The Effect of SRA Intron-1 Splicing on Differential Ratio of SRA-SRAP Levels and on ER-Mediated Transcription in Breast Cancer Cells

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biochemistry and Medical Genetics

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Abstract

The steroid receptor RNA activator gene (*SRA1*) generates two distinct entities. SRA RNA coactivates several NRs whereas SRA protein (SRAP) is suspected to regulate the activity of several transcription factors, including estrogen receptors (ER). Splicing of SRA intron-1 is the major event defining SRAP coding frame. Fully spliced, coding SRA and intron-1 retained, non-coding SRA coexist in breast cancer cells. The relative proportion between the two types of SRA RNA maintains a balance between two genetically linked entities, SRA and SRAP.

In this study, a minigene model was used to demonstrate that the primary sequence of SRA exon-1-intron-1-exon-2 is sufficient for alternative splicing of SRA intron-1. In addition, a modified oligoribonucleotidic construct promotes SRA intron-1 retention in breast cancer cells. This oligoribonucleotide differentially alters estradiol-induced transcription of ER regulated genes. Together, results presented herein demonstrate that the SRA-SRAP balance, which can be artificially modified by targeting alternative splicing of SRA intron-1, might be a new critical target to treat breast cancer patients.

Acknowledgements

It is my pleasure to acknowledge a list of people to whom I would like to express my appreciation for their contributions to make this thesis possible.

Thank you, Dr. Etienne Leygue, for being a role model who presented to me enthusiasm and seriousness toward scientific research and for your guidance, inspiration and encouragement, even when sometimes my gel is crappy. Etienne, you are a great mentor! Drs. Francis Amara and Jiuyong Xie, thank you for valuable discussions and thoughtful advice, which helped me to keep my directions during the thesis research and for my future career. I was lucky to have you on my advisory committee. Mr. Mohammad Hamedani, thank you for sharing expertise and for all the happy times in the laboratory. You said your goal was to be a good man. I think you have achieved way beyond that. Dr. Florent Hube, thank you for your encouragement regarding being a good researcher when I embarked on graduate studies. I am trying my best to get to there. My laboratory colleagues and members of the Department of Biochemistry and Medical Genetics, thank you for your helping hands and accompany. However, as I am afraid of carelessly leaving any of you out of this list, I will simply say thank you very much to you all.

I can not finish without expressing how grateful I am to my family and my extent family for creating a loving and supportive environment. Special thanks go to my wife, who showed great patience during the tenure of my studies and provided me with mental support to keep my head up during the easy and hard times.

For my wife and my parents.

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Introduction

1. Estrogen Action in Breast Cancer

Breast cancer is a common cause of cancer-related death among women worldwide and in North America. In Canada, about 1 in 9 women is expected to be diagnosed with breast cancer in her lifetime, and about 1 in 27 will die of it (1). Most breast tumors arise from uncontrolled cell proliferation in the epithelium of mammary gland (2). In addition, breast cancer cells are often found to metastasize to the bone and the lung, and form secondary tumors. These secondary tumors indicate late stage of breast tumorigenesis and poor prognosis (3;4). Estrogen, a hormone that regulates growth and development of normal mammary gland, is known to participate in uncontrolled breast cancer cell growth. It can indeed promote breast cancer progression and metastasis through inducing mitosis and transformation of mammary epithelial cells (5;6). Estrogen action is mainly mediated through two estrogen receptors (ERs), α and β (7;8), which act as ligand-dependent transcription factors. Besides ERs, a series of factors termed coregulators also participate in estrogen action, by regulating ER mediated gene transcription.

1.1. Estrogen Receptors (ERs)

ERs belong to the steroid/thyroid/retinoic acid receptors superfamily of nuclear receptors (NR) (9). Based on ligands, this superfamily of NRs can be classified into three classes (10). Class I receptors are activated by steroids, and consist of ERs, glucocorticoid receptors (GRs), androgen receptors (ARs), progesterone receptors

(PRs) and mineralocorticoid receptors (MRs). Class II receptors are activated by non-steroid hormones, and consist of thyroid hormone receptors (TRs), all-trans retinoic acid receptors (RARs), 9-cis retinoic acid receptors (RXRs), and vitamin D receptor (VDR). Class III receptors are considered as orphan receptors, as their ligands remain to be determined. All receptors in this superfamily share a same functional and structural organization: a variable N-terminal region containing a hormone-independent activation domain (AF-1), an identical DNA binding domain and a C-terminal region containing the ligand binding domain and a hormone-dependent activation domain AF-2 (9) (Figure 1).

ERs participate in regulating critical cellular events mainly through directly or indirectly mediating gene transcription. Ligand binding induces ERs to undergo conformational changes and phosphorylation (11). Consequently, ERs dimerize, and specifically bind to cis-acting elements (estrogen responsive element, ERE) in promoter regions of target genes (12;13). In turn, the primary ERE sequences may regulate ER activity: Different EREs have various affinity to ligand-bound ER and can direct different conformational changes of the ERs bound to them (14-16). The different conformations, in turn, determine the profiles of co-regulators that interact with ERs (13). (Roles of coregulators are discussed in Section 1.3) Through dynamic interplays with coregulators and ERE, activated ER directs the assembly and stabilization of a pre-initiation complex that ultimately conducts gene transcription (17). ERs can also indirectly regulate gene transcription through transactivating a transcription factor or a transcription coregulator. For example, ERs control expression of progesterone receptor (PR) by binding to the ERE on PR promoter and activate PR transcription (18). PR, in turn, functions as a transcription factor. In

mammary epithelial cells, PR transactivates a series of genes involved in adhesion, signaling transduction and metabolism (19).

In addition to their role in directly regulating transcription, ERs are also found to cross-talk with other signaling pathways. For example, in an estradiol-dependent manner, ERα and ERβ-1 can compete with a transforming growth factor beta (TGF-β) signaling pathway messenger, Smad3, for the recruitment of an AP-1 family transcription activator c-Jun (20). This competition presumably sequesters Smad3 mediated transcription, hence may alter multiple cellular events regulated by TGF-β signaling, such as cell cycle control and growth inhibition. Also, a G Protein Coupled Receptor (GPCR) termed GPR30 was identified to contain a ligand binding domain identical to those of nuclear ERs. GPR30 is predominantly expressed on the endoplasmic reticulum membrane and, distinct from nuclear ERs, can activate the phosphoinositide 3-kinase (PI3K)–Akt pathway, which in turn regulates cell proliferation (21). In conclusion, emerging findings have revealed more complex roles of estrogen signalling in the regulation of cellular events (Figure 2).

1.2. Relevant of ERs in Breast Cancer

ERs are found in about two thirds of breast tumors, in which they are believed to potentially regulate cancer cell proliferation and ultimately metastasis (22). However, ERα and ERβ may have different roles in breast cancer pathology. ERα is a well-established risk factor as well as prevention/treatment target, whereas ERβ has been proposed as a tumor suppressor gene for breast cancer (23-26). The elevated levels of ERα in benign breast epithelium appears to indicate an increased risk of breast cancer;

in addition, ERα expression correlates with better prognosis and the likelihood of response to hormonal therapy (see Section 1.5 for more details) (27). On the other hand, in breast cancer cells, ERβ can antagonize the effects of ERα through mechanisms involving the c-Fos c-Jun transcription activator complex. For example, expression of ERβ reduces the occupancy of ERα on PR promoter, as well as the recruitment of c-Fos and c-Jun to the promoter by ERα (28;29). In addition, expression of ERβ further increases E2-dependent degradation of ERα (29). Loss of ERβ expression is a common step during progression of estrogen-dependent breast tumor, pinpointing its potential role as a tumor suppressor (24).

1.3. Nuclear Receptor (NR) Coregulators

In addition to transcription factors, the complex mechanism underlying gene transcription also requires transcription coregulators, such as CREB binding protein (CBP/p300) and P/CAF. These factors were found to regulate transactivation by NRs as well as other types of transcription factors such as p53 and NFκB (30). They usually function as a bridge between transcription factors and the basal transcription machinery through protein-protein interactions and regulate the assembly and activity of the transcription initiation complex (31).

The observation that two NRs decrease each other's transactivation in cells led to the awareness of the potential critical role played by NR specific coregulators (32). Ever since the characterization of the first NR coregulator, the steroid receptor coactivator-1 (SRC-1) (33), the list of these factors grows rapidly. In about a decade, nearly 300 factors were characterized to be able to regulate NR activity (34). These factors can

alter basal transcription mediated by the receptors. Based on the outcomes of their regulations, they are categorized into two large groups: coactivators and corepressors (35-39). Coactivators are defined as molecules that are directly recruited by NRs to enhance NR-mediated gene expression, usually in a ligand-dependent manner (40), whereas corepressors function as counterparts of coactivators to attenuate NR-mediated transcription, primarily through their interaction with unliganded, or antagonist-bound NRs (40).

NR coregulators usually form complexes, which are dynamically recruited by the NRs (31). Figure 3 illustrates several well-characterized complexes and their mechanisms of action. The binding and exchange of coregulatory complexes on NRs probably follow specific orders. Taking ERα as an example, a gene promoter seems to recruit ERα and coregulatory complexes in a cyclic manner: ERα binds to and disassociates from the promoter reciprocally, and binding of ER directs the recruitments of SRC-3/AIB1, CBP and pCAF in a temporal order (41). In addition to directly regulating NRs, some NR coregulators can also recruit general transcription coregulators through protein-protein interactions. For example, p160 family coactivators (SRC-1/NCoA1, SRC-2/TIF2, SRC-3/AIB1) physically interact with CBP through a domain adjacent to the NR binding domain, recruiting this transcription coactivator to NRs (31).

Depending on the cellular environment, a given factor can act as a coactivator or a corepressor. For example, CBP/p300 is conventionally viewed as a coactivator that modulates chromatin structure in favor of transcription through its histone acetyltransferase (HAT) activity. However, prolonged exposure to estradiol promotes

the acetylation of p160 family NR coactivators (SRC-1/NCoA1, SRC-2/TIF2, SRC-3/AIB1) by CPB/p300, and consequently leads to the disassociation of these coactivators from the NRs, ultimately attenuating transactivation (42). In this case, CBP/p300 switches its role from a coactivator to a repressor, and presumably provides a negative feedback loop to regulate NR transactivation.

Besides transcription regulation, specific coregulators are also found to participate in other transcriptional/RNA processing events such as RNA chain elongation, precursor RNA splicing and mRNA translocation (43-47).

1.4. Implications of NR Coregulators in Disease

The crucial roles of NR coregulators in regulating multiple transcriptional events implicate them as players in the development of a wide range of hormone-dependent pathologies. Indeed, a recent survey showed that altered expressions of over 1/3 of known NR coregulators are involved in human diseases, including various types of cancer such as breast and prostate cancers (48). Hence, many of these coregulators are believed to behave as oncogenes or tumor suppressor genes (49). In breast cancer, through regulating ER activity, NR coregulators were found to participate in breast tumorigenesis and cancer progression, as well as apoptosis (50;51).

1.5. Estrogen Signalling Pathway as a Target of Breast Cancer Therapy

The awareness of estrogen action in breast cancer pathology has guided the development of breast cancer therapeutic and preventive strategies that function

through modulating ER activity (25;52). Commonly, the modulation is achieved through hormone therapies, which aim to either block ER activation or decrease estrogen production. For example, the selective estrogen receptor modulators (SERMs), such as Tamoxifen and Raloxifen, are synthetic non-steroidal compounds that can bind to ER with high affinity. Tamoxifen acts as an anti-estrogen by competing with estrogen for the binding to ER, and antagonizes hormone-induced transactivation in breast cancer (53). On the other hand, ovarian shutdown or removal is sometimes suggested to treat pre-menopausal patients with hormone-responsive breast cancer, as most estrogen in these patients is synthesized by the ovaries (54). A similar approach consists of using aromatase inhibitors (AI) to treat breast cancer. AIs, in postmenopausal women, block estrogen production through inhibiting the activity of aromatase, an enzyme that convert androgen to estrogen (55). The resulting lowered estrogen level leads to decreased ER activity.

Alternative to the hormone therapies, NR coregulators have opened new avenues to design novel therapeutic and preventive approaches of breast cancer, due to their crucial roles in regulating ER activity and implications in breast cancer pathology. It is perceivable that attenuating the functions of NR coactivators or promoting the functions of NR corepressors in breast cancer cells may modulate ER activity and ultimately slow down cancer cell proliferation and invasion. Therefore, it is highly meaningful to not only identify new NR coregulators but also to characterize their mechanisms of action in regulating ER activity.

2. Steroid Receptor RNA Activator (SRA)

Among the NR coregulators identified so far, the steroid receptor RNA activator (SRA) is unique due to two reasons. First, SRA is a functional RNA that acts as a coactivator of steroid receptors as well as several other NRs (56-58). Second, even though SRA was initially identified as a non-coding RNA transcript (56), specific SRA variants encode a protein, termed SRAP, which appears to regulate ER activity (59-61).

2.1. Characterization of SRA

SRA was originally identified from a short open reading frame (ORF) present in two positive clones obtained in a Yeast two hybrid (Y2H) screening aimed at identifying proteins that interact with the AF-1 domain of progesterone receptor (PR). Intriguingly, the Gal-SRA fusion constructs in these clones contained a stop codon upstream of the SRA sequence, hence no GAL-SRA fusion protein was produced (56). As such, the fact that these clones are viable can not result from the interaction between the AF-1 of PR and a protein encoded by SRA. It was therefore speculated that SRA acts as an RNA molecule interacting with the AF-1 and potentially recruiting endogenous yeast transcription activators (56).

Further identification of SRA transcripts from other sources defines SRA as a family of RNA transcripts highly homologous in a core region (56). As a result, three human SRA transcripts were described to have a same 687bp core region but 5' and 3' extremities of various lengths (56).

2.2. SRA is an RNA Coactivator of a Wide Range of NRs.

SRA was initially found to specifically coactivate steroid receptors as an RNA molecule (56). Indeed, it coactivates PR in an open reading frame (ORF) independent manner, suggesting that translation is not necessary for the coactivation function. Furthermore, cycloheximide, a *de novo* protein synthesis inhibitor, had no effect on the coactivating property of SRA on GR-mediated transcription, but efficiently reduced the activity of peptide coactivators such as SRC-1 and CBP (56).

Subsequently, SRA was found to coactivate other NRs besides steroid receptors, such as TRs (57;58). Different classes of NRs might utilize distinct mechanisms to recruit SRA for coactivation (56-58). Class 1 receptors such as AR, ER and PR associate with SRA through their AF-1 domains in a ligand dependent manner (56;62;63). However, since the AF-1 regions of these receptors lack sequence homology, the interactions between SRA and the AF-1 domains are unlikely to be direct (56). In contrast, class II receptors TRα1, TRα2 and TRβ physically bind to SRA through a 41 amino acid RNA binding motif located between their DNA binding domain and ligand binding domain (57;58). TRα1 lacking this motif could not be coactivated by SRA, but retain full response to its ligand. Hence, it appears that for TRs, the direct binding is crucial for SRA coactivation (57).

Besides the above NRs, SRA was also found to coactivate MyoD, another transcription factor involved in skeletal myogenesis (64). In addition, SRA was also found to coactivate type II receptor RAR-γ in given cell contents (65). SRA by itself does not increase the activity of RAR-γ. However, co-expressing a pseudouridine synthase Pus1p with SRA potentiates RAR-γ transactivation whereas in the absence

of SRA Pus1p does not coactivate RAR-γ. These findings pinpoint the role of pseudouridylation in SRA's coactivating function (also see Section 2.4) (65).

2.3. Functional Core Region of SRA

The SRA core region is necessary and sufficient to coactivate steroid receptors; and the coactivation function is attributed to several RNA secondary structures distributed throughout the core, instead of one distinct motif (56;66;67). Serial truncations from both ends of the core region reduced SRA coactivation. However, the removed sections by themselves were not sufficient to coactivate steroid receptors (67). By RNA secondary structure prediction followed by mutations, 7 motifs in the core region were identified as participating in SRA's coactivator property (67). They are illustrated in Figure 4 (68). These secondary structures may serve as targets of RNA binding proteins presumably recruited to steroid receptors by SRA and also function as coregulators. Indeed, the largest and relatively the most stable structure, SRA stem-loop structure 7 (STR7), was found to interact with distinct arrays of nuclear proteins in breast cancer cells (66).

2.4. Mechanism of Action

An emerging model of SRA's function in regulating NR transactivation has been reviewed and illustrated in Figure 5 (68). The formation of proper secondary structures in the SRA core region is presumably achieved through a post-transcriptional modification, pseudouridylation. These secondary structures enable SRA to physically or indirectly interact with other NR coregulators. SRA was found

to associate with coregulators of both AF-1 and AF-2 domains. Specific coactivators and corepressors may compete for binding to SRA. Depending on associated coregulator(s), SRA may synergize with coactivators, or function as a platform to recruit corepressors. As such, SRA forms ribonucleoprotein complex with coregulators and NRs (56;62-64;66;69;70). However, SRA does not seem to have a vital structural role in these complexes, at least in those containing SRC-1, as digesting SRA with RNase does not alter the presence of SRC-1 in cell fractions separated by gel filtration chromatography (56). Taken together, SRA may serve as a "gasket" molecule to facilitate protein-protein interactions in coregulator complexes; and in some cases it can bridge AF-1 and AF-2 activities.

2.4.1. Association with Coactivators

SRA's coactivation function is activated by two pseudouridylases, Pus1p and Pus3p, which also have been characterized as NR coactivators (65;71). Pseudouridylation is the post-transcriptional replacement of specific uridines (U) by pseudouridines (Ψ) on non-coding, functional RNA such as t-RNA and ribosomal RNA (72-77). This modification alters the secondary structure and rigidity of the target RNA molecules to promote proper folding and correct functioning (78;79). Pus1p but not Pus3p associates with class I receptors whereas they both bind to the first Zinc finger of the DNA binding domain of the class II receptor RAR. In these scenarios, SRA can synergize with the pseudouridylase to coactivate the associated receptors.

Pus1p and Pus3p pseudouridylate SRA at distinct, as well as several overlapping, sites (65;71). A common pseudouridylation site of both enzymes, located in the STR5

substructure of the SRA core region (Figure 4), is involved in the regulation of SRA's coactivation property (65;67;68). Mutating this site led to overall hyper-pseudouridylation of SRA, and switched SRA from a coactivator to a dominant negative regulator (65). Hence, Pus1p and Pus3p are likely crucial regulators of SRA action on NR mediated transcription.

In addition, SRA was found to associate with and synergize with several well-characterized NR coactivators such as SRC-1 and the p72/p68 proteins. SRC-1 belongs to the p160 family coactivators (SRC-1, SRC-2/TIF2 and SRC-3/AIB1). These factors bind to the AF-2 domain of nuclear receptors through an LxxLL motif in which L stands for Leucine (80-84). They can recruit other coregulators to steroid receptors as well as promote a functional synergy between AF-1 and AF-2 domains (58;85;86). Using coimmunoprecipitation from an expression system consisting of *Xenopus* oocytes programmed with *in vitro* generated RNA, SRA was found to associate with SRC-1, but not with two general coactivators, p300 and CBP. In addition, the presence of SRC-1 also enabled SRA to associate with an AR mutant lacking the AF-1 domain (56). Together, these results suggest that SRA and SRC-1 belong to a common ribonucleoprotein complex recruited by steroid receptors (56).

The p72/p68 proteins are DEAD-box RNA binding helicases that can physically interact with p160 family proteins and with the AF-1 region of ERα, but not other NRs (including ERβ) (87). In addition, they can bind to SRA through a well-conserved motif in the DEAD box (69). These factors can potentiate the AF-1 activity of ERα irrespective of ligand, and can synergize with SRA and SRC-2/T1F2 to coactivate ERα under the presence of estradiol. However, abolishing the binding of

p72/p68 to SRA disrupts this synergy and brings down ERα transactivation close to the base level (69). These results indicate that SRA bridges the coactivations of AF-1 and AF-2 by interacting with their specific coregulators: physical interaction with SRA maybe necessary for p72/p68 proteins to coactivate AF-1(68), whereas p72/p68 proteins in return may serve as adaptor proteins to integrate SRA with the AF-2 coactivators p160 proteins (69).

2.4.2. Association with Corepressors

On the other hand, SRA may serve as a platform to recruit corepressors such as SHARP (SMRT/HDAC1 Associated Repressor Protein) and SLIRP (SRA stem-loop interacting RNA binding protein). SHARP was found to physically interact with a NR corepressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) as well as SRA through distinct domains (88). It contains a repression domain (RD) and an RNA binding domain consisting of three RNA recognition motifs (RRM). Through the RD domain SHARP can recruit SMRT and histone deacetylase activities hence act as a potent transcription repressor. SHARP binds to the STR7 (Figure 4) of SRA (66;67;88). Full length SHARP, but not RD alone, represses SRA-potentiated, ligand-dependent transactivation of ER and GR, but does not alter the transcription potentiated by SRC-1. Altogether, this suggests that SHARP represses NR-mediated transcription by sequestrating SRA and its associated factors from NRs or by recruiting corepressors such as SMRT and HDACs to NRs through binding to SRA (88).

SLIRP mainly consists of an RNA binding domain highly homologous to that of SHARP. Similarly, SLIRP specifically binds to SRA SRT-7 (66). It can be recruited to endogenous NR target promoters, and can attenuate SRA-mediated transactivation of a wide range of NRs. It has been suggested that SLIRP conducts the repressor function through recruiting nuclear receptor corepressor (NCoR) to the target promoter, as knocking down SLIRP by siRNA led to a reduced level of association between NCoR and ER on TFF1/pS2 promoter in the absence of E2. Interestingly, SLIRP and SRC-1 associate with SRA in a competitive manner to drive NR activity to opposite directions (66).

2.4.3. Synergy with Coregulators

SRA and the associated NR coregulators can regulate NR transactivation in a cooperative/synergic manner (56;62;70). For example, co-transfecting non-coding SRA and individual p160 family coactivators into COS-1 cells led to additive increases of ERα and ERβ activities (62). On the other hand, knocking down endogenous SRA RNA by siRNA impairs ligand-dependent transactivation mediated by ERα, whereas simultaneous inhibition of SRA and either SRC-1 or T1F2 led to a more-than-additive effect comparing to individually inhibit these coactivators (70). However, it is necessary to note that specific SRA RNA transcripts can indeed encode a protein (Section 3). The siRNA approach simultaneously decreases both SRA RNA and SRA protein, and can not separate the function of one from the other. Therefore it is not sufficient to correlate results from the siRNA study to SRA's function.

2.5. Regulation of ER Signalling by SRA

Several research groups have reported that SRA can coactivate full length ERα and ERβ in a ligand-dependent manner (56;62;63;69;70;88). Unlike PR or GR, of which AF-1 truncated mutants did not respond to SRA coactivation (56), both ER subtypes can be coactivated by SRA through the AF-2 domain under the presence of E2 (62;63;69). This distinct response of ER AF-2 is presumably due to differential SRA-mediated mechanisms of ERs and/or differences between cell systems/HRE constructs used to observe action of SRA on a given receptor (68).

SRA can also coactivate the AF-1 activity of ERα but not ERβ. However, conflicting reports have been published regarding the role of estradiol and phosphorylation of a specific serine residue (S118) in the AF-1 of ERα (62;63). S118 phosphorylation allows AF-1 to be fully functional in COS-1 and breast cancer cells (11;89). In one study, SRA moderately increased the transactivation of an AF-2 truncated ERα construct (containing the AF-1 and the DBD) when estradiol was added (62). This coactivation required S118 phosphorylation. It was suggested that although the truncated ERα construct did not bind to estradiol, estradiol somehow could activate the mitogen-activated protein kinase (MAPK) pathway, and consequently induce S118 phosphorylation (62;90). Therefore, it was concluded that SRA requires the presence of S118 to coactivate AF-1 of ERα (62). In another study, SRA enhanced the transactivation of a fusion protein containing the AF-1 of ERα downstream of the GAL4 DBD but not an identical construct containing the AF-1 of ERβ. Estradiol was not required for SRA to coactivate the ERα fusion construct. In addition, mutating the S118 did not alter the response to SRA, although simultaneously mutating all 3 serine

phosphorylation sites attenuated the response (63). Both studies pinpointed the importance of serine phosphorylation in the coactivation of ERα AF-1 by SRA. It has been suggested that the "contrast" findings on the involvements of estradiol and S118 in the process of ERα coactivation by SRA may be due to different model systems used (truncated ER vs. fusion construct, different cell contents etc.). It was also suggested that ERα sequences present in the first report, but absent in the second study (mainly the DBD) can be a target of alternative coregulatory mechanisms (68).

2.6. SRA in Steroid-Dependent Cancers

SRA expression is up-regulated in tumors from steroid-dependent tissues such as the breast, uterus and ovary (91-94). This over-expression could therefore characterize particular subgroups of lesions among different tumors (68). For example, across ovarian cancers, serous ovarian tumors expressed higher levels of SRA than granulose cell tumors or mucinous cystadenocarcinoma (94).

