Development of Novel Therapeutics Targeting Splicing Machinery in Ovarian Cancer

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THESIS

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LIST OF ABBREVIATIONS

APMs	Assay Performance Measures
AS	Alternative Splicing
ASOs	Antisense Oligonucleotides
AVR	Assay Variability Ratio
BPS	Branch Point Sequence
BRCA1	Breast Cancer Type 1
CV	Coefficient of Variation
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOX	Doxycycline
EOC	Epithelial Ovarian Cancer
ESE	Exonic Splicing Enhancers
ESS	Exonic Splicing Silencers
EST	Expressed Sequence Tags
GFP	Green Fluorescent Protein
HMECs	Human Mammary Epithelial Cells
hnRNP	Heterogeneous Nuclear Ribonucleoproteins
HRT	Hormone Replacement Therapy
HTS	High Throughput Screening
IRES	Internal Ribosomal Entry Site
ISE	Intronic Splicing Enhancers
ISS	Intronic Splicing Silencers
LMP	Low Malignant Potential

LIST OF ABBREVIATIONS (continued)

MBNL	Muscle Blind-Like Protein
MEM	Minimum Essential Medium
mRNA	Messenger RNA
NMD	Nonsense Mediated Decay
OC	Ovarian Cancer
PKC	Protein kinase C
PTB	Polypyrimidine Tract-Binding Protein
RRM	RNA Recognition Motif
SELEX	Systematic Evolution Of Ligands By Exponential Enrichment
shRNA	Small Hairpin RNA
siRNA	Small Interfering RNA
snRNP	Small Nuclear Ribonucleoproteins
snRNA	Small Nuclear Ribonucleic Acid
SR	Serine/Arginine-rich
SW	Signal Window
TMAs	Tissue Microarrays
U2AF	U2 Auxillary Factor
UsnRNP	Uridine-Rich Small Nuclear Ribonucleoproteins
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor

Chapter 1

1. 1. Summary

Ovarian cancer (OC) is the most lethal gynecologic malignancy. OC strikes more than 23,000 American women annually, and more than half of these women will lost their lives from this disease. To develop new therapeutic approaches and discover early diagnostic markers in OC, it is necessary to better understand the molecular events that are associated with the neoplastic transformation of the ovary. The origin and pathogenesis of epithelial ovarian cancer (EOC) have long been investigated but still poorly understood because of lack of suitable models. Recently developed procedures to immortalize and transform human ovarian surface epithelial (HOSE) cells provide a novel chance to investigate the molecular basis of EOC. In cultured HOSE cells, disruption of four distinct signaling pathways can mediate neoplastic transformation and the phenotypic diversity associated with this state. Alternative splicing (AS) of pre-messenger RNA (pre-mRNA) is one explanation that the cells can generate this phenotypic diversity.

Pre-mRNA splicing is one of the essential steps in the maturation of eukaryotic genes, and AS is an efficient mechanism that involves in generation of transcriptome and protein diversity from a single gene. Defects in mRNA splicing

are an important cause of disease; at least 15% of all disease-causing single base-pair mutations affect splicing. Hence, the splicing process and its regulation are of great interest. Many genes associated with tumorigenesis, such as oncogenes, tumor suppressor genes, cell cycle-related genes, and apoptosis-related genes undergo alternative splicing. It has been shown that cancer-specific aberrant splicing often interferes with genes involved in mRNA processing, which suggests that splicing regulatory factors are not expressed at proper levels and/or that their functions are impaired in cancer compared to normal tissues. These alterations in the splicing machinery and its regulation are key to cancer progression. Defects can arise in the splicing machinery, as in the breast cancer, resulting from inappropriate creation of splice site signals. An AA to AG mutation creates a 3′ splice site that adds 11 basepairs to BRCA1 mRNA, which encodes a truncated protein [9-11]. Mutations that cause exon skipping/retention without maintaining the original reading frame unavoidably introduce premature stop codons, such mis-spliced transcripts subject to non-sense mediated decay (NMD). Another similar mutation in breast cancer is an AT to GT change in the estrogen receptor gene, which creates a 5′ splice site in an intron. This causes to the insertion of a 69-basepair long extra exon into the reading frame. In many cases, altered splicing patterns in cancer are very likely

due to changes in *trans*-acting splicing factors rather than *cis*-element mutations. Alterations in the levels of various *trans*-acting splicing factors within a cell could change the ratio of certain splice events. Such SFs are SR proteins and hnRNPs that are known to antagonize each other's activity in a concentration-dependent manner. A change in their expression of these SFs may lead to the production of proteins of altered function and confer a growth or other advantage to the cell expressing them. Indeed, several splicing factors has been found upregulated in tumors and one splicing factor, SF2/ASF, was shown to be an oncogene. Our previous work suggested that splicing factors PTB and SRp20 are overexpressed in human ovarian tumors and their expression was associated with tumor malignancy. Moreover, knockdown of PTB expression by shRNA impaired ovarian tumor cell growth and malignant properties in vitro. Consistent with our results with *in vitro* loss-of-function studies, we see slower tumor growth in a xenograft mouse model of ovarian cancer when PTB was knocked down (unpublished), thus suggesting that splicing factors may represent a class of novel therapeutic targets for cancer treatment in epithelial ovarian cancer (EOC). Based on these findings and the role that upregulated PTB has on cell proliferation and malignant properties, we propose that targeting splicing factor PTB for inhibition holds a promising strategy for cancer therapy. Therefore we

hypothesize that PTB is a druggable therapeutic target in this disease. We further hypothesize that PTB activity in live cells can be monitored by measuring the splicing of a PTB target gene. To test these hypotheses, I propose the following four specific aims:

In Aim 1, we tested whether overexpression of PTB had effects on maintaining ovarian tumor cell growth and malignant properties by using vector-based siRNA technology to knock down the expression of PTB in A2780 ovarian tumor cells. Our data reveal that PTB overexpression in ovarian cancer is associated with malignancy of EOC. We also demonstrated that overexpressed PTB plays functional roles in maintaining ovarian tumor cell growth and malignant properties. Moreover we showed that PTB regulates the expression and splicing pattern of hundreds of genes, and it does this using a variety of alternative splicing modes. Also, we observed an increase in sensitivity to the ovarian cancer chemotherapeutic agents, carboplatin and paclitaxel, in A2780 ovarian cancer cells after PTB knockdown. Importantly, our results with PTB knockdown provide a strong proof-of-principle that PTB is a valid therapeutic target.

Being able to monitor PTB activity via a cell-based assay will enable us to develop new drug discovery platforms and to identify novel splicing modulators. With the hypothesis that manipulation of AS by targeting PTB may have therapeutic potential for ovarian cancer treatment, we first needed to measure PTB activity in live cells. To perform this, we developed in Aim 2 a cell-based fluorescent reporter assay to monitor PTB-mediated RNA splicing using an EGFP minigene. This EGFP-tagged reporter is based on differential splicing of a PTB target gene, identified in Aim 1, and depends on the PTB levels in cells; it is visualized by resulting changes in EGFP intensity. These experiments revealed that the cell-based reporter system is robust and sensitive enough to monitor PTB activity in live cells by different detection platforms. We then adapted our splicing-sensitive EGFP reporter minigene for high-throughput screening (HTS) assay.

Accordingly, in Aim 3 we completed the development of the reporter system to use with our HTS campaign. All validation and optimization screens were performed in engineered A2780 cells. Our results demonstrated that all the assay evaluation parameters were in the range considered acceptable for HTS-suitable assays.

To follow up on work in Aim 3 (development of the HTS assay), in Aim 4 we performed multiple assay demonstrations in 96-well format. We tested the reproducibility and variability of the maximum and minimum plate controls within the assay plate itself (intra-plate), from plate to plate (inter-plate) and from day to day.

In conclusion, we demonstrated that: (i) PTB is overexpressed in ovarian cancer and is associated with malignant properties of EOC; (ii) PTB knockdown results in inhibition of tumor cell growth in vitro and in vivo and colony formation and invasiveness in vitro (iii) causes an altered splicing pattern and expression profile in the cell; (iv) PTB activity can be monitored through its downstream target genes that exhibit PTB-depended splicing pattern changes; and (v) the ability to monitor PTB activity in living cells allowed us to develop a high-throughput screening assay to identify small molecules from chemical libraries.

Overall, this thesis seeks to develop novel small molecule therapeutic strategies for ovarian cancer with high clinical translational potential based on targeting PTB activity, which inhibits of tumor cell growth in vitro and in vivo and colony formation and invasiveness in vitro by multiple pathways. Further, it seeks to

enhance the therapeutic effect of current chemotherapy strategies by identifying and employing novel small molecule inhibitors of PTB.

1.2 Introduction

1.2.1 Overview: Ovarian Cancer

Ovarian cancer is the most lethal gynecological malignancy, and also the fifth leading cause of cancer death among women in the United States. About 1 in every 67 women in the United States will develop ovarian cancer. The American Cancer Society estimates 23,722 new cases of ovarian cancer being diagnosed, and about 16,522 deaths from ovarian cancer in the United States in 2012 (Fig 1.1) [4]. The risk factors of ovarian cancer include family and personal history, age, diet and hormonal exposure, which is influenced by child bearing, hormone replacement therapy (HRT), contraceptive pills and fertility drugs [4]. Having first-degree relatives with ovarian cancer, or a family or personal history of breast or colon cancer are associated with increased risk of developing ovarian cancer. As for hormone status, most ovarian cancers occur in women over 50 years of age, after the onset of menopause. Moreover, some studies suggest that reductions in the number of ovulations, through child-bearing breast-feeding, and oral contraceptive use, may lower risk [12, 13]. In contrast, women who have never had children are more likely to develop ovarian cancer. Furthermore, evidence suggests that women who use hormone replacement therapy (HRT) after menopause, especially for 10 or more years, have a higher incidence of the disease [14-16]. Long-term use of the fertility drug, clomiphene citrate, also has been once suggested to increase the risk [17]. The relationship between ovarian tumorigenesis and hormone status continues to be an active area of further investigation. Other factors that have been suggested to lower the risk for ovarian

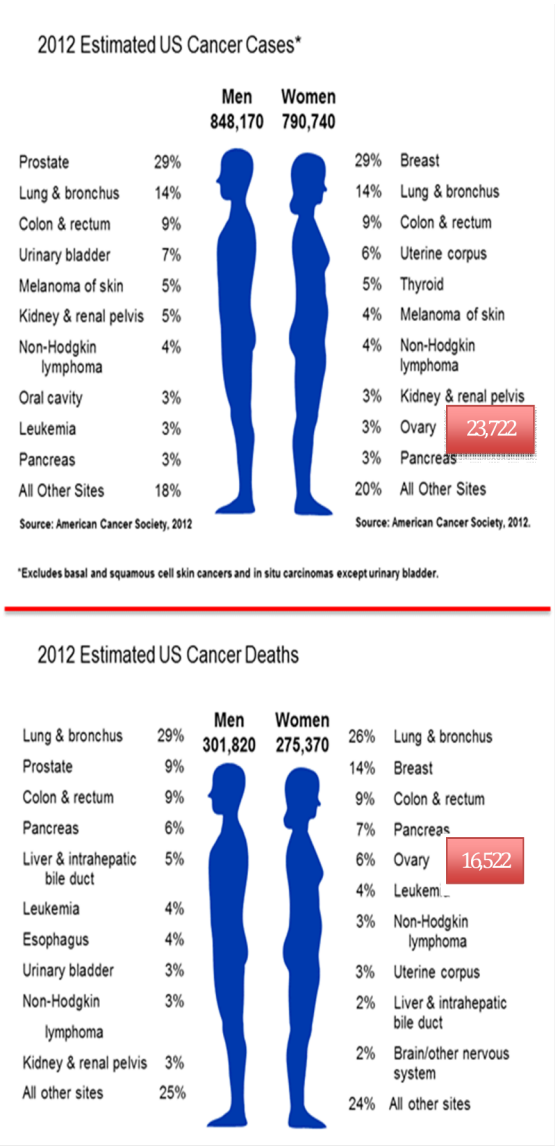

Fig 1.1 Distribution of the ten leading cancer types for the estimated new cancer cases and deaths, by sex in United States [4].

cancer include reductions in consumption of animal fat [18, 19], and the use of aspirin and acetaminophen [20, 21]. While the surgical removal of one or both ovaries (oophorecotomy) certainly reduces the incidence of ovarian cancer, it is only recommended as an option for high-risk patients.

Survival rates for ovarian cancer can be favorably affected by patient age (< 65 years old) and early detection and treatment. With diagnosis and treatment at early stages of the disease, the 5-year survival rate can reach 94% [4]. Unfortunately, only 19% of all ovarian cancers are diagnosed at an early stage. In 80% of cases, patients experience few symptoms early in the course of the disease and are not hospitalized until the cancer has progressed to an advanced grade. As a result, the 5-year survival rate can be as low as 28%. The high mortality of ovarian cancer is mainly due to its poor diagnosis and its aggressive nature. Ovarian cancer can be classified into three types: epithelial ovarian cancer, which originates from ovarian surface epithelial (OSE) cells and accounts for 90% of ovarian cancers, germline ovarian cancer, which develops from the cells that produce the eggs (ova), and stromal ovarian cancer, which starts from connective tissue cells that hold the ovary together and produce the female hormones. Epithelial ovarian cancer is graded in 4 stages, depending on the malignancy level [4]. In Stage 1 cancers, the tumor cells are still confined within the ovaries. Stage 2 cancer has spread to other organs in the pelvis, such as bladder, rectum or uterus. Stage 3 disease is characterized by further spread to the lining of the abdomen or to the lymph nodes. In Stage 4, the most advanced stage, the cancer has disseminated to distant organs, such as the liver or lungs. The low early detection rate of ovarian cancer contributes to its having the worst prognosis of all gynecological cancers. Although several techniques are used to detect ovarian cancer, including pelvic exam, ultrasound exam, CT (computed tomography) scans, X-rays, and a blood test for CA-125 antigen, none on its own

can reliably detect early ovarian cancer. These existing methods must be used in combination for early detection or to confirm the presence of recurrent ovarian cancer. Understanding of the pathogenesis for ovarian carcinoma is still incomplete. Steroid hormone balance is considered one factor in ovarian carcinogenesis. It has been speculated that elevated risk of ovarian cancer is related to high serum androgen levels which results from the post-menopausal shift in the balance of ovarian steroids from estrogens to androgens. Ninety-percent of ovarian cancer tissues were shown to be positive for androgen receptor (AR), and chemotherapy decreased androgen production from cancer cells [22]. Epidemiological evidence suggests that elevated levels of the pregnancy hormone progesterone might reduce ovarian cancer incidence. *In vitro* data has demosntrated that chronic progesterone treatment caused reduction of cyclindependent kinase (CDK) activity, loss of anchorage-independence, suppression of transformed cellular phenotype, and reduction of tumor [23]. Angiogenesis is a critical process in tumorigenesis. Vascular endothelial growth factor (VEGF), an essential angiogenic protein, has been found to be highly expressed in ovarian cancer cells and associated with ovarian cancer progression [24]. Moreover, the inhibition of VEGF suppressed tumor growth, invasion, and metastasis in an animal model [25]. Like most malignancies, ovarian cancer is a heterogeneous disease characterized by multiple molecular defects. These include elevation of cellular survival signaling pathways, such as HER-2/neu and PI3K, which have been been correlated with ovarian malignancy, and defects in *Ras* and *PTEN* which have proven to be

especially important in the development of ovarian endometrioid cancer, a subtype of epithelial ovarian cancer [26].

1.2.1.1 Current Clinical Therapies for Ovarian Cancers

The current therapies for ovarian cancers include surgical removal of tumors, radiation therapy, and chemotherapy. For chemotherapy, platinum drugs, such as cisplatin and carboplatin, are the first-line choice drugs [27]. Combination therapy using a platinum drug and paclitaxel (taxane) is also a standard approach. The second-line salvage chemotherapy agents include Doxil (liposomal doxorubicin), topotecan, anthracyclines, such as doxorubicin (Adriamycin), gemcitabine, cyclophosphamide, vinorelbine (Navelbine), hexamethylmelamine, ifosfamide, and etoposide [28]. These second- line treatments are used in recurrent and metastatic patients after cisplatin- and paclitaxel- based chemotherapy have failed.

Beyond the currently approved chemotherapeutic agents, other therapeutic choices are under clinical trials. Some of these include immunotherapy, such as OvaRex Mab, a murine monoclonal antibody targeting CA-125 antigen, which has completed phase II trials in which patients have shown longer remission times [29, 30]. It is proposed that OvaRex Mab stimulates the immune system to kill tumors that express CA-125. Based on the phase II results, a phase III trial of OvaRex Mab has started in patients with stage III or IV ovarian cancers.

In addition to development of new drugs, the improvement of drug administration is also a focus of investigation. It has been recently reported that the intraperitoneal (IP) injection of chemotherapy drugs yielded longer survival times than the typical intravenous route in ovarican cancer [31].

1.2.2. Overview: Pre-mRNA Splicing

The process of removal of intervening sequences or introns from the nascent transcripts is called pre-mRNA splicing which is an important step of eukaryotic gene expression. Pre-mRNA splicing forms the messenger RNA (mRNA), from the nascent transcripts also known as precursor messenger RNA (pre-mRNA). The borders of exons and introns are defined by the 5' splice site at the upstream end of an intron and the 3' splice site at the downstream end (Fig1.2). Pre-mRNA splicing consists of two SN2-type transesterification reactions, involving functional groups from three reactive regions in the pre-mRNA. The first step, the phosphodiester bond at the 5′ splice site is attacked by the 2′-hydroxyl of an adenosine of the branch point sequence (BPS) in the intron, which generates a free 5′ exon and an intron lariat-3′ exon. In the second step, the 3′-hydroxyl of the 5′ exon attacks the phosphodiester bond at the 3′SS, leading to exon ligation and excision of the lariat intron.

