of 10 genes (Figure 8A). Nine genes (B-cell lymphoma 2: BCL2, Non-metastatic cells protein: NME1, Thrombospondin 1: THBS1, Trefoil factor 1: TFF1, Deleted in liver cancer 1: DCL1, Keratin 18: KRT18, Serpin peptidase inhibitor 3: SERPINA3, Fas, Catenin beta 1: CTNNB1) are up-regulated whereas a single gene (Fibronectin Leucine-rich transmembrane protein 1: FLRT1) is downregulated. Expression of only five of the up-regulated genes (BCL2, THBS1, TFF1, TGFA and DCL1) remain significantly increased when cells are pre-treated with SRA–AS oligoribonucleotide (Figure 8B). In these latter conditions, estradiol treatment also led to a significantly increase in Heat shock 27 kDa protein 1 (HSPB1) and Cathepsin D (CTSD) expression and a decrease in Nuclear transcription factor Y-beta (NFYB) and Mucin 1 (MUC1) mRNA levels. Interestingly, a significant difference in estrogen effect when comparing directly SRA–AS or βgl–AS oligoribonucleotides pre-treatment is observed for NME1, KRT18, FAS, CTNNB1 and FLRT1 (Figure 8 C).

Modulation of the balance coding/non-coding endogenous SRA RNAs alters T5 breast cancer cells growth

To further assess the relevance of the balance coding/non-coding SRA transcripts, we have compared the viability of cells treated with SRA–AS, βgl–AS or without oligonucleotides (Mock). Cells were treated as described earlier, re-seeded after 24 h and allowed to grow in normal medium for an additional 24 h. Viability of cells was measured at this time (T0, corresponding to 48 h after transfection) and following 1, 3 or 5 days, as described in the ‘Materials and Methods’ section. It should be noted that we have confirmed, using fluorescent oligonucleotides, that more than 70% of re-seeded cells still contained SRA–AS and βgl–AS (data not shown). As shown in Figure 9, the number of viable cells remains identical in cells treated without (Mock) or with a control oligonucleotide (βgl–AS). In contrast, significantly less viable
cells are present at 1, 3 and 5 d, which corresponds to 72, 120 and 168 h following SRA–AS treatment, respectively.

**DISCUSSION**

The full sequencing of the human genome has led to a growing interest in alternative splicing events. Indeed, originally thought to possibly result from ‘hiccups’ of the splicing machinery, it became evident that these events are highly controlled and define a new level of complexity in gene expression (23–26). The critical participation of alternative splicing in regulating normal major biological events, such as sex determination or tuning of brain receptor sensitivity, has been underlined in many different systems (27–29). It is now also suggested that alteration of splicing can also be involved in many

| Symbol | Description | ΔCT | Percentage expression | P-value |
|--------|-------------|-----|-----------------------|---------|
| PLAU   | Plasminogen activator, urokinase | 2.87 | 729 | 0.021 |
| ESR2   | Estrogen receptor 2 (ER beta) | 1.73 | 331 | 0.029 |
| STC2   | Stanniocalcin 2 | 1.20 | 230 | 0.009 |
| JUN    | V-jun sarcoma virus 17 oncogene homolog (avian) | 1.15 | 222 | 0.164 |
| VEGF   | Vasculat growth factor | 0.98 | 197 | 0.034 |
| SERPIN A3 | Serpin peptidase inhibitor, clade A, member 3 | 0.84 | 179 | 0.187 |
| ID2    | Inhibitor of DNA binding 2 | 0.72 | 165 | 0.234 |
| THBS1  | Thrombospondin 1 | 0.58 | 149 | 0.043 |

**Table 1. Change in gene expression at t: 24 h following treatment of T5 cells with SRA–AS**

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*Boldface depicts significant P-values.*
pathological situations including but not limited to skin diseases (30), neurodegeneration (31), and cancer (32–37). Interestingly, it has even been proposed that specific splicing events may be associated to tissue specific cancer (38).

We have previously reported that different breast cancer cell lines grown in culture have different relative levels of coding/non-coding SRA transcripts (12). This balance ultimately controls two functional entities (SRA RNA and SRAP) modulating the action of ER and PR, major players in human breast tumorigenesis and tumor progression (39). It was critical to establish whether the relative proportion of these transcripts differed in vivo within tumor tissues. The selected cohort consisted of tumors with ER and PR values higher than 3 and 10 fmol/mg of total prot, respectively (40), therefore belonging to the clinically established ER+/PR+ subgroup. This subgroup has been selected as it represents the main subtype of cases contained in the Manitoba Breast Tumor Bank (MBTB). Indeed, within more than 5000 specimens collected in the MBTB, 48% of tumors are ER+/PR+, whereas 25%, 5% and 22% are ER+/PR−, ER−/PR+, and ER−/PR−, respectively.