The involvement of SRA in estrogen action pinpoints its possible roles in breast cancer pathology. Indeed, SRA expression is elevated in ER+/PR- and ER-/PR+ breast tumors compared to ER+/PR+ or ER-/PR- tumors whereas not different between Tamoxifen-sensitive and resistant ones (92;93). In addition, certain SRA transcripts might serve as standards to characterize phenotypes of breast tumors. For example, ratio of a transcript corresponding to exon-3 deleted SRA in total SRA positively correlates with breast tumor grade score (92). This transcript is not a coactivator of steroid receptors as artificially deleting partial SRA exon-3 abolishes SRA's coactivating activity (56). It has been proposed that this mutant transcript

interferes with the regulation of steroid receptor signaling by SRA, resulting a more aggressive behavior of the tumor (92). Characterizing the correlation between exon-3 deleted SRA and breast tumor grade, as well as this transcript's mechanism of action, can provide new understanding of breast cancer pathology and may facilitate breast cancer diagnosis, grading and prognosis.

SRA, through its ability to modify ER activity, has been suspected to participate in breast tumorigenesis and tumor progression (91;92). By itself, it was however insufficient to transform breast epithelial cells into a malignant phenotype (91). In a transgenic mouse model over-expressing SRA in mammary glands, elevated cellular proliferation in the mammary epithelium was observed, resulting in the formation of neoplastic-like lesions. In addition, the expression of PR, an ER downstream gene, was increased in these transgenic mammary glands, presumably due to an increase of ER activity by SRA. However, the increased proliferation and PR expression was not potent enough to induce breast tumorigenesis, possibly due to an elevated level of apoptosis coupled with these events (91). Presumably, in response to the increase of proliferation caused by SRA, apoptosis was utilized as a compensation mechanism to eliminate cells that are abnormally highly-proliferative and potentially inclined to undergo mutagenesis and tumorigenesis. Alternatively, the impotency of the SRA core to lead to a full malignant phenotype by itself may result from the lack of other necessary mechanisms (68).

3. SRAP, a Second Player of *SRA1* Functions

Initially, SRA was described as a group of non-coding, functional RNA transcripts (56). The first SRA sequence submitted to the NCBI database (AF092038) corresponds to a transcript properly spliced but not translatable (56;95). Nevertheless, from breast cancer cells, three SRA variants identical to the original SRA in the core region but containing extended exon-1 sequences were detected. These extended exon-1 sequences contain 2 additional methionine codons that define the start of a 236/237 amino acid open reading frame (60). These transcripts are translatable *in vitro* and in cells to generate a polypeptide that localizes in both the nucleus and cytoplasm (60). Hence, it was proposed that specific SRA transcripts can encode a protein.

3.1. Characterization of SRAP

A highly conserved SRAP primary sequence with two consensus regions located at the N and C termini was established from putative SRAP sequences of 20 species (59) (see Figure 6). These two domains represent the typical signature of this family of proteins, and likely contribute to SRAP function (68).

Endogenous SRAP was detected as a doublet of the predicted size from skeletal muscle extracts of most species tested as well as from a series of human breast cancer and prostate cancer cells, by Western blot (59;60;96). These proteins are endogenous and intact, as knocking down SRA with siRNA led to a decrease of SRAP expression (59;96). The doublet can presumably be due to the two methionine codons used for translation, or alternatively, due to post-translational modifications.

3.2. SRAP Function and Implication in Cancer

SRAP's functions and mechanisms of action remain to be fully characterized at this moment. It has recently been shown that SRAP interacts with several transcription factors and transcription regulators (68;97). Accumulating data suggests that SRAP, similar to SRA, participates in the regulation of NR transactivation.

In some cases, SRA can be a repressor of ER activity (61): in MCF-7 breast cells stably transfected with coding SRA (MCF-7-S151), an ERE-luciferase reporter construct showed decreased response to estradiol (but no altered sensitivity to tamoxifen), suggesting that SRAP might play a role to repress the ligand-induced activity of ER in these cells (61). However, two ER downstream genes, PR and trefoil factor-1 (TFF1) responded differentially to the simultaneous over-expression of SRA and SRAP: while estradiol-induced transcription of PR was elevated in the MCF-7-S151 cells, no effect was observed for transcription of TFF1. These results suggested that the SRA-SRAP system may regulate different ER-target genes in distinct manners (61).

SRAP expression correlates with better prognosis among Tamoxifen treated, ER-positive breast tumors: patients whose primary breast tumors were positive for SRAP expression had a significantly lower risk of dying from recurrent disease than those who are SRAP-negative (61). As such, SRAP expression is likely and indication of less aggressive ER-positive breast cancer, and SRAP could be a prognostic marker to predict disease outcome (61).

In prostate cancer cells that lack androgen receptors (AR) or contain mutated ARs, co-transfecting siRNA targeting endogenous SRA with AR leads to a decrease of AR-mediated transcription (96). The authors concluded that SRAP plays a role in activating ARs (96). However, as discussed in Section 2.4.3., the siRNA approach decreases both SRA RNA and SRAP. It is not clear if the decreased AR activity was due to knocking down SRA RNA or SRAP. Nevertheless, this study demonstrated that endogenous SRA is a relevant factor in AR-mediated transcription in prostate cancer cells (96).

3.3. Rat SRAP

A fusion cDNA clone containing an SRA-homologous portion with 78 % identity was identified from a rat prostate library. This portion defines an ORF for a 16.3kD protein translatable *in vitro* (98). However, compared to the putative SRAP primary sequence, this rat SRAP lacks the N-terminal consensus region; hence the integrity of the rat cDNA as well as the endogenous presence of the rat SRAP remains to be determined.

The rat SRAP was reported to be a transcription activator (98). Recombinant rat SRAP can associate with AR, likely through direct interaction with the AF-2 domain, and can activate transcription as well as can coactivate AR in a ligand-dependent manner (98). Introducing mutations or premature stop codons to the rat SRAP coding frame suppressed its production, and led to attenuated coactivation of AR transactivation (98). This effect is likely attributed to the decrease of the recombinant rat SRAP but not the rat SRA-like RNA, since the RNA transcript is unlikely a

functional coactivator due to the absence of the STR5 secondary structure at the 5' end of the core (see Figure 4 for SRT5) (68). However, as mentioned, the rat SRAP lacks the N-terminal domain which is strongly conserved across Chordates (59). It is reasonable to postulate that it may have distinct functions from the full-length SRAP containing both the N- and C-terminal domains.

4. Alternative Splicing of SRA Intron-1

As discussed in the beginning of Section 3, three fully spliced SRA transcripts with extended exon-1 encode SRAP. An alignment of SRA transcripts in the NCBI nucleotide database revealed that the extended exon-1 is contained in 6 sequences (Figure 7, sequences 2-7) (95). As the two methionine codons initiating SRAP open reading frame are present in all these RNAs, these SRA transcripts, in addition to be functional RNA molecules, also have the ability to encode SRAP. On the other hand, 4 sequences have a full or partial retention of intron-1, introducing a frame shift or a premature stop codon to the SRAP reading frame (Figure 7, sequences 9-12). These transcripts, although unable to encode SRAP, are still capable to be coactivators as they contain the intact core region. Taken together, accurate splicing of SRA intron-1 appears to be a major event to determine SRAP coding frame.

4.1. Coding and Non-Coding SRA Coexist in Breast Cancer Cells.

Previous investigation in our laboratory demonstrated the co-existence of non-coding SRA containing intron-1 and coding SRA fully spliced in a series of breast cancer cells. However, the relative ratio between the two species varies (95) (Figure 8). As a

result, relative ratios between SRA and SRAP molecules are likely not the same between different breast cancer cell lines. We hypothesized that, breast cancer cells utilize alternative splicing of SRA intron-1 to regulate ER transactivation through SRA and SRAP functions.

4.2. Alternative RNA Splicing

Intron splicing involves both cis-acting elements on precursor RNA as well as a trans-acting RNA/protein macromolecular machinery termed the spliceosome (99). In a constitutive splicing model, an intron is defined by a 5' donor site, a branch point followed by a polypyrimidine tract and a 3' acceptor site. The spliceosome consists of 5 ribonucleoprotein subunits, the uridine-rich small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5, and U6. In addition, RNA splicing also requires a dynamic array of RNA-binding proteins, namely, heterogeneous nuclear ribonucleoproteins (hnRNP) and serine/arginine-rich proteins (SR proteins) (100;101).

Traditionally, the assembly of spliceosome on the intron is considered a highly-ordered pathway, consisting of step-wise binding of spliceosome subunits to splicing sites and interactions between the subunits, using ATP as energy source. Splicing is initiated by the binding of U1 snRNP to the donor site. At the main time, distinct hnRNP/SR proteins recognize and bind to the branch point/polypyrimidine tract, and then are replaced by U2 snRNP. Afterwards, U1 snRNP is displaced by a trimer, consisting of U4, U5 and U6 snRNPs. Then the interaction between U2 and U6 snRNPs displaces U4 snRNP and brings close the donor and acceptor sites. Following two transesterification interactions on the backbone of pre-mRNA, the intron is

removed from the RNA in the form of a lariat and the two flanking exons are joined (102). This process is briefly illustrated in Figure 9. However, emerging findings also suggest that in higher eukaryotes, the spliceosome machinery can be fully or partially pre-assembled, and load onto pre-mRNA as a whole complex (103;104).

The selections of different splicing sites dictate different definitions of an intron, leading to alternative splicing of precursor RNA. Alternative intron definitions can be due to several mechanisms such as RNA secondary structure, strength of splicing sites and the presence of splicing enhancer/repressor elements on the RNA primary sequence. These elements are specifically recognized by hnRNP and/or SR proteins, which regulate splicing site selection presumably through mechanisms such as interfering/facilitating the recognition of a site, looping out a part of the precursor RNA, or facilitating the assembly or binding of the spliceosome (105-107).

4.3. Alternative Splicing as a Cause of Human Pathologies

Alternative splicing enables a gene to execute multiple functions, usually through the generation of proteins conducting diverse or even contrasting functions. An example consists of the Bcl-X precursor RNA which can be alternatively spliced to produce two mRNAs. The two proteins encoded, bcl-xL and bcl-xS, act as anti- and pro-apoptotic factors and their relative levels are critical for cell fate determination (108). It is perceivable that for certain genes, an alternative splicing event in favor of the other(s) can promote pathologies. Indeed, an example can be the exon-3 deletion of SRC-3/AIB1, an alternative splicing event commonly observed in breast tumors. The exon-3 deleted mRNA generates a shorter protein found to be more potent to

coactivate ER than the full length SRC-3/AIB1, hence might participate in breast tumorigenesis (109;110). Also as an example, human *tau* gene mutations leading to aberrant splicing have been identified in Hereditary Frontotemporal Dementia with Parkinsonism-17 (FTDP-17), an autosomal dominant hereditary neurodegenerative disorder (111;112). Therefore, altered regulations of splicing events can contribute to the development of diseases.

5. Summary

Estrogen signalling plays a central role in breast tumorigenesis and breast cancer progression. It is mainly mediated through transactivation of gene transcription by the ERs. ER activity is regulated by coregulators, among which SRA holds a unique position. SRA is the only functional RNA coregulator identified so far, and can coactivate ERs by recruiting other coregulators through its secondary structures in the core region. Specific SRA transcripts, besides being coactivators, are able to encode SRAP, which is also likely a regulator of ER activity.

In breast cancer cells, alternative splicing of SRA intron-1 determines the balance between coding and non-coding SRA, hence dictates the equilibrium of SRA and SRAP functions. This equilibrium represents the overall regulation of ERs by the *SRA* gene products.

Hypothesis

Modulating the balance between coding and non-coding SRA in breast cancer cells alters the SRA-SRAP balance and consequently changes estradiol-induced gene transcription.

Rational

Modulating ER activity has been proved a successful approach to treat breast cancer. SRA and SRAP, both regulators of ER transactivation, provide a new prospective for the modulation. In breast cancer cells, the balance between SRA and SRAP is partially controlled by the alternative splicing of SRA intron-1. As such, the present study was designed to establish a model to study mechanisms regulating SRA intron-1 splicing, as well as to investigate the potential consequences of challenging the SRA-SRAP equilibrium on expressions of certain genes involved in ER signalling pathway.

Study Design

In order to test the hypothesis, the following specific aims are investigated:

Aim 1: to validate SRA minigene as a model to study SRA intron-1 splicing.

Aim 2: to establish an approach to alter SRA intron-1 splicing in breast cancer cells.

Aim 3: to examine the consequences of altering SRA intron-1 splicing on estradiol-induced gene transcription.

Materials and Methods

1. Cell Culture

1.1. Bacteria Cell Culture

Medium: Luria-Bertani (LB) broths were autoclaved and stored at -4^{o}C for up to one month. Before use, the LB broths were warmed to room temperature and supplemented with Carbenicillin (Sigma) at a final concentration of 20μg/mL. To prepare LB-agar plates, 1.5 % (weight by volume) agar was added to LB before autoclave. Autoclaved LB-agar was allowed to cool down to about 40-50^{o}C and then supplemented with Carbenicillin at a final concentration of 40μg/mL before being poured onto plates. LB-agar plates were stored at 4^{o}C, away from light.

Antibiotic selection: After cloning, 50μl E. coli. Suspension was spread on a 10cm LB-agar plate supplemented with 40μg/mL Carbenicillin. The bacteria were allowed to grow for about 16hr in a 37^{o}C incubator. Then the plate was sealed with elastic tape and stored at 4^{o}C.

Culture: To prepare a bacterial cryo-culture, a positive bacteria colony was picked from an agar plate, verified by PCR for desired plasmid, and transferred to 2ml Carbenicillin supplemented LB medium and cultured at 37^{o}C for about 16h with vigorous shaking (250rpm). Then 800μl active bacterial culture was mixed with 200μl sterile glycerol in cryovials (Nalgene). Cryo-cultures were vigorously vortexed or pippeted to ensure even mixing, and are stored at -80 ^{o}C.

To prepare a culture for mini-prep, a positive bacteria colony was picked from agar plate, verified by PCR for desired plasmid, and transferred to 2ml Carbenicillin supplemented LB medium and cultured at 37^{o}C with vigorous shaking (250rpm) for about 16h. To prepare a culture for maxi-prep, a trace amount of bacteria from the glycerol stock was transferred to 100ml Carbenicillin supplemented LB medium and cultured at 37^{o}C with vigorous shaking (250rpm) for about 16h.

1.2. Mammalian Cell Culture

Breast cancer cells (BT-20, MDA-MB-468 and T47D-5) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 5 % Fatal Bovine Serum (FBS, Hyclone or Cansera), 0.3 % D-Glucose (Fisher), 2mM L-Glutamine (GIBCO), 1000 units/ml pennicilline and 100µg/ml streptomycine (GIBCO). For experiments involving estradiol treatment, cells were cultured in phenol red free DMEM (Sigma) supplemented with 5 % charcoal-stripped fetal bovine serum (FBS, Hyclone or Cansera), 0.3 % D-Glucose (Fisher), 2mM L-Glutamine (GIBCO), 1000 units/ml penicillin and 100µg/ml streptomycin (GIBCO). Cells were grown at 37^{o}C in a humidified atmosphere containing 5 % CO_2, in a water-jacketed incubator.

2. Transfection and Estradiol Treatment

2.1. Plasmid Transfection

Lipofectamine (Invitrogen) was used to transfect plasmid into cultured mammalian cells. Five hundred thousand cells were plated 48h prior transfection to allow 60 % confluence and incubated in antibiotic-free DMEM 2 h prior to transfection. Transfections were performed at a ratio of 4µl transfection reagent per µg of DNA in Opti-MEM reduced serum medium (Invitrogen), and according to the manufacturer's instructions. Five hours post-transfection the medium containing Lipofectamine was replaced by regular DMEM containing serum and antibiotics, in order to reduce the cytotoxicity on prolonged incubation with the polycationic transfection reagent.

Transfections were performed in 6-well plates with 1.65µg plasmid DNA (transfected in 1ml Opti-MEM) per well, except for minigene protein studies, where cells were transfected in 10 cm dishes and transfected with 9.9 µg plasmid DNA (transfected in a volume of 6 ml Opti-MEM).

2.2. Oligoribonucleotides Transfection

DMRIE-C (Invitrogen) was used to transfect the modified oligoribonucleotides into cultured mammalian cells. Cells were plated and incubated as described above. Transfections were performed at a ratio of 4µl transfection reagent per µg DNA in Opti-MEM reduced serum medium (Invitrogen), and according to the manufacturer's instructions. The medium containing DMRIE-C was replaced by regular medium containing serum and antibiotics 5 hours post-transfection. Sequences of the oligoribonucleotides are illustrated in the Result section and Figure 14.

2.3. Estradiol Treatment Following Transfection

Cells were seeded into 6-well plates. Twenty four hours post seeding, the cells were washed twice with room temperature PBS and then cultured in phenol red free DMEM (Sigma) supplemented with 5 % charcoal-stripped FBS. Cells were allowed to grow for up to another 24 h until reach about 50-60 % confluence.

Before transfection, cells were washed twice with PBS and incubated with phenol red free, serum free DMEM for 2 hours. The 2'-O-Methyl oligoribonucleotide (2'-OMe) constructs were transfected in the same medium, as described in Section 2.2. The transfection medium was replaced by phenol red free DMEM supplemented with 5 % charcoal-stripped FBS 5 hours post transfection. Cells were allowed to grow for 24 h before estradiol treatment.

Before estradiol treatment, cells were briefly washed with PBS. Estradiol (Sigma) was 1:1000 dissolved from a pre-made 10^{-5}M stock into phenol red free DMEM supplemented with 5 % charcoal-stripped FBS to a final concentration of 10^{-8}M. Cells in 1 well on a 6-well plate were incubated with 1ml of the medium containing estradiol for 4 h, 8 h, 24 h and 48 h.

3. Western Blot Analysis

3.1. Protein Extraction

Cells cultured on 10 cm dishes were washed twice PBS at room temperature and lysed with 100 µl SIB lysis buffer on ice. Cell lysates were scraped off the surface of the dishes with a plastic cell scraper (Costar) and transferred to 1.5 ml Eppendorf tubes. The lysates were then sonicated with a Sonicator (Fisher Scientific) to sheer genomic DNA. Three rounds of sonication were performed; each lasted for 10 seconds at the setting of 4. Between each sonication, lysates were placed on ice for at least 10 seconds. Sonicated lysates were centrifuged at over 10,000 g on a personal centrifuge (Eppendorf). Supernatants were transferred to a new tube and stored at -20^{o}C. In the experiments analyzing SRAP expression following 2'-OMe treatments, protein samples were extracted by All-prep kit (Qiagen), as described in Section 7.

3.2. Micro BCA Assay

Total protein concentration in a cell lysate was determined using a Micro BCA Protein Assay Kit (Pierce), according to the manufacturer's instructions. Briefly, the cell lysates, alongside with a series of concentration of bovine serum albumine (BSA), were diluted 1:500 in ddH_2O and incubated with Cu^{2+} in an alkaline medium for 30 minutes at 37^{o}C. A Spectra Max 190 spectrophotometer (Molecular Devices) was used to detect colorimetric absorbance at 562nm. The SpectraMax software bundled with the spectrophotometer performed a linear regression statistics on the readings of BSA to calculate the equation of the best fit line as a standard. The Y-intercept and the slope of the standard line were used to calculate total protein concentrations.

3.3. Sample Preparation

Cell lysates were thawed on ice. The amounts of lysates containing 100 µg of total protein were transferred to 1.5 ml Eppendorf tube. The lysates were then adjusted to equal volume with the SIB buffer. Appropriate amount of 4X sample buffer was combined with lysates to a final concentration of 1X. Prior to electrophoresis, samples were placed in a boiled water bath for 5 minutes.

3.4. SDS-PAGE and Immuno-detection

Samples containing 100µg total protein were separated on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN Electrophoresis System (Bio-Rad). Samples were stacked at 80V in 5 % polyacrylamide gel for 15-20 minutes and then separated at 150V in 15 % polyacrylamide gel for 2-2.5h.

Following electrophoresis, proteins were transferred to 0.2µm nitrocellulose membrane (Bio-Rad) in a mini gel transfer apparatus (Bio-Rad), according to the manufacturer's instructions. Transfers were done in 1X pre-chilled CAPS transfer buffer (0.22 % 3-[cyclohexylamino]-1 propane sulfonic acid, 10 % v/v methanol, pH 10.5) at 4^{o}C, 80V for 1h. After transfer, the membrane was baked at 65^{o}C for 30 minutes to stabilize the attachment of proteins, and then blocked in TRIS buffered saline (TBS) containing 5 % non-fat milk powder (Nestle) for at least 30 minutes.

The membrane was then probed with a primary antibody diluted in blocking buffer (1:3000 or 1:5000, depending on the antibody) and incubated at 4^{o}C for 16-20h with gentle shaking. Primary antibody mixture was then removed and the membranes were washed 3 times for 5 minutes each in TBS buffer containing 0.1 % Tween-20 (TBST).

Secondary antibody compatible with the primary antibody was diluted in blocking buffer (1:5000) and incubated on the membrane for 1h at room temperature. Similarly, secondary antibody mix was removed and the membranes were washed. The membranes were placed in a plastic bag and covered with Super Signal HRP substrate (Pierce) for 5 minutes at room temperature. Finally, membranes were exposed on a ChemiDoc imaging system (Bio-Rad) and then stored at -20^{o}C.

4. RNA Analysis

4.1. RNA Extraction

RNA samples were extracted using Trizol reagent (Invitrogen) in most experiments, except the experiments studying time course effects of modified oligoribonucleotides on the splicing of endogenous SRA, in which the All-prep RNA/Protein Kit (Qiagen) was used (see Section 7).

Cells cultured on 6-well plates were washed twice with room temperature PBS prior to extraction. The entire surface of one well was covered by 1ml Trizol reagent and allowed 5 minutes for incubation. The slurry was then pippeted up and down for several times and transferred to a DNase/RNase-free 1.5ml Eppendorf tube. The slurry was immediately processed for RNA extraction, or stored at -80^{o}C for no more than 1 week prior to extraction.

RNA extractions were carried out according to the manufacturer's instructions, with one minor modification. Briefly, 0.2 ml chloroform (Fisher Scientific) was added to

the Eppendorf tube containing the slurry prepared with 1ml Trizol. The tube was vigorously inverted for 15 seconds and set at room temperature for 2-3 minutes. The sample was then centrifuged at 12,000 g for 15 minutes at 4^{o}C. Four hundred micro liters from the aqueous phase was transferred to DNase/RNase-free 1.5 ml Eppendorf tube and then mixed with 0.5ml Isopropanol (Fisher Scientific). The mixture was vigorously inverted for 15 seconds and incubated at room temperature for 10 minutes. The sample was then centrifuged at 12,000 g for 10 minutes at 4^{o}C to precipitate RNA. After centrifugation, the supernatant was discarded. The RNA pellet was re-suspended in 1ml 75 % ethanol, which replaced the isopropanol, and centrifuged at 12,000 g for 5 minutes at 4^{o}C. The RNA pellet was air-dried at room temperature. Depending on size, the pellet was dissolved in 20 to 50 µl ddH_2O and stored at -80^{o}C. The minor modification was that, 400 µl from the aqueous phase was transferred to a new tube, instead of the entire volume of this layer. This modification aimed to minimize contaminations from genomic DNA from the intermediate phase or proteins from the lower phase.

4.2. RNA Quantification

RNA stock was removed from -80^{o}C, placed in a 65^{o}C water bath for 10 minutes. The tube was briefly centrifuged and placed on ice. In most experiments, to quantify RNA concentration, the stock was 1:50 diluted by mixing 4µl RNA with 196µl ddH_2O in a UV transparent 96-well plate (Falcon or Costar). Each sample was processed in duplicates to control pipetting accuracy. As a blank, 4µl ddH_2O was processed in the same way. In some cases when the RNA yield was expected to be low, a dilution factor of 200 was used by mixing 1µl RNA with 199µl ddH_2O.

The Spectra Max 190 spectrophotometer (Molecular Devices) was used to measure optical density at 260 nm (OD260). Averages of two OD260 readings from the duplicates (OD260ave) were calculated and used to determine the concentration the RNA stock by the following equation:

$$Concentration(\mu g / \mu l) = OD260ave \times 40 \times dilu.factor \Big/ 1000.$$

An optical density of 1 is equivalent to 40 μg/μl of single-stranded RNA and the dilution factor is 50 (or 200 in some experiments). RNA yield was typically 10-20 μg (from cells plated in a well on 6-well dishes). Purity of the samples was determined by the ratio of readings at 260 nm and 280 nm. Typical purities range from 1.6 to 2.0.

4.3. Adjusting RNA Concentration for Reverse Transcription

Following quantification, a portion of the RNA stock was transferred to a 200 μl PCR tube, diluted with ddH_2O to 0.2μg/μl and stored at -80^oC. Before use, the samples were thawed and centrifuged as described in Section 4.2.