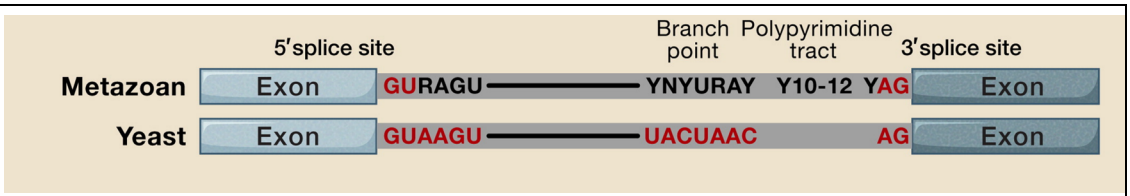

Fig1.2. Conserved Sequence elements of pre-mRNAs. The two exons (blue) are separated by an intron (Gray). The consensus sequences at the 5' splice site, branch point, and 3' splice site is indicated, N is any nucleotide, R is purine, and Y is pyrimidine [3]. Reprinted by permission from Elsevier , The Cell [3].

The spliceosome is the largest macromolecular ribonucleoprotein complex in the pre-mRNA splicing process that catalyzes the biochemical reaction of pre-mRNA splicing. This spliceosome consists of the pre-mRNA substrate and several uridine-rich small nuclear ribonucleoproteins (UsnRNP) particles as well as a large number of auxillary proteins [32-38]. The spliceosome consists of U1, U2, U4/6 and U5 snRNPs[36, 39, 40]. Splicing of a minor class of introns (also called U12-type) occurs in the spliceosome containing U11 and U12, in addition to U4atac, U6atac, and U5 snRNPs [3, 37, 41-45].

Essential subunits of splicing machinery are spliceosomal UsnRNPs which contain uridine-rich snRNA and associated proteins. U1, U2, U4, and U5, and are transcribed by RNA polymerase II as precursors containing additional 3' nucleotides [37, 46, 47]. The precursor snRNAs acquire a mono-methylated guanosine cap as they are exported to the cytoplasm where the pre-UsnRNA interact with Sm proteins including B/B', D3, D2, D1, E, F and G to form the snRNP core structure. Following the UsnRNP Sm core complex assembly and subsequent processing of the pre- UsnRNA these UsnRNPs are imported into the nucleus to assemble into spliceosome [47-53].

Based on in vitro studies (i.e. native gel electrophoresis, affinity selection, and glycerol gradient centrifugation), the consensus view on spliceosome assembly suggests that the pathway of spliceosomal assembly follows a sequential association and dissociation of spliceosomal snRNPs ([54, 55]). This model suggests that each successive stage in the transcript-dependent association of snRNPs and other proteins factors is a necessary precursor for the subsequent step (Fig1.3) [54].

Assembly begins with E complex which involves binding of the U1 snRNP through the base-pairing interaction of the 5' end of the U1snRNA to the 5' splice

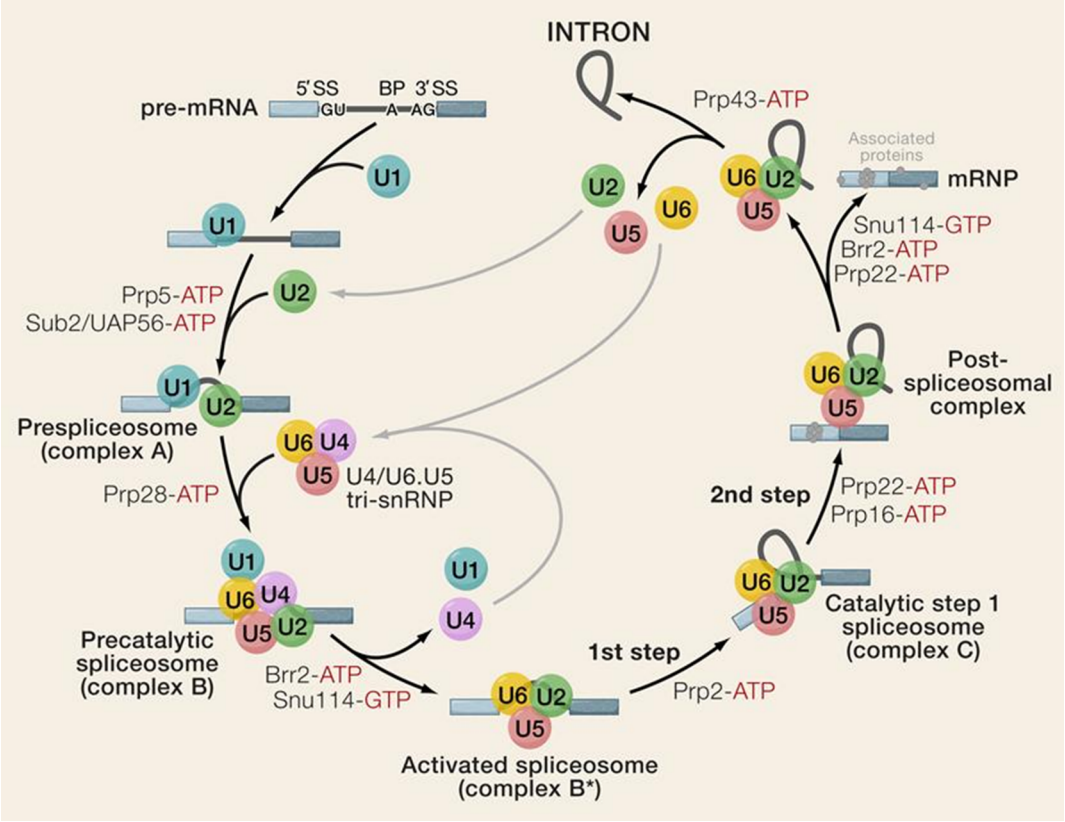

Fig1.3. Cross-intron assembly and disassembly cycle of the spliceosome. The stepwise interaction of the snRNPs to the pre-mRNA is required for the removal of an intron from a pre-mRNA. The assembly and recruitment of snRNPs requires the aid of several non-snRNP proteins [3]. Reprinted by permission from Elsevier , The Cell [3].

site of the intron. This step progresses in an ATP-independent manner. These described interactions in higher eukaryotes are stabilized by members of the

serine-arginine-rich (SR) protein families and proteins associating with the U1snRNP. In addition to the U1-5' splice site interaction, this step also involves binding of the SF1/BBP protein and the U2 auxillary factor (U2AF) to the BPS (Fig1.3). These proteins co-operate with each other during the binding, and recruits U2AF65 through its C-terminal RNA recognition motif (RRM), while the U2AF35 interacts with U2AF65 and binds to the AG dinucleotide of the 3' splice site.

After the formation of the E complex, the U2 snRNA undergoes an ATP-dependent base-pairing interaction with the BPS, which leads to the formation of the A complex (Fig1.4B). U2AF65 and heteromeric protein complexes of U2 snRNP consisting of SF3a and SF3b, stabilize this base pairing interaction [56, 57] [58]. The interaction of U2 casues to the displacement of SF1/BBP from the BP followed by the association of SF3b14a/p14 with the BPS adenosine [59].

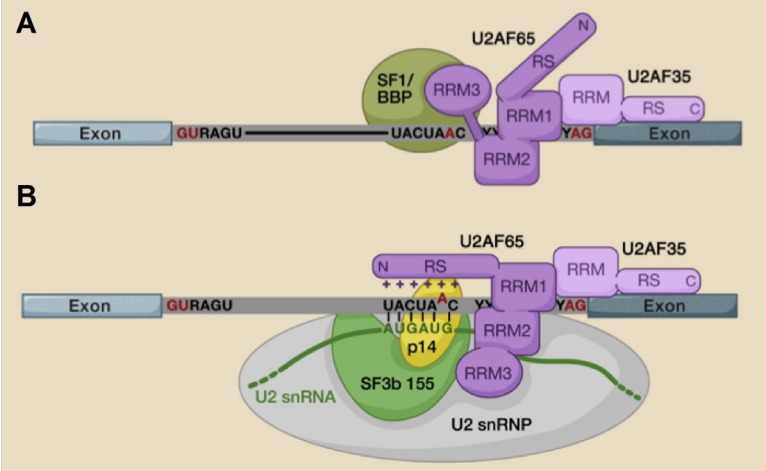

Fig 1.4 **A**. Molecular Interactions at the branch point and the 3' splice site. The pre-mRNA is bound by SF1/BBP, whereas the well-defined tracts and the 3' splice site are bound by U2 auxillary factor (U2AF) [3]. **B.** Molecular interaction at the branch point and the 3' splice site in the "spliceosomal A" complex. Upon stable binding of U2snRNP, SF1/BBP is displaced to allow U2-associated protein p14 to interact with the BPS and U2AF65 to SF3b155 [3]. Reprinted by permission from Elsevier , The Cell [3].

Following to the A complex formation, the U4/U6 and U5 snRNPs are recruited as a pre-assembled tri-snRNP, leading to the formation of the B complex (Fig1.4). The snRNA component of the U4/U6 U5 tri-snRNP engage in base pairing interactions with the pre-mRNA, specifically U5 contacts the 5' and 3' splice sites while U6 base pairs with the U2 [60]. Nucleotides of U6 snRNA, which contains the catalytically active sites, are transferred in an inactive state to the spliceosome because of the base pairing with U4 snRNA. Following the formation of the B complex the catalytically inactive spliceosome undergoes a conformational and compositional rearrangement (i.e. catalytic activation) to facilitate the first transesterification step of splicing.

The splicing reactions are carried out by the UsnRNA components of the spliceosome, but proteins in the spliceosome make up two third of the mass of a spliceosome. Hence in addition to RNA-RNA interactions, the RNA-protein and protein-protein interactions play an important role in this process. The human spliceosome contains numerous non-snRNP proteins complexes [39]. Mass spectrometric analyses of different assembly intermediates as well as comparative studies on the human A, B and C complexes uncover a dynamic exchange of proteins during spliceosomal assembly and activation [39, 61-63].

These detailed analyses suggest that several different groups of spliceosomal proteins are present in the spliceosome throughout the splicing cycle, that includes U2-associated proteins, members of the SR-protein family and hnRNPs. Additionally, during the transition from the A to B or B to C complexes numerous proteins are recruited while others are destabilized at each different stage of the pre-mRNA splicing process (Fig 1.5).

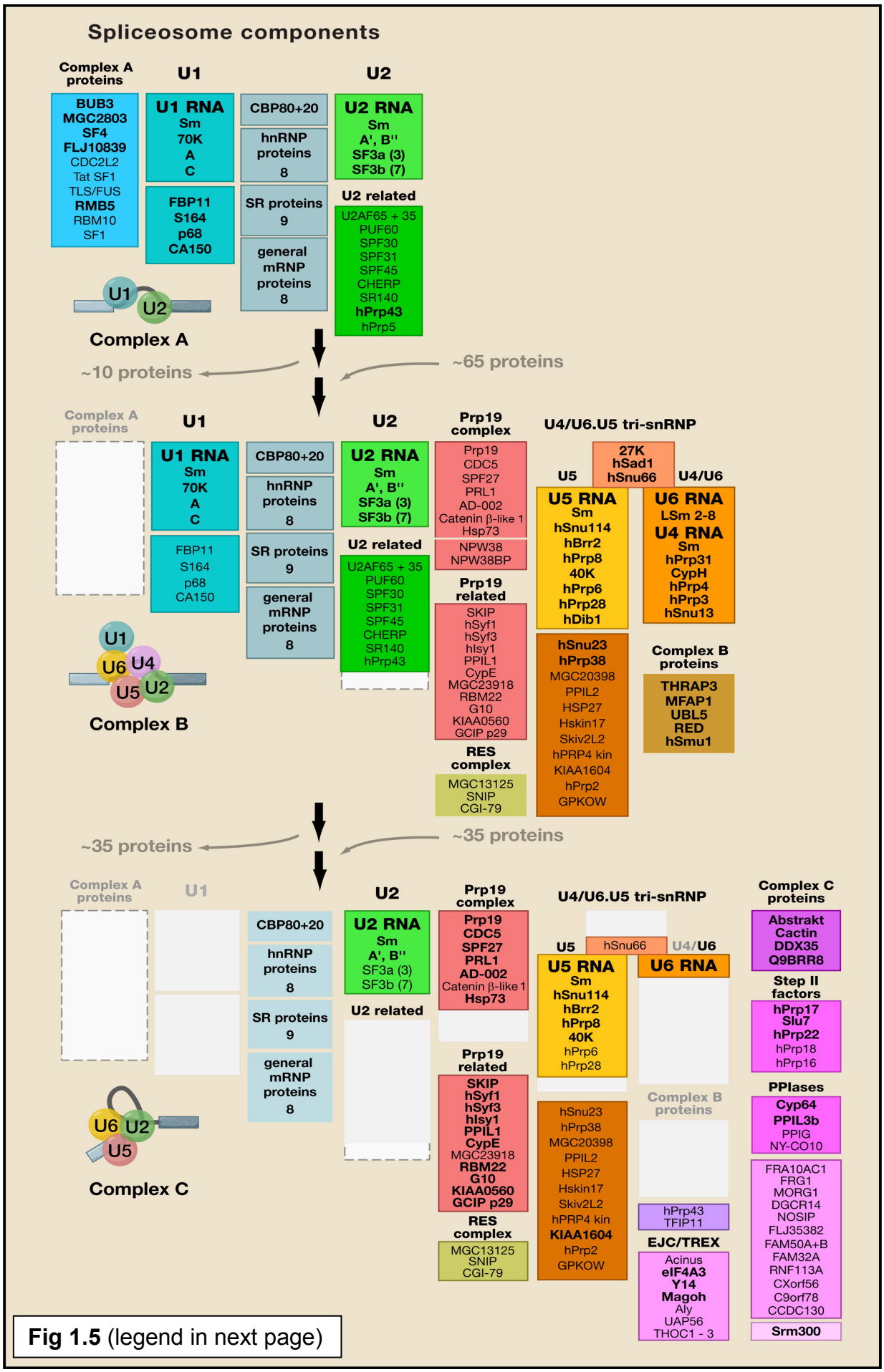

Fig 1.5 (legend in next page)

Fig1.5. Compositional Dynamics of Human A, B, and C spliceosomal complexes as identified from earlier analyses [3, 39, 61-63] Reprinted by permission from Elsevier , The Cell [3].

Thus pre-mRNA splicing is a complex and a highly dynamic process which involves interplay of multiple networks of RNA-RNA, RNA-protein, and Protein-protein interactions at every step of the splicing reaction. However much still remains to be learned about the intricacies of the spliceosome and the carefully controlled pathways that determine the function of these components [3].

1.2.3. Overview: Alternative splicing

Alternative splicing (AS) is a well-characterized mechanism by which multiple transcripts are generated from a single mRNA precursor due to variations in incorporation of exons, and because of that contributing to proteomic diversity [64, 65]. The proteomic diversity helps organisms to maximize the coding capacity of their limited genomic materials. When alternative splicing occurs within the protein-coding region of a gene, it can alter the protein product in numerous ways such as adding or removing peptide segments (in-frame AS events), while other which change the downstream reading frame (out-of-frame AS events or frame-shifting AS events) causing slight changes in the amino acid

sequence or influence mRNA stability via Nonsense mediated decay (NMD) by inserting of pre-mature termination codons. AS of untranslated regions or untranslated region (UTR) can also determine mRNA localization, stability and translational efficiency. These changes in the peptide sequence can have significant changes in protein function, biochemical characteristics, subcellular localization, and may influence its ability to interact with other cellular components [66, 67].

Intron removal during pre-mRNA splicing in higher eukaryotes requires the accurate identification of two splice sites at the end of the exons (exon definition) [68]. However the four main splicing signals that delineate the proper exon-intron boundary such as 5', 3' splice site, branch point and the polypyrimidine tract provide insufficient information to distinguish between true and cryptic splice sites. *In vitro* studies on eukaryotic pre-mRNA splicing showed that additional information required for exon recognition resides in a large number of positively and negatively cis-acting elements that lies both in the exon and surrounding introns. These elements interact with trans-acting factors and impact splice site recognition/selection. These regulatory elements may be required for both constitutive as well as AS. The cis-acting elements are relatively short, usually 5-

20 nucleotides, and are classified as exonic or intronic splicing enhancers or silencers (ESE, ESS, ISE, and ISS). This high degree of degeneracy in the splicing regulatory elements in the mammalian pre-mRNA transcripts provides the flexibility for alternative selection and pairing of different splice sites, a profound mechanism for regulating AS [68-71].

AS events can be classified into four main subgroups namely, (i) exon skipping (cassette exons), where by the exon is spliced out of the transcript together with its flanking introns; (ii) alternative 5' splice site or 3' splice site selection, where two or more splice sites exist at one end of exon and any one of them can be used; (iii) intron retention, which involves inclusion of an intron in a mature mRNA molecule; (iv) other less frequent events such as mutually exclusive splicing, alternative transcription start sites and multiple polyadenylation sites [8] (Fig1.6).