Observed relative levels of non-coding intron-1-containing RNA varied from one sample to another, ranging from 0.71 to 1.61 a.u. (Figure 2C). Frozen tissue sections analyzed are intrinsically heterogeneous, containing cancer cells but also normal epithelial, fibroblastic and blood cells. These values could therefore represent an average of the relative SRA transcripts expression within all these cell types. These numbers are however fully consistent with the ones obtained on isolated cancer cell lines (between 0.80 and 2 a.u. for MCF-10A1 cells and MDA-MB-468 cells, respectively) (12). We have also shown that SRA expression was higher in breast cancer tissue than in normal breast tissue (41). The relative expression of SRA transcripts assessed on tumor tissues therefore likely reflects the balance coding/non-coding SRAs in breast cancer cells rather than in normal other cells contained in these sections. However further in-situ experiments, specifically highlighting individual SRA splicing variants, are needed to confirm this hypothesis.

Surprisingly, with such a small tumor subset, a significant higher relative intron-1-retention in tumors with higher PR levels was identified. Indeed this suggests that within these ER+/PR+ cases, the balance coding/non-coding SRA transcript might characterize particular subgroups. It is well established that patients with ER+/PR−/PR+ tumors have a higher chance to respond to endocrine therapy than other patients (42). It is also known that within this group, higher ER and PR level are associated with a better response to tamoxifen (40). Unfortunately some patients, even though defined through ER and PR levels as most likely to benefit, will not respond to endocrine therapy. This reflects the heterogeneous biology of tumors belonging to the currently established ER+/PR+ subgroup and underscores the need to further characterize these lesions to better predict their behaviour upon treatment. It is believed that understanding the exact connections between traditional prognostic/predictive factors, gene expression signatures and patient outcome will allow the establishment of more adequately targeted treatment (43). Our observation that SRA intron-1 retention potentially characterizes a particular tumor subgroup fits with the current idea that assessing alternative splicing events might help profiling cancer sub-types (38,44–46).

We have herein successfully shifted the relative levels of endogenous coding/non-coding SRA transcripts in the
estrogen receptor alpha positive breast cancer cell line T5. Our approach, consisted in masking a splicing donor site with a modified oligonucleotide, and has been used successfully by many different groups to redirect splicing events involving specific target RNAs (19,47–49). Such an approach presents several advantages. As an anti-sense method, it benefits from the fact that 2’-O-modified (either methyl or methoxy-ethyl) oligoribonucleotide phosphorothioates bind to pre-mRNA target sequences in vivo with high specificity but do not form RNase H competent RNA-oligonucleotide hybrid complexes (50). Therefore, unlike more classical anti-sense oligonucleotide techniques that result in mRNA degradation, these modified anti-sense oligoribonucleotides are not expected to

Figure 8. Differential estrogen regulation of gene expression in T5 cells following SRA–AS treatment. T5 cells were treated with 0.5 µM of βgl–AS or 0.5 µM SRA–AS as described earlier. Forty hours later, cells were treated for 4 h by E2 10^{-7} M or vehicle (ethanol) alone, RNA was extracted, reverse-transcribed, and assessed for the expression of a series of 57 genes as described in the ‘Materials and Methods’ section. Three independent experiments were performed, and the expression of each gene normalized as detailed in the ‘Materials and Methods’ section. The average expression of 15 genes upon E2 treatment (filled circle) and ethanol (hollow circle) are depicted. Bars represent standard deviation. (A) Cells have been treated with 0.5 µM βgl–AS. Genes expression of which is significantly (p < 0.05, Student’s t-test, two-sided) different between E2 treatment and Ethanol, are indicated by blue stars. (B) Cells have been treated with 0.5 µM SRA–AS. Genes expression of which is significantly (P < 0.05, Student’s t-test, two-sided) different between E2 treatment and Ethanol are indicated by orange stars. (C) To visualize the differential effect of E2 in cells treated with SRA–AS (orange) and βgl–AS (blue), E2 corresponding dots from A and B have been plotted on the same graph. Genes whose expression is significantly (P < 0.05, Student’s t-test, two-sided) different upon E2 treatment are indicated by green stars.
PLAU (plasminogen urokinase activator also called uPA), is known to play a critical role in the development of metastases through the activation of several metalloproteinase (22,51) and was among the genes whose expression was modified after SRA–AS oligonucleotide treatment. Interestingly, we have previously reported that invasive MDA-MB-231 and MDA-MB-468 breast cancer cells expressed significant more SRA RNAs retaining intron-1 than non-invasive MCF-7, T47D or BT20 cells, whereas the more ‘normal’ MCF-10A1 breast cell line expressed the lowest relative level of SRA intron-1 RNA (12). This suggests that a balance ‘tipped’ toward the production of non-coding SRA1 RNA in breast cells might affect growth and/or invasion properties. Altogether, this observation suggests that non-coding SRA RNA, through the over-expression of genes such as PLAU, might directly participate in the establishment of an invasive phenotype in breast cancer cells. Further studies are needed to corroborate this hypothesis.