4.4. DNase Treatment

DNA in the RNA sample was digested by the RQ1 RNase-free DNase (Promega), according to the manufacturer's instructions with minor modifications. Briefly, in a 200 μl PCR tube, 0.5 to 1.5 μg RNA sample was mixed with 4 μl RT buffer (Invitrogen), 2 μl (1 Unit) RQ1 DNase and proper amount of ddH_2O in a total volume of 20 μl. The tube was then placed in a 37^oC water bath for 45 minutes to allow digestion of DNA. Then, 2 μl Stop Solution was added and the tube was incubated in

a 65 ^{o}C water bath for 10 minutes to inactivate DNase. Samples were than placed on ice for at least 2 minutes prior reverse transcription. Two modifications were applied to the manufacturer's recommended protocol. First, the reverse transcription buffer (RT buffer, from the MML-V kit, see Section 4.5) was used instead of the original DNase buffer, and second, the time for digesting DNA was 45 minutes instead of 30 minutes. As the RQ1 DNase was competent in the RT buffer (preliminary result, not shown), using this buffer for DNase treatment eliminated inconsistency of buffer in the downstream reverse transcription step. A longer incubation time, suggested by the manufacturer as an option, aimed to obtain optimal digestion.

4.5. Reverse Transcription

The RNA was reverse transcribed to cDNA by Moloney Murine Leukemia Virus (MML-V) Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Briefly, in a 200 µl PCR tube, one half of the DNase-treated RNA was mixed with 4 µl RT buffer, 3 µl DTT (Dithiothreitol, 0.1 M), 0.3 µl random primer (0.2µg/ml), 1.5 µl dNTPs (10mM each), 0.3µl (12 Units) RNaseOUT Ribonuclease Inhibitor, 1.5 µl (300 Units) MML-V and appropriate amount of ddH_2O in a total volume of 30 µl. The mixture was incubated at room temperature for 10 minutes and then in a 37 ^{o}C water bath for 1 h. Following incubation, the mixture was transferred to a thermocycler (TECHNE, model number FGEN02TP) where it was heated to 99 ^{o}C for 10 minutes and then cooled to 4 ^{o}C. The resulting cDNA was stored at -20 ^{o}C. To control the efficiency of DNase treatment, the rest of the DNase-treated RNA sample was subjected to the same procedures in the absence of MML-V. For real-time

PCR, 0.75µg RNA was reverse transcribed. The cDNA was then 1:5 diluted by adding 120µl ddH_2O into the tube.

5. PCR and Electrophoresis

Sequences and brief description of PCR primers are listed in Table 1, by the end of Section 5.

5.1. Radioactive PCR and Triple-Primer PCR (TP-PCR)

Radioactive PCR was used to detect transcripts derived from SRA minigene. The Xie1-U and Ex2-L primers were designed to target the 5' and 3' ends of SRA minigene sequence (Table 1). In a 200 µl PCR tube, 1 µl cDNA was mixed with 1.5 µl PCR buffer (10 X), 0.6µl $MgCl_2$ (50 mM), 0.3 µl each primer (0.2 µg/µl), 0.3 µl dNTPs (10mM each), 0.08 µl (0.4Units) Platinum TAQ polymerase (Invitrogen), 11.5 µl ddH_2O and 0.1 µl fresh α-^{32}P-dCTP (GE Healthcare). The mixture was then transferred to the TECHNE thermocycler in which it was processed 1 cycle of 94 ^oC for 4 minutes; 30 cycles of 94 ^oC for 30 seconds, 62 ^oC for 30 seconds, 72 ^oC for 30 seconds, and 1 cycle of 4 ^oC hold.

Triple-Primer PCR (TP-PCR) (113) was used to detect endogenous fully spliced and intron-1 retention SRA transcripts. Laz-L is a common primer designed to target the beginning of SRA exon-3; Prot-U and Laz-U are designed to target the end of SRA exon-1 and intron-1, respectively (Table 1). In a 200 µl PCR tube, 1 µl cDNA was mixed with 1.5 µl PCR buffer (10 X), 0.6 µl $MgCl_2$ (50mM), 0.3 µl each primer (0.2

µg/µl), 0.3 µl dNTPs (10 mM each), 0.08 µl (0.4 Units) Platinum TAQ polymerase (Invitrogen), 11 µl ddH_2O and 0.1 µl fresh α-^{32}P-dCTP (GE Healthcare). The mixture was then transferred to the TECHNE thermocycler in which it was processed 1 cycle of 94 ^{o}C for 4 minutes; 30 cycles of 94 ^{o}C for 30 seconds, 62 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds, and 1 cycle of 4 ^{o}C hold. The PCR products were separated on acrylamide gels immediately (see Section 5.2) or stored at -20^{o}C.

5.2. Denaturing Acrylamide Gel Electrophoresis

The radioactive PCR product was 1:5 diluted in loading blue (80 % deionized formamide, 1 mM EDTA, trace xylene cyanol and bromophenol blue), boiled for 5 minutes and briefly centrifuged. 10 µl of the sample was migrated on denaturing polyacrylamide gel (4.8 % acrylamide, 0.16 % bis-acrylamide, 42 % Urea, 1 X TBE) in a Sequi-Gen or a Sequi-Gen Model S2 apparatus (Bio-Rad) at 50^{o}C for 1.5 to 2 hours. The gel was transferred on a piece of Waterman paper, dried in a Slob Gel Dryer (Savant, model number SDG-4050) and exposed to a Phosphorimager screen (Bio-Rad) for either 2 hours or overnight. Autographs were developed by a Personal Molecular Imager FX (Bio-Rad). Quantity1 software (Bio-Rad, version 3.1) was used to quantify the intensity of each band. A rectangle was set to enclose a band and an identical rectangle was set just above the band as background. When it is not feasible to set a background right above the band, a background right below the band was set. The intensity of a band was calculated by subtracting the signal in the background rectangle from the signal in the rectangle containing the band.

5.3. Preparing PCR Templates from Acrylamide Gel

The position of a band on a denaturing acrylamide gel was visualized by exposing the dried gel to a film (Kodak) in a cassette for at least 16 h. The autograph was developed and used as a reference to locate the positions of bands. A band was marked with a pencil in a rectangle, and the piece of the gel was cut with a surgery scalp. The gel was then transferred to a 1.5 ml Eppendorf tube containing 100 µl ddH_2O and boiled for 10 minutes. The sample was then briefly centrifuged to bring down debris from the gel and the Waterman paper. From the top, 50 µl of supernatant was transferred to a new tube to serve as PCR template.

5.4. PCR for T-A Cloning

The template prepared was re-amplified by Platinum TAQ polymerase (Invitrogen). The Xie1-U and Ex2-L primers were described in 4.6. In a 200 µl PCR tube, 1 µl PCR template was mixed with 2.5 µl PCR buffer (10 X), 1µl $MgCl_2$ (50 mM), 0.5 µl each primer (0.2 µg/µl), 0.5 µl dNTPs (10 mM each), 0.1 µl (0.5 Unit) Platinum TAQ polymerase (Invitrogen), and 18.9 µl ddH_2O. The mixture was then transferred to the TECHNE thermocycler in which it was processed 1 cycle of 94 ^oC for 4 minutes; 30 cycles of 94 ^oC for 30 seconds, 54 ^oC for 30 seconds, 72 ^oC for 30 seconds, and 1 cycle of 4^oC hold. To check the sizes of the amplicons, a portion of the PCR product was separated on 1.5 % agarose gel. The gel was stained in TBE containing ethidium bromide for 10-20 minutes after the separation. The image was captured under a UV light box.

This protocol was also used to select bacteria colonies containing the desired plasmid for sequencing.

5.5. Real Time PCR and Data Analysis

As described in Section 4.5, to prepare templates for real time PCR, 0.75 µg RNA was reverse transcribed and the resulting cDNA was 1:5 diluted. The final cDNA concentration is 50 ng/10 µl.

The real-time PCR reaction consists of 0.56 µl LightCycler FastStart DNA Master SYBR Green I (Roche, final concentration is 1 X), 10 µl template cDNA. 2.8 µl PCR buffer (10 X), 1.4 µl $MgCl_2$ (50 mM), 2.24 µl dNTPs (2.5 mM each), 0.7 µl DMSO (Sigma), 0.35 µl each primer (0.2 µg/µl), 0.14 µl (0.7 Unit) Platinum TAQ polymerase (Invitrogen), 0.28 µl Fluorescin (1 µM, Bio-Rad) and 11.6 µl ddH_2O.

The real-time PCR was performed in an iCycler (Bio-Rad) for 1 cycle of 95 °C for 10 minutes; 40 cycles of 30 seconds at 95 °C, 60 seconds at 61 °C, and 30 seconds at 72 °C, with fluorescence acquisition at the end of each cycle, and 1 cycle of a melt curve program consisting of 1 minute at 60 °C, and gradual increase to 95°C at a rate of 0.5 °C after every 10 seconds of hold at the previous temperature. A fluorescence acquisition was performed at the end of each hold.

Each cDNA sample was analyzed for 3 genes, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), TFF1 (Trefoil Factor 1) and PR (Progesterone Receptor), in 3 adjacent wells on the same PCR plate. Primers were previously described by another research group (114). For each sample, the threshold cycle of GAPDH was first arbitrarily normalized to the median of all GAPDH readings from the real-time PCR analysis. Then the threshold cycles of PR and TFF1 were normalized according to the

differences between the GAPDH threshold cycle and the median. Finally, the delta Ct method was used to calculate the level of induction (difference between estradiol-treatment and ethanol-treatment, for cells transfected by either AS-SRA or AS-Bgl). Then the difference between the inductions from cells transfected by AS-SRA and with AS-Bgl was calculated.

6. Cloning and Plasmid Preparation

6.1. T-A Cloning for Sequencing

For sequencing the minigene RNA transcripts, PCR was performed as described in Sections 5.3 and 5.4. TOPO TA Sequencing Cloning Kit (Invitrogen) was used to ligate the PCR products into PCR4-TOPO vector for sequencing, according to the manufacturer's instructions.

Briefly, 2 µl of fresh PCR product was mixed with 1µl salt solution, 1µl PCR4-TOPO vector and 2 µl ddH_2O. The mixture was incubated at room temperature for 20 minutes and placed on ice. For each cloning, 1 vial of OneShot chemically competent E. coli. (Invitrogen) was allowed to thaw on ice. Then 2 µl of the mixture was gently added to the bacteria. The vial was incubated on ice for 20 minutes. Following the incubation, a heat-shock (42 ^{o}C, 30 seconds) was performed. Then the vial was immediately placed on ice. 250 µl of room temperature SOC Medium was added into the vial and the vial was incubated at 37 ^{o}C for 1 h with vigorous shaking (250 rpm). Finally, 50 µl of the actively growing bacterial culture was spread on a room temperature LB-Agar plate containing carbenicillin (described in Section 1.1) and

cultured at 37 ^{o}C for 16-20 h. For each cloning experiment, a blank control mixture without PCR product was processed alongside to control for the efficiency of antibiotic selection.

Presence of the desired plasmid in a colony was checked by PCR as described in Section 5.4. The colony was lifted with a sterile toothpick and re-suspended in 50 µl of ddH_2O. PCR template was prepared by boiling 25 µl of the suspension for 5 minutes, followed by a brief centrifugation. For each cloning, at least 5 colonies were checked by PCR, and one colony presenting a strong signal of correct size was selected to be cultured. For this, the remaining 25 µl of suspension was transferred into 5ml LB medium supplemented with carbenicillin, and cultured at 37 ^{o}C for 16-20h with vigorous shaking (250 rpm).

The Mini-prep Kit (Invitrogen) was used to prepare plasmid for sequencing, according to the manufacturer's instructions. Before sequencing, 1 µg of the plasmid was subjected to a restriction digest by two restriction enzymes to check for the presence of the correct insert size. Sequencing was done by University of Calgary DNA Services.

6.2. Plasmid Preparation

Bacteria cultures were described in Section 1.1, and processed with either the Maxi-prep Kit (Qiagen) or the Mini-prep Kit (Qiagen), according to the manufacturer's instructions. Isolated plasmid DNA was dissolved in ddH_2O.

6.3. Quantification of Plasmid Yield

Dissolved plasmid DNA was 1:200 diluted by mixing 1µl plasmid DNA with 199µl ddH_2O in UV transparent 96-well plate (Falcon or Costar). Each sample was processed in duplicates to control for pipetting accuracy. As background, 1µl ddH_2O was subjected for the same procedure.

The Spectra Max 190 spectrophotometer (Molecular Devices) was used to measure optical density at the wavelength of 260 nm (OD 260). Averages of the OD 260 readings (OD 260ave) from the duplicates were calculated and used to determine plasmid yield by the following equation:

$$Concentration(\mu g/\mu l) = OD260ave \times 50 \times dilu.factor \Big/ 1000 .$$

An optical density of 1 is equivalent to 50µg/µl of double-stranded plasmid DNA, and the dilution factor is 200. Yield of a Maxi-prep was typically >300 µg while yield of a mini-prep was typically >10 µg. Purity of the sample was determined by the ratio of 260 nm and 280 nm, and typically ranges from 1.6 to 1.8.

6.4. Restriction Digest

Hind III or Xho I restriction enzymes (Invitrogen) were used to digest 1µl of plasmid DNA, individually or together, in the compatible buffer React 3 (Invitrogen). Samples were incubated at 37^{o}C for 16-20h and then separated on 1.5 % agarose gel.

7. All-prep and Acetone Precipitation

All-prep: The All-prep Kit (Qiagen) was used to extract RNA and protein from the same sample. Cells cultured in 6-well dishes were washed twice with room temperature PBS and lysed in 200µl APL buffer (lysis buffer from the kit) on ice. The cell lysate was then processed, as illustrated in the flowchart (Figure 10A).

Acetone precipitation: Protein extracted by the All-Prep kit forms a yellow precipitate when resolved on SDS-PAGE. The APL buffer is suspected to contain high concentration of potassium ions that may precipitate SDS. Therefore, an acetone precipitation to purify protein sample is preferred. The 200 µl protein sample was mixed with 800 µl ice cold acetone and incubated on ice for 15 minutes. The mixture was then centrifuged at 12,000 g for 10 minutes at 4^{o}C. The supernatant was discarded and the pellet was dissolved in 40 µl SIB buffer.

8. Fluorescence Microscopy

Cells were cultured on cover-slips placed in 6-well dishes and transfected with 0.5 µM fluorophore-tagged oligoribonucleotides (AS-SRA-flu or AS-Bgl-flu, as described in Section 2.2). Then 24 h, 48 h or 72 h post-transfection, the cover-slips were briefly washed with PBS and the cells were fixed with 3.7 % formaldehyde in PBS for 15 minutes at room temperature. The fixed cells were then rinsed with PBS and treated with 0.2 % Triton-X100 in PBS for 1 minute, in order to be permeable. Cell nuclei were stained with 1µg/ml of DAPI (4', 6-diamidino-2-phenylindole). Finally, the cover-slip was mounted onto microscopy slides with FluorSave™Reagent

(Calbiochem). Fluorescent images were captured and visualized with an Eclipse E1000 epifluorescent microscope and the ACT-1 software provided with the microscope (v.2.63; Nikon).

Results

1. SRA Minigene as a Model to Study SRA Intron-1 Splicing

Prior to this study, it has been established that the relative proportion of intron-1 retention, non-coding SRA varies among breast cancer cells. Hence, alternative splicing of SRA intron-1 determines the differential ratios between coding and non-coding SRA in these cells, and ultimately the differential balances between SRA and SRAP. To study whether the primary genomic sequence in and around intron-1 is sufficient for the splicing of this intron, an SRA minigene, which was previously constructed by Dr. Florent Hubé (95), was used.

1.1 Designing SRA Minigene

The minigene is designed to fuse SRA exon-1-intron-1-exon-2 downstream to a modified portion of β-globin gene containing a constitutively splice-able intron. Successful removal of the β-globin intron serves as an indication that the minigene RNA transcript has been at least partially processed by the splicing machinery (115). An adaptor was fused downstream to the SRA sequence to introduce V5 tags to three possible open reading frames. The minigene was then cloned in to pcDNA4/HisMax TOPO TA vector (Invitrogen), which offers 6 His tags upstream of the construct (Figure 11A). The pcDNA4/HisMax TOPO TA vector is an expression vector driven by a CMV promoter, allowing the transcription of the recombinant gene in mammalian cells. Minigene RNA transcripts can be detected by RT-PCR; their

putative proteins can be detected by Western blot, targeting the His or V5 tags (Figure 11B).

The minigene plasmid was prepared by Maxi-prep. After each preparation, to check the identity of the plasmid, 1 μg plasmid (by OD) was subjected to restriction digestion by two restriction enzymes with sites flanking the insert, EcoR1 and HindIII (Invitrogen). The expected pattern of digestion by these two enzymes consists of 2 fragments of 4970bp and 1144bp.

1.2 Splicing Patterns of Minigene RNA

Transfecting the minigene into a series of breast cancer cell lines led to the production of 3 RNA products detectable by RT-PCR (Figure 12A). According to their sizes, the signals correspond to RNA transcripts containing both introns (741bp), containing only the SRA intron (612bp) and containing no intron (457bp). In T47D-5 cells, the relative intensity of the smallest band (corresponding to fully spliced, coding SRA) in the total signal appeared to be greater than that of the other two cell lines. This is compatible with the previous finding that T5 cells have a relatively higher ratio of fully spliced SRA (95).

However, splicing of precursor RNA can generate transcripts in low abundance or with approximate sizes. In order to observe other possible transcripts, radioactive PCR was performed as this method provides higher sensitivity and better separation. As such, two additional bands at 517bp and 443bp were detected (Figure 12B, while the top band Figure 12A was not shown). Sequencing of these bands revealed that the

two major products migrated at 457bp and 612bp corresponded to a fully spliced transcript and a transcript with SRA intron-1 retention. The 517bp band corresponded to a transcript with partial SRA intron-1 retention (60bp on the 5' termini of intron-1) and the 443bp band corresponded to a transcript in which the SRA intron-1 was spliced from an alternative donor site 14 bases upstream of the conventional site (Figure 12B). The partial intron-1 retention event has previously been observed from endogenous SRA (Figure 7, transcript 12). The alternative donor site, even though not observed for endogenous SRA, suggests the possible use of a cryptic splicing site.

1.3 Minigene SRA Intron-1 Retention in Breast Cancer Cells

Quantification (performed as in Section 5.2 of Materials and Methods) shows that the relative ratio of the minigene transcript containing SRA intron-1 retention was significantly higher in MDA-MB-468 cells than in T5 cells, whereas that of the fully spliced signal was significantly lower in MDA-MB-468 cells than in T5 cells (Figure 12C). There is no significant difference between the two types of cells, for the relative ratio of transcripts containing the partial intron-1 or the alternative donor site.

1.4 Minigene Protein Detected by Western blot

By Western blot, a minigene protein with an approximate molecular weight of 23-25 kD was detected by a mouse monoclonal anti-V5 antibody from MDA-MB-468 and T47D-5 cells 24 hours after transfecting the cells with the minigene. This protein is approximate to, in size, the putative protein encoded by the fully spliced minigene RNA transcript. As a control, the anti-V5 antibody successfully detected the SRAP-

V5 recombinant protein from MCF-7 cells permanently transfected with a construct encoding V5 tagged SRAP (MCF-7-S151) (Figure 13 upper panel).

However, the minigene protein was not detected by a mouse polyclonal antibody targeting a peptide sequence encoded by the junction of SRA exon-1 and exon-2, whereas the antibody recognized endogenous SRAP doublet (Figure 13 lower panel).

2. Using 2'-OMe Oligoribonucleotides to Alter SRA Intron-1 Splicing

Short antisense 2'-O-Methyl oligoribonucleotide phosphorothioates (2'-OMe) hybridize to the target RNA, and form double-stranded RNA complex resistant to RNase H-mediated degradation (116). These constructs have been used previously to modulate intron splicing (117;118). Similar strategy was utilized in this study to modify the alternative splicing of SRA intron-1.

2.1 Designing 2'-O-Me Oligoribonucleotides

A 20mer 2'-O-Me construct, AS-SRA, was designed to be antisense to the 5' donor site (GU) of SRA intron-1, and 18 bases upstream of this site (in SRA exon-1). It was designed to hybridize to both the donor site and the alternative donor site, 14 bases upstream, blocking the access of splicing machinery from binding to any of these sites. Similarly, the AS-Bgl construct was designed antisense to the donor site and 18 upstream bases on the β-globin intron. In addition, to visualize the intake of the 20mer constructs by cells, two identical fluorescent constructs, AS-SRA-flu and AS-Bgl-flu, were constructed by tagging AS-SRA with CY3 and AS-Bgl with FAM, both on the

3'. The four constructs were synthesized and purified by Tri-Link Technologies (San Diego, USA). Their sequences and targeting sites are illustrated in Figure 14.

The 2'-OMe constructs were tested for their ability to enter breast cancer cells. AS-SRA-flu and AS-Bgl-flu were delivered into T47D-5 cells. Transfected at a dose of 0.5μM, the robustness of fluorescent signals of AS-SRA and AS-Bgl appeared to be on the same level over time. These signals could be detected in both the cytoplasm and nuclei in virtually all cells 24 hours post-transfection, with relatively stronger peripheral nuclear distribution. Over time the peripheral signals attenuated, as well as the overall signal in a cell. Fluorescence was still detectable 72 hours post-transfection (Figure 15).

2.2 2'-OMe Oligos Specifically Promote Target Intron Retention.

To test if the 2'-OMe constructs are capable to specifically promote the retention of their target introns, the minigene plasmid and either 0.5μM AS-SRA or AS-Bgl were co-transfected into T47D-5 or MDA-MB-468 cells, by Lipofectamine. In MDA-MB-468 cells, the 3 major RNA variants (Figure 12) were detected (Figure 16A, identical result in T47-D5 cells was not shown). As expected, while AS-SRA increased the relative intensity of the signal that corresponds to SRA intron-1 retention (Figure 16B), AS-Bgl led to the production of an additional band corresponding to a minigene RNA transcript containing the β-globin intron but not the SRA intron (Figure 16A).

Lipofectamine is a classic cationic-lipid reagent for delivering DNA into cells; however, information regarding its efficiency to deliver single stranded construct was

not provided by the manufacturer at the time this study was performed (communications with Invitrogen). In order to obtain optimal results, Lipofectamine was compared with DMRIE-C (Invitrogen), a newer generation of cationic-lipid reagent optimized to deliver single-stranded RNA as well as DNA, for their ability to co-transfect the minigene plasmid and the 2'-OMe constructs (Figure 17A). At each concentration, AS-SRA co-transfected with the minigene using DMRIE-C provided more robust SRA intron retention on minigene RNA transcripts (Figure 17B). DMRIE-C was therefore used in the following experiments to co-transfect the minigene plasmid with 2'-OMe constructs, or in further experiments to transfect the 2'-OMe constructs alone.

In T47D-5 cells, AS-SRA and AS-Bgl transfected by DMRIE-C specifically promoted the retention of their corresponding introns in SRA minigene RNA transcripts in a dose dependent manner (Figure 18). In addition, AS-SRA led to the production of a unique minigene RNA transcript. Sequence of this transcript revealed that it corresponded to an exon skipping transcript (containing only β-globin exon-1 and SRA exon-2). These result demonstrated that AS-SRA and AS-Bgl are capable to alter the splicing of their target introns.

2.3 Modifying Endogenous SRA Intron-1 Splicing and SRAP Level.

Since we established that the 2'-OMe constructs are capable to alter intron splicing, we further utilized this approach to alter endogenous SRA intron-1 splicing. We were also interested to investigate whether an alteration at the RNA splicing level will result in a change of SRAP expression. Therefore, RNA/Protein All-prep Kit (Qiagen)

was used to simultaneously obtain RNA and protein samples from one sample. A flowchart of the kit is illustrated in Figure 10. It was found that protein samples obtained by this kit require an additional purification step in order to be separated on SDS-PAGE (Figure 10B, also see Section 7 of Materials and Methods).

A TP-PCR method was used to co-amplify SRA transcripts with and without intron-1 (113). A common lower primer (Laz-L) designed in SRA exon-3 recognizes both types of transcript while two upper primers (Prot-U and Laz-L) specifically recognize SRA exon-1 and intron-1 sequences. As such, two signals expected are a 377bp signal corresponding to SRA transcripts contain intron-1 and a 360bp signal corresponding to transcripts without intron-1 (See Figure 8.) (95). Using this method, we were able to detect these two signals from T47D-5 cells transfected by the 2'-OMe constructs or mock transfected (Figure 19A). Comparing to the mock transfection controls (Figure 19A lanes 7-9), AS-SRA shifted the splicing of SRA intron-1 towards its retention at each time point (lanes 1-3), whereas no alteration was observed in cells transfected with an equivalent amount of AS-Bgl (lanes 4-6).