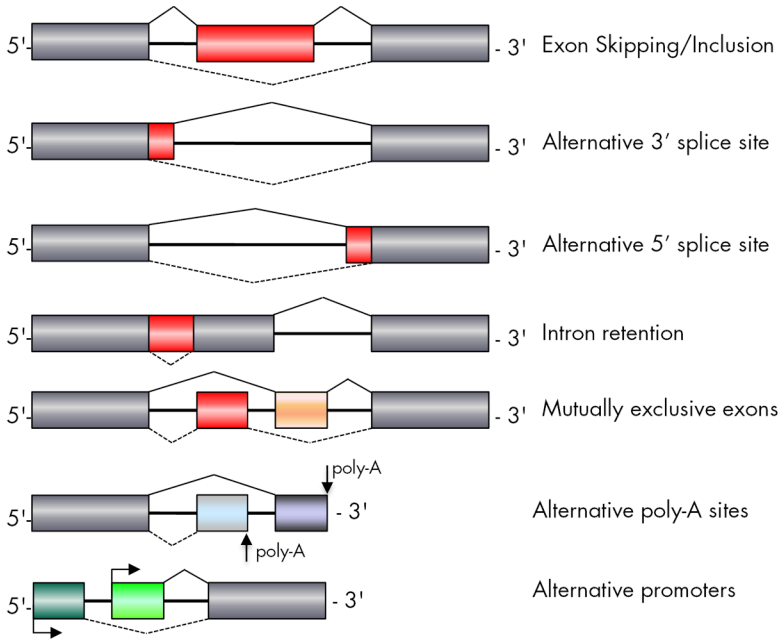

Fig1.6. Patterns of alternative splicing. Transcripts from a gene can undergo many different patterns of alternative splicing, Transcriptional initiation at different promoters generate alternative 5'-terminal exons which are joined to downstream 3' exon. Similarly alternative 3'exons, with alternative polyadenylation sites can be joined to a common upstream exon. Alternative 5' or 3' splice sites, exons can be extended or shortened in length. The most common pattern of alternative splicing is a cassette exon in which exons are included or skipped thus inserting or deleting a portion of amino acid sequences. Specific paired exons show mutually exclusive splicing, where certain exons are included only if the other is excluded, or certain exons are included only if a specific exon is included. Finally, the excision of an intron can be suppressed leading to retained intronic sequences in mRNA. (Adapted from Maniatis *et al.* [8])

The fundamental mechanism underlying alternative splicing is a complex interaction between trans-acting factors and cis-elements in the pre-mRNA substrates, leading to differential selection of splice sites [72]. The recognition of splice sites and cis-acting elements is mediated by networks of interactions between pre-mRNA and trans-acting factors, including snRNP and non-snRNP splicing factors. The recognition and pairing of splice sites is influenced by intronic and exonic sequence elements which can enhance or suppress splice site recognition [72-80].

Most of the exonic splicing elements (ESE) have been identified by biochemical, genetic and bioinformatics approaches including systematic evolution of ligands by exponential enrichment (SELEX) [72-80]. A/G-rich (purine rich) and A/C rich elements are among the exonic splicing enhancers identified that interact with proteins containing SR domains and that function by recruiting other spliceosomal components (such as; snRNPs and other protein factors), hence promoting the usage of the neighboring splice sites [66]. Intronic splicing enhancer (ISE) elements have been studied in a number of genes, including c-src, b-tropomyosin, calcitonin/calcitonin gene-related peptide gene (CGRP), fibronectin, non-muscle myosin heavy chain, cardiac troponin T (cTNT), FGFR-2,

Alpha-2 subunit of glycine receptor, microtubule associated protein Tau (Tau) and other genes. Such ISE may contain sequences similar to 5' splice site, U-rich elements adjacent to regulated 5' splice site, UGCAUG elements, (UCAUY) 3-containing sequences or CUG-containing motifs [81-99].

Similarly, studies have also identified a number of intronic and exonic splicing suppressor (ISS and ESS respectively) elements. A number of ESS elements have been characterized in genes such as, HIV tat, bovine papillioma virus and rous sarcoma virus, beta-tropomyosin and others. The suppressor activity of such cis- elements is modulated via the interaction with proteins of the hnRNP family such as hnRNPA1, hnRNPH and hnRNPF. A variety of ISS have been identified to regulate alternative splicing of genes such as FGFR2, Caspase 2, clathrin light chain B, and others. Many of these ISS contain extended polypyrimidine tracts, while others have authentic or decoy splice sites. The proposed function of these elements is to mediate nonproductive interactions to suppress the usage of the upstream 5' splice site. Several hnRNP proteins including polypyrimidine tract binding protein (PTB), hnRNPA1, hnRNPH play an important role in suppression of splicing via interaction with ISS [95, 100-109].

In certain genes such as IgM, exon M1 and M2 splicing, Tau exon10 and Caspase 2 exon 9, cis-regulatory elements usually contain both enhancers and suppressor domains and in some cases, contain pre-positioned enhancer and suppressor elements, decisions of exon splicing are regulated by a complex interaction between multiple trans-acting factors [84, 110]. Studies on alternative splicing of caspase2 exon9 identified a 100 nucleotide long region in intron10 that is responsible for suppression of exon9. This 100 nucleotide element also known as intron 100 (In100) has sequence features of an authentic 3'-splice site including a branch point, poplypyrimidine tract and the AG dinucleotide. Detailed biochemical and *in vivo* cell culture based experiments show that this decoy 3'-splice site interacts nonproductively with the 5' splice site and this suppresses the exon9 splicing [83, 87].

The recognition and usage of splice sites are determined during the early steps of spliceosome assembly. The key to understanding this is splice site selection, which occurs in two separable steps, first the initial commitment of transcripts to splicing pathway, followed by the committed pairing of a particular 5' and 3' splice site. These steps usually occur in vitro during the formation of E complex. It has been shown that in alternatively spliced transcripts, splice sites only become

irreversibly paired in the A complex, so that splice site pairings could be affected even after spliceosome assembly at E complex in a splicing regulator dependent manner. This suggests that splice site pairing is a weak or dynamic process prior to ATP- dependent irreversible selection of the 3' element in the A complex [111]. Splicing activators or suppressors that regulate these commitment steps can either facilitate or inhibit the interaction of U1snRNP to the 5' splice site, and of U2snRNP with the 3' splice site.

Tissue-specific alternative splicing has been reported for specific genes such as FGFR, tropomyosin,and c-src [112-121]. However, only a limited number of tissue-specific splicing regulators have been identified; these include Nova-1 and -2, nPTB and Fox2/Fox1 [122-130] . This suggests that perhaps no single factor is sufficient for dictating tissue specificity of alternative splicing of any gene. Instead, multi-component interactions among tissue specific splicing regulators, pre-mRNA and general splicing factors together may determine the fate of a tissue specific splicing event [131-133]. Tissue- specific combination of different splicing regulators, relative concentrations of distinct splicing factors, and differential modifications of splicing regulators likely all contribute towards a tissue-specific splicing event. For example, PTB levels are lower in neural tissue

as compared to other tissues, thus providing a permissive environment for exon inclusion of several PTB-regulated neuron specific exons such as c-src N1 [134]. Similarly, although most SR proteins are expressed in a wide range of tissues, many SR proteins themselves have different isoforms due to alternative splicing or post-translational modifications in particular tissues that can affect function of these SR proteins in regulated splicing events [135-138]. It is, however, unclear how these extremely complicated alternative splicing patterns of different genes in a given cell or specific tissue are coordinated.

Aberrant pre-mRNA splicing has been implicated in the pathogenesis of a number of human diseases. Mutations that cause disease by affecting splicing either disrupt cis-acting splicing signals or trans-acting components that are required for recognition or modulation of splice site. Usually mutations in cis-acting elements alter expression of only the mutated allele, while mutations in splicing factors can affect both constitutive or regulated splicing that they regulate [139-142].

Myotonic dystrophy (DM) is caused by CTG repeats expansions in the 3'UTR of the DMPK gene (DM1) or in CCTG repeat expansion in intron1 of ZNF9 gene (DM2). However, it has been shown that the underlying cause of DM is not the

disruption of DMPK and ZNF9 gene expression, but expanded repeat transcripts [117]. A major feature of DM is disruption of regulation of alternative splicing, which is caused by the expanded RNA repeats that alter the activities of splicing regulatory factors, hence leading to a transdominant misregulation of alternative splicing [143-148]. The expression of CUG and CCUG repeat containing RNA disrupts the regulatory activities of at least two splicing factors, Muscle blind protein (MBNL) and CELF (CUG-BP and ETR-3-like factors) which are involved in regulating alternative splicing of heart- and muscle-specific exons. The proposed hypothesis suggests that CUG-and CCUG-repeat interacting proteins such as MBNL interact with or are recruited to these repeat expansions and form foci; these associations deplete the nuclear pool of MBNL and thus cause aberrant regulation of MBNL1-sensitive splicing events [143, 148-155]. In contrast, other studies have shown that CUG / CCUG repeat expansion causes hyperstabilization of CUG-BP1 by PKC-mediated phosphorylation, thus affecting splicing events regulated by CUG-BP1 [147, 156-158]. These findings highlight the complex and delicate roles of alternative splicing regulation in general human physiology and pathogenesis.

1.2.4 Alternative splicing and disease status

1.2.4.1 Alternative splicing of genes associated with tumorigenesis

Many genes associated with tumorigenesis, such as oncogenes, tumor suppressor genes, cell cycle-related genes, and apoptos is related genes, have alternative splice forms. Also of interest is the growing role of mis-regulated alternative splicing events associated with tumor formation and metastasis [159-164] For example, the BC-related genes BRCA1, BRCA2, ERBB2, ER-α, ER-β and PR have splice variants ranging from 3 up to 25 [165-167]. Although the functions of most splice variants are unknown, many have distinct or antagonistic functions from each other. For example, two mRNAs with or without the alternative exon IDX can be generated from the c-H-ras proto-oncogene premRNA. One encodes the familiar protein p21 with strong transformation activity and the other encodes a smaller form, p19, which lacks transformation potential and can act as a negative regulator of the p21 [168, 169]. Moreover, the trans-acting splicing factors hnRNP A1, SR proteins SC35 and SRp40, as well as helicase p68, regulate this alternative splicing [170]. Another example is the oncogene HER2/neu, whose overexpression frequently coincides with poor prognosis in some human cancers, including ovarian and breast cancers [171] [46, 47]. Some splice variants of HER2/neu can inhibit the oncogenic activity of

this gene, while others possess increased transformation activity [172-175]. It is not clear at present what kind of splicing factors are involved in the regulation of HER2/neu pre-mRNA splicing. However, these two examples show clearly that alternative splicing is an important mechanism involved in regulation of the function of genes associated with tumorigenesis. Therefore, it is possible that oncogenic processes can be altered (suppressed or enhanced) through splicing manipulation.

1.2.4.2 Alteration of pre-mRNA splicing in cancer

Defects in pre-mRNA splicing have been shown to be causes of a variety of human diseases [139, 176]. Cancer-associated alterations in pre-mRNA splicing have been the subject of several recent reviews [161, 177, 178]. Although it is still not clear whether altered premRNA splicing causes cancers, there is mounting evidence showing that altered splicing is associated with and possibly involved in tumor progression and/or metastasis [179-184]. The most studied example is the correlation between CD44 splice variants and tumor progression. CD44 splice variants are aberrantly expressed in many human tumors including ovarian and breast cancers [179, 180, 185]. Some CD44 variants are associated with the metastatic potential of cells, and their expression levels are an indicator of poor prognosis [179, 180]. Besides CD44, an alternatively spliced cadherin-11

was found to enhance breast tumor invasiveness, and aberrantly spliced mdm2 was correlated with shortened overall survival of BC patients [186, 187]. Therefore, controlling or modifying these alternative or aberrant splicing events may present a novel target for tumor therapy. For example, the production of mRNAs for bcl-XS and bcl-XL, which are alternatively spliced forms of the same gene, but have completely opposite functions (apoptotic and anti-apoptotic, respectively), can be altered through the use of antisense oligonucleotides (ASOs) that move splicing factors positioning from one splicing site on the gene to another [188]. In most cancers, the misregulated splicing of CD44 is not the result of gene mutation; rather, altered splicing patterns are very likely due to changes in expression of trans-acting splicing factors. Indeed, in a mouse mammary gland model, expression of some SR protein family members were found to be altered during tumor progression, and CD44 alternative splicing correlated with the expression of these SR proteins [182]. Finally, we observed by western blot that two splicing factors, PTB and SRp20, were highly expressed in human ovarian tumors, compared to matched normal ovarian tissues. Additionally, we also found on average twice as many splice variants in the gene encoding the multidrug resistance-associated protein 1 (MRP1/ABCC1) in ovarian tumors than in matched normal ovarian tissues [189]. Similarly, we

observed more CD44 splice variants in most ovarian tumors than in their matched normal tissues. Our recent work further indicates that the overexpressed PTB plays functional role in maintaining ovarian tumor cell growth and malignant properties [2]. In addition, we also observed that PTB and SRp20 were up-regulated when HMECs were transformed from normal to malignant state (unpublished data). Given the functions of splicing factors in regulating alternative splicing, the roles of splicing factors in tumorigenesis are likely mediated by splice variants of various genes. Our preliminary study of global alternative splicing using splicing-sensitive microarray technology revealed that splicing of thousands of genes could be influenced by a single splicing factor (chapter 5). Together, these results suggest that neoplastic transformation is very likely accompanied by broad changes in alternative splicing and such changes may constitute an important component of the multi-step tumorigenic process.

1.2.5 Overview: SR protein family and SRp20

Several families of splicing activators have been identified; the most notable are SR proteins (Table1.1). The interaction of SR proteins and ESEs play a critical role in exon recognition by the spliceosome.

Classification	Factors	Key Domains	Functions
Classic SR Proteins	SRp20, SF2/ASF, SC35, 9G8, SRp40, SRp55/B52, SRp75	One or two RRMs plus an RS domain	Constitutive and alternative splicing
Additional SR proteins	hTRA2α, hTRA2β, RNPS1, SRp38, SRp30c, p54, SRrp35, SRrp53, SRp86	One or two RRMs plus an RS domain	Positive and negative regulation of alternative splicing
RNA binding SR related factors	U2AF65, U2AF35, Urp, HCC1/CAPER, U1-70K, hsWAP, Pinin, Sip1, SR-A1, ZNF265, SRm160, SRm300	RRM, PWI domain, Zn finger plus an RS domain	Splicing factor of co-activators
Enzymes and regulators carrying and RS domain	hPRP5, hPRP16, Prp22/HRH1, U5-100K/hPRP28, ClkSty-1, 2, 3, CLASP, Prp4K, CrkRS/CRK7/CDK12, CDC2L5, 39 CCNL1, CCNL2, SR-cyp,	DEATH box, kinase domains, peptidyl- prolyl isomerase domain	Spliceosome rearrangement and modification of splicing factors

Table1.1 SR proteins and SR-related splicing factors (RRM: RNA Recognition Motif, RS domain: C-terminal arginine/serine-rich domain). Reprinted by permission from Springer, Advances in Experimental Medicine and Biology, and Landes Bioscience [5].

The early function of SR proteins in splicing is mediated by their sequence-specific binding to cis-acting elements, or ESE and recruitment and stabilization of factors such as U1snRNP at the 5' splice site and U2AF at the 3'splice site. These proteins have been implicated in promoting the communication between the 5' and the 3' splice site an essential aspect of the exon definition model of splicing (Fig1.6). Also in certain cases SR proteins function via preventing or displacing other RNA binding proteins such as hnRNPA1 from exonic splicing silencers (ESS) [190-194]. In the cell these mechanisms of SR protein mediated processes function synergistically to favor spliceosomal assembly on functional splice site. Based on studies, SR proteins mainly bind and promote selection of proximal splice sites over distal alternative 5' or 3' splice sites (Fig1.7). In such circumstances the selection of splice sites is decided by intrinsic strength of the competing splice sites and the frequency of the competing exonic splicing enhancer or suppressor sequences. Binding of SR proteins can enhance complex assembly on both strong and weak splice sites making them equally competitive [195-197].

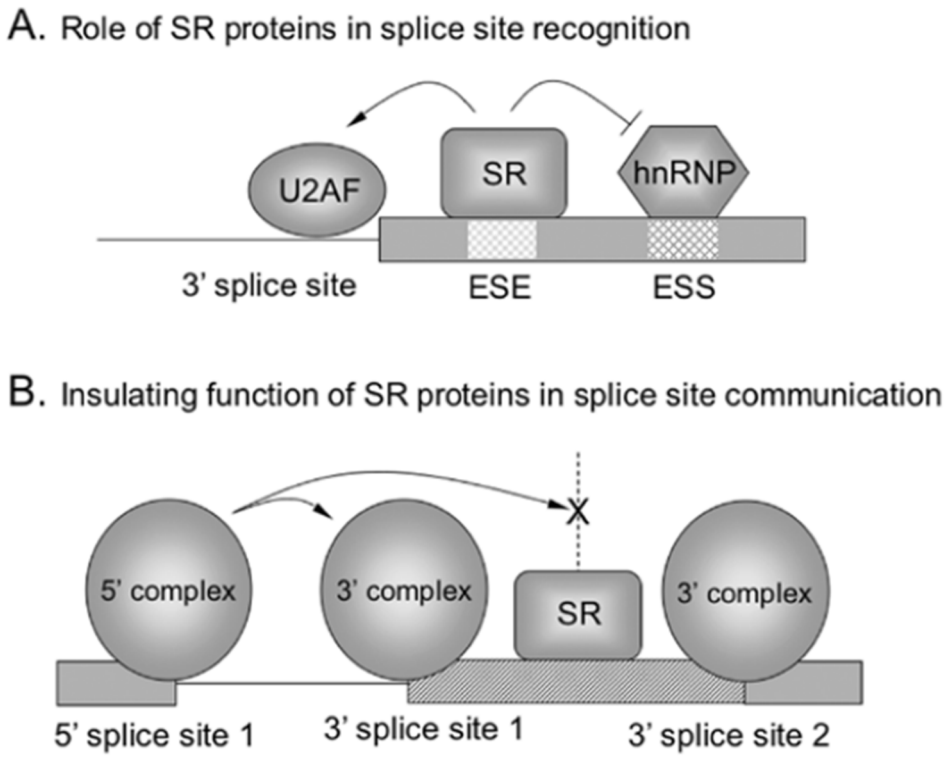

Fig 1.7 Role of SR proteins in splice site selection. **A,** An ESE bound SR protein stimulates complex assembly at a nearby functional splice site and or blocks the negative activity of hnRNP on spliceosomal assembly. **B,** An insulating function of SR protein promotes selection of proximal splice site and blocks usage of a distal splice site. Reprinted by permission from Springer, Advances in Experimental Medicine and Biology, and Landes Bioscience [5].