A significant increase in ESR2, and to a lesser extent in Stanniocalcin 2, Vascular endothelial growth factor and Thrombospondin 1 expression were observed upon SRA–AS treatment. All these genes have previously been shown to be up-regulated by estrogens in breast cancer cells (52–54). Their altered expression following the artificial shift in endogenous coding/non-coding SRA transcripts outline the relevance of this balance in mediating estrogen effects in breast cancer cells. Interestingly, increasing the relative levels of non-coding SRA had distinct potential effects on estrogen mediated gene regulation. For genes such as Bcl2, THSB1, TFF1, TGFA, DLC1 the significant and previously described estrogen induced over-expression (55–58) remains significant (Figure 8A and B). This could result from an absence of effect of SRA RNA and SRAP on the estrogen regulation of these particular genes. As outlined earlier, our approach modifies both functional RNA and protein levels. This lack of effect could therefore also reflect a neutralization of opposite individual actions. For genes like CTSD or MUC1, the respective trend toward an increase or a decrease in expression upon estrogen treatment becomes significant (Figure 8A and B). This amplification of estrogen action fits with the concept that increasing the relative levels of the functional non-coding RNA co-activator enhances estrogen receptor activity. It should however be noted that for these genes, no significant differences in estrogen effect is observed upon modifying coding/non-coding SRA transcripts (Figure 8C). Additional time-course experiments as well as experiments designed to investigate the respective actions of non-coding SRA and SRAP are needed to further characterize the effect the balance of coding/non-coding transcripts plays on the estrogen regulation of these genes.

Most interestingly, altering the balance of coding/non-coding SRA significantly disrupts estrogen’s effect on NME1, CTNNB1, KRT18, Fas and FLRT1 genes (Figure 8C). CTNNB1 or beta catenin, an inherent component of the Wnt/β-Catenin canonical signalling pathway, is a critical player in development as well as cell-cell contact and migration processes (59–61).
NME1 (non-metastatic cells 1), whose expression is regulated by estradiol in breast cancer cells (62), is also believed to itself modulate estrogen receptor alpha action (63). These results overall suggest that relative levels of SRA transcripts still containing intron-1 have the potential to control selectively estrogen effect on specific genes highly relevant to development and cancer. Further experiments are warranted to establish the exact mechanisms behind these effects and to assess the full extent of the impact of the balance of SRA transcripts on estrogen action.

We have herein used a colorimetric method for determining the number of viable cells following modification of the balance of endogenous SRA towards the production of more non-coding transcripts. It should be stressed that the number of viable cells at a given time is a direct result of cell proliferation and cell death. Our experiments strongly suggest that the balance of coding/non-coding transcripts participates to the growth of breast cancer cells. Indeed, compared to both our controls, less viable cells are present following SRA–AS treatment. We cannot however, at the present stage, establish whether enhancing the relative amount of non-coding SRA RNA increases apoptosis or decreased cell proliferation. Additional experiments are needed to address this issue.

Herein, we have shown that the balance of coding/non-coding SRA RNAs could be altered through the use of modified oligoribonucleotides in breast cancer cells. This led to a change in expression of genes likely to have an important impact on two critical aspects of breast cancer cell phenotype, namely invasion and response to estrogen. It also led to a decrease in cell growth/viability. It has been previously hypothesized that strategies aimed at favouring the production of a given splice variant could be developed and proposed as new therapeutic tools (64–66). We propose that modifying SRA splicing events might lead to establishing potential new breast cancer treatments.

While SRA remains one the best characterized bi-functional RNA to date, other studies have highlighted the existence of similar other cases (67–69). Such systems, even though they challenge the paradigm classifying RNA as either strictly coding or as non-coding, provide a unique opportunity to further explore the mechanisms used by nature to control gene expression. There is an urgent need to design new experimental approaches to address the respective functions of these peculiar bi-faceted RNAs.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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