An identical study performed in our laboratory found that the altered SRA intron-1 splicing is associated with an increase in urokinase plasminogen activator (uPA) mRNA, as detected by a real-time PCR based profiling (personal communications with Mr. Charlton Cooper). By Western blot, a corresponding decrease of SRAP, coupled by an increase of urokinase plasminogen activator (uPA), was detected from total cell lysates of T47D-5 cells transfected by AS-SRA (Figure 20). As uPA is an important player involving in breast cancer metastasis, the altered uPA expression suggested a possible role of SRA and SRAP in regulating breast cancer cell invasion.

To further establish the effect of AS-SRA to alter SRA intron-1 splicing in breast cancer cells, different doses of AS-SRA and AS-Bgl were transfected into MDA-MB-468 cells for 24 hours. TP-PCR detected two additional bands in addition to the 377bp and 360bp signals (Figure 21A, top). These bands were amplified as the primer designed for fully spliced SRA (in exon-1) can also hybridize to SRA transcripts containing full and partial intron-1. The principle of TP-PCR aims to generate close PCR efficiencies for the two products, hence favors the co-amplification of the two types of templates. However, MDA-MB-468 cells has the highest relative ratio of SRA transcripts with intron-1 retention among a breast cancer cells tested (95). In addition, AS-SRA may further increase this ratio. As such, amplification of the intron-1 retention transcripts by the primer targeting exon-1 is not avoidable. Therefore, to simplify data analysis, radioactive PCR using Prot-U and Laz-L primers was performed (Figure 21A, bottom). Three bands were amplified from SRA transcripts containing full intron-1, partial intron-1 and without intron-1. Similar as in T47D-5 cells, AS-SRA appeared to increase SRA intron-1 retention in MDA-MB-468 cells (Figure 21B).

2.4 Fluorescent Modified Oligos Alters SRA Intron-1 Splicing.

In addition to visualizing transfection efficiency, the fluorescent 2'-OMe constructs were designed to hybridize to the same sites as the non-fluorescent constructs. Hence we determined if AS-SRA-flu is also potent to alter intron splicing. AS-SRA-flu and AS-Bgl-flu were transfected into T47D-5 and MDA-MB-468 cells. Presence of the

fluorescent tag did not seem to interfere with the capability of AS-SRA-flu to promote SRA intron-1 retention (Figure 22).

In summary, the 2'-OMe construct targeting the donor site of SRA intron-1 is capable to promote retention of this intron in breast cancer cells. As a consequence, SRAP expression is decreased. Hence, we successfully modified the balance between SRA and SRAP levels toward less SRAP.

3. Altered Estradiol-induced Gene Transcription following 2'-OMe Oligos Treatment

SRA and SRAP can both regulate ERα activity in mediating gene transcription. To determine if altering the SRA-SRAP equilibrium may change ER-mediated gene transcription, we analyzed the expressions of two known ERα downstream genes, PR and TFF1/pS2, following transfecting T47D-5 cells (ER positive) with the 2'-OMe constructs. Three conditions were compared to obtain minimum endogenous estrogen background in the experimental system without introducing observable stress to the cells. These conditions defer in the time that cells are cultured in phenol red free medium supplemented with charcoal-stripped serum (during the whole process, or only after transfection), and the medium used during transfection (no serum, or low serum) (Figure 23 A). Under these conditions, T47D-5 cells were mock-transfected and treated with 10^{-8}M estradiol. Cells did not present observable defects under all of the conditions (light microscopy observations). At both 4 and 24 hours post estradiol treatment, inductions of PR and TFF1 in condition 3 were the highest (Figure 23B). Hence, this condition was chosen for further experiments.

In T47D-5 cells transfected with either AS-SRA or AS-Bgl, estradiol treatment induced PR and TFF1 transcription, whereas PR appeared to be more responsive than TFF1. The inductions could be observed at 4 hours and lasted over 48 hours post treatment. Inductions of gene transcription between cells transfected with AS-SRA and AS-Bgl were compared. While AS-SRA did not change TFF1 transcription at any time points, 48 hours post estradiol treatment, RNA level of PR in cells transfected with AS-SRA was significantly lower than that in cells transfected with AS-Bgl (Figure 24). These results suggest that ERα downstream genes responded differentially to the alteration of the balance between SRA and SRAP levels.

4. Summary of Results

This study is based on the following preliminary findings. 1. Splicing of SRA intron-1 is the event that dictates the coding frame of SRAP. 2. SRAP is a regulator of ERα activity. 3. Intron-1 retention, non-coding SRA and fully spliced, coding SRA co-exist in breast cancer cells but at various relative ratios. Hence in a cell, the overall function of *SRA1* products on ERα is dictated by the splicing of SRA intron-1.

An SRA minigene was designed as a model to study SRA intron-1 splicing. In breast cancer cells, successful removal of the β-globin intron in minigene RNA transcripts indicates that these transcripts have been, at least partially, processed by the splicing mechanism. The minigene RNA transcripts presented identical SRA intron-1 splicing patterns as endogenous SRA: intron-1 retention, partial intron-1 retention and intron-1 spliced. Moreover, the relative ratio of minigene RNA with SRA intron-1 retention

was higher in MDA-MB-468 cells than in T47D-5 cells, consistent to the previous finding for endogenous SRA in these cells.

A 2'-OMe construct was used as an approach to alter SRA intron-1 splicing in breast cancer cells. The specificity of this approach was validated as AS-SRA and AS-Bgl increased the retention of their target introns on minigene RNA transcripts. In MDA-MB-468 and T47D-5 cells, AS-SRA promoted retention of SRA intron-1, leading to the generation of more intron-1 retained non-coding SRA transcripts. In T47D-5 cells, altering this balance resulted in a corresponding decrease in SRAP. Also in these cells, estradiol induced transcriptions of two ERα-regulated genes (TFF1 and PR) responded differentially to the alteration of SRA intron-1 splicing.

Discussion

Traditionally, functional RNA and mRNA are perceived as two exclusive groups. SRA, so far the only identified functional RNA encoding a protein, bridges the gap between the two groups. As such, our conventional assumption about the distinct roles of the two types of RNA has been challenged. We are encouraged to embrace a newly-revealed complexity that a gene, such as *SRA*, can regulate cellular processes through two products at the RNA and the protein levels, presumably for the sake of making more efficient use of cellular resources.

The correlation between SRA over-expression and breast tumorigenesis, together with the correlation between SRAP expression and breast cancer patient prognosis under Tamoxifen treatment, justify the study of these molecules highly relevant to breast cancer research. Full understanding of the bifaceted SRA-SRAP system might identify new targets for curative or preventive strategies to fight breast cancer.

1. Minigene as a Model to Study SRA Intron-1 Retention

In this study we described an SRA minigene model to study SRA intron-1 splicing as well as established an approach to artificially alter the balance between coding and non-coding SRA toward the production of more intron-1 retention, non-coding SRA. Two cell lines used, MDA-MB-468 and T47D-5, have respectively the highest and the lowest relative ratio of intron-1 retention SRA among a series of breast cancer cells tested.

Our findings validated the SRA minigene as a model to study SRA intron-1 splicing. Transfecting the minigene construct containing the SRA exon-1-intron-1-exon-2 into breast cancer cells generated RNA transcripts with identical SRA intron-1 splicing patterns as those of the endogenous SRA: intron-1 retention, partial intron-1 retention and intron-1 spliced. This result suggests that the primary sequence of this region is sufficient for splicing of SRA intron-1. Further more, similar to endogenous SRA, the relative proportion of minigene RNA with SRA intron-1 retention is higher in MDA-MB-468 cells than in T47D-5 cells, indicating cell type specific regulations of SRA intron-1 splicing.

In addition, using an anti-V5 antibody, a protein encoded by the minigene RNA was detected by Western blot, indicating that specific minigene transcript is indeed translatable (Figure 13 upper panel, V5-SMG). However, it was not detected by an anti-SRAP antibody targeting a peptide encoded by the junction of SRA exon-1-exon-2, which is capable to recognize proteins derived from SRA intron-1 spliced transcript, such as the V5-tagged SRAP (Figure 13 lower panel).

This minigene protein has an approximately similar molecular weight to the putative protein of fully spliced minigene transcript (Figure 11B). Failure to be detected by anti-SRAP may be due to the downstream V5 tag. Presumably, detection of V5-tagged minigene protein can be more interfered by the tag, comparing to that of V5-tagged SRAP, as the coding frame between the V5 tag and SRA exon-1-exon-2 junction is much smaller (exon-2 only, versus exon-2 to exon-5). It is possible that the V5 tag interferes with the detection of the minigene protein by anti-SRAP antibody, even if the targeted epitope of the antibody is presented in the protein.

2. 2'-OMe Oligoribonucleotides Alter Splicing of Minigene Introns

The second goal of this study was to establish an approach to artificially alter SRA intron-1 splicing. Anti-sense 2'-O-Methyl phosphorothioated oligoribonucleotides have long been used to design molecular beacons for cell imaging and quantification of gene expression (119). This backbone modification allows the antisense construct to hybridize to target quicker than another commonly-used modification, 2'-deoxy, while minimizes degradation of the double-stranded RNA duplex by RNase H (116). Therefore, unlike antisense RNA interference technologies, 2'-OMe oligoribonucleotides do not aim to affect the stability of its target. Several previous research findings utilized this principle to re-direct intron splicing. By masking a splicing site to block the access of spliceosome machinery, 2'-OMe oligoribonucleotides were used to abolish abnormal splicing due to a mutation-generated splicing site, or to favor the selection of one splicing site to its alternative (117;118).

This principle was utilized to reprogram SRA alternative splicing in breast cancer cells. AS-SRA is a 2'-OMe oligoribonucleotides construct designed to mask both the canonic donor site of SRA intron-1 as well as an alternative donor site 14 bases upstream. Masking these sites should block the access of U1 snRNP, a key player of spliceosome that binds to the donor site during splicing (Figure 9). AS-Bgl is a control construct designed to target the identical region on human β-globin precursor RNA. This control is competent to reprogram splicing, but is not functional in the breast cancer cells due to absence of its target intron. Using the minigene model, we demonstrated the specificity of the two constructs, as co-transfecting AS-SRA or AS-

Bgl with the minigene led to relative increases of minigene RNA transcripts containing the corresponding intron.

Masking the SRA donor sites on minigene precursor RNA also promoted the pairing of the β-globin donor site with the SRA acceptor site or the alternative acceptor site (for partial intron-1 retention), generating exon-skipping minigene RNA transcripts. (The pairing between the β-globin donor site and the SRA alternative acceptor site was only detectable on film as a weak signal hence was not shown.) In fact, even without masking the SRA donor site, a basal level of the pairing between the β-globin donor site and the SRA acceptor site can be detected by film (personal observations not shown). These findings indicate that the SRA intron-1 acceptor site is unlikely a weak site not competent for recognition by the spliceosome.

3. A Possible Model of SRA Intron-1 Splicing

In fact, the differential ratio of SRA intron-1 splicing among the breast cancer cells was not mainly determined by SRA's primary sequence. First, SRA exon-1-intron-1-exon-2 region did not show polymorphism across breast cancer cell lines (95). Second, minigene RNA underwent the cell-type specific regulation of SRA intron-1 splicing, as endogenous SRA. Hence, it is reasonable to postulate that differential expressions of splicing regulators among the cells may play a role in the cell type specific alternative splicing of SRA intron-1.

A Rainbow splicing database analysis (120) of the SRA exon-1-intron-1 primary sequence reveals that the exon-intron junction, which contains the SRA intron-1

donor site, is a putative binding site of hnRNP F/H (6 Gs out of 9 bases) (Figure 25). Preliminary data demonstrated that, in three breast cancer cell lines (MDA-MB-468, ZR75 and T47D-5), a higher ratio of SRA intron-1 retention is coincident with a higher hnRNP F/H expression (personal observation, not shown). These factors are conventionally viewed to enhance generic intron splicing: they can form homo or heterodimers and bind to intronic G-rich sequences and facilitate donor site definition (121;122). However, the overlapping of the hnRNP F/H binding site and the SRA intron-1 donor site may raise an alternative hypothesis that hnRNP F/H, in the form of monomer, dimer or polymer, bind to the SRA exon-1-intron-1 junction and interfere with the definition of SRA intron-1 by blocking the access of U1snRNP to the donor site. However, to test this hypothesis, the binding of these factors to the SRA exon-1-intron-1 junction *in vitro* and in cells should be validated through UV crossing-linking and oligoribonucleotide competition. Also, it should be established whether these factors fulfill the proposed role in regulating SRA intron-1 splicing, presumably using the minigene as a model in hnRNP F/H over-expression/knock-down/rescue cell contexts.

4. AS-SRA Alters the Balance between SRA and SRAP Levels

Furthermore, this study showed that alternative splicing of SRA intron-1 in breast cancer cells can be artificially reprogrammed by AS-SRA. Transfecting AS-SRA into T47D-5 breast cancer cells led to the production of more intron-1 retained, non-coding SRA, consequently a decrease in SRAP expression. Binding by AS-SRA presumably does not drastically change stability of the target SRA RNA. Further, AS-SRA does not overlap with the SRA functional core region. Hence AS-SRA was

designed to change the relative ratio between coding and non-coding SRA without decreasing the population SRA transcripts or attenuating SRA function.

Besides the precursor SRA RNA and intron-1 retention, non-coding SRA, AS-SRA may also hybridize to fully spliced, coding SRA through 18 bases antisense to the 3' of SRA exon-1. However, the decrease of SRAP observed is not likely due to blockage of translation by the hybridization. In a parallel study, AS-SRA was delivered into MCF-7-S151 cells, which are permanently transfected with a V5-tagged SRAP coding construct. While an expected decrease of endogenous SRAP was observed, the level of recombinant V5-SRAP remained constant (communication of results, Mr. Yi Yan). This result supports that AS-SRA alters the SRA-SRAP equilibrium by regulating alternative splicing of SRA intron-1.

5. Changes in ER Signalling Following AS-SRA Treatment

A further goal of this study was to investigate the consequences on expression of genes that involve in breast cancer and estrogen signalling. In an identical study, transfecting the AS-SRA construct led to an increase in urokinase plasminogen activator (uPA) mRNA level, as detected by a real time PCR based profiling (communication of results, Mr. Charlton Cooper). Consistently, uPA protein was found to increase in T47D-5 cells transfected with AS-SRA. uPA is a predictor of the aggressive phenotype of breast cancer (123), as well as other cancers (124-126). Its expression is associated with increased activities of metalloproteinases (MMPs) such as MMP-1 and MMP-9 (126;127). Up-regulated uPA expression following the alteration of SRA intron-1 splicing suggests that the SRA-SRAP balance, through

regulating cell invasiveness, may play critical roles in breast cancer metastasis. Indeed, previous results in our lab indicated that a relative higher ratio of intron-1 retention, non-coding SRA seems to correlate to the metastatic phenotype of breast cancer cells (95).

Our data also suggests that estradiol-induced gene transcriptions may respond differentially to the alteration of SRA-SRAP equilibrium. In T47D-5 cells, the alteration did not change estradiol-induced transcription of TFF1 at any time point designed for the study, whereas that of PR was significantly down-regulated 48 hours post-estradiol treatment. These results are consistent with previous findings that, induction of PR transcription by estradiol was faster and PR protein expression was elevated in the MCF-7-S151 cells comparing to control cells (MCF-7 cells permanently transfected with empty vector and MCF-7 cells with low level recombinant SRAP detected), whereas the induction of TFF1 transcription did not alter (61). Indeed, unlike p160 family members, non-coding SRA was found not to be required for hormone-dependent transcription of TFF1 or DNA synthesis (70).

Different involvements of SRA and SRAP in transcriptions of the two genes may attribute to distinct ERE sequences on the promoters. TFF1 and PR promoters both contain a consensus half-site ERE (5'-AGGTCA-3'), however, the other half-site of PR (5'-GCTCTT-3', on PRα promoter) has distinct variations to the consensus sequence (5'-TGACCT-3') comparing to that of TFF1 (5'-TGGCCA-3'). (Non-consensus nucleotides are underlined.) Besides ligand and co-regulators, ERE sequence is also a player in the regulation of ER activity. Different ERE sequences have different affinity to ERα and direct conformational changes of ligand-bound

ERα once the receptor dimer loads on DNA (14-16). The different conformations, in turn, determine the profiles of coregulator complexes that interact with the receptors (13). It is possible that the confirmations of ligand-bound ERα on the EREs of TFF1 and PR lead to differential recruitments of SRA and/or SRAP-containing complex, hence altering the SRA-SRAP equilibrium by decreasing SRAP may have differential effects on the transcription of the two genes.

An alternative hypothesis involves factors, besides ER, that have regulatory roles on the transcription of PR but not TFF1: it is also possible that SRA or SRAP is not directly involved in ER-mediated transactivation of these genes, however, may coregulate the expression/activation of transcription factors/coregulators that directly or indirectly associate with the promoter of PR but not TFF1. As such, 48 hours was needed to observe the effect of altering the balance between coding and non-coding SRA on PR transactivation.

A major challenge in testing the above hypotheses comes from the co-existence of SRA and SRAP functions in cells: transient/stable transfection of SRA or antisense RNA interference will increase or decrease both SRA and SRAP simultaneously, introducing a complexity to the correlation of experimental results with either SRA or SRAP. An ideal experimental system should allow the study of SRA and SRAP functions individually. Such a system is being proposed in our laboratory, consisting of constructs that are capable to express, in cells, either SRA or SRAP, or none of both. The construct expressing SRA but not SRAP shall generate RNA transcripts containing the full SRA core region but not the 2 methionine codons of the SRAP coding frame. The construct expressing only SRAP shall contain silent mutations, in

its sequence, that will drastically modifying the predicted RNA secondary structures STR1, 9, 5, 10 and 7 (shown in Figure 4). It has previously been proved that abolishing these structures decreases the function of SRA RNA (67). Besides, a control construct expressing none of SRA or SRAP is designed to have none of the methionine codons but contain all the silent mutations.

6. Conclusion

In conclusion, we designed an SRA minigene that can be used as a model to study alternative splicing of SRA intron-1. In breast cancer cells, minigene RNA transcripts underwent the splicing events of endogenous SRA. We have also established an approach, utilizing the principle of 2'-O-Me oligoribonucleotide phosphorothioates, to change the balance between coding and non-coding SRA in breast cancer cells toward the production of more intron-1 retention, non-coding SRA. This change, in turn, altered the SRA-SRAP equilibrium in these cells. Evidence presented here demonstrated that this alteration can change the expression of certain gene involved in breast cancer pathology, as well as genes regulated through estrogenic activity.

At this moment, the mechanisms regulating alternative splicing of SRA intron-1 and the physiological change in breast cancer cells following the alteration of the SRA-SRAP balance remain to be investigated.

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List of abbreviations

2'-OMe: 2'-O-Methyl oligonucleotides phosphothioates

AD: alternative donor site

AF-1, AF-2: activation function-1, activation function-2

AR: androgen receptor

CBP/p300: CREB binding protein, and p300

DBD: DNA binding domain

DMEM: Dulbecco's Modified Eagles Medium

ER: estrogen receptor

ERE: estrogen responsive element

GPCR: G-protein coupled receptor

GR: glucocorticoid receptor

HDAC: histone deacetylases

hnRNP: heterogeneous nuclear ribonucleoproteins

LB medium: Luria-Bertani medium

LBD: ligand binding domain

MAPK: Mitogen-activated protein kinase

MR: mineralocorticoid receptor

NCBI: National Center for Biotechnology Information

NCoR: nuclear receptor corepressor

NR: nuclear receptor corepressor

ORF: open reading frame

P/CAF: p300/CBP-associated factor

PCR: polymerase chain reaction

PIR: partial intron retention

PR: progesterone receptor

RAR: Retinoic Acid Receptor

RRM: RNA recognition motif

RT-PCR: reverse transcription-PCR

RXR: retinoid X receptor

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SERM: slective estrogen receptor modulator

SHARP: SMRT/HDAC1-associated repressor protein

SLIRP: SRA stem-loop interacting RNA binding protein

SMRT: silencing mediator of retinoic acid and thyroid hormone receptor

snRNP: small nuclear ribonucleoproteins

SR proteins: serine/arginine-rich proteins

SRA: steroid receptor RNA activator

SRAP: SRA protein

SRC-1: steroid receptor coactivator-1

STR: SRA stem-loop structure

TFF1: trefoil factor 1

TGF-β: transforming growth factor-β

TP-PCR: triple primer-PCR

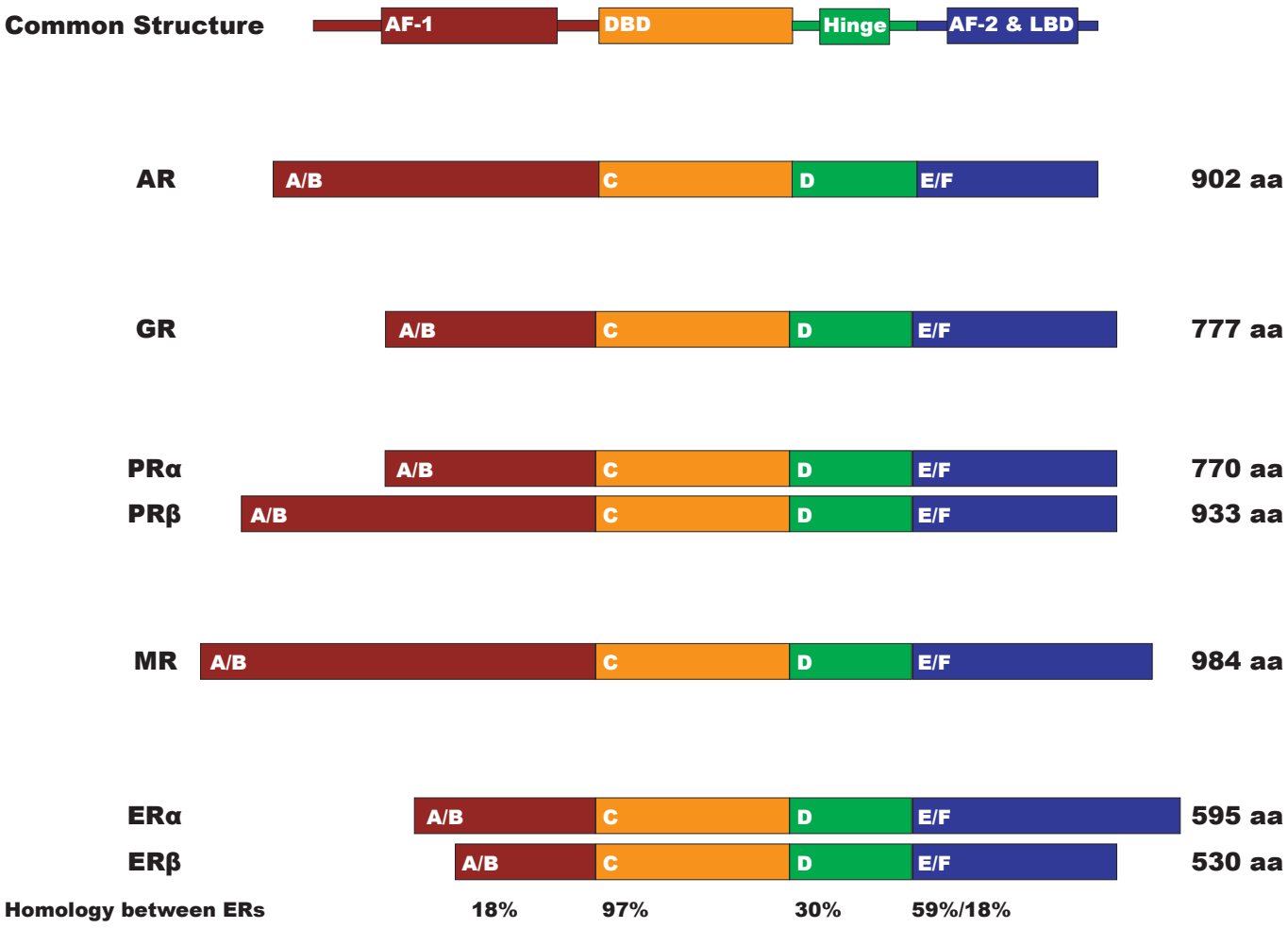
TR: thyroid hormone receptor

Y2H: yeast 2 hybrid

Table 1. Primers used for radioactive PCR, TP-PCR and real-time PCR.