Though SR proteins have primarily been shown to act as activators of splicing, studies have suggested SR protein dependent splicing inhibition, e.g. SR proteins might recognize certain intronic sequences resembling ESE and activate

cryptic splice sites such as in Caspase 2 exon9 splicing [83, 198]. In certain substrates such as hnRNPA1, CamKII delta SR proteins have been shown to actively suppress splice sites; though the exact mechanism is unknown [199, 200]. Studies have suggested that this function may depend on factors such as location of binding sites with respect to splice sites, or certain SR protein interactions being more productive in splice site recognition than others and less productive interactions interfering with more productive ones in a competitive manner (Fig 1.8) [201-206]. However there are a subset of SR proteins like, SRp86, SRp54, SRp35 and SRp40 have been shown to antagonize typical SR proteins in splice site recognition [207-213].

One of the important structural elements of SR proteins is the RS domain, which are involved in both protein-protein and RNA-protein interaction necessary for SR protein function. Differential phosphorylation of the RS domain has been shown regulate the protein-protein interaction, subcellular localization/ distribution and activities of SR proteins, thus providing level of regulation in gene expression which can be modulated by various cellular events [214-221].

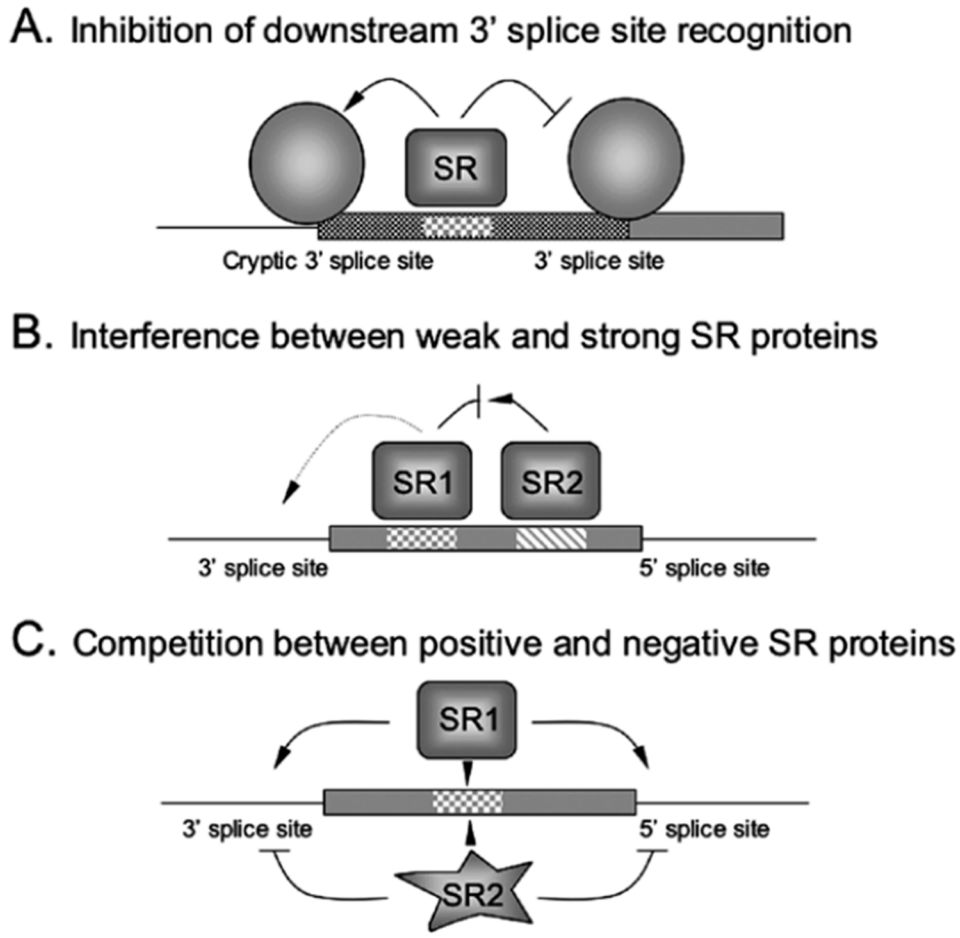

Fig1.8. Positive and negative effects of SR proteins on splice site selection. **A,** SR protein may bind to an intronic sequence resembling an ESE, thereby activating an upstream cryptic 3' splice site and thus inhibiting the downstream authentic splice site. **B,** Function of a ESE bound SR protein may blocked by the binding of another SR protein with weaker ability of spliceosomal activation. **C,** Binding of both positive and negative SR protein to the same cis-element. Reprinted by permission from Springer, Advances in Experimental Medicine and Biology, and Landes Bioscience [173].

1.2.6 Overview: HNRNP protein family and PTBP1

Heterogenous nuclear ribonucleoparticles (hnRNPs) family of proteins are a diverse group of RNA binding proteins implicated in splicing regulation, especially repression by directly antagonizing the recognition of splice sites, or interfering with proteins associating with enhancer elements (Fig1.8). HnRNP A1 and PTB are the best studied proteins in this group. HnRNPA1 has been shown to alter 5' splice site choice and promote skipping via its interaction with G-tracts [105, 193, 222]. The mechanism of splicing repression by A1 involves either interaction with the pre-mRNA and hindering spliceosomal complex formation at 5' splice site, or by binding and antagonizing the recruitment of SR proteins thus preventing the recruitment of factors at the splice sites [105, 193, 222]. Also in some cases where one high affinity hnRNPA1 binding sites are flanked by low affinity sites, cooperative interactions could create a zone of local repression thus preventing interaction of spliceosomal components to the splice sites (Fig1.8) [193]. PTB or hnRNPI has been shown to regulate splicing repression in alpha-tropomyosin (alpha-TM) exon3, c-src exon N1, Fas exon6, caspase2 exon9 etc. In alpha-TM exon3 splicing is repressed via interaction of PTB to UCUU sites both upstream and downstream of exons via preventing binding of U2AF at the 3' splice site [223-225]. Similarly in c-src N1 exon splicing is repressed in non-

neuronal cells by PTB via cooperative binding to a CUCUCU element upstream and downstream of the exon [101, 103]. However this splicing repression event mediated by PTB involves the recruitment of hnRNPF, hnRNPH, KSRP which blocks the binding of U2AF at the 3'splice site of the exon downstream of exonN1 thus uncoupling the intron definition complex formation and thus leading to repression of exonN1 [91, 104, 134, 226, 227]. In the neuronal cells, a brain specific paralog of PTB, nPTB can replace PTB complex at the binding site, this relieves PTB mediated repression and thus enhances N1 splicing in neurons [228]. In case of Fas receptor pre-mRNA, interaction of PTB to exon6 does not alter the U1snRNP recruitment to the 5'splice site of exon6 or U2AF recruitment at the 3'splice site of the downstream exon, however it blocks the U1snRNP mediated recruitment of U2AF65 to the 3'splice site of exon6, thus blocking the exon- definition [7, 229]. Thus PTB does not seem to prevent the basal interaction of splicing factors rather compromises subsequent steps on intron or exon definition (Fig1.9).

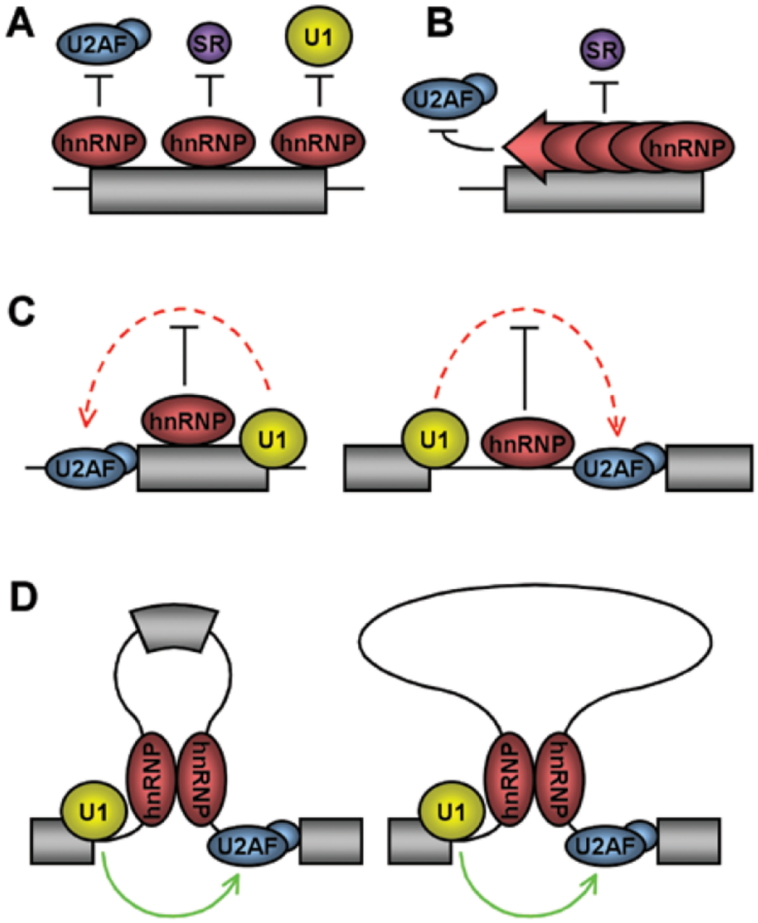

Fig 1.9 Stategies used by hnRNP proteins to control splice site selection. **A,** Binding of hnRNPs close to splicing signals such as ESEs can block the recruitment of factors to these sites. **B,** The propagation of hnRNP binding from high affinity site located in an exon (as in the case of hnRNPA1) may occlude binding of splicing factors. **C,** As in the case of PTB, hnRNP inhibit exon definition when bound to an exon, or block intron definition is bound to intron. D, Interaction between bound hnRNP proteins may loop out portions of pre-mRNA thus either promoting skipping or in some cases stimulating inclusion. Reprinted by permission from Springer, Advances in Experimental Medicine and Biology, and Landes Bioscience [7].

Similar to SR proteins hnRNP also have dual roles in alternative splicing, for e.g. in c-src N1 splicing, hnRNP F and H interact with PTB in non-neuronal cells to block N1 splicing, while in neuronal cells they interact to the same cis-element via nPTB to enhance exonN1 splicing [228]. Also in several other pre-mRNA hnRNP F and H have been shown to bind intronic sequences and activate splicing of a nearby 5' splice site, while binding of hnRNPH to an exon can stabilize the interaction U1snRNP with the 5'splice site [230-233]. Another interesting mechanism for splicing activation is looping out model, which suggests that interaction of hnRNP bound to distinct sites in the pre-mRNA such as ends of long introns could cause looping out and thus contribute to intron definition by reducing the distance separating two exons [234]. However this mechanism can also occur between two different introns and can trap an exon in the looping out thus blocking the splicing of the trapped exon (Fig1.9) [235].

Also like SR proteins hnRNPs can be post-translationally modified such as by phosphorylation, arginine methylation and sumolyation in response to various stimuli, which can affect sub-cellular localization, RNA- protein and protein-protein interaction of hnRNPs and subsequently influence splicing events regulated by these hnRNPs [236-239].

Other groups of RNA binding trans-acting factors consist of proteins such as hnRNP with K-type homology domains or KH domains. These proteins such as SF1, KSRP, NOVA-1 and 2 and rSLM-2 can enhance splicing by interacting with ISEs. SF1 is important for branch point recognition via interacting with a GGGGCUG repeats in the cardiac troponin T (cTNT) gene [240]. KSRP interacts with UGCAUG sequences and activate neuronal specific splicing in c-src [104] [227, 241]. Nova-1 enhances splcing of exon E3A in alpha-2 subunit of glycine receptor gene (GlyRalpha-2) [242, 243], while the rat Sam68 like proteins rSLM2 can influence CD44v5 splicing, Bcl-x, and Tau splicing [244-246].

1.2.7 Overview: High Throughput Screening

HTS is a scientific method to assay large numbers of various compounds against biological targets in a relatively short period of time [247]. Recently, the use of high-throughput screening (HTS) technology has substantially accelerated the evaluation of molecularly targeted drugs [248]. HTS provides the opportunity to identify biologically active small molecules as candidates for further biological or pharmacological experiments by rapidly testing hundreds of thousands to millions of small molecules. HTS utilizes the discovery and design of large compound libraries, sophisticated automated liquid handling platforms and innovated detection to expose potential lead compounds.

Fig 1.10 Modern HTS facilities. The Kalypsys robot at the NIH Chemical Genomics Center's (NCGC) enables ultra-high throughput screening of small molecules against diverse types of assays.(Credit: Maggie Bartlett/NHGRI)

HTS assays are either entirely or partially automated. Today's HTS assays can be carried out in the 96-well, 384-well or 1536-well format [249]. Robotic automation in HTS helps speed up the process of drug discovery and facilitates the generation of a large amount of scientific data in a short interval of time. Increasing numbers of different libraries of synthetic and drug-like compounds are available for HTS (Fig 1.10) [249]. Significant advances in combinatorial chemistry and genomics have helped drive the rapid growth in HTS. Combinatorial chemistry and structural biology provide the technology to target a specific protein of interest and design structurally related molecules *in silico*.

Combinatorial chemists have helped generate large and diverse libraries of molecules that can be exploited with HTS to identify inhibitors or mimetics of a specific protein. The BCL-2 prosurvival protein inhibitors ABT-737 and ABT-263 are examples of small molecule inhibitors identified by combinatorial chemistry and HTS [250]. Advances in genomics have substantially expanded our knowledge of human development, physiology, evolution and medicine with the sequencing of the human genome at the beginning of 2000s. Using HTS, protein sequencing technology, and combinatorial chemistry, we can design and develop novel small molecule inhibitors that inhibit proteins from the human genome project that specifically affect cancer cells and not normal cells. There are over 30,000 genes encoding proteins of the human proteome, a specific subset of which yield gene products that are good druggable targets: the druggable genome [6].

The targeting of aberrant splicing is an emerging strategy to combat certain types of cancers [251]. There are methods that use direct targeting of alternatively spliced protein isoforms [252, 253]. Recent studies discussed other methods that alter the alternative splicing process itself as a therapeutic intervention in cancer [254, 255]. The key difference in these methods is whether small molecules target transcripts directly or work by affecting *trans*-acting splicing factors. One of my proposed aims focuses on designing a versatile cell-based assay for screening large numbers of small molecule compounds for their effects on the alternative splicing of a PTB target gene. However, finding small

molecules that alter alternative splicing requires an effective, robust and fast assay. Thus, HTS is a promising and rapid methodology to identify potential modulators of the biological activity of the target from a large number of compounds.

1.2.8 Success of High Throughput Screening

The screening of chemical compounds for pharmacological activity has been ongoing in various forms for at least 30 years. The screening paradigm proposes that when a chemical interacts with a target in a desired way, that chemical then passes the first milestone on the long way of drug discovery process. Chemicals that cannot pass this initial screen go back into the library. High-throughput screening has become an important part of drug discovery at most pharmaceutical and many biotechnology companies worldwide, and use of HTS technologies is expanding into new areas such as: target validation, assay development,secondary screening, ADME/Tox, and lead optimization. In the last 10 years, high-throughput screening instruments, assays, and services have emerged as a significant growth market. As a measure of success of recent HTS studies, a report has been published in 2006 [256], 54 participants were asked to report on number of leads originally found through HTS methods that are being tested in humans. Data from the published report based on interviews with 54 high-throughput screening directors, 26 of them reported having a total of 104 drugs in the clinic or on the market that were originally screened in HTS from 1991 through 2006 (Table 1.2). The largest number of drug candidates from any one HTS laboratory is 12. Four drug candidates originally screened in an HTS

laboratory were reported as having made it to the commercial market: 1 originally screened in 1984, 2 screened in 1989, and 1 screened in 1999. The 104 drug candidates generated by HTS laboratories are listed in Table 1.2 [256].

Table 1.2 Drug candidates originally screened in HTS by individual HTS laboratories. Reprinted by permission from SAGE Journals: Journal of Biomolecular Screening [256].