Primer	Sequence	Target
Prot-U	5'-GCCAAGCGGAAGTGGAGAT-3'	3' terminal of SRA exon-1
Laz-U	5'-CCCCAGTATAAGCTAACAGT-3'	3' terminal of SRA intron-1
Laz-L	5'-GACGTCTTCCAATGCCTGTT-3'	3' terminal of SRA exon-3
Xie1-U	5'-GTGCACCTGACTCCTGAGGAGAA-3'	β-globin exon-1
Ex2-L	5'-CTCTGGGGGATCCATCCTGGGGTG-3'	3' terminal of SRA exon-2
PR-U	5'-ACAGGACCCCTCCGACGAAAA-3'	exon-1
PR-L	5'-AGCTGTCTCCAACCTTGCACC-3'	exon-1
TFF1-U	5'-GCCCAGACAGAGACGTGTACAGT-3'	flanking exon-1-exon-2
TFF1-L	5'-CTGGAGGGACGTCGATGGTATTAG-3'	exon-2

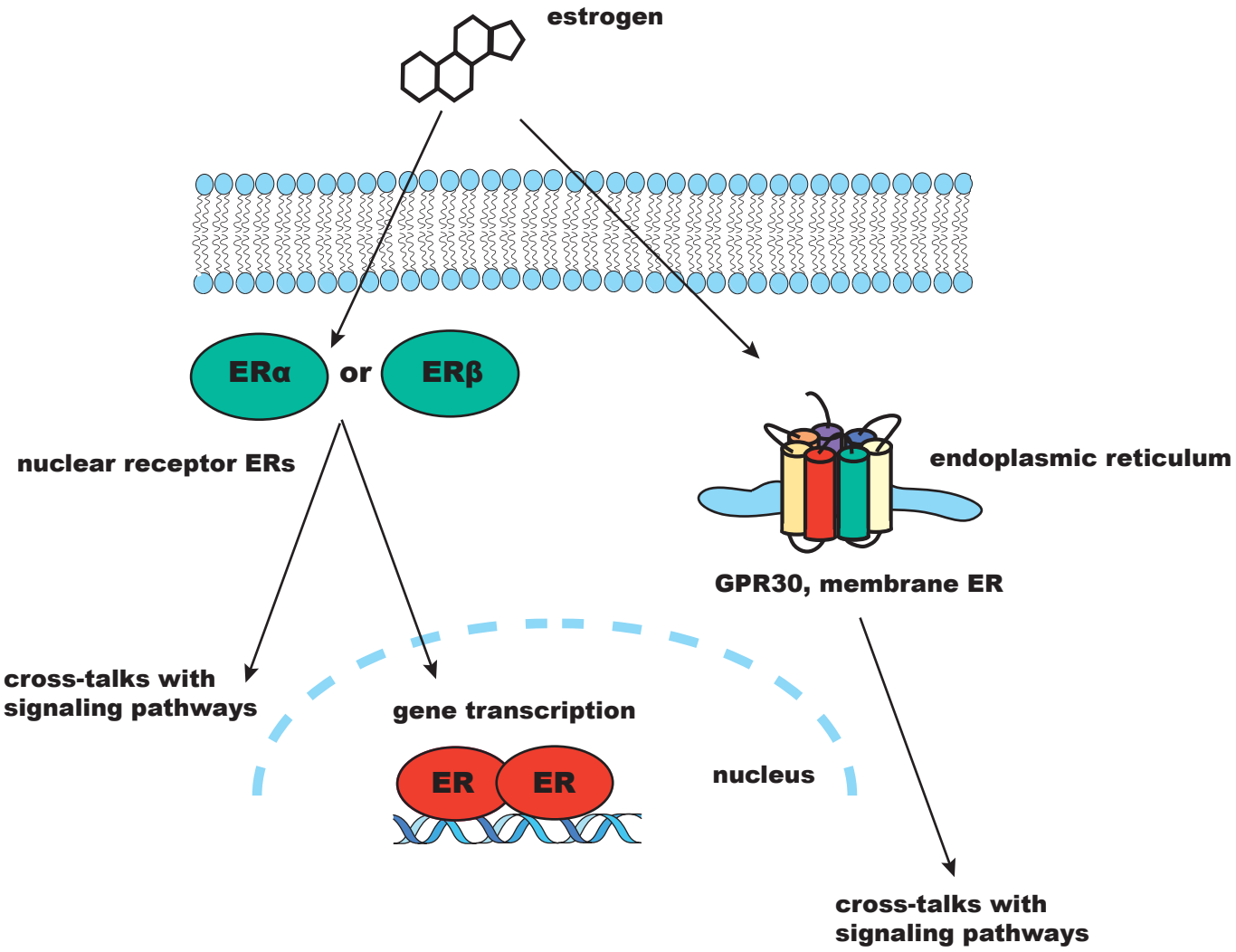
Figure 1. Structure of Steroid Receptors.



All steroid receptors are composed of a variable N-terminal domain (A/B) containing the AF-1 transactivation region, a highly conserved DNA binding domain (C), a flexible hinge region (D), and a ligand binding domain (E) containing the AF-2 transactivation region. The C-terminal F domains of the receptors are not well-defined due to the lack of sequence homology, hence are illustrated together with the E domains. AF: activation function; DBD: DNA binding domain; LBD: ligand binding domain.

Summarized and reproduced from Griekspoor, A. *et. al.* Nuclear Receptor Signaling. 2007 5, e003 and Gustafsson, J.A. J. Endocrinology .1999 163:379-383

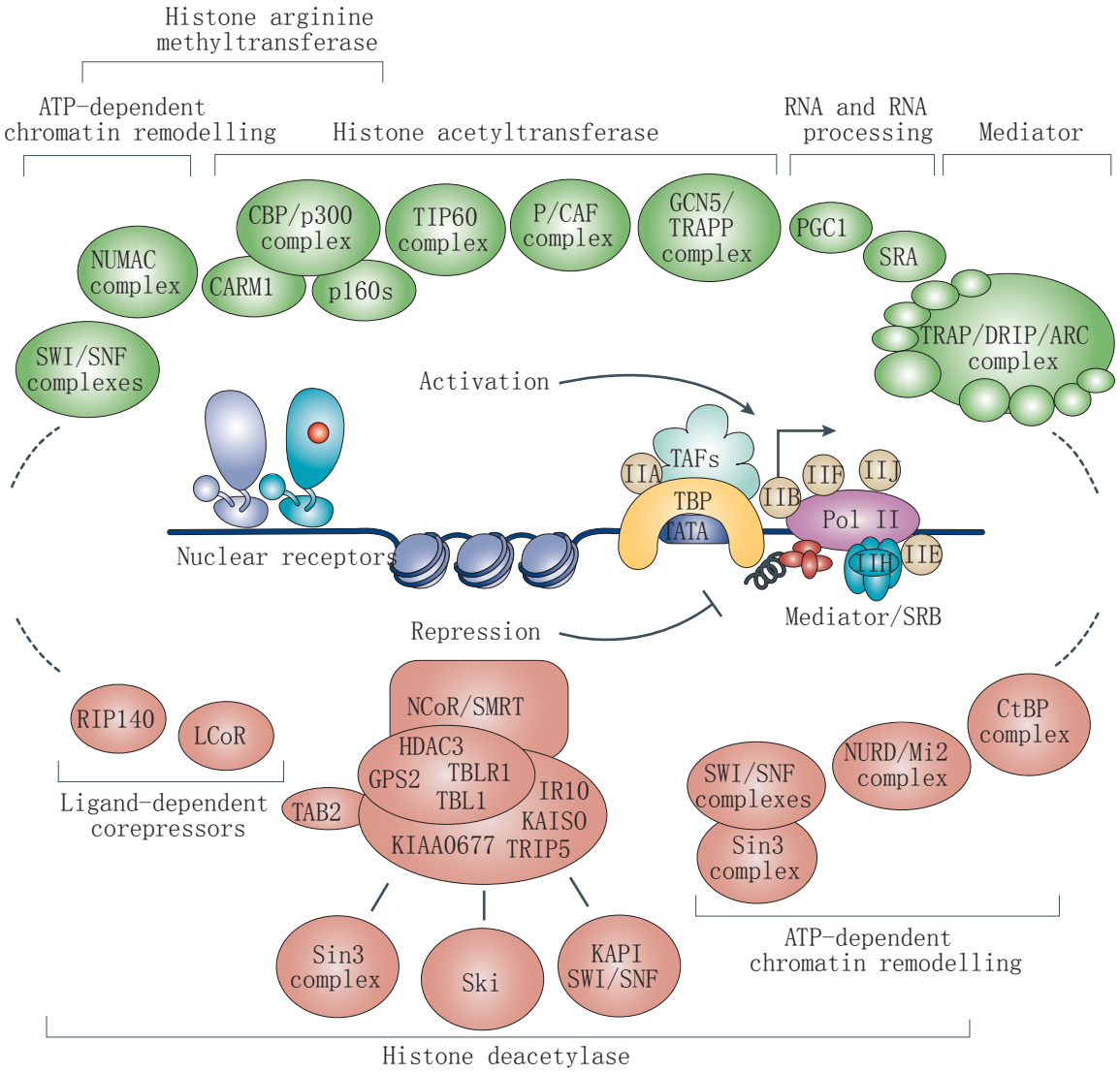
Figure 2. Estrogen signaling pathways in a cell.



Estrogen can signal through either the nuclear receptor ERs to induce gene transcription or crosstalk with other signaling pathways, or through a recently identified membrane ER, GPR30, to regulate other pathways.

Modified from Weatherman, R. Nature Chemical Biology, 2006, 2:175-176

Figure 3. Coactivator and corepressor complexes involving in NR-mediated transcription.



Coregulatory complexes that have various functions and enzymatic activities may participate in the regulation of NR transactivation. Coactivator complexes and their mechanisms of action are shown on the top; corepressor complexes are shown on the bottom. Complexes/factors cited in this thesis are briefly described as the following:

CBP/P300: CREB binding protein/E1A binding protein p300 are two closely related proteins able to coactivate various transcription factors.

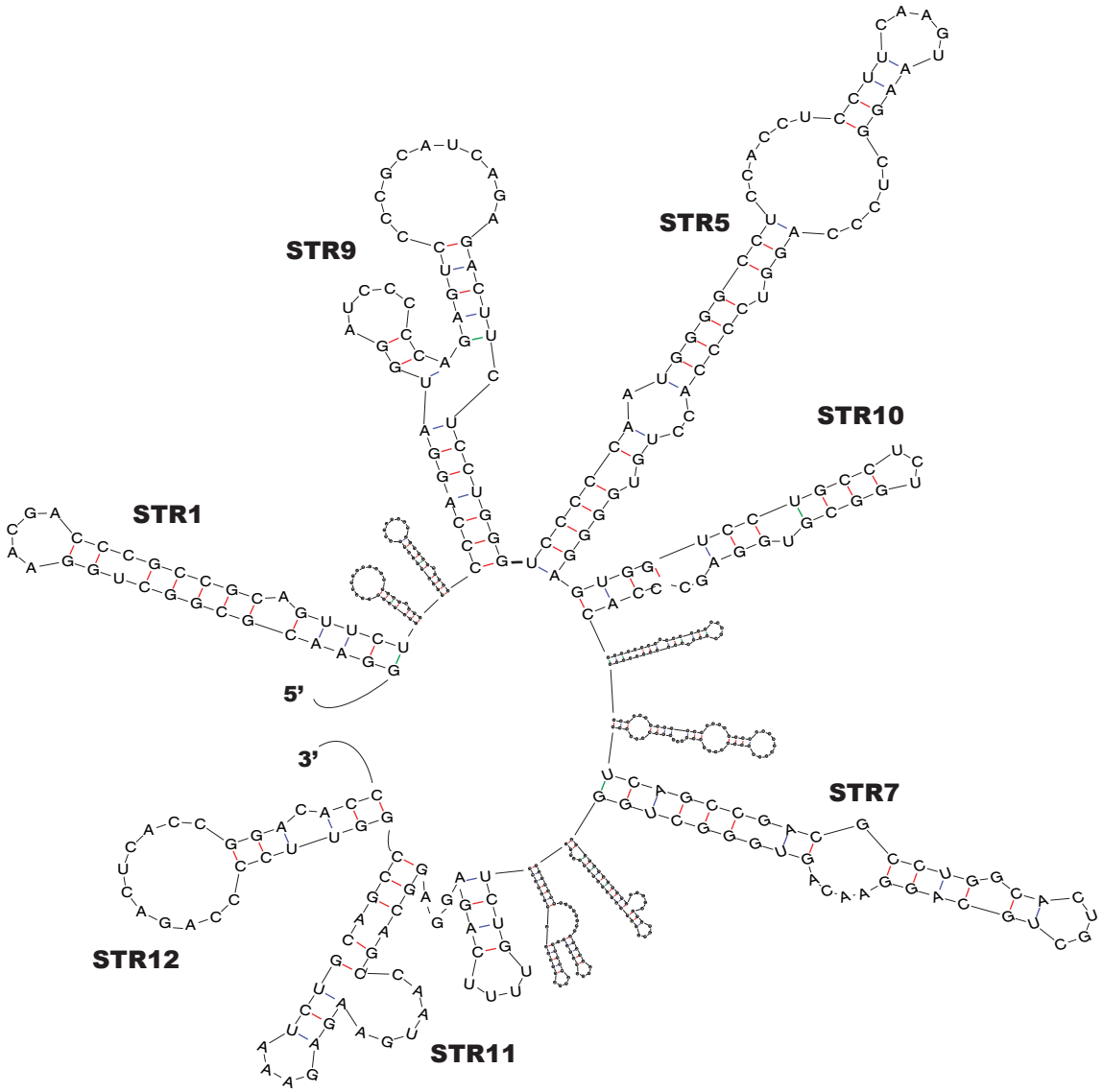
pCAF: p300/CBP-associated factor binds to p300 and CBP in vitro and in vivo. It has histone acetyl-transferase (HAT) activity.

p160 family proteins: SRC-1/NCoA1, SRC-2/TIF2 and SRC-3/AIB1 are homologous coactivators. They bind to the AF-2 domain of ligand-bound NR through an LXXLL domain.

NcoR/SMRT: nuclear receptor co-repressor/silencing mediator for retinoid and thyroid-hormone receptors is a corepressor complex that physically interacts with NR and can recruit histone deacetylase (HDAC) activities.

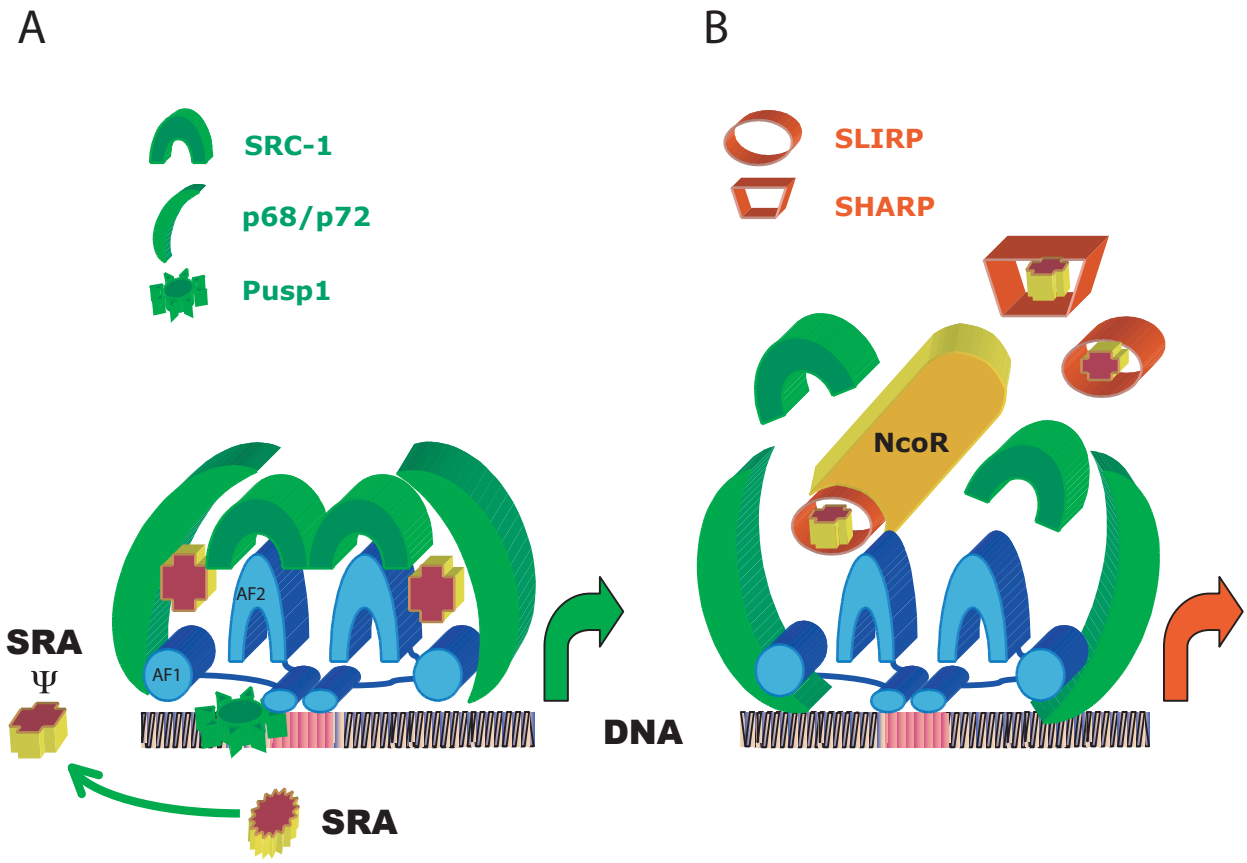
Cited from Perissi, V. and Rosenfeld, M.G. Nat Rev Mol Cell Biol. 2005 Jul; 6(7):542-54.

Figure 4. Predicted secondary structures of the SRA core region.



These secondary structures are modeled using the Mfold software. Detailed structures of STR1, 9, 5, 10, 7, 11 and 12 are illustrated. Ψ indicates the position of a Uridine residue 207 in STR-5, found to be a common pseudouridylation site of Pusp1 and Pusp3. Default parameters of the Mfold software are ΔG = -9.8 kcal/mol at 37 ^{o}C. Cited from (68).

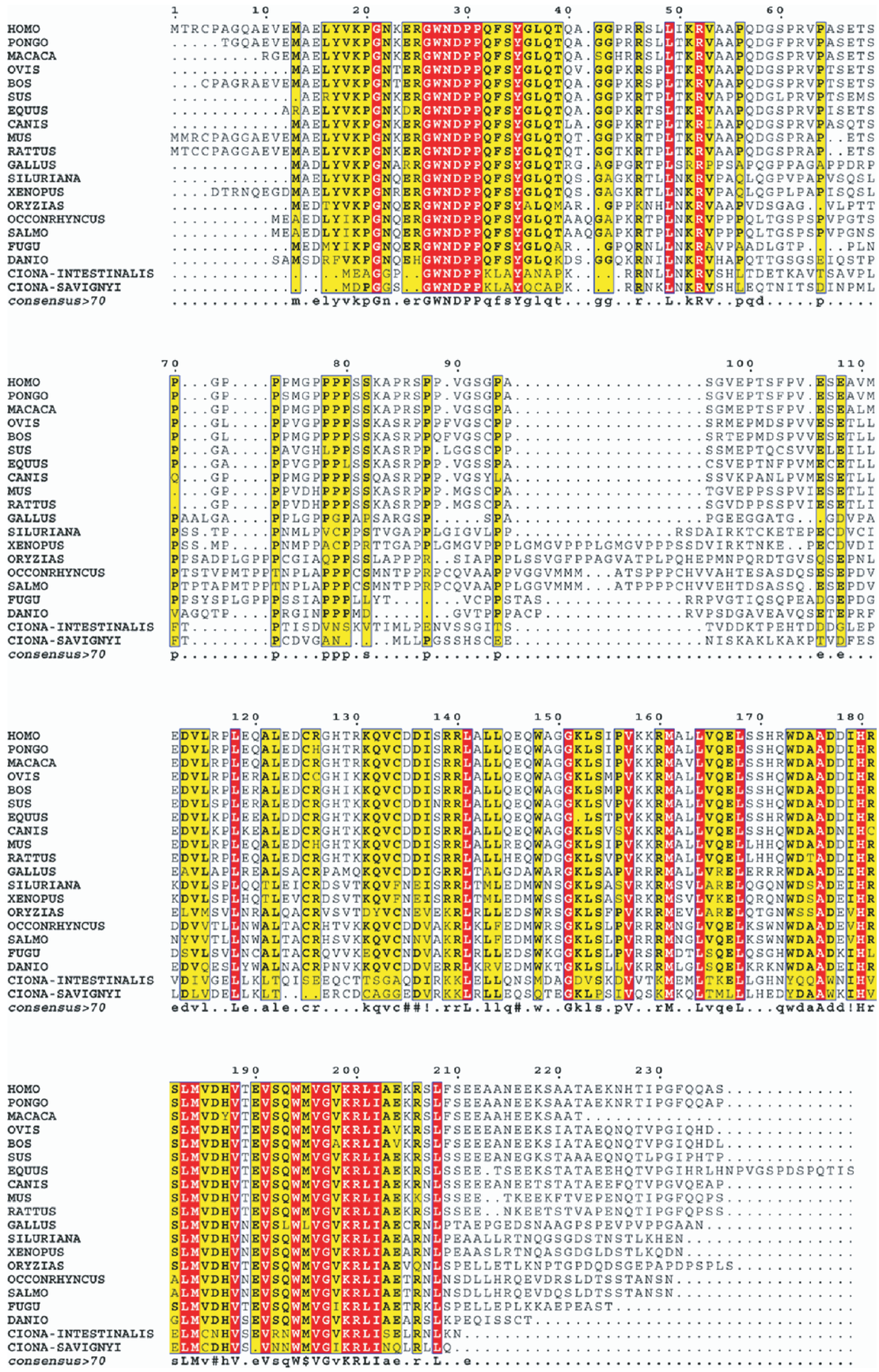
Figure 5. Model of SRA in regulating ERα activity.



A. Coactivation of ERα gene expression by SRA. Pus1p (green wheel), which is able to bind the DNA binding domain of all nuclear receptors, pseudouridylates specific SRA uridine residues, leading to an optimum configuration of this RNA. The resulting SRA-ψ (cross), could now form a stabilizing complex with p68 (green crescent able to bind SRC-1 and ER-α AF-1 region) and SRC-1 (green horseshoe). Transcription of genes with suitable EREs (red elements on DNA) will be activated. It should be stressed that the physical presence of SRA at the level of promoter has not yet been established experimentally. Similarly, the kinetics of events involving these molecules at the promoter site, as well as the possible effect of specific ligands (red sphere), remain to be determined.
B. Inhibition of SRA coactivation. SLIRP (hollow red cylinder) and SHARP (hollow trapeze) act as negative regulators. It has been proposed that they might act by sequestrating SRA, by destabilizing the complex containing SRA and SRC-1 or by recruiting the nuclear receptor corepressor N-CoR to the promoter region. ERα ligand binding domain, DNA binding domain and AF-1 domain are shown in blue horseshoe, flat elliptic cylinder and blue cylinder, respectively.

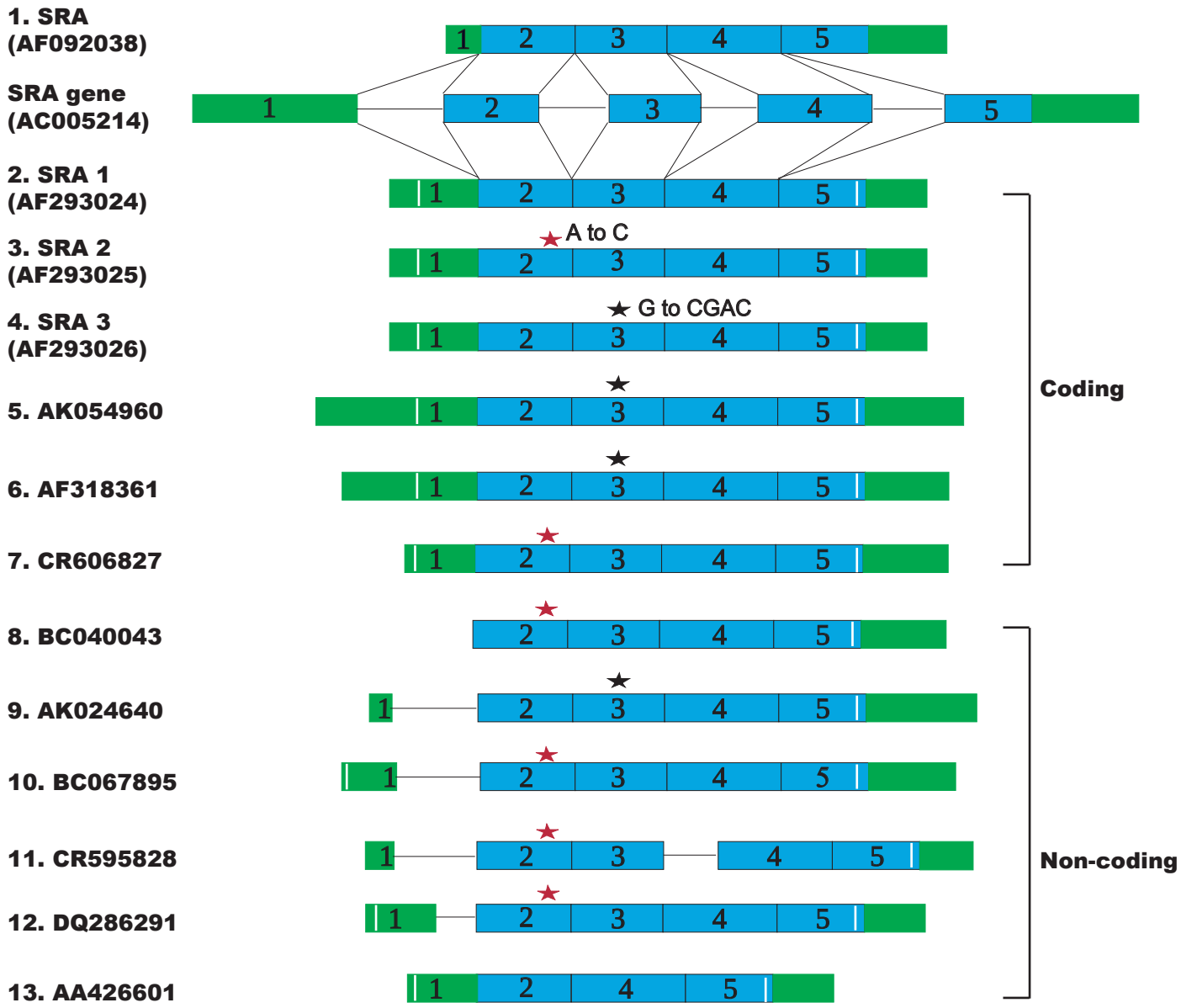
Cited from (68).

Figure 6. Alignment of putative SRAP sequences.



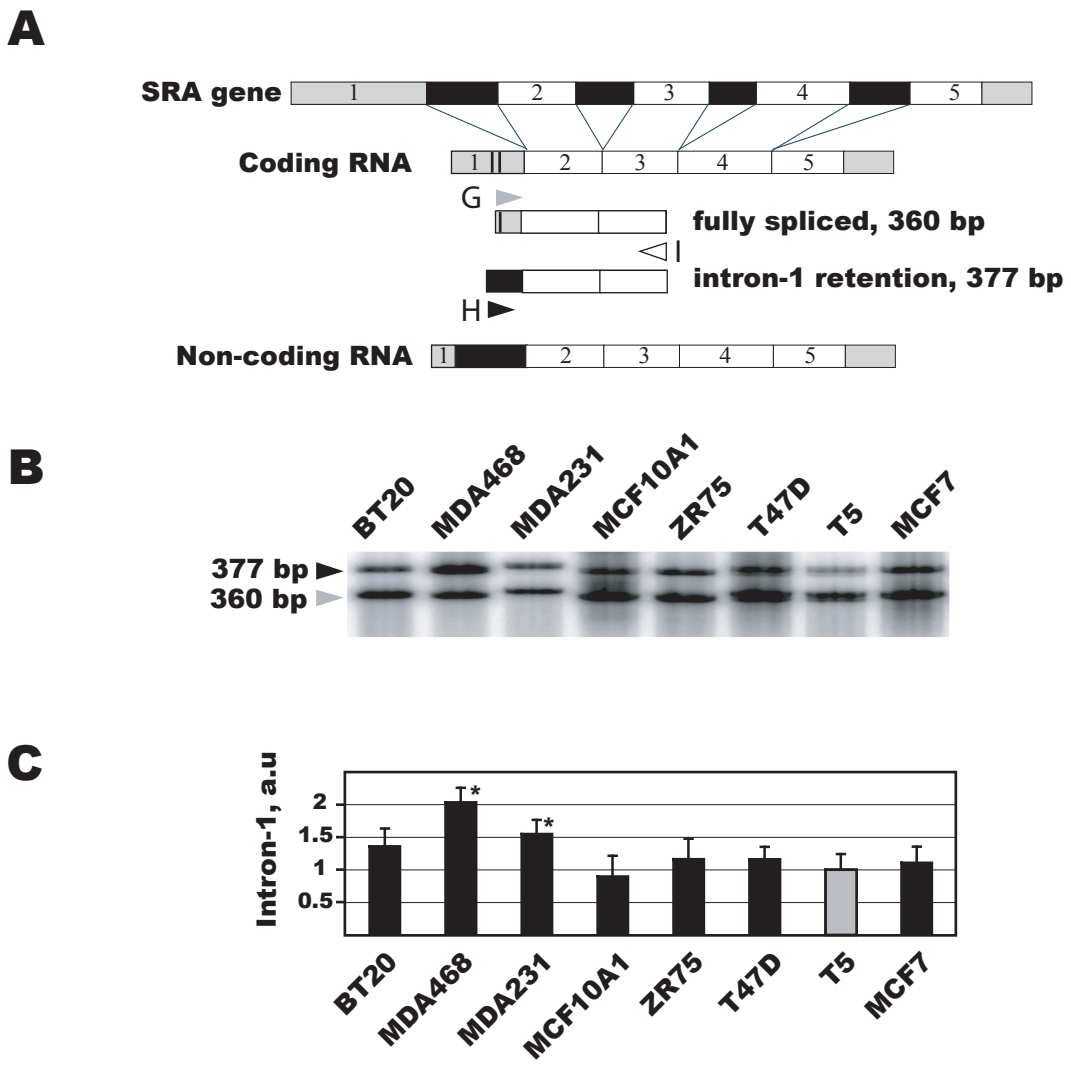
Putative SRAP of B. taurus, M. musculus, R. norvegicus, S. scrofa, E. caballus, M. mulata, G. gallus, O. latipes, O. mykiss, Fugu rubripes, S. tropicalis, X. laevis, D. rerio, C. intestinalis and C. savignyi are aligned. The numbers indicated on top of the alignment show positions of amino-acids in human SRAP sequence corresponded to the core region. Fully conserved amino acids are shown in red; partial conserved (over 70%) ones are in yellow. Cited from (68).

Figure 7. A schematic alignment of SRA mRNA homologues in the NCBI database.



Boxes with numbers indicate exons, while lines indicate introns. Blue boxes define the exons in the core sequence essential for SRA's coactivation function. Green boxes show sequences of various lengths to the 5' and 3' of the core sequence. Red stars represent an A to C point mutation, and black stars represent a G to C point mutation followed by an insertion of a full codon (GAC). Strips in exon-1 and exon-5 indicate the positions of translation start codon and stop codon, respectively. Except sequences 11 and 13, all other transcripts contain the complete SRA core sequence. Transcripts 9 and 11 have relative shorter exon-1 sequences missing the SRAP start codons.

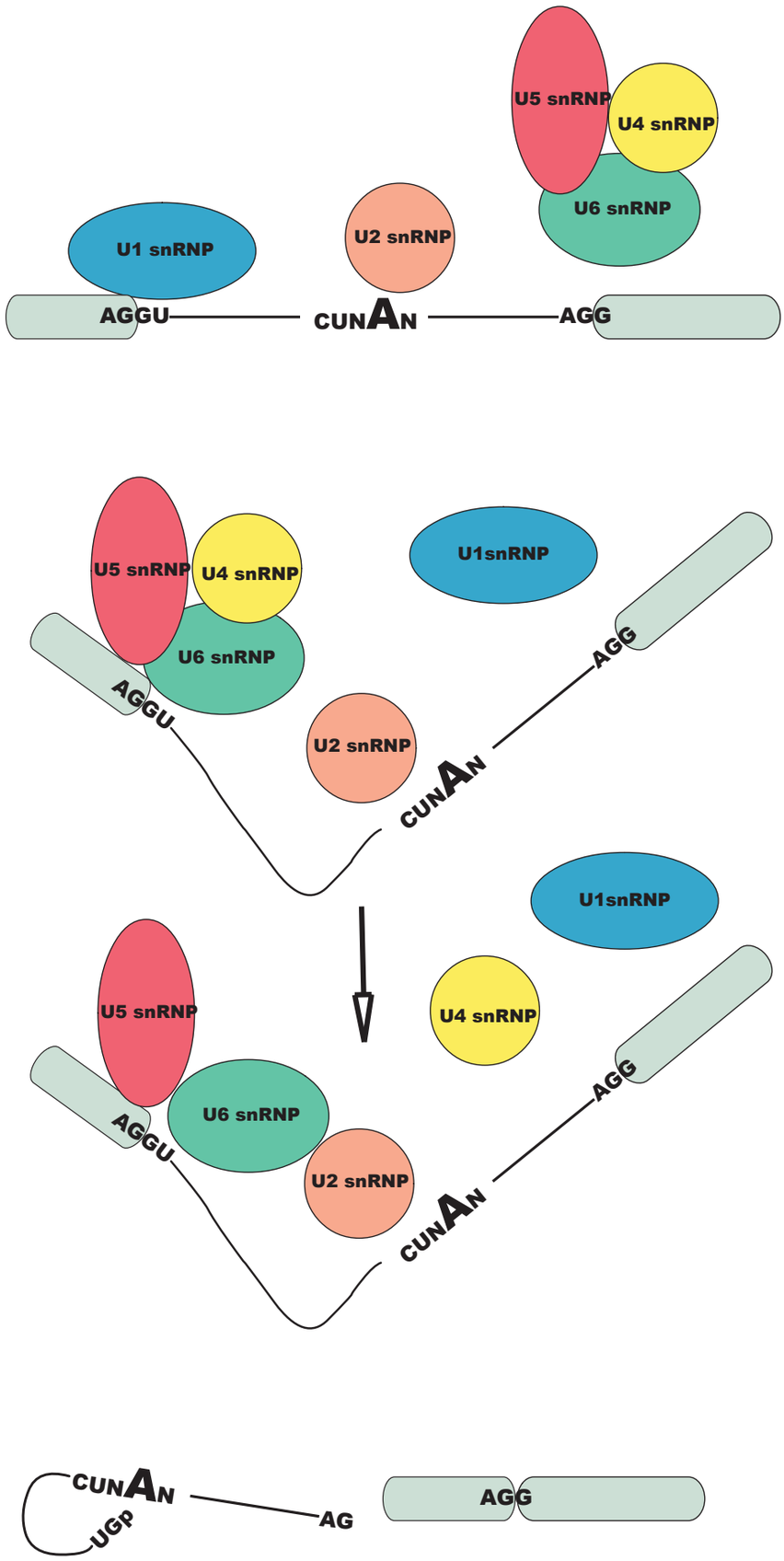
Figure 8. Detection of coding and non-coding SRA transcripts from breast cancer cells.



A: Principle of TP-PCR: Three primers are used for co-amplifying intron-1 retention SRA and fully spliced SRA. The lower primer targeting the end of exon-3 recognizes the two kinds of splicing variants whereas the two upper primers are specific for exon-1 (gray) and intron-1 (black).
B: Total RNA was extracted from 8 types of breast cancer cells, reverse-transcribed, and amplified by TP-PCR. Bands migrated at 377bp and 360bp correspond to intron-1 retention SRA and fully spliced SRA, respectively.
C: The relative proportion of the signal representing intron-1 retention, non-coding SRA for each type of cell is expressed in arbitrary unit, using that of T47D-5 cells as a standard. The statistical differences between T5 (gray bar) and other cell lines (black bars) have been tested using the Student's t-test. Stars indicate a significant difference ($P < 0.05$). Standard deviations are shown.

Cited from (95).

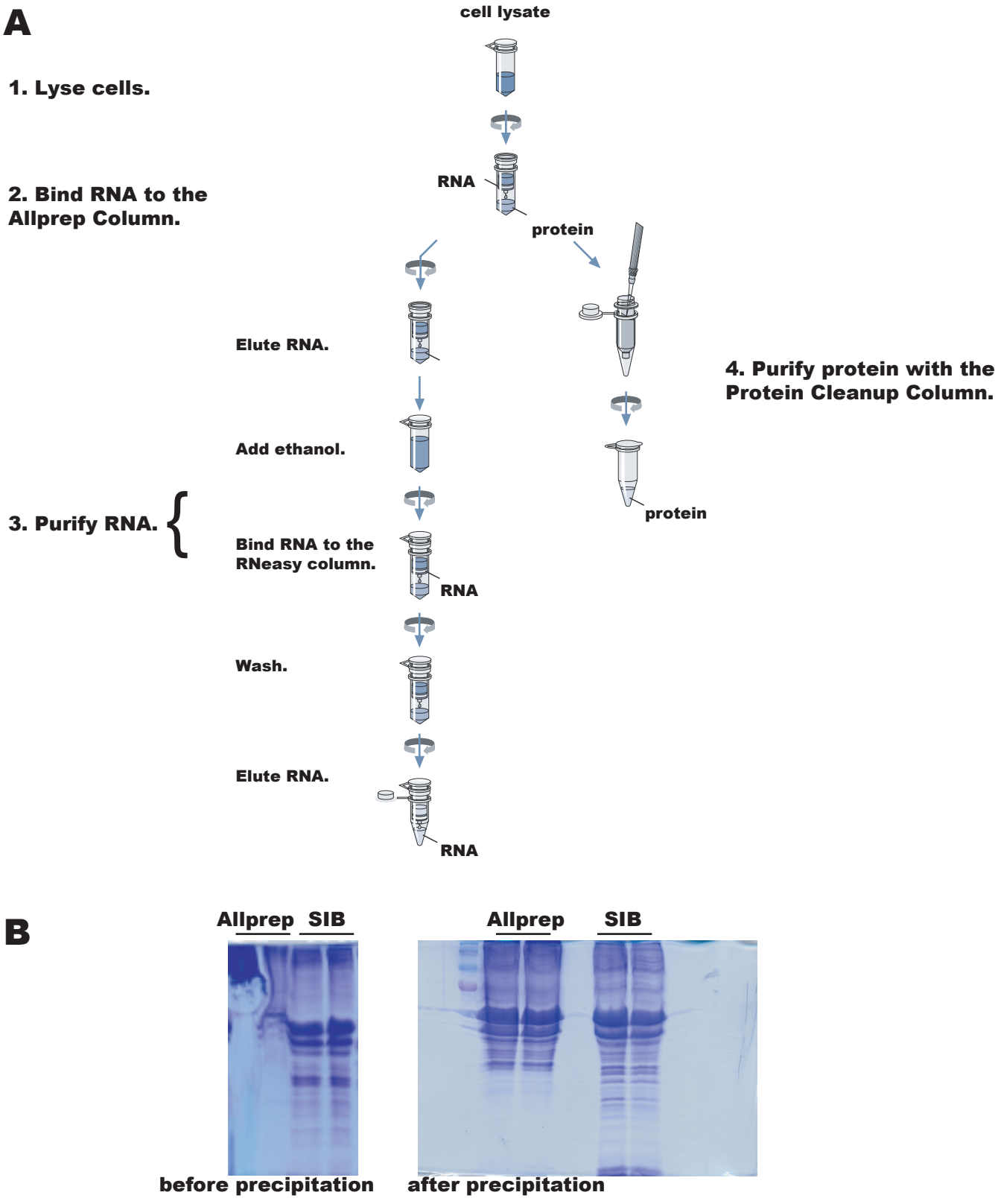
Figure 9. Conventional model of pre-mRNA intron splicing.



Upper panel: The intron is shown by the lines with specific sequences marked out, and upstream and downstream exons are shown in boxes. The specific sequences defining the intron are a 5' donor site (GU), a branch point (A, in bold, flanked by sequences marked where N stands for any nucleotide) and a 3' acceptor site (AG). Spliceosome subunit U1 snRNP specifically recognize and bind to the donor site, while U2 snRNP can recognize and bind to the branch point.
Middle panel: The assembly of spliceosome consists of a series of protein-protein and RNA-protein interactions.
Lower panel: After two transesterification reactions, the intron is removed in the form of lariat and the two exons are joined.

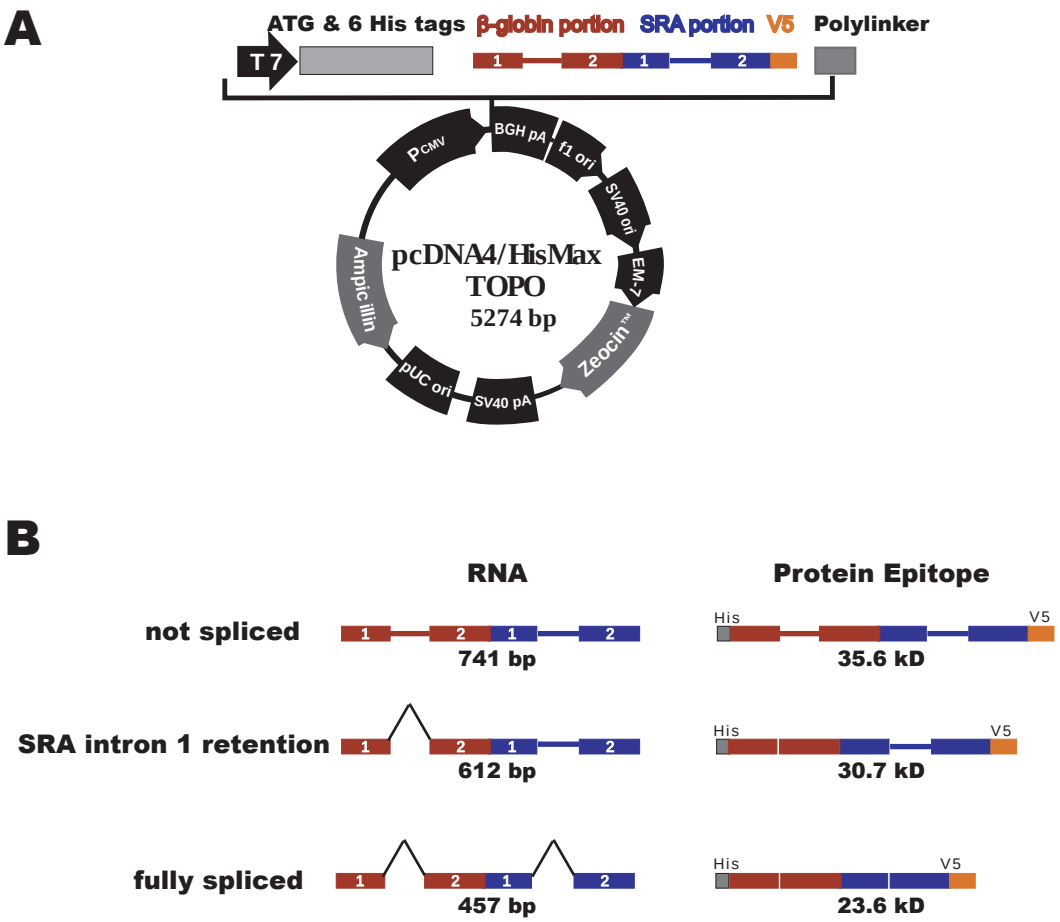
Modified from http://genome.imim.es/courses/Madrid04/exercises/ensembl/images/splicing.jpg.

Figure 10. Using All-prep Kit to extract RNA and protein from the same sample.



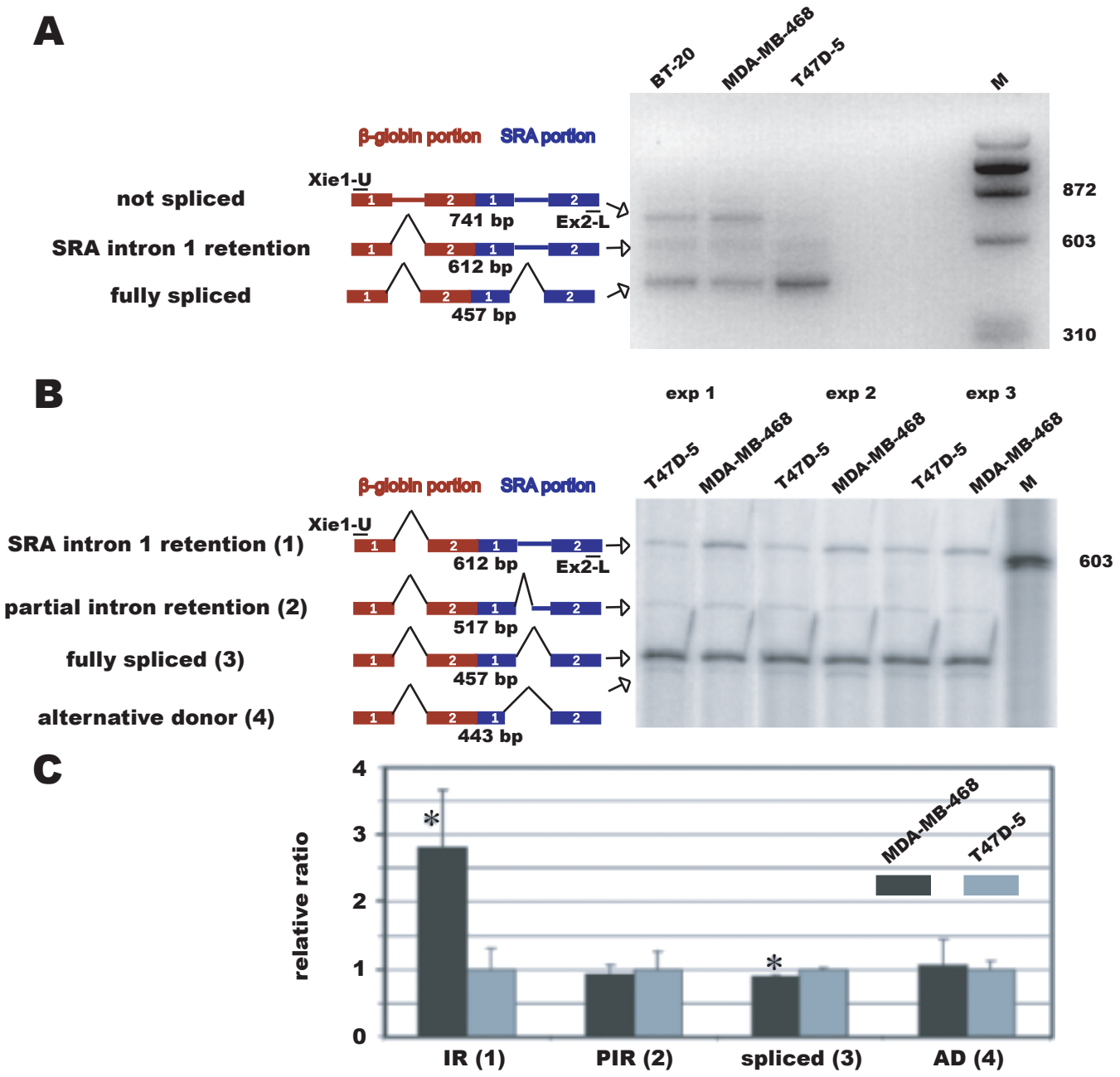
A: A flowchart of the extractions. RNA molecules longer than 200bp bind to the first column, then are eluted and finally purified in an RNeasy column. On the other hand, total proteins in the flow-through are purified by a Cleanup column. (Modified from the All-prep instructions.)
B: Total proteins from T47D-5 cells were extracted either by the All-prep Kit or by SIB buffer (cell lysis buffer containing 10% SDS) according to the descriptions in Section 3.1 of Materials and Methods. The All-prep samples failed to be separated on SDS-PAGE (left). This problem is solved by acetone precipitation (right).

Figure 11. Designing of SRA minigene.



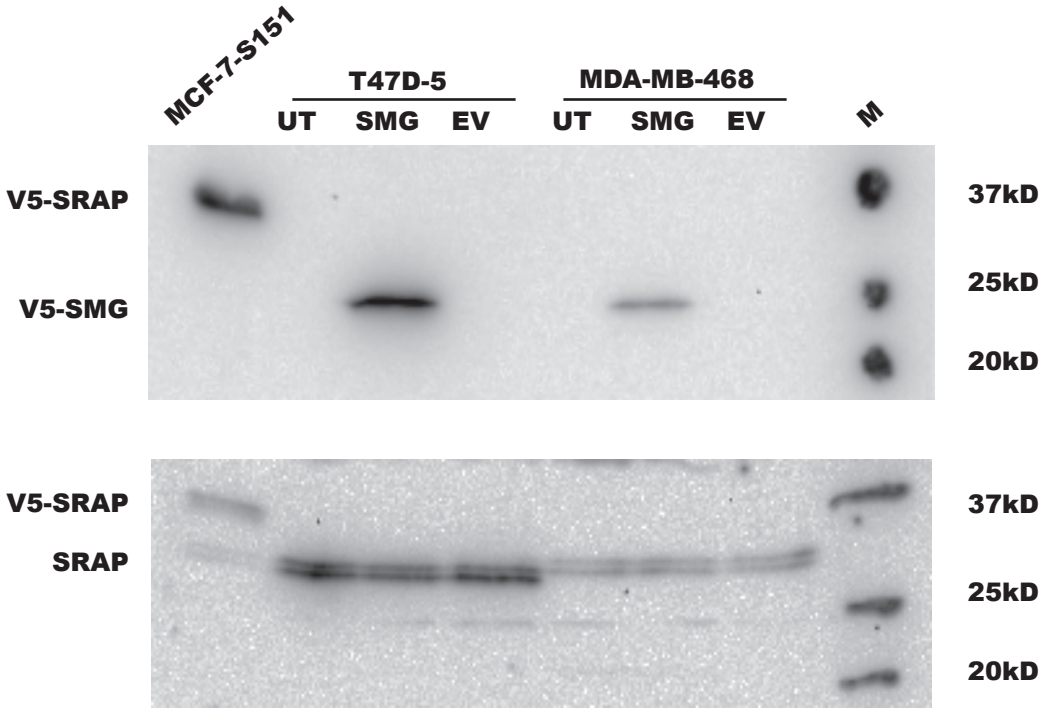
A: A schematic illustration of the minigene. The minigene has a modified β-globin portion fused upstream to SRA exon 1-intron-1-exon-2. The β-globin portion contains a constitutively splice-able intron.
B: Putative RNA and protein products generated from the minigene.