Number of Drug Candidates	*Year Originally Screened*	*Type of Targets*	*Status*
12	1994-1999	Kinases	Clinic
		GPCRs	Clinic
11	—	Tyrosine kinases	Clinic GPCRs Clinic
		Proteins	Clinic
		Cytokines	Clinic
10	1989 (2)	Nuclear receptors (2)	On the Market
	1993	Nuclear receptor	Clinic
	1995	Nuclear receptor	Clinic
	1996	Nuclear receptor	Clinic
	1998	Nuclear receptor	Clinic
	1999	Transcription factor	Clinic
	2000 (2)	Nuclear receptors (2)	Clinic
	2001	Nuclear receptor	Clinic
9	1995 (2)	Enzyme inhibitors (2)	Clinic
	1994	Receptor	Clinic
	1999	Polymerase	Clinic
	1999	Receptor	Clinic
	1999	Ion channel	Clinic
	2001	Receptor	Clinic
	2003	Receptor	Clinic
	2003	Kinase	Clinic
5	2000 (2)	Kinases (2)	Phase 1 (2)
	1999 (2)	Kinases (2)	Phase 2 (2)
	1999	Kinase	Phase 3
5	2000	Cytokines (5)	Phase 2
	2001		Phase 2
	2001		Phase 1
	2002 (2)		Phase 1 (2)
5	1984	Reverse transcriptase	On the Market
	1988	Kinase	Clinic
	1989	Kinase	Clinic
	1990	LFA adhesion protein (2)	Clinic
4	1999	Kinase	Phase 1/2
	1999 (3)	GPCR (3)	Phase 1/2
4	1996	Kinase	Phase 3
	1999	Kinase	Phase 2
	1999	GPCR	Phase 2
	1999	Protease	Phase 2
4	—	Inhibitor	Phase 3
		Protease inhibitor	Phase 1
		Other inhibitors (2)	Early clinical
4	1999	Antiviral	Just launched
	1998	Kinase	Phase 3
	1999 (2)	Antiviral (2)	Phase 3
4	1998	Metabolic enzyme	Clinic
	2000	Cell cycle progression	
	2001 (2)	Cell cycle progression (2)	
3	2001	Enzyme	PreClin/IND 2005
	1998	Enzyme	Clinic
	1999	Enzyme	Clinic
3	1999	Nuclear receptor	Phase 2
	2000	GPCR	Phase 2
	2000	GPCR	Phase 1b
	(1996-2001)	(Cancer target, GPCR, antiviral)	(Dropped after clinical trials)
3	1999	GPCR	Phase 1
	1999	Kinase	Phase 1
	2000	Phosphatase	Phase 1
3	—	Multitarget inhibitor	Phase 1
		Receptor ligand (2)	Phase 1
2	2003	MEK inhibitor	Phase 1
	2004	Cancer target	Phase 1
2	1995	Kinase	Phase 2
	1998	Kinase	Phase 2/3
2	1993	—	Phase 3
	1995	—	Phase 2
		(10 others)	(Dropped after clinical trials)
2	1999	Kinase	Phase 1
	2001	Kinase	Phase 1
2	1998 (2)	—	Phase 1
1	2003	Enzyme	Phase 1
1	2003	—	Phase 1
1	2000	GPCR	Phase 1
1	2002	GPCR	Pre–phase 1
1	2003	Kinase	Pre-IND

GPCR = G protein–coupled receptor; LFA = lymphocyte-function-associated anti- gen; IND = investigational new drug application; MEK = mitogen-activated protein kinase/extracellular signal-regulated kinase. There was a total of 104 drug candi- dates. Twenty-six HTS directors reported drug candidates or commercial products from HTS.

1.2.9 The druggable genome

Proteins, polysaccharides, lipids and nucleic acids are the four major types of macromolecules that can be targeted in biological systems [6]. Due to toxicity, lack of specificity and the inability to obtain potent compounds against polysaccharides, lipids and nucleic acids, proteins are clearly the most popular target for drug discovery. A subset of the greater than 30,000 genes in the human genome encode proteins that are known to or are theorized to bind small molecules with appropriate affinity and specificity; these genes comprise

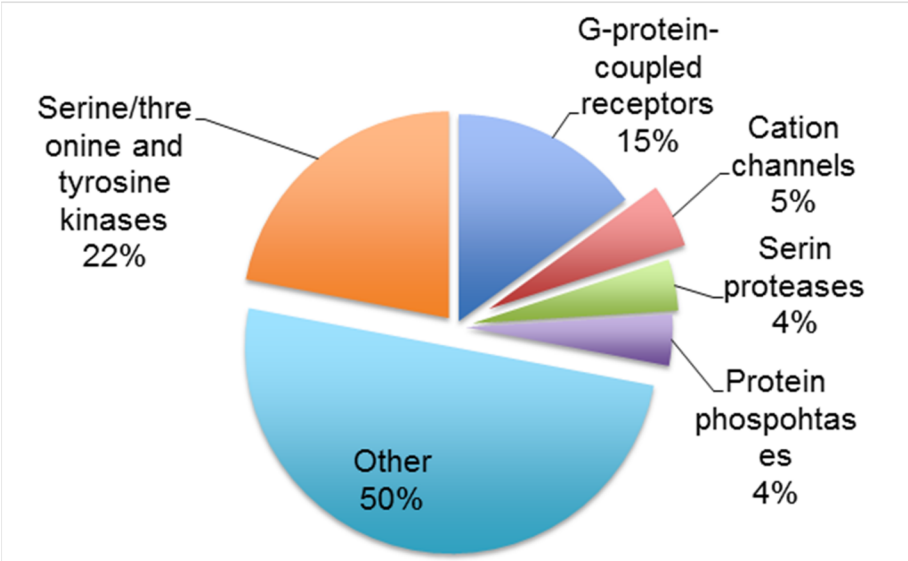

Fig 1.11 The gene family distribution of the human druggable genome. The human druggable genome consists of 5,520 different genes distributed through various gene families including serine/threonine and tyrosine kinases (22%), G-protein-coupled receptors (15%), cation channels (5%) serine proteinases (4%), protein phosphatases (4%) and other (50%). Other includes, but is not limited to, zinc peptidases, cytochrome P450s, and nuclear hormone receptors. Adapted from [6].

what has been termed the druggable genome [257]. Using a combination of genomics, proteomics and combinatorial chemistry, approximately 5,000 proteins have been classified as druggable (Fig 1.11).

1.2.10 Small molecule libraries

There are currently over 1 million commercially available compounds that could be screened in an HTS campaign. It would be impractical for an academic lab to purchase, curate and screen such a large number of compounds. There is also precedent for performing HTS campaigns on chemical libraries of FDA-approved drugs in that they are safe for use and do not have to undergo extensive toxicity testing [258]. As indicated by Jones and Diamond [258], when designing cell-based assays for HTS of small molecules, it is best to start with small libraries of biologically-active molecules. As these authors further indicate, the molecules should be able to get into cells, meaning that they should not be too lipophilic; they should have good bioavailability and not be mutagenic or toxic. Some types of chemical libraries suitable for such cell-based screens would be from three, small commercially available libraries composed of FDA and Foreign approved drugs, natural products and bioactive compounds (e.g. JHCCL version 1, Prestwick Chemical Library, LOPAC Library and the Spectrum Collection). This type of library consists of agents that have already been through toxicology testing and whose toxicities and possibly mechanisms are known.

1.2.11 Dissertation overview

As described above, alternative splicing of the pre-mRNA represents a growing optimism that this research area may allow new opportunities in cancer treatment. Since a most of the GOG ovarian cancer patient tissue samples showed the overexpression of the PTB, I believe that PTB is a viable drug target to treat OC disease by inhibiting its function. The goal of my project was to identify small molecular inhibitors of the PTB activity and validate these compounds in secondary confirmation assays.

The first phase of this work was initiated by William Beck, PhD, developed by Xiaolong He, MD, PhD, currently, at the University of Illinois at Chicago, College of Pharmacy, Rockford Campus. We previously observed that human ovarian tumors overexpressed PTB and another splicing factor, SRp20, compared to matched normal ovarian tissues. Correspondingly, we found more splice variants of the multidrug resistance-associated protein 1 (MRP1/ABCC1) as well as CD44 in ovarian tumors than in matched normal ovarian tissues [189]. We expanded this observation to more ovarian cancer specimens and confirmed the overexpression of PTB in ovarian tumors at the cellular level. We showed that knockdown of PTB expression by small interfering RNA (siRNA) substantially impairs ovarian tumor cell growth, colony formation, and invasiveness in vitro [2]. We also observed that knockdown of PTB in cells enhances the cytotoxicity of drugs used to treat ovarian cancer [259]. Furthermore, we examined the effects of SRp20 knockdown in ovarian cancer cells and found that this manipulation

impairs ovarian tumor cell growth and malignant properties, similar to what we have demonstrated with PTB [1]. Moreover, we found that substantial suppression of SRp20 expression caused apoptosis in ovarian cancer cells [1]. These efforts are detailed in Chapters 3 and 4 respectively.

Given the promising results of the knockdown studies, we initiated a search for PTB inhibitors. We first identified PTB regulated splicing events by developing a splicing sensitive microarray. To measure PTB activity in live cells we developed a cell-based fluorescent reporter assay to monitor PTB-mediated RNA splicing using an EGFP minigene. This EGFP-tagged reporter is based on differential splicing of a PTB target gene, identified by microarray, depends on the PTB levels in cells and is visualized by resulting changes in EGFP intensity. These efforts are detailed in Chapter 5.

I screened a combination of 4 libraries of small molecules using our splicing-sensitive EGFP reporter minigene to identify inhibitors of PTB. The compound libraries were screened in collaboration with the Cellular Screening Center at the Institute for Genomics & Systems Biology of University of Chicago. We adapted the screen to automated use. From the combination library of a ~4000 compounds, we identified ten small molecules as actives and we established several methods to test these compounds for their activity on PTB inhibition; these are described in Chapter 6.

The nature of my dissertation work presents an opportunity to comment on the prospect of finding PTB inhibitors using these approaches. Chapter 7 is a discussion of the impact and relevance of my work.

Chapter 2

2.1. Hypothesis and Specific Aims

The alternative splicing (AS) of RNA as a target for cancer therapy has not been exploited. As I have detailed in Chapter 1, the precision in splicing is achieved by multiple interactions that exist between regulatory elements in the pre-mRNA and trans-factors that recognize these regulatory elements in the pre-mRNA. The regulatory elements in the pre-mRNA that assist the spliceosome in the process of definition of an exon are exonic splicing enhancers (ESEs), and intronic splicing enhancers (ISEs). In contrast, the regulatory elements that interfere with the process of exon definition are exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs). The ESEs and ESSs are present in the exons, whereas ISEs and ISSs reside in the intronic region of the pre-mRNA. . Alterations in the levels of various trans-acting splicing factors, such as SR proteins and hnRNPs, within a cell could change the ratio of certain splicing events. A change in their expression may produce proteins of altered function and confer a growth or other advantage to the cell expressing them. Indeed, several splicing factors have been found to be upregulated in tumors, and one splicing factor, SF2/ASF, was shown to be an oncogene. Our previous work demonstrated that the splicing factors PTB (polypyrimidine tract-binding) and SRp20 (arginine/serine-rich 3) are overexpressed in human ovarian tumors and their expression is associated with tumor malignancy.

Based on these preliminary data, we speculated that targeting the splicing factor PTB for inhibition by small molecules holds a promising strategy for cancer therapy. Therefore we **hypothesized** that PTB is a druggable therapeutic target in this disease. We further **hypothesized** that PTB activity in live cells can be monitored by measuring the splicing of a PTB target gene.

To test these hypotheses, I proposed the following specific aims:

1. Determine the functional role of PTB in ovarian cancer cells in vitro and in vivo.
2. Develop a minigene reporter to monitor PTB activity in live cells.
3. Develop a high throughput-screening (HTS) assay to identify inhibitors of PTB mediated splicing.
4. Perform full-scale HTS screen using selected libraries of small molecule compounds.

The overall aim of my PhD study was to develop a novel cell-based high-throughput screen to identify small molecule inhibitor(s) of the splicing factor, PTB. Identification of these PTB inhibitors may result in novel drugs for the treatment of ovarian and possibly other cancers. Moreover, I also aimed to shine some light on the mechanisms by which OC cells survive chemotherapy and subsequently develop chemoresistance.

2.2. Significance

The research conducted in this thesis allows us to monitor PTB activity in live cells, a novel accomplishment, thereby enabling us to find potential PTB inhibitor(s). The work in this thesis also seeks to develop novel small molecule therapeutic strategies for ovarian cancer with high clinical translational potential based on targeting PTB activity, which inhibits of tumor cell growth in vitro and in vivo and colony formation and invasiveness in vitro by multiple pathways. A better understanding of splicing in normal and cancer tissue can lead to development of useful tools for future cancer diagnostics and therapies. Further, the work in this thesis seeks to enhance the therapeutic effect of the current chemotherapy strategies by employing small molecule inhibitors of PTB.

Chapter 3

Knockdown of Polypyrimidine-Tract Binding Protein suppresses ovarian tumor cell growth and invasiveness *in vitro*

3.1. Background

PTB is widely expressed in many cells and tissues. It is mainly localized to the nucleus and distributed diffusely throughout the nucleoplasm with high concentration in a nuclear structure called the perinucleolar compartment (PNC) [260], which is much more prevalent in tumor cells than in normal cells [261]. Recently, it has been reported that higher PNC prevalence significantly correlates with higher malignancy and poorer prognosis of breast cancer [262].

We previously observed that human ovarian tumors overexpressed PTB and another splicing factor, SRp20, compared to matched normal ovarian tissues. Correspondingly, we found more splice variants of the multidrug resistance-associated protein 1 (MRP1/ABCC1) as well as CD44 in ovarian tumors than in matched normal ovarian tissues [189]. It remains to be determined whether these two splicing factors directly participate in the splicing of the MRP1 and CD44 genes. Others found that overexpression of PTB in glioblastoma tissues coincided with the increased exclusion of the α-exon of fibroblast growth factor receptor 1 in transformed glial cells [114, 263]. Nevertheless, it is unknown whether PTB plays any functional role in tumor progression.

In this chapter. we expanded our previous observation to more ovarian cancer specimens and confirmed the overexpression of PTB in ovarian tumors at the cellular level. We also found that knockdown of PTB expression by small interfering RNA (siRNA) substantially impairs ovarian tumor cell growth, colony formation, and invasiveness *in vitro*.

3.2 Methods

3.2.1 Cell lines and cell culture conditions

Human ovarian surface epithelial (HOSE) cells were kind gift from Dr. Nelly Auersperg of the Canadian Ovarian Tissue Bank (University of British Columbia, Vancouver, Canada). Briefly, HOSE cells were isolated from four healthy pre-menopausal women undergoing tubal ligations pooled, and then cultured for one passage. From HOSE cells (not used in this study), she derived line IOSE 398, SV40 large T antigen transduced HOSE [264], which senesce at about passage 20 and line IOSE 120T, HOSE cells sequentially transduced with SV40 large T antigen and human telomerase reverse transcriptase [265], which can be propagated indefinitely. These cells were grown in media 199 and MCDB105 (1:1) (Sigma, St Louis, MO), containing 5% FBS and 50μg gentamycin/ml. Epithelial ovarian tumor cell lines PA-1, SKOV3, OVCAR8 were obtained from ATCC and A2780 was received as a generous gift from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. All were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and antibiotics.

3.2.2 Western Blot

10 mg to 20 mg of tissue specimens were homogenized with Kontes ground glass tissue grinder in 100–200 µl T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) supplemented with protease inhibitor mixture [2 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 1 mM EDTA, 130 µM bestatin, 14 µM L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine *N*-(*trans*-epoxysuccinyl)-L-leucine, 4-guanidinobutylamide *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 1 mM leupeptin, and 0.3 µM aprotinin (Sigma, St. Louis, MO)]. The homogenates were then centrifuged at 10,000 rpm at 4°C for 5 min to remove cell/tissue debris. Supernatants (20 µl) from each specimen were applied to SDS-PAGE on a 4–20% gradient gel, and proteins were transferred to a nitrocellulose membrane using a semidry electroblotter. The blot was then blocked in 5% milk and probed with monoclonal antibodies against PTB (Ab-1; Oncogene Research Products, San Diego, CA) and SRp20 (7B4; Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated donkey antimouse IgG. Signals were detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

3.2.3 Preparation of lentiviruses carrying tetracycline-inducible expression cassette of small hairpin RNA (shRNA)

The sequences of two oligonucleotides for making PTB shRNA 1 are 5’GATCCCC**AGGTGACAGCCGAAGTGCA**TTCAAGAGA**TGCACTTCGGCTGT**

CACCTTTTTTGGAAA3’ and 5’AGCTTTTCCAAAAA**TGCACTTCGGCTGTCACCT**TCTCTTGAA**AGGTGACA GCCGAAGTGCA**GGG3’, and the sequences of two oligonucleotides for making PTB shRNA 3 are 5’GATCCCC**TGACAAGAGCCGTGACTAC**TTCAAGAGA**GTAGTCACGGCTCT TGTCA**TTTTTGGAAA3’ and 5’AGCTTTTCCAAAAA**TGACAAGAGCCGTGACTAC**TCTCTTGAA**GTAGTC ACGGCTCTTGTCA**GGG3’. The siRNA sequences targeting to PTB mRNA at nucleotides 231-249 and 807-825 (relative to start codon), respectively, are in bold. The annealing of the two oligonucleotides generates the DNA fragment with protruding ends compatible with Hind III and Bgl II restriction enzyme sites respectively. The DNA fragment was first cloned into pSuper vector (OligoEngine, Seattle, WA) at Hind III and Bgl II sites downstream of H1 promoter and then along with H1 promoter was subcloned into a third generation of lentiviral vector, LV-THM, by replacing the existing H1 promoter in the vector. The resultant lentiviral vector is called LV-THsiPTB. LV-THM harbors both the tetracycline operon (tetO) and H1 promoter within 3’ LTR/SIN region and the GFP gene as a reporter driven by the EF-1α promoter [266]. Once integrated into the chromosome, the activities of both the H1 promoter and the EF-1α promoter are regulated by tetracycline [266]. This vector, as well as LV-tTR/KRAB-Red, a lentiviral vector expressing a fusion protein of tet repressor (tTR) and the silencing domain (KRAB module) of human Kox1 protein and red fluorescent protein, were generous gifts of Dr. Didier Trono (University of Geneva,

Switzerland). Lentiviruses were generated by cotransfection of HEK293T cells with four plasmids: a lentiviral vector plus pMD2.G (expressing envelop protein VSV-G), pMDLg/pRRE (expressing Gag and Pol) and pRSV Rev (expressing Rev) (The latter three plasmids were also generous gifts of Dr. Didier Trono). Media were changed 16 h after transfection and harvested 48 h after transfection. Cell debris in the media was removed by centrifugation at 2500g for 10 min. The titers of lentiviruses in the media were determined by flow cytometry and ranged from 1 to 5 x 10^6 transducing units/ml.

3.2.4 Cell proliferation assay

Briefly, 100 cells per well were seeded in triplicate in a 96-well plate and grown in a humidified incubator at 37°C, 5% CO_2 for four days. Then, 25 μl of MTT solution (2mg/ml in PBS) were added to each well. After incubation at 37°C for 4 h, medium was removed and 100 μl of DMSO were added to each well to dissolve the formazan produced in the cell. Color density at 560nm was determined using a microplate reader (Molecular Devices, Sunnyvale, CA). To obtain a cell growth curve, 10^4 cells/well were seeded in a 24-well plate at day 0. Cells in the wells of columns 1 to 6 were trypsinized and counted at day 1 to day 6, respectively. Cells in the top two rows of wells were grown with DOX (2μg/ml) and cells in the bottom two rows were grown without DOX.

3.2.5 Establishment of stable cell lines expressing tetracycline-inducible PTB siRNA

We first established cell lines transduced by lentiviruses LV-tTR/KRAB-Red, and then re-infected them with lentiviruses LV-THsiPTB. Clones expressing both red fluorescent protein and GFP were selected and expanded. The regulation by DOX of siRNA expression in these clones was verified by measuring PTB expression by both RT-PCR and Western Blotting.

3.2.6 *In vitro* invasiveness assay

This assay was modified from Kleinman *et al*. [267]. Before analysis, each subline was split into two groups with one grown with DOX and the other without DOX for two days. Cells were then trypsinized and resuspended in serum free DMEM. 300μl of cell suspension at $5x10^5$ cells/ml were added to the inner compartment of the ECM-coated culture insert and 500μl of DMEM, containing 10% FBS, were added to the lower companion-plate well. After incubation for 48 h at 37°C, 5% CO_2, the non-invading cells were removed from the interior of the inserts by wiping with cotton-tipped swabs and the cells that invaded to the lower side of the membrane were stained in the Cell Stain Solution from the kit. After washing in water and drying in air, those cells that invaded into the lower side were counted under a light microscope with a high magnification objective lens.

3.2.7 Soft agar colony formation

The assay was performed in 60mm dishes or 6-well plates that contained two layers of soft agar. The top and bottom layers were 0.3% and 0.4% agarose, respectively, in DMEM with 5% FBS. 5000 cells together with or without DOX were added to the top agarose before pouring. Colonies were counted manually after two to three weeks incubation at 37°C, 5% CO2.

3.3 Results

3.3.1 The splicing factor PTB is overexpressed in ovarian tumors and it is not a general phenomenon for all splicing factors

To further verify our previous observation [189] that PTB was overexpressed in human epithelial ovarian cancer (EOC), another 19 pairs of matched ovarian tumor and normal tissues examined by western blot. 17 out of 19 pairs showed overexpression of PTB. Moreover, this overexpression has also been observed at cellular levels by immunohistochemical staining, as exemplified in Fig 3.1. It can be seen that staining for PTB in normal ovarian epithelia was very weak or negative, while matched ovarian tumor cells stained strongly positive. In contrast, both normal ovarian surface epithelial and ovarian tumor cells stained strong positive for other two splicing factors, ASF and U2AF65. This result suggests that the overexpression of splicing factors in EOC is not universal; rather, it is restricted to certain splicing factors.

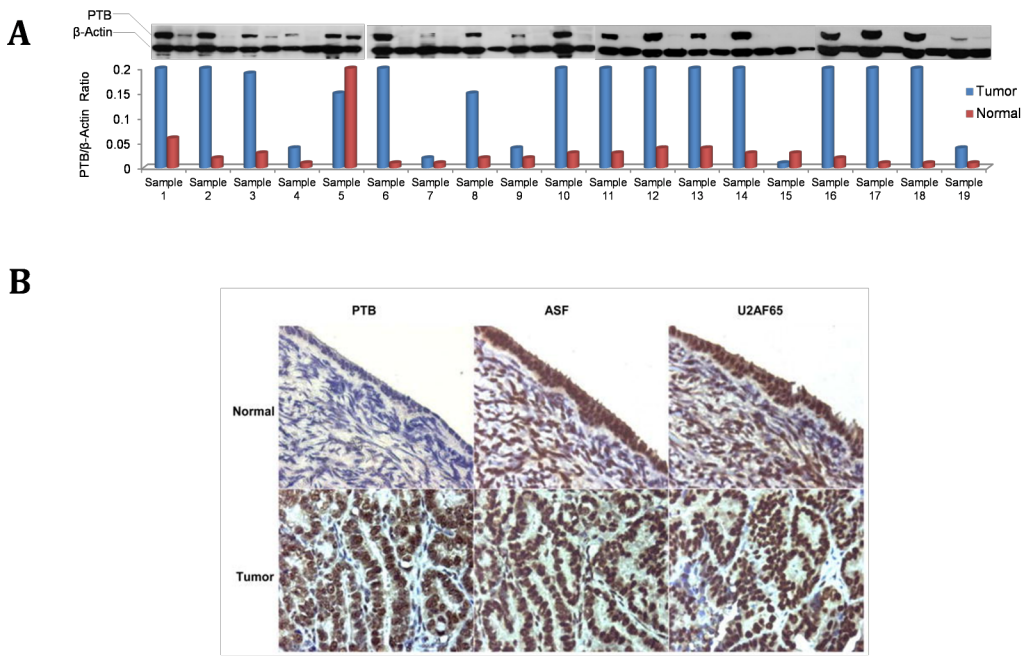

Fig 3.1 PTB is overexpressed in EOC. **A.** Western blot analysis of PTB expression in matched ovarian tumor and normal tissues. Blue:Tumor; Red:Normal. The expression levels of PTB are quantified as a ratio of PTB to β-actin expression. **B.** Immunohistochemical staining for PTB and two other splicing factors, ASF and U2AF65, on human EOC TMA. Arrows indicate ovarian surface epithelial cells. Note negative PTB staining in normal ovarian epithelial cells. Magnification, 400x. Reprinted by permission from Nature Publishing Group: Oncogene [2, 3].

3.3.2 Immortalization of ovarian epithelial cells increases the expression of PTB

The observation that normal ovarian tissues express low levels of PTB compared to ovarian tumors raised the question of when during oncogenesis this splicing factor becomes overexpressed. Hence, we examined by Western Blotting the

expression of PTB in normal human ovarian surface epithelia (HOSE), life-extended HOSE (IOSE398) [264], truly immortalized HOSE (IOSE120T) [265]

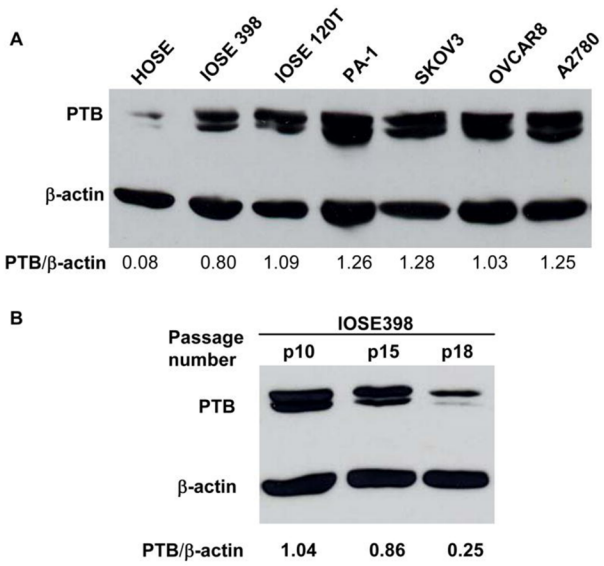

Fig 3.2 PTB is overexpressed in immortalized HOSE cells and ovarian tumor cell lines. **A.** Immunoblotting analysis of PTB expression in cell lines. **B.** PTB expression in IOSE398 cells at different passages. Multiple PTB bands are different splice variants of PTB. The expression levels of PTB are quantified as a ratio of PTB to β-actin expression. Reprinted by permission from Nature Publishing Group: Oncogene [2]

and ovarian epithelial tumor cell lines PA-1, SKOV3, OVCAR8 and A2780. As shown in Fig. 3.2A, the expression of PTB is substantially overexpressed in life-extended IOSE398 cells and maintained at high levels in IOSE120T and ovarian tumor cell lines, compared to normal HOSE cells. Further, we compared the PTB levels at different passages of IOSE398 cells, which senesce at around passage

20. As can be seen in Fig. 3.2B, PTB levels were gradually reduced when cells were approaching senescence. These results suggest that the up-regulation of PTB is an early event in the neoplastic transformation of ovarian epithelial cells and might be required for cell growth.

3.3.3 Knockdown of PTB expression by vector-based siRNA

Our above observation raised another question of whether overexpressed PTB plays any functional role(s) in maintaining ovarian tumor cell growth. To address this, we used siRNA technology to knock down the expression of PTB in tumor

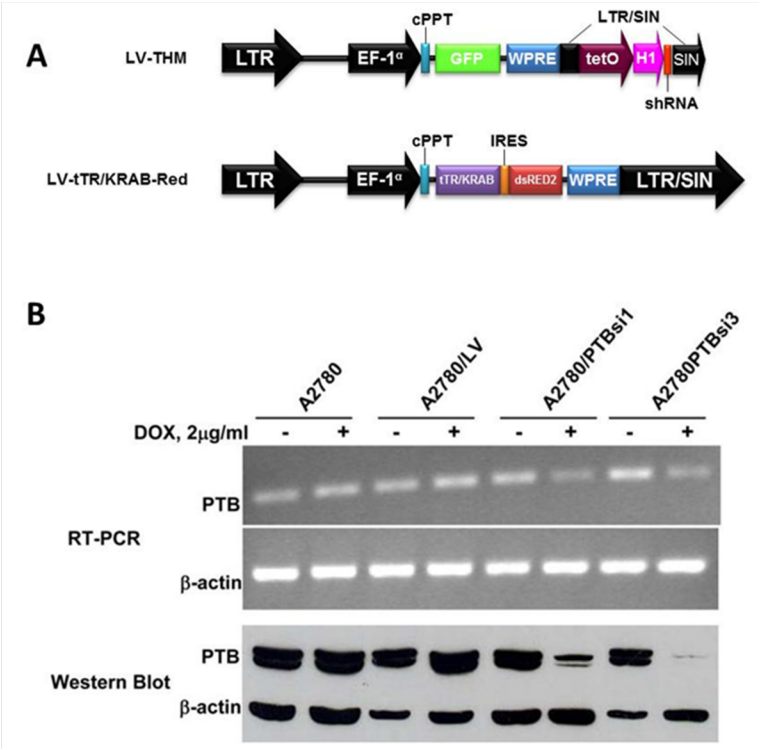

Fig 3.3 Knockdown of PTB expression by vector-based DOX-inducible PTB siRNA. **A.** Schematic structure of lentiviral vectors. **B.** Expression of PTB at mRNA and protein levels in A2780 sublines carrying tTR/KRAB-Red and THMsiPTB (A2780/PTBsi1 and A2780/PTBsi3) in the presence or absence of DOX. Reprinted by permission from Nature Publishing Group: Oncogene [2]

cells and then examined the effects of such manipulations on cell growth and malignancy, the latter assessed in an in vitro invasiveness assay. In order to significantly knockdown PTB expression, we tested over ten vector-based siRNAs targeting different regions of PTB mRNA and found three to be very effective in suppression of PTB expression. In this study, we chose two of them (PTB small hairpin RNAs 1 and 3 (shRNA1 and shRNA3)) and transferred them individually to a lentiviral vector as described in the Methods and Materials. We then established sublines of the epithelial ovarian tumor cell line A2780 that express tetracycline-inducible PTB siRNAs. The sublines (named as A2780/PTBsi1 and A2780/PTBsi3 respectively) carry both expression cassettes of tTR/KRAB-Red and PTB shRNA1 or PTB shRNA3 (Fig. 3.3A). In the presence of doxycycline (DOX), the fusion protein tTR/KRAB-Red is bound by DOX and dissociated from the tetO, thus unblocking the downstream PTB shRNA, allowing it to be expressed. Shown in Fig. 3.3B is the expression of PTB at mRNA and protein levels in the absence and presence of DOX. It can be seen that the expression of PTB is controlled by DOX: in the presence of DOX, PTB expression is significantly knocked down at both mRNA and protein levels. In contrast, the expression of PTB in the control subline A2780/LV, which carries LV-tTR/KRAB-Red and the lentiviral vector without PTB shRNA, is not influenced by DOX.

3.3.4 Knockdown of PTB expression suppresses the growth of ovarian tumor cells *in vitro*

Using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay [268], we compared the proliferation of A2780/PTBsi1 and A2780/PTBsi3 in the presence and absence of DOX. As shown in Fig. 3.4A, the growth of A2780/PTBsi3 was suppressed substantially in the presence of DOX, while the control subline A2780/LV, as well as the parental A2780 cells grew similarly in both conditions (with and without DOX). It is worth noting that in the absence of DOX, there is no significant difference in cell growth among the four cell lines, indicating that the introduction of lentiviral vectors into the cell itself did not influence cell growth. We counted cell numbers at 24 h intervals, using a Coulter Counter (Beckman Coulter, Fullerton, CA). The result was highly consistent with that obtained by MTT assay. It can be seen in Fig. 3.4B that A2780/PTBsi1 and A2780/PTBsi3 grows substantially more slowly in the presence of DOX. By contrast, we observed no such difference in the control subline or parental A2780 cells. We also observed similar results in a breast cancer cell line, MCF7, following knockdown of PTB, indicating that growth suppression following PTB knockdown is likely a general phenomenon and not confined to one tumor histotype (TT Ho *et al*, unpublished).

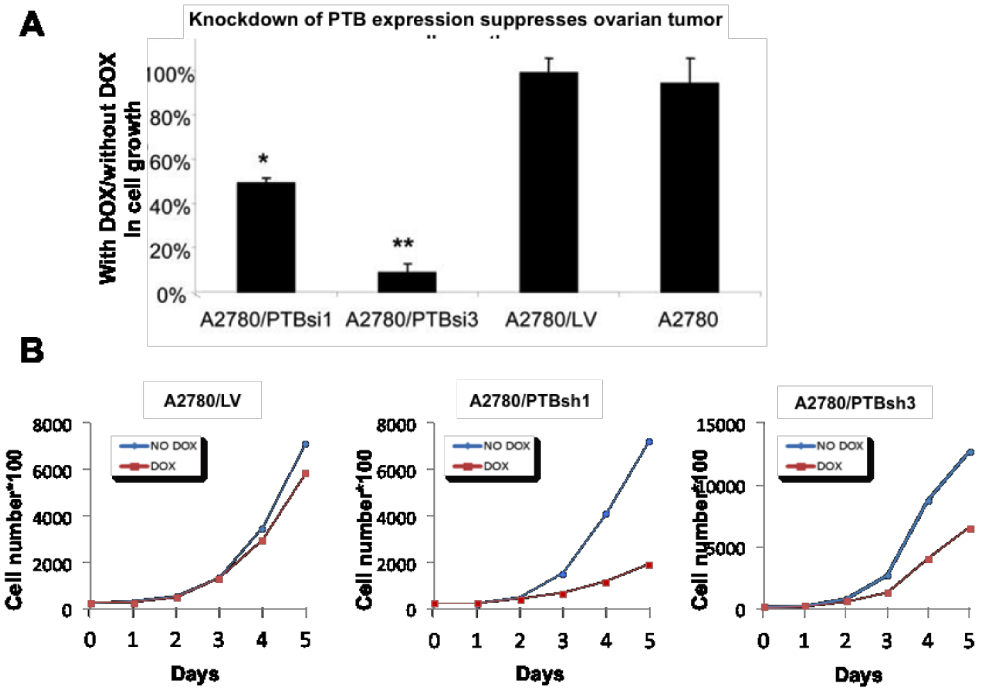

Fig 3.4 Knockdown of PTB expression suppresses ovarian tumor cell growth. **A.** MTT assay of cell proliferation. Shown is the average of three separate experiments. Error bars represent the standard error (SE). * and ** indicate $P<0.05$ and $P<0.01$, respectively, when compared to either control cell line. **B.** Cell growth curves. Two separate experiments were done and exhibited similar trends. Shown are the results of one experiment. Reprinted by permission from Nature Publishing Group: Oncogene [2]

3.3.5 Knockdown of PTB expression decreases the invasiveness of ovarian tumor cells

To determine whether overexpression of PTB makes any contribution to the invasiveness, and therefore, the malignancy of ovarian tumor cells, we examined two malignant properties, anchorage-independent growth (AIG) and invasiveness, in the A2780/PTBsi1 and A2780/PTBsi3 cells when their PTB expression was manipulated by treatment with DOX. The AIG of tumor cells was measured by the formation of colonies in soft agar. As shown in Fig. 3.5A, the colonies formed when A2780/PTBsi1 and A2780/PTBsi3 grown with DOX were only 10.4±3.1% and 19.5±4.4%, respectively, of that formed when grown without DOX, indicating that knockdown of PTB expression substantially impaired the AIG of A2780 cells. By contrast, both the control subline and the parental A2780 cells exhibited little differences in their ability to form colonies in soft agar when grown with DOX and without DOX (The percentages were 90.1±2.9% and 87.4±2.3%, respectively). The invasiveness of tumor cells was measured by their abilities to degrade the basement membrane matrix proteins in the coating layer, which serves as a barrier to discriminate invasive cells from non-invasive cells, and ultimately pass through the pores of a polycarbonate membrane. As shown in Fig. 3.5B, when A2780/PTBsi1 and A2780/PTBsi3 cells were grown with DOX, the number of invasive cells (indicated by arrows in the figure) was far less (23.5±14.1% and 14.0±3.3%, respectively) than when they were grown without DOX, indicating that knockdown of PTB expression also greatly reduced the invasive potential of A2780 cells. In contrast, DOX treatment produced little or no

effect on the invasive abilities of the control cell lines A2780/LV and A2780 (The percentages were 103.3±11% and 81.6±8.0%, respectively).