Figure 12. Detection of minigene RNA products.



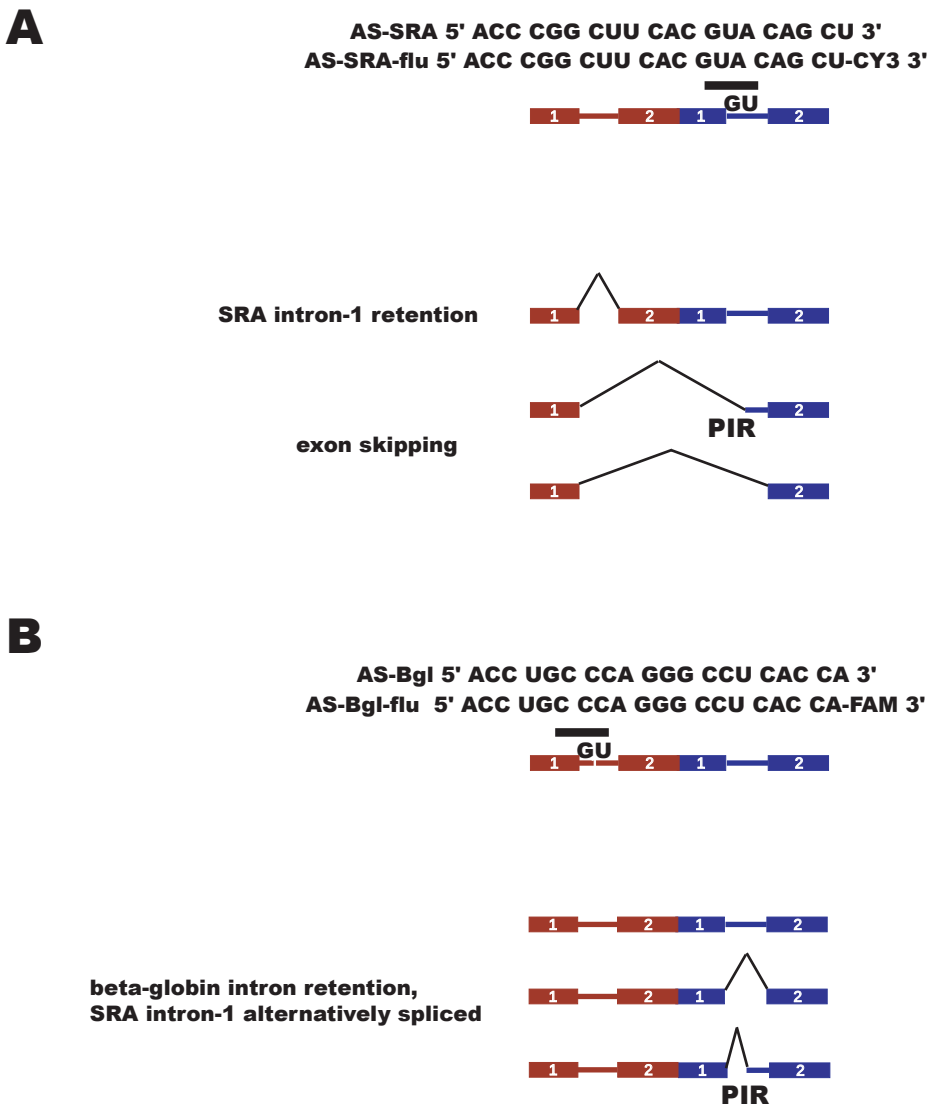
A. Detection by PCR. Minigene was transfected into BT-20, MDA-MB-468 and T47D-5 breast cancer cells. Total RNA was extracted 24h post-transfection, reverse transcribed and amplified with the minigene specific primers (Xie1-U and Ex2-L). PCR products were separated on 1.5% agarose gel.
B. Detection by radioactive PCR. Minigene was transfected into MDA-MB-468 and T5 cells. cDNA was obtained as described above and subjected for radioactive PCR with the minigene specific primers. PCR products were separated on polyacrylamide gel. Each PCR product was sequenced and illustrated on the side. (PIR: partial intron retention; AD: alternative donor site)
Marker (M) is Marker ΦX 174 (Invitrogen). Red and blue boxes indicate beta-globin and SRA exons, respectively.
C: For B, the relative intensity of each signal in the total four signals was quantified. The graph shows the average of three experiments and standard deviation. Differences in each signal between the two cell lines were tested using the Student's *T*-test. Stars indicate significant differences ($P<0.05$).

Figure 13. Detection of a protein derived from the SRA minigene.



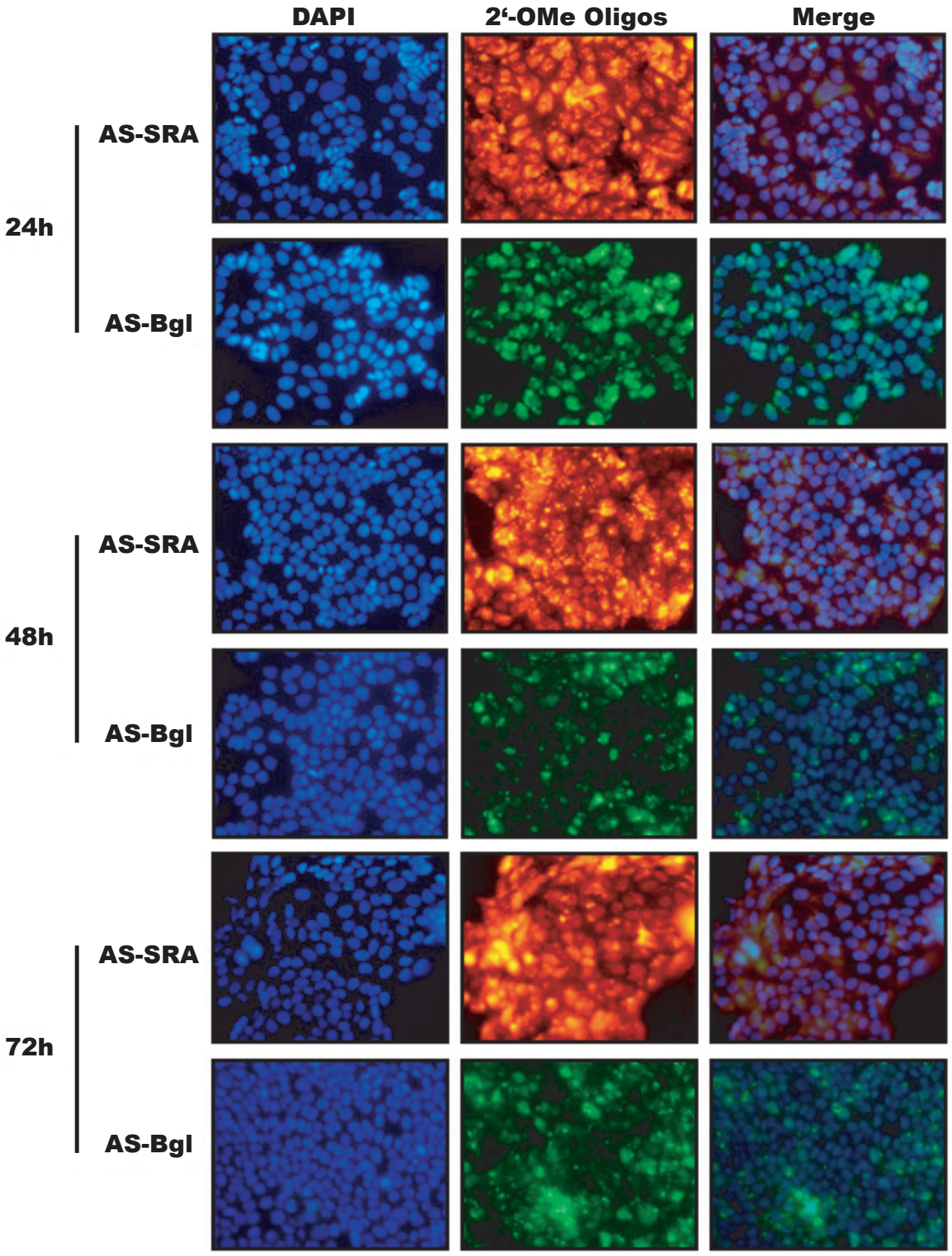
T47D-5 and MDA-MB-468 cells were transfected with minigene plasmid (SMG) or empty vector (EV) or without plasmid (untransfected, UT). Cells were lysed 24h post-transfection and cell lysates containing 100µg total protein (quantified by the BCA method) was separated on an SDS-PAGE gel. Lysate containing the same amount of total protein from MCF-7 cells permanently transfected by V5-SRAP (MCF-7-S151) was used as a positive control for detection of V5 tag and SRAP.
Upper panel: Immuno-detection was performed using a mouse anti-V5 antibody, which recognizes a V5 tagged minigene protein (V5-SMG) as well as the V5-SRAP.
Lower panel: The same blot was stripped and re-probed with a rabbit polyclonal anti-SRAP antibody. The V5-SMG was not detected by the anti-SRAP antibody. (Also consult Section 1.4 in Results and Section 1 in Discussion for more information.)
Molecular weight marker (M) is Kaleidoscope marker (Bio-rad).

Figure 14. Principle of the 2'-OMe Oligoribonucleotide constructs.



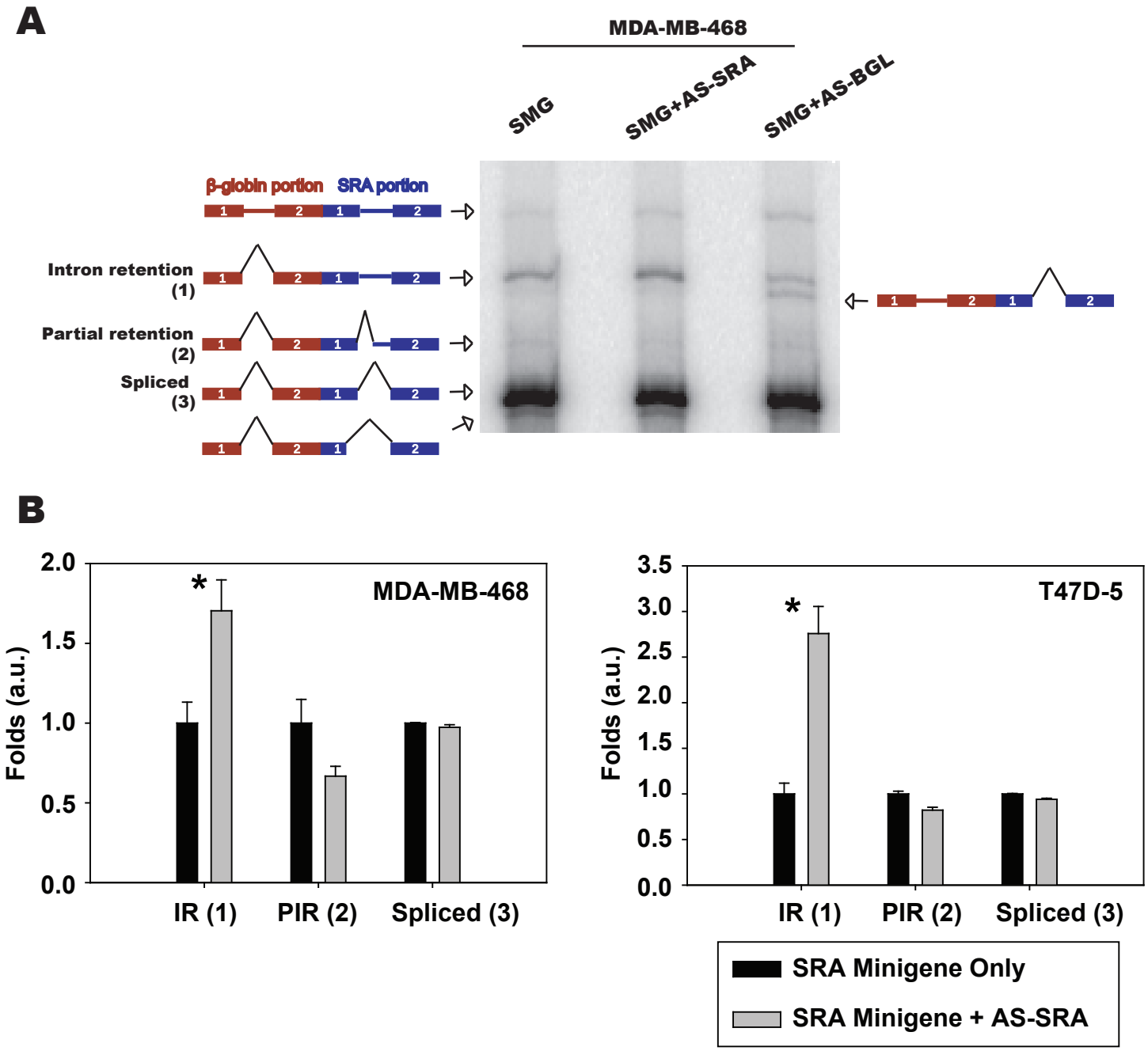
A: Sequence and target site of AS-SRA (and its fluorescent form), using the minigene as a reference. Three splicing events, as illustrated, are expected to be promoted by AS-SRA.
B: Sequence and target site of AS-Bgl (and its fluorescent form).
(Note that AS-SRA and AS-Bgl are antisense constructs. For the ease of reading they are shown from 5' to 3'.)

Figure 15. Intake of fluorophore conjugated 2'-OMe constructs by cells.



T47D-5 cells were transfected with 0.5uM AS-SRA-flu (red signal) or AS-Bgl-flu (green signal) using Lipofectamine. Direct fluorescent microscopy (40X) on formaldehyde fixed cells was performed at 24 h (upper panel), 48 h (middle panel) and 72 h (lower panel) post-transfection. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole-dihydrochloride).

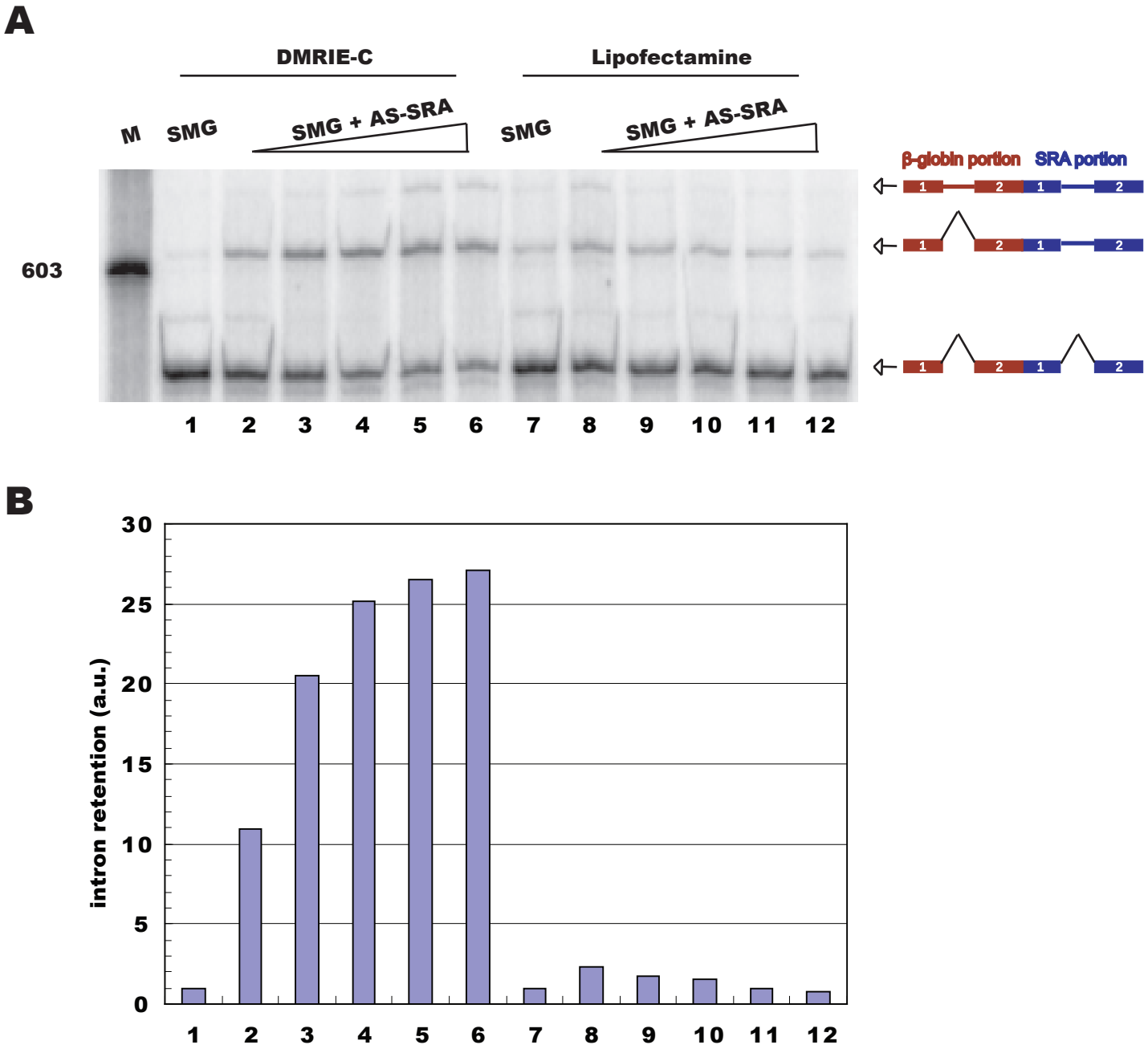
Figure 16. Alteration of minigene RNA splicing by AS-SRA (preliminary studies).



A: The minigene was co-transfected with 0.5uM AS-SRA or AS-Bgl into MDA-MB-468 cells by Lipofectamine. Total RNA was extracted 24 hours post-transfection, treated with DNase, reverse transcribed and subjected for radioactive PCR using the minigene specific primers. PCR products were separated on a polyacrylamide gel. Identities of major bands are shown on side of the autograph. An identical autograph for co-transfection in T47D-5 cells is not shown.

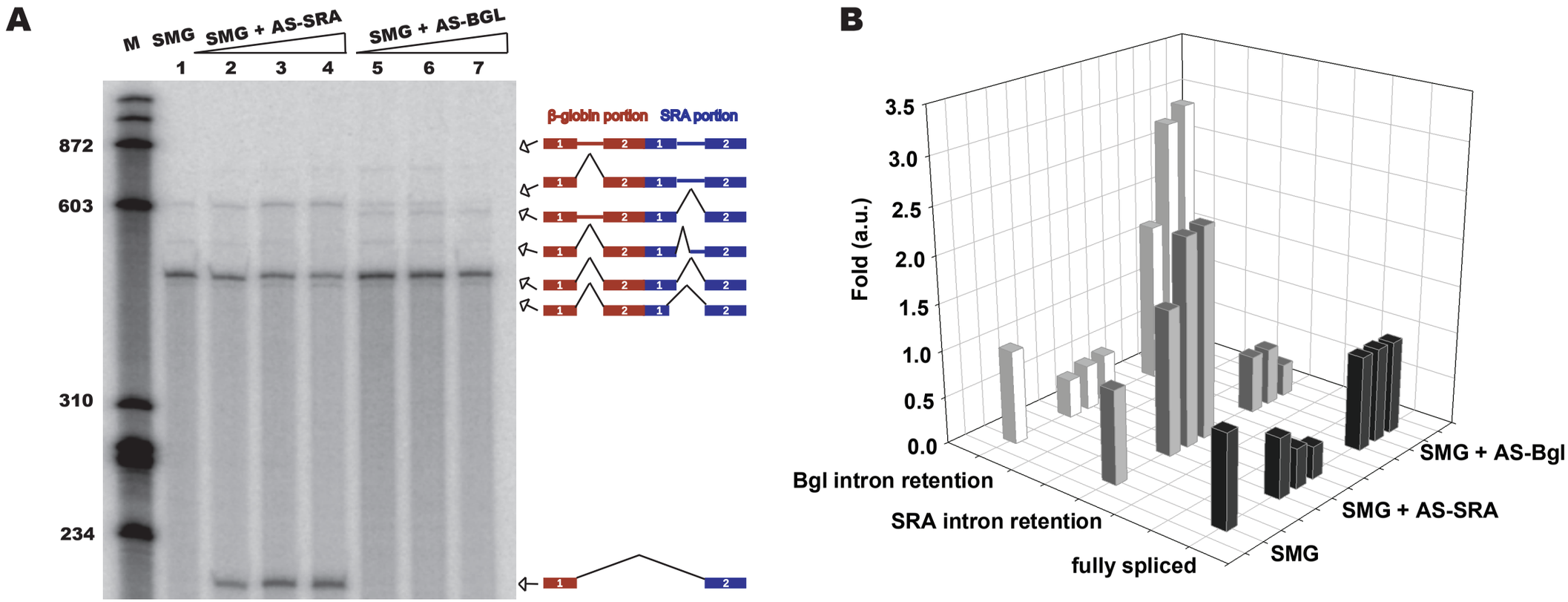
B: The ratio of each signal in total signals detected was quantified for cells co-transfected with AS-SRA and the minigene, or the minigene only. The figures show the relative ratio of the three major products, averaged from three individual experiments. The folds are shown in arbitrary units, with the intensity of each signal detected from minigene-transfected cells as 1. Statistical significance is tested by Student's T-test and indicated by *. In order to illustrate the fold changes in MDA-MB-468 cells, the standards in the two figures are different.

Figure 17. Comparing DMRIE-C and Lipofectamine for co-transfection.



T47-D cells were co-transfected by a fixed amount of minigene plasmid (1.65 ug per well on 6-well plate) and increasing amount of AS-SRA (0 uM, 0.1 uM, 0.25 uM, 0.5 uM, 0.75 uM, 1 uM), using either DMRIE-C or Lipofectamine at a ratio of 4 ul reagent per ug of total DNA/RNA.
A: Total RNA was extracted 24 h post-transcription, DNase treated, reverse transcribed and subjected for radioactive PCR.
B: Quantification of the relative ratio of the signal corresponding to SRA intron-1 retention transcript to the total intensity of the signals corresponding to not spliced, SRA intron-1 retention and fully spliced minigene transcripts.

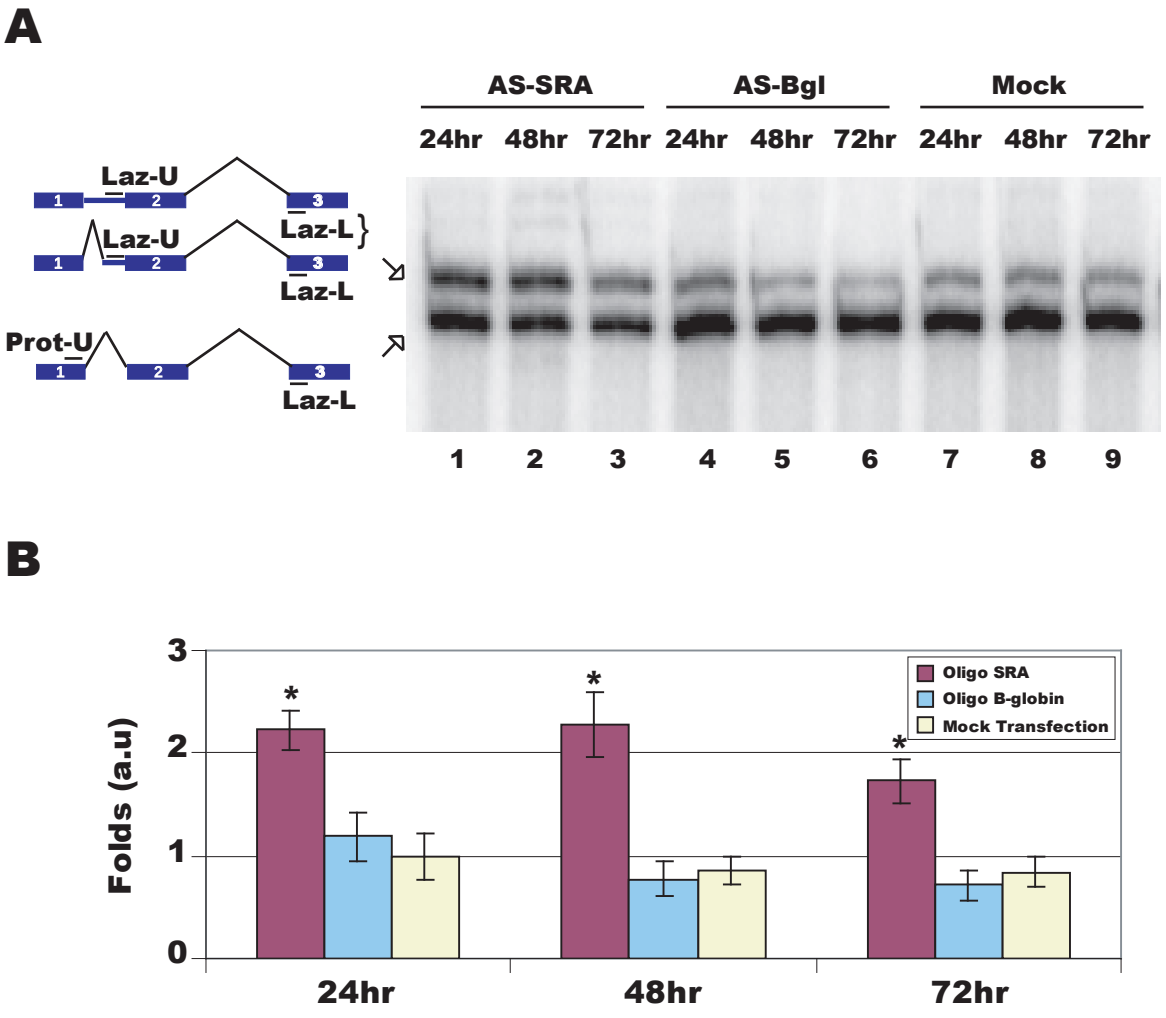
Figure 18. Alteration of intron splicing on SRA minigene RNA by AS-SRA and AS-Bgl.



A: T47D-5 cells were co-transfected with a fixed amount of minigene (1.65ug per well on 6-well plate) and increasing amounts of AS-SRA or AS-Bgl (0.05 uM, 0.1 uM and 0.5 uM). DMRIE-C was used for co-transfection and was maintained at 8ul per well (6-well plate). Total RNA was extracted 24 hours post-transfection, treated with DNase, reverse transcribed and amplified by radioactive PCR using the minigene specific primers. Identity of each band is illustrated on the side.

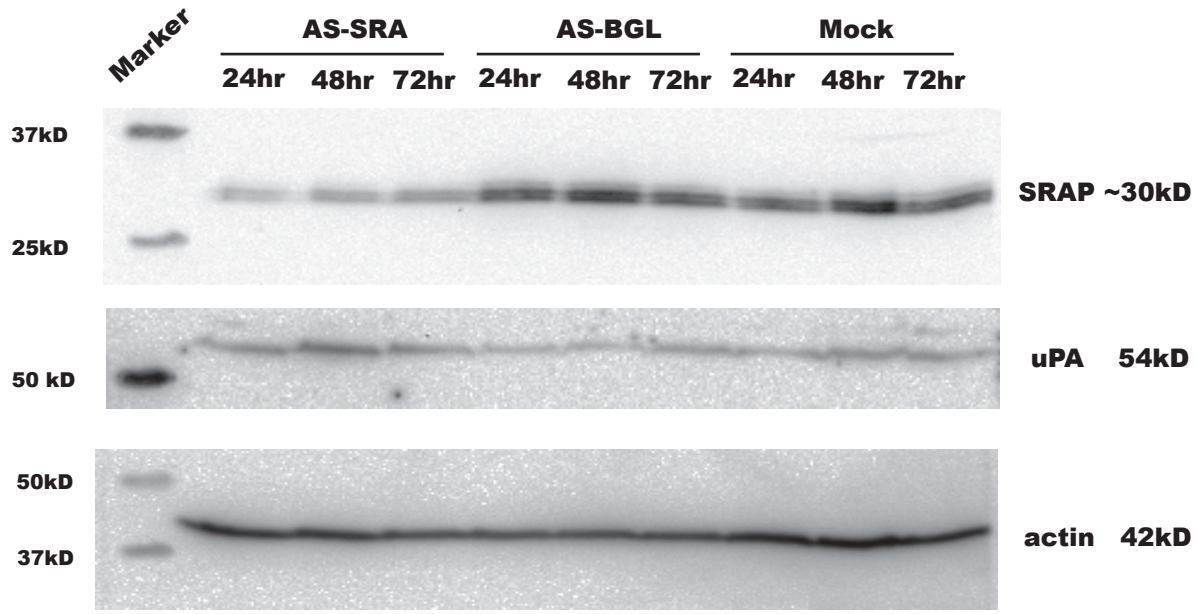
B: Quantification of the relative ratio of the signal corresponding to fully spliced, SRA intron retention and β-globin intron retention in the total signal detected. For lanes 1, 5, 6, 7, total signal refers to the sum of the not spliced, the SRA intron-1 retention, the β-globin intron retention, the partial SRA intron-1 retention, the fully spliced and the alternative donor site signals. For lanes 2-4, total signal refers to the sum of above signals and the exon skipping signal. Results for each signal are shown in arbitrary units, with those from cells transfected with only the minigene as 1.

Figure 19. Time course effect of AS-SRA on altering intron-1 splicing of the endogenous SRA gene.



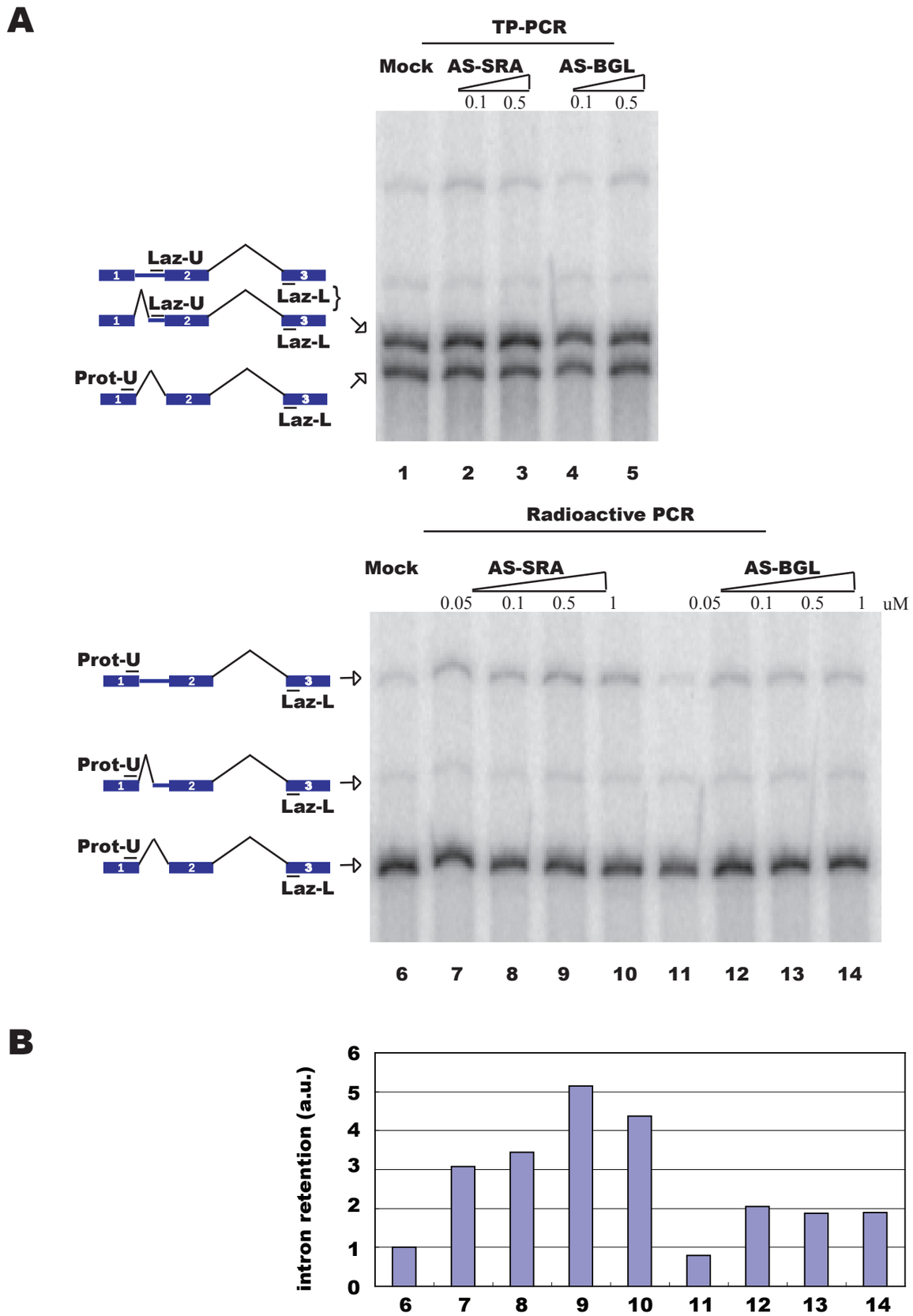
A: T47D-5 cells were transfected with no oligonucleotides (Mock), 0.5 uM AS-SRA or 0.5 uM AS-Bgl. Total RNA was extracted 24 hours, 48 hours and 72 hours post-transfection, treated with DNase, reverse-transcribed and subjected for TP-PCR. PCR products were separated on a polyacrylamide gel. B: The relative proportion of the signal corresponding to intron-1 retention transcripts was expressed in arbitrary unit (a.u) using that of the 24 hours, mock-transfection sample as a standard. The values represent the average of triplicated experiments. A star indicates significant difference calculated by Student's t-test ($P<0.05$). Error bars show standard deviations.

Figure 20. Changed expression of SRAP and uPA following the alteration of SRA intron-1 splicing.



Total proteins extracted from T47D-5 cells by the All-prep kit were acetone precipitated and subjected for SDS-PAGE and immunodetection. From the same blot, SRAP was detected by a polyclonal rat antibody (Bethyl Laboratories, catalogue number A300-743A, top panel); uPA was detected by a monoclonal mouse antibody (Abcam, catalogue number H77A10, middle panel); β-actin was detected as loading control (lower panel). The molecular weight marker is Kaleidoscope marker (Bio-rad).

Figure 21. AS-SRA promotes SRA intron-1 retention in MDA-MB-468 cells.

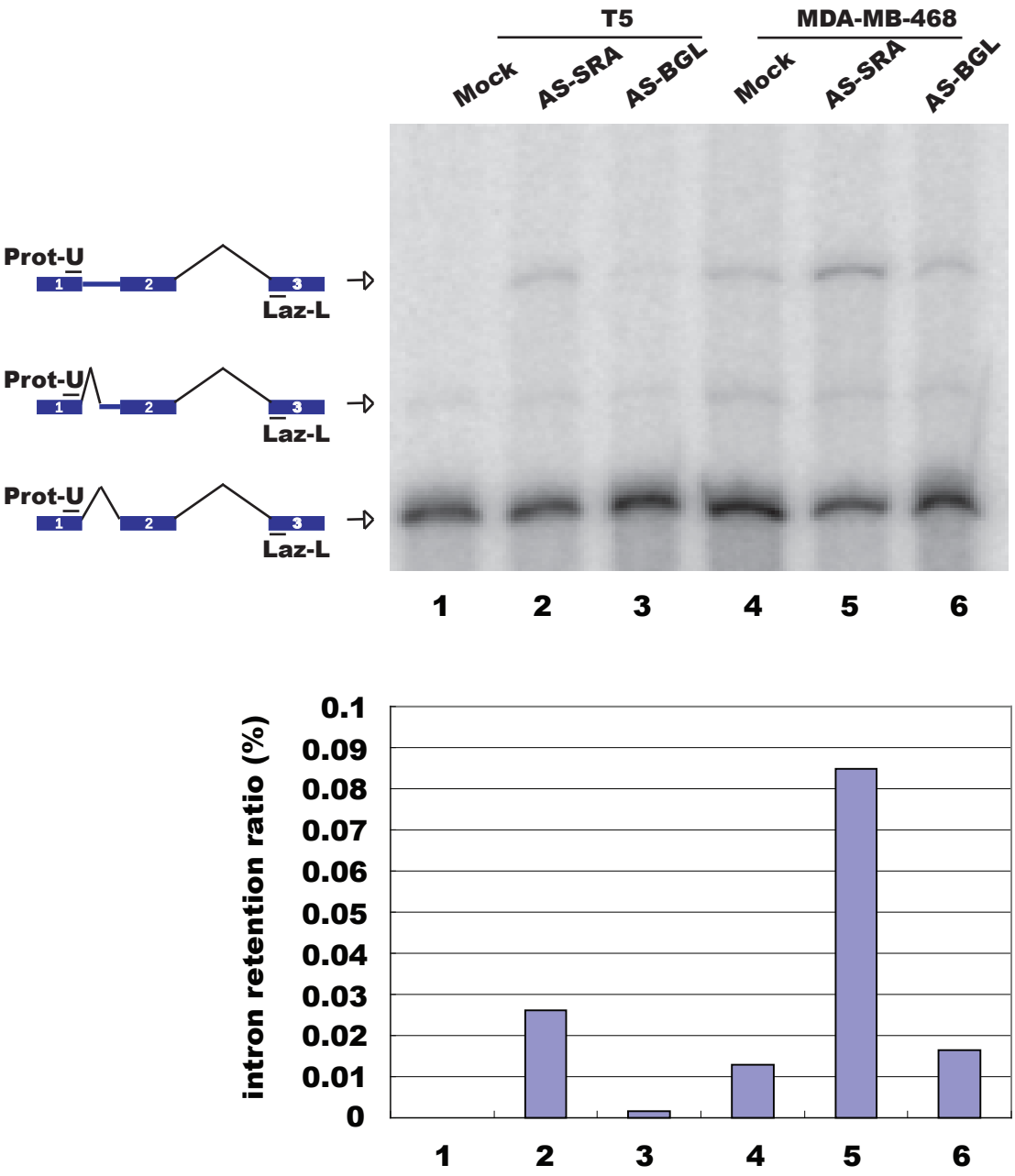


Cells were transfected with increasing amount of either AS-SRA or AS-Bgl as described, or mock transfected. Total RNA was extracted 24h post-transfection, treated with DNase, reverse transcribed and subjected for either TP-PCR (selected samples) or radioactive PCR. Positions of primers and putative PCR products are illustrated on the side.

A: Lanes 1-5: Detecting intron-1 retention and fully spliced SRA transcripts using TP-PCR. Note that beside the fully spliced templates, the Prot-U primer can also hybridize to templates containing intron-1. Lanes 6-10: Radioactive PCR using the Prot-U and Laz-L primers.

B: Quantification of the relative ratio of the signal corresponding to intron-1 retention transcript to the total intensity of the signals corresponding to intron-1 retention, partial intron-1 retention and fully spliced minigene transcripts.

Figure 22. AS-SRA-flu also promotes SRA intron-1 retention in cells.



Cells were mock transfected or transfected with 0.5uM AS-SRA-flu or 0.5uM AS-Bgl-flu. Total RNA was extracted 24 hours post-transfection, treated with DNase, reverse transcribed and subjected for radioactive PCR. The quantification show ratios of the signal corresponding to intron-1 retained SRA transcripts in the total of the three signals detected.

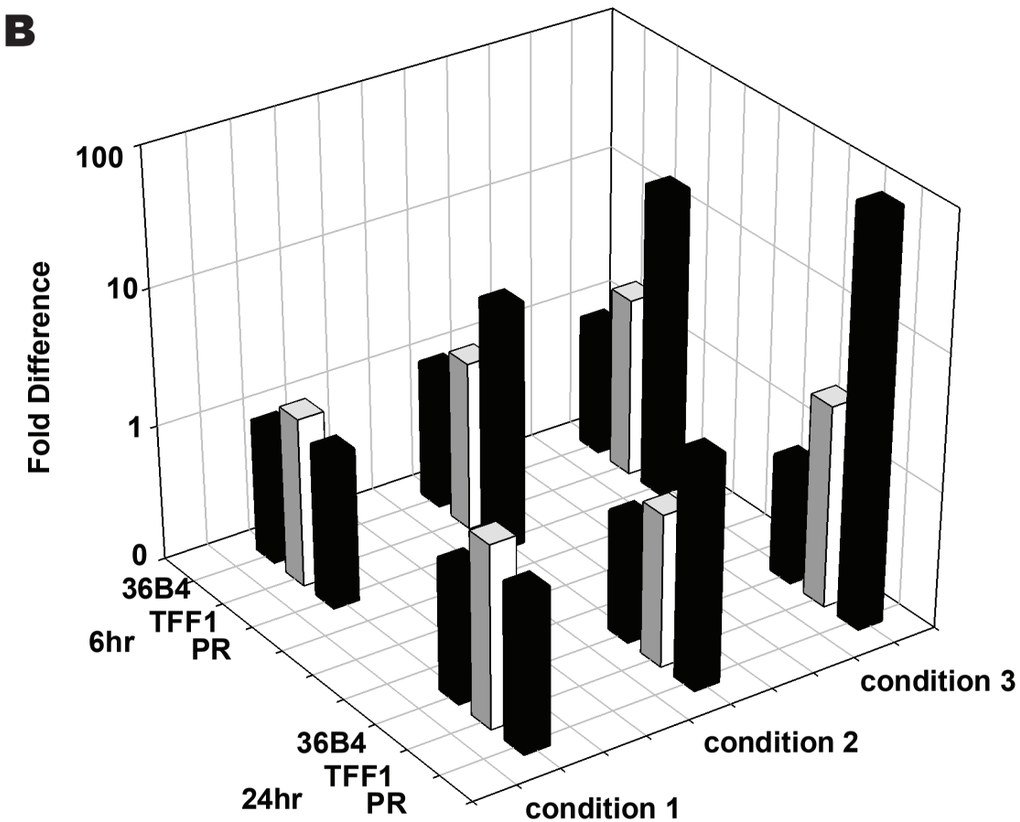
Figure 23. Optimizing conditions for estradiol treatment following 2'-OMe transfection.

A

	Condition 1	Condition 2 (no serum)	Condition 3 (stripped serum)
Day 1	Seed 200, 000 cells/well in DMEM+5%FBS. .	Seed 200, 000 cells/well in DMEM+5%FBS.	Seed 200, 000 cells/well in DMEM+5%FBS.
Day 2	Change medium to DMEM+5% FBS.	Change medium to DMEM+5% FBS.	Change medium to DMEM+5% charcoal stripped FBS.
Day 3	1. Equilibrate cells in Opti-mem. 2. Transfect in Opti-Mem. 3. Change Medium to DMEM+5%FBS.	1. Equilibrate cells in Opti-mem. 2. Transfect in Opti-Mem. 3. Change Medium to DMEM+5%FBS.	1. Equilibrate cells in no serum PRF. 2. Transfect in no serum PRF. 3. Change Medium to PRF+5% charcoal stripped FBS.
Day 4	Change medium to DMEM+5%FBS.	Change medium to no serum PRF.	Change Medium to PRF+5% charcoal stripped FBS.
Day 5	1. E2 treatment in DMEM+5% FBS. 2. Collect RNA after 6h.	1. E2 treatment in no serum PRF. 2. Collect RNA after 6h.	1. E2 treatment in PRF+5% charcoal stripped FBS. 2. Collect RNA after 6h.
Day 6	Collect RNA after 24 h.	Collect RNA after 24 h.	Collect RNA after 24 h.

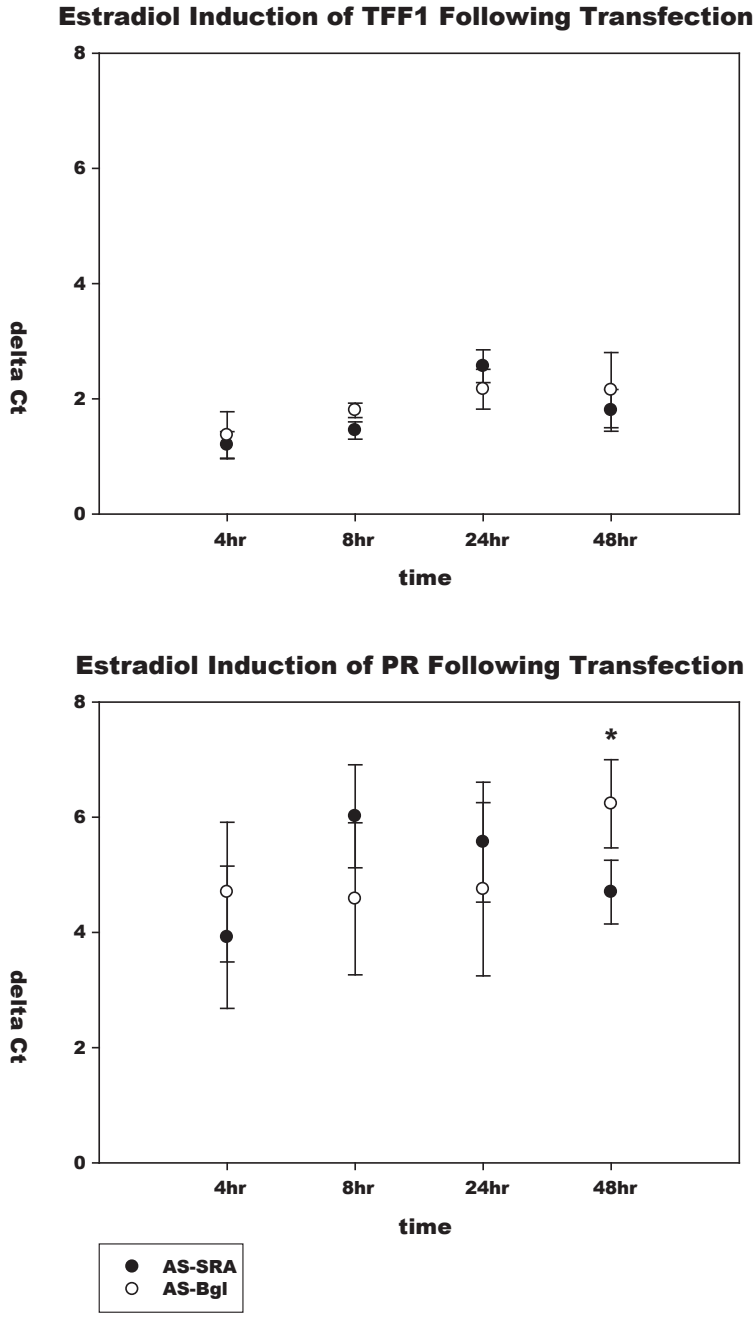
PRF: phenol red free DMEM.

B



A: A flowchart of the three experimental conditions compared. Briefly, the conditions differ in the periods of time that cells were cultured prior estradiol treatment, and, in the transfection medium used. B: T47D-5 cells were cultured and mock transfected according to the three conditions. Total RNA was extracted, treated with DNase, reverse transcribed and subjected for real time PCR to analyze the estradiol-induced transcriptions of PR and TFF1, with GAPDH as the normalizing control. The delta Ct method was used to calculate folds of induction and the PCR efficiency was arbitrarily considered as 2. While estradiol induced the expression of the two ERα downstream genes, the expression of a control gene, 36B4 (ribosomal phosphoprotein P0), remained constant.

Figure 24. Estradiol-induced TFF1 and PR transcriptions following 2'-OMe transfection.



T47D-5 cells were cultured in Phenol Red Free DMEM (Sigma) supplemented with 5% charcoal-stripped serum and transfected with 0.5uM AS-SRA or AS-Bgl under no serum conditions. Transfected cells were then treated with either 10nM estradiol (Sigma) or vehicle (ethanol) for 4 hours, 8 hours, 24 hours and 48 hours. Total RNA was extracted, DNase treated, reverse transcribed and subjected for real time PCR. The ΔCt method was used to calculate the level of induction (difference between estradiol-treatment and ethanol-treatment, for cells transfected by either AS-SRA or AS-Bgl). Then the difference between the inductions from cells transfected by AS-SRA and with AS-Bgl was calculated.

Figure 25. Potential binding sites of hnRNPs and SR proteins in the SRA exon-1-intron-1 region.

SRA exon-1-intron-1 primary sequence was analyzed by the Splicing Rainbow program (http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8) to predict potential splicing factor binding sites. Numbers in the counter line show positions of nucleotides. The vertical dash shows the boundary between SRA exon-1 and intron-1. Exon-1 ends at position 61 (before the dash). Binding sites of hnRNP F/H at the junction between exon-1 and intron-1 are highlighted by a red square.

Modified from the original webpage display.