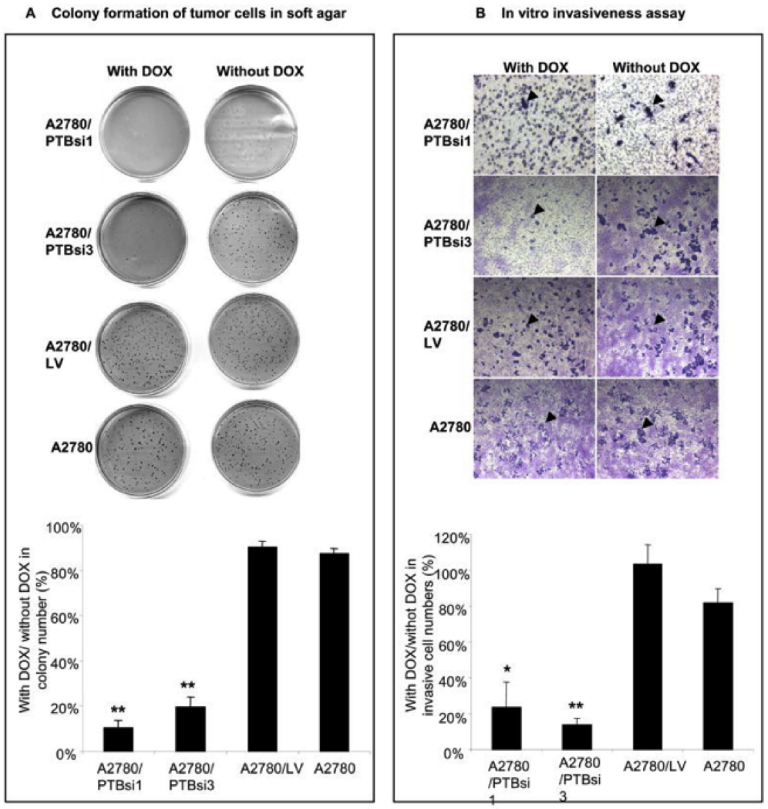

Fig. 3.5 Knockdown of PTB expression lowers malignant potential of ovarian tumor cells. **A.** Colony formation of tumor cells in soft agar. Upper part shows colonies in the soft agar of one experiment and lower part shows average ratios (in percentage) of colony numbers formed with DOX vs without DOX (n=3). **B.** In vitro invasiveness assay of ovarian tumor cells. Upper part shows invasive cells under microscope (40x) of one experiment and lower part shows average ratios (in percentage) of invasive cells grown with DOX vs without DOX (n=3). Invasive cells were counted under microscope with high magnification (150×). Arrows indicate the invasive cells. * and ** indicate $P<0.05$ and $P<0.01$, respectively, when compared to either control cell line. Error bars represent the standard error (SE). Reprinted by permission from Nature Publishing Group: Oncogene [3]

3.3.6 Knockdown of PTB sensitizes ovarian cancer cells to chemotherapeutic agents *in vitro*

After PTB was fully knocked down (~5 days after treatment with DOX; see Fig5.6), we compared the proliferation of A2780/LucshRNA, A2780/PTBshRNA1 and A2780/PTBshRNA3 in the presence of chemotherapeutic agents, carboplatin and paclitaxel, frontline agents in the treatment of OC, by using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. As shown in Fig. 3.6, the growth of A2780/PTBshRNA3 and A2780/PTBshRNA3 were suppressed substantially in the PTB knockdown , while the control subline A2780/LucshRNA cells grew similarly in both conditions (with and without PTB knockdown). In summary, knockdown of PTB conferred 3- to 6-fold increase in sensitivity to carboplatin and paclitaxel, current standard of care pharmaceuticals (Table 3.1). Findings here support the idea that PTB and other splicing factors may be novel drug targets in the treatment of ovarian cancer.

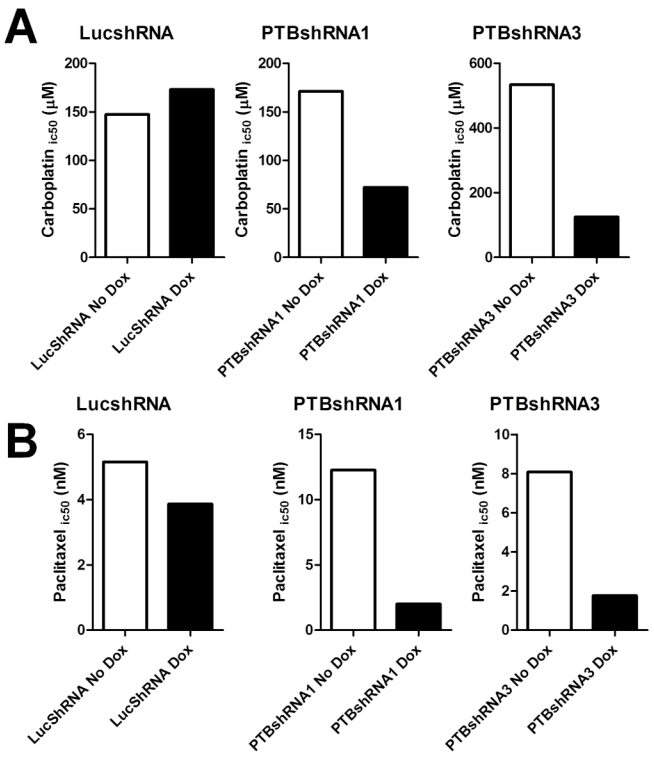

Fig. 3.6 PTB induced cytotoxicity. Knockdown of PTB expression sensitizes OC cells (A2780) to Carboplatin **(A)** and to Paclitaxel **(B)** in a 48 h MTT cytotoxicity assay. Shown are the results of three independent experiments.

Table 3.1

	Fold decrease in relative IC50 (T/C)		
	LucShRNA (control)	PTBshRNA1	PTBshRNA3
Paclitaxel	1.33	6.1	4.3
Carboplatin	0.99	2.4	4.5

3.4 Discussion

In this chapter, we confirmed, by immunohistochemical staining of EOC TMA, our earlier observation [189] that PTB is overexpressed in human EOC. Moreover, we found that PTB expression was up-regulated in immortalized ovarian epithelial cells as well as in ovarian tumor cell lines, compared to normal or untransformed ovarian epithelial cells, a novel observation. Additionally, we demonstrated that knockdown of PTB expression by siRNA impaired the growth of ovarian tumor cells, diminished their malignant potential, and sensitizes them to chemotherapeutic agents *in vitro*. Together, these results suggest to us that overexpression of PTB could be an important component of a multi-step process of tumorigenesis and might be required for the development and maintenance of EOC.

As indicated in the Introduction, PTB is a key regulator of alternative splicing, and is involved in the repression of inclusion of many alternative exons [269]. Therefore, overexpression of PTB is very likely to cause alterations in splicing patterns that may have such effects as we have shown herein, and we are presently examining this in our system. Defects in pre-mRNA splicing have been shown to be causes of a variety of human diseases [270]. Mounting evidence suggests that altered splicing is associated with and possibly involved in tumor progression and/or metastasis [161]. Recent computational analyses, based on alignments of expressed sequence tags (EST) to human RefSeq mRNAs or human genomic DNA, revealed that many alternatively spliced variants were

significantly associated with cancer, and the majority of these genes have functions related to cancer [271, 272]. In general, the direct causes behind the alteration of pre-mRNA splicing can be divided into two categories: mutations in cis-elements and changes in trans-acting factors. At present, it is not clear whether mutations make significant contributions to aberrant splicing found in tumors. Our finding of overexpresssion of PTB in ovarian tumors indicates that aberrant splicing is at least partially due to changes in trans-acting splicing factors. Since pre-mRNA splicing is a complex cellular process involving many other splicing factors, to have a better understanding of why and how splicing patterns change in tumor cells, it is necessary to have a global view of expression of all splicing factors in tumor and normal cells, and some of our current effort addressing this matter is detailed in Chapter 5.

Overexpression of PTB in the majority of ovarian tumors raises possibilities that PTB could be a novel therapeutic target in OC. Ovarian cancer is the deadliest disease among all gynecological cancers [273]. Two facts account for this dismal outcome: one is the absence of reliable early detection markers; the other is inadequacy of present therapy for advanced disease [274]. My work is focused on the latter, development of novel targets and novel therapeutic agents to treat this disease.

The results that knockdown of PTB expression causes suppression of tumor cell proliferation, suppression of AIG, and suppression of invasiveness strongly

support the notion that PTB is important in maintaining ovarian tumor cell growth and malignant potential. At present, it is not clear what mechanisms mediate these effects. Given the multiple functions of PTB, these effects could be related to changes in alternative splicing, mRNA stability or IRES-driven translation of certain genes. However, current knowledge about the targets of PTB cannot entirely explain our observations. Therefore, it is very likely that there exist some unidentified substrates of PTB that are involved in the maintenance of ovarian tumor cell growth and malignancy by PTB. Despite these gaps in our knowledge, our results support the idea that PTB might have potential as a therapeutic target for the treatment of ovarian cancer, and my work in Chapter 5 is devoted to this aspect.

Chapter 4

Knockdown of splicing factor SRp20 causes apoptosis in ovarian cancer cells and its expression is associated with malignancy of epithelial ovarian cancer

4.1. Background

Our previous study revealed that two splicing factors, polypyrimidine tract-binding protein (PTB) and SRp20, were up-regulated in epithelial ovarian cancer (EOC) and knockdown of PTB expression inhibited ovarian tumor cell growth and transformation properties. In this chapter, we show that knockdown of SRp20 expression in ovarian cancer cells also causes substantial inhibition of tumor cell growth and colony formation in soft agar and the extent of such inhibition appeared to correlate with the extent of suppression of SRp20. Massive knockdown of SRp20 expression triggered remarkable apoptosis in these cells. These results suggest that overexpression of SRp20 is required for ovarian tumor cell growth and survival. Immunohistochemical staining for PTB and SRp20 of two specialized tissue microarrays (TMAs), one containing benign ovarian tumors, borderline/low malignant potential (LMP) ovarian tumors as well as invasive EOC and the other containing invasive EOC ranging from stage I to stage IV disease, reveals that PTB and SRp20 are both expressed differentially between benign tumors and invasive EOC, and between borderline/LMP tumors and invasive EOC.

4.2 Methods

4.2.1 Cell lines and cell culture conditions

Ovarian cancer cell lines IGROV1 and SKOV3 were obtained from the National Cancer Institute and human ovarian cancer cell line A2780 was received as a generous gift from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA, and were maintained in DMEM (A2780 and SKOV3) or RPMI1640 (IGROV1) supplemented with 10% FBS under a humid environment at 37°C, 5% CO_2.

4.2.2 Western blot analysis

Whole cell lysates were prepared in 1x sample buffer (50mM Tris pH 6.8, 2%SDS, 10% glycerol, 5% β-mecaptoethanol and 0.002% bromophenol blue) and resolved by electrophoresis on NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA). Afterwards, proteins were transferred to nitrocellulose membranes using XCell II™ Blot Module (Invitrogen). The membranes were then probed with diluted primary antibodies in blocking buffer (5% nonfat milk, 50mM Tris pH 7.4, 0.87% sodium chloride and 0.05% Tween 20). The primary antibodies and dilutions are as follows: mouse anti-human SRp20 monoclonal antibody (7B4) (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500; rabbit-anti human antibodies against cleaved caspases 3, 7 or 9 (Cell Signaling Technology, Danvers, MA), 1:1000; mouse anti-human monoclonal antibodies against caspases 2 or 8 (Cell Signaling Technology), 1:1000; mouse anti-human β-actin monoclonal antibody (AC-15) (Sigma-Aldrich, St. Louis, MO), 1:10000. Antibodies binding to proteins on the membranes were detected by horseradish

peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and ECL™ Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ).

4.2.3 Preparation of lentiviruses carrying tetracycline-inducible expression cassette of small hairpin RNA (shRNA)

We used the lentiviral system developed in Dr. Didier Trono's lab [266] to achieve Dox-induced siRNA expression in the cell. The sequences of two oligonucleotides whose transcripts can be processed into effective SRp20-targeted siRNAs, SRp20si1 and SRp20si2, respectively, are 5'-CGCGCCGG**CGAGAGCTAGATGGAAGAACA**CTCGAG**TGTTCTTCCATCTAG CTCTCG**TTTT-3' and 5'-CGCGCCGG**GACGGAATTGGAACGGGCTTT**CTCGAG**AAAGCCCGTTCCAAT TCCGTC**TTTTT-3' with SRp20si1 and SRp20si2 sequences in bold. The above oligonucleotides and their complementary oligonucleotides were annealed in a thermocycler to form double-stranded DNA fragments with protruding ends compatible with Mlu I and Cla I restriction enzyme sites, respectively. The resulting DNA fragments were then cloned into a third generation lentiviral vector, LV-THM (Wiznerowicz & Trono, 2003), between *Mlu* I and *Cla* I sites downstream of the tetracycline operon (tetO) and H1 promoter. Lentivirus preparation and establishment of sublines of ovarian cancer cells were done as described in Chapter 3.

4.2.4 Establishment of stable cell lines expressing tetracycline-inducible SRp20 siRNA

We first established cell lines transduced by lentiviruses LV-tTR/KRAB-Red, and then re-infected them with lentiviruses LV-THsiPTB. Clones expressing both red fluorescent protein and GFP were selected and expanded. The regulation by DOX of siRNA expression in these clones was verified by measuring SRp20 expression by both RT-PCR and Western Blotting.Cell proliferation assay.

4.2.5 Cell proliferation assay

Cells were seeded at a density of 1000 per well in triplicate into 96-well plate and grown in media supplemented with or without 1 μg/ml of DOX at 37°C, 5% CO_2. Viable cell numbers were determined daily for 6 days using an MTT assay as described previously in Chapter 3.

4.2.6 Tissue microarray (TMA)

Two types of TMA were used in this study: one was an ovarian disease status TMA and the other was an ovarian cancer stage TMA. These TMAs were created by and obtained from the GOG Tissue Bank (Columbus, OH) utilizing a 0.6 mm core and each case is represented by three cores, which are distributed randomly on the TMA slide. This study was performed with the approval of Institutional Review Board (IRB) of The University of Illinois at Chicago. All of the patients who provided tumor tissue for these TMA provided consent for specimen banking and future research via GOG protocol 136, and individual GOG institutions were required to have annual IRB approval to participate in GOG 136. Specimens were labeled with coded identifiers and immunohistochemistry

assays were performed without knowledge of patient identities. The disease status TMA contained 173 unique cases of benign ovarian tumors (n=42), borderline/ low malignant potential (LMP) ovarian tumors (n=61) and invasive EOC (n=70) including mucinous tumors (n=88), serous tumors (n=50), clear cell carcinomas (n=32), endometrioid adenocarcinomas (n=1) and poorly differentiated adenocarcinomas (n=2). The ovarian cancer stage TMA contained 175 unique cases of endometrioid adenocarcinoma (n=77), serous adenocarcinoma (n=90) or mixed epithelial carcinoma (n=8) with stage I (n=46), stage II (n=32), stage III (n=57) or stage IV (n=40) disease. All cases were represented with formalin-fixed and paraffin-embedded primary tumor removed during primary cytoreductive surgery which preceded administration of first-line chemotherapy.

4.2.7 Soft agar colony formation

Colony formation was assessed in 6-well plates containing two layers of soft agar. The bottom layer was 1.5 ml of 0.4% agarose in DMEM supplemented with 5% FBS and the top layer was 1.5 ml of 0.3% agarose mixed with 2000 cells in DMEM supplemented with 5% FBS and with or without 1 μg/ml of DOX. After incubation for two to three weeks at 37°C, 5% CO_2, the plates were photographed under a dissecting microscope and colonies bigger than 0.5 mm in diameter were counted manually with computer assistance.

4.2.8 Apoptosis assay

Cells were seeded at a density of 10^5/well in 12-well plates and grown in the presence or absence of 1μg of DOX/ml for 5 days at 37°C, 5% CO_2. Cells were then fixed in 4% paraformaldehyde for 10 min and stained in a solution of Hoechst 33342 (10 μg/ml) for 15 min. Apoptotic cells and non-apoptotic cells were counted manually with computer assistance.

4.3 Results

4.3.1 SRp20 knockdown inhibits ovarian cancer cell growth

We previously reported that splicing factors PTB and SRp20 were overexpressed in human ovarian tumors compared to normal ovarian tissues (Fig 4.1A-B) [189].

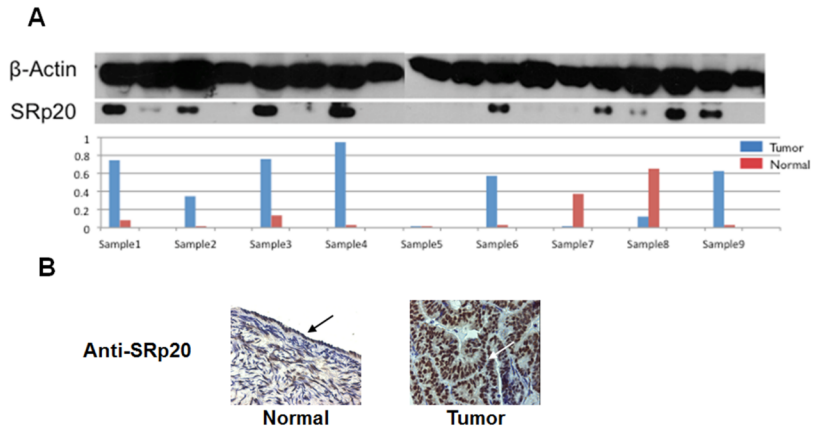

Fig 4.1 SRp20 is overexpressed in EOC. **A.** Western blot analysis of SRp20 expression in matched ovarian tumor and normal tissues. Blue:Tumor; Red:Normal. The expression levels of SRp20 are quantified as a ratio of SRp20 to β-actin expression. **B.** Immunohistochemical staining for SRp20 on human EOC TMA. Arrows indicate ovarian surface epithelial cells. Note negative SRp20 staining in normal ovarian epithelial cells. Magnification, 400x

As indicated in Chapter 3, we showed that suppression of PTB expression by siRNA substantially inhibited ovarian cancer cell growth and malignant properties [2]. However, what role, if any, SRp20 may have in ovarian cancer remained unanswered, because we have not succeeded in manipulating SRp20 expression by siRNA until recently. After testing over 15 siRNAs (designed by ourselves or by commercial companies) targeting various regions of human SRp20 mRNA, we have now identified two effective siRNAs, SRp20si1 and SRp20si2, which can knockdown SRp20 expression by about 50% and about 90%, respectively, in A2780 cells.

We constructed a DOX-inducible lentiviral system to deliver and express SRp20si1 or SRp20si2 in the cell as we had done for siRNAs against PTB [2]. Following lentiviral infection, we established A2780 sublines A2780/SRp20si1 and A2780/SRp20si2 to express SRp20si1 and SRp20si2, respectively, under the control of Dox. The Dox-induced suppression of SRp20 expression is shown in Fig 4.2A. The effects of SRp20 knockdown on cell growth was determined by cell growth curve. As shown in Fig 4.2B, the growth of A2780/SRp20si1 and A2780/SRp20si2 cells was inhibited in the presence of Dox compared to the growth without Dox, but the growth of control subline A2780/LUCsi, which expresses Dox-induced luciferase siRNA, was not affected by Dox treatment (Fig 4.2B). The growth inhibition was detectable by day 4, corresponding to the time when SRp20 started to decrease, which occurred by day 3 (data not shown). In addition, the extent of growth inhibition apparently correlated with the extent of

suppression of SRp20 expression. Growth inhibition by SRp20 knockdown was also observed in two other ovarian cancer cell lines, SKOV3 and IGROV1 (data not shown).

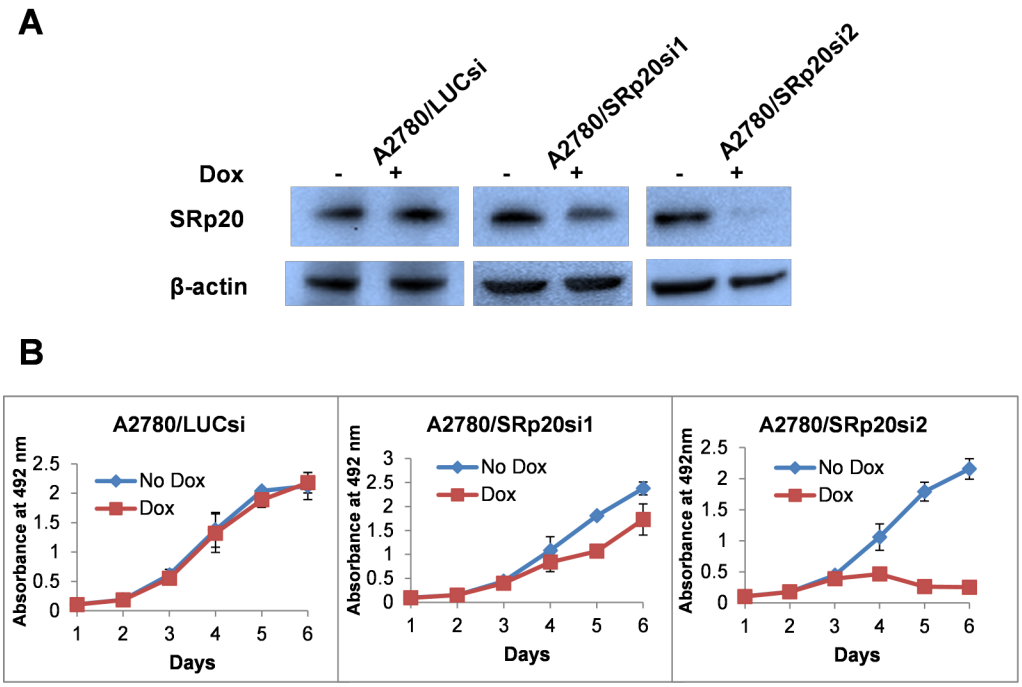

Fig 4.2 SRp20 knockdown inhibits ovarian cancer cell growth. **A:** Western blot showing the suppression of SRp20 expression by Dox induction of SRp20 siRNAs in A2780 subline cells. **B:** Cell growth curve. Shown are the results of three independent experiments. Error bars represent standard error (SE). Reprinted by permission from Nature Publishing Group: Oncogene [1].

4.3.2 SRp20 knockdown inhibits anchorage-independent growth (AIG) of ovarian cancer cells

AIG is a characteristic phenotype of transformed cells and is measured by colony formation assay in soft agar. To determine whether SRp20 overexpression is required to maintain AIG of ovarian cancer cells, we examined the effect of SRp20 knockdown on A2780 cells' capability to form colonies in soft agar. As shown in Fig 4.3A, A2780/SRp20si1 and A2780/SRp20si2 cells formed substantially fewer and smaller colonies when they were grown in the presence of Dox (i.e. SRp20 expression was suppressed) than when they were grown in the absence of Dox. Moreover, the degree of AIG inhibition correlated with the extent of SRp20 suppression by siRNA. In A2780/SRp20si1 cells, DOX reatment induced knockdown of SRp20 expression about 50% (Fig 4.2A), which subsequently led to inhibition of colony formation-- fewer and smaller colonies (Fig 4.3A). By contrast, in A2780/SRp20si2 cells, SRp20 expression was suppressed more than 90% by DOX induction (Fig 4.2A), which resulted in almost complete abolition of colony formation (Fig 4.3B).

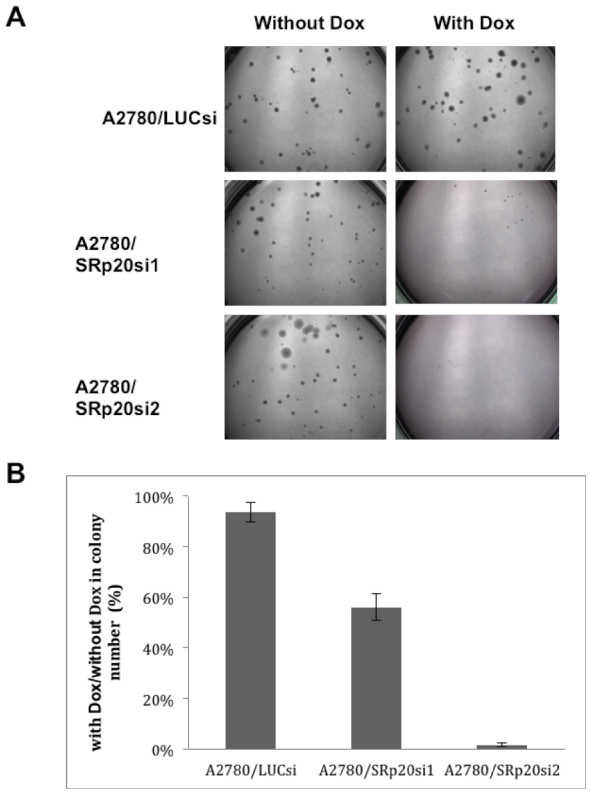

Fig 4.3 SRp20 knockdown inhibits anchorage-independent growth of ovarian cancer cells. **A:** Sample pictures showing colonies formed in soft agar. **B:** Average ratios (expressed in percentage) of colony numbers formed in the presence vs in the absence of Dox (n=3). Error bars: standard error (SE). Reprinted by permission from Nature Publishing Group: Oncogene [1].

4.3.3 SRp20 knockdown induces apoptosis in ovarian cancer cells

To determine whether the inhibition of cell growth by SRp20 knockdown was due to increased apoptosis, we stained A2780 sublines treated with or without Dox for five days with Hoechst 33342 and counted apoptotic cells. As shown in Fig 4.4, the percentage of apoptotic cells in A2780/SRp20si2 subline cells was remarkably increased after Dox treatment, which induced substantial

suppression of SRp20 by more than 90% (see Fig 1A). By contrast, moderate suppression by Dox treatment of SRp20 expression (~ 50% reduction, see Fig 4.1A) in the A2780/SRp20si1 subline did not trigger apoptosis, which was maintained at similarly low levels in both Dox-treated and untreated cells. Consistent with these results, we also observed substantial increases in apoptosis in the SKOV3/SRp20si2 and IGROV1/SRp20si2 sublines after Dox treatment but not in those sublines expressing SRp20si1 or control siRNA (data not shown).

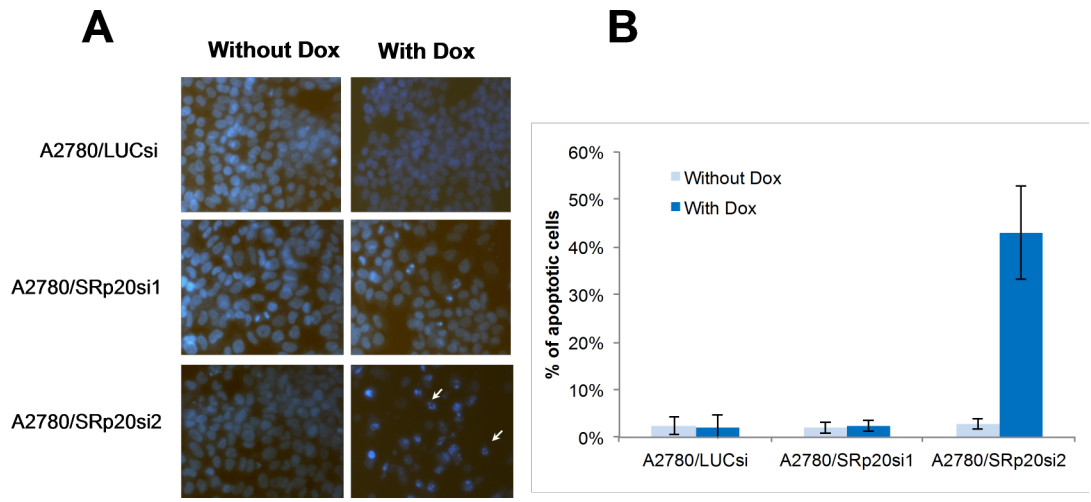

Fig 4.4. SRp20 knockdown induces apoptosis in ovarian cancer cells. **A:** Sample micrographs of Hoechst 33342-stained nuclei of A2780 subline cells. Arrow indicates the typical apoptotic cells. **B:** Percentage of apoptotic cells. Shown are averages of three independent experiments. Error bar: standard error. Reprinted by permission from Nature Publishing Group: Oncogene [1].

4.3.4 SRp20 knockdown activates intrinsic apoptotic pathway

Apoptosis is triggered through three major cellular pathways, i.e. the extrinsic pathway, the intrinsic pathway and the PIDDosome-mediated pathway [275]. Caspase 8, caspase 9 and caspase 2 are corresponding initiator caspases in these pathways. To determine which pathway is activated in SRp20 knockdown-induced apoptosis, we examined the activation of caspase 8, caspase 9 and caspase 2 by Western Blotting. The antibodies against caspases 2 and 8 can detect procaspases as well as cleaved fragmants and the antibody against caspase 9 detects only cleaved caspase 9. As shown in Fig 4.5, cleaved caspase 9 was detected in Dox-treated A2780/SRp20si2 cells, which showed substantial increase in apoptosis (Fig 4.4), but not detected in Dox-treated A2780/SRp20si1 cells and in untreated cells. In contrast, no cleaved caspases 2 and 8 but procaspases 2 and 8 were detected in any of these cells. We further examined the activation of downstream effector caspases 3, 6 and 7 using antibodies against the cleaved fragments of these caspases and found that caspase 3 and caspase 7 were activated in Dox-treated A2780/SRp20si2 cells but not in other cells (Fig 4.5B). We failed to detect the cleaved caspase 6 in any of these cells (data not shown). These results indicate that the intrinsic apoptotic pathway is activated in SRp20 massively knocked-down A2780 cells. Since Bcl-2 is a major anti-apoptotic regulator of the intrinsic pathway [276], we asked whether the apoptosis induced by SRp20 knockdown is mediated through its action on Bcl-2. Therefore, we examined the expression of Bcl-2 in A2780/SRp20si1 and A2780/SRp20si2 cells by RT-PCR and Western Blotting.

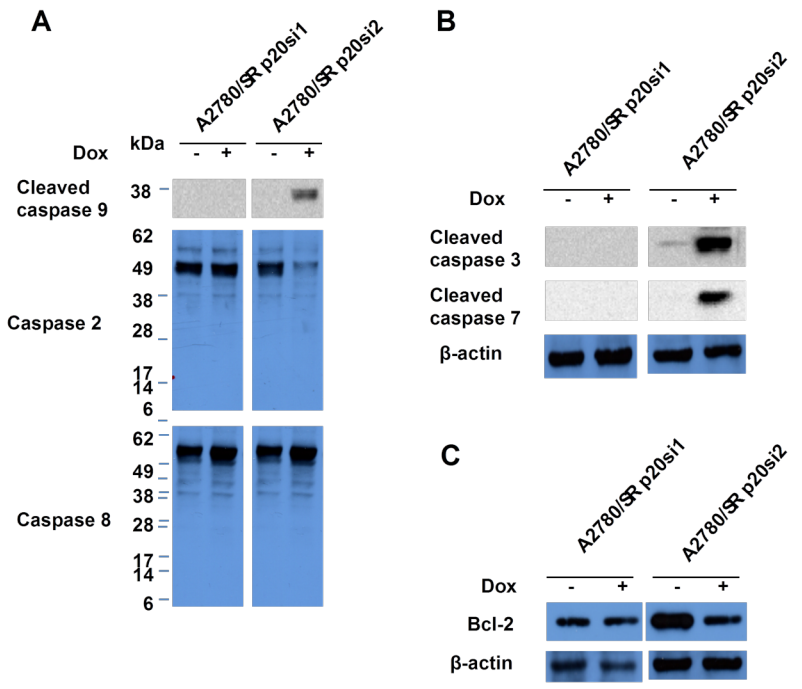

Fig 4.5 SRp20 knockdown activates the intrinsic apoptotic pathway. **A:** Western blot of initiator caspases, caspase-9, -2 and -8. **B:** Western blot of effector caspases, caspases-3 and -7. **C:** Western blot of Bcl-2. Reprinted by permission from Nature Publishing Group: Oncogene [1].

As shown in Fig 4.5C, Bcl-2 is indeed down-regulated at both mRNA and protein levels in Dox-treated A2780/SRp20si2 cells but not in other cells.

4.3.5 Expression of PTB and SRp20 is associated with malignancy of human ovarian tumors

Our above and previously reported results [2] indicate that overexpression of PTB and SRp20 is required for growth and maintenance of transformed

properties of ovarian tumor cells. To further assess the clinical significance of these two splicing factors, we studied the expression of PTB and SRp20 in non-malignant epithelial ovarian tumors in comparison with malignant tumors by immunohistochemical staining of ovarian disease status TMAs, which contain benign, borderline/LMP and invasive ovarian tumors. After staining, we had 133 valid cases for analysis of PTB expression and 117 valid cases for analysis of SRp20 expression. Our rule for valid cases was that there were a minimum of 2 satisfactory cores for each case. Unsatisfactory cases were those with missing core(s), scant/insufficient tumor cells, increased background or folded/wrinkled/torn sections. Representative staining for PTB and SRp20 in benign tumor, borderline/LMP tumor and invasive EOC are shown in Fig 4.6A. We categorized the average staining for each case into three groups: all negative (all evaluable cores were negative), all positive (all evaluable cores were positive), and mixed (at least one evaluable core negative and one evaluable core positive).

As shown in the graphs in Fig 4.6B, the percentage of cases that stained all positive increased while the percentage of cases stained all negative or mixed decreased in the order of benign tumor, borderline/LMP tumor and invasive EOC. Approximately 85% and 97% of invasive EOC stained all positive for PTB and SRp20, respectively, whereas a great majority of benign ovarian tumors stained all negative or mixed for PTB with only 17.6% stained all positive. The percentages of borderline/LMP ovarian tumors that stained all positive, all

negative, or mixed fell between those of benign and invasive tumors. Statistical analyses indicated that the differences in PTB staining among benign, borderline/LMP and invasive ovarian tumors were significant in both overall comparison and all pair-wise comparisons ($p<0.01$ for all comparisons). SRp20 staining varied significantly between benign and invasive tumors ($p<0.01$) and between borderline/LMP and invasive tumors ($p<0.01$) but not between benign and borderline/LMP tumors.

Further analysis focusing on mucinous tumors generated results consistent with the above conclusion, i.e. both PTB and SRp20 were expressed differentially between benign and invasive tumors ($p<0.05$) and between borderline/LMP and invasive tumors ($p<0.05$) but not between benign and borderline/LMP tumors. Other subtypes on this TMA could not be further analyzed because of the limited number of valid cases retained after staining.

Among cases with at least one positive core, we calculated the average frequency of positive cancer cells for each case, which is shown in Fig 5C. When this average frequency was categorized into low (<50% positive tumor cells) or high (≥50% positive tumor cells), a significant association was observed between disease status and categorized staining frequency for PTB ($p=0.001$ for the overall comparison between the three groups, $p=0.01$ for the pairwise comparison between benign and invasive tumors, and $p=0.003$ for the pairwise comparison between borderline/LMP and invasive tumors) or SRp20 ($p=0.005$ for

the overall comparison between the three groups, p=0.01 for benign vs. invasive tumors, and p=0.03 for borderline/LMP vs. invasive tumors), as shown in Table 2.

As shown in the graphs in Fig 4.6D, staining intensity for each case with at least one positive core was categorized as low (weak to light brown staining; score<2.0) or high (moderate to intense dark brown staining; score≥2.0). The results are shown in Table 3. There was statistical evidence of an association between disease status and categorized staining intensity for PTB (p=0.027 for the overall comparison between the three groups and p=0.022 for the pairwise comparison between benign and invasive tumors), but not for SRp20 (p>0.05).

In summary, both PTB and SRp20 were differentially expressed between benign tumors and invasive EOC, and between borderline/LMP tumors and invasive EOC, but the modest differences between benign and borderline/LMP tumors were not always statistically significant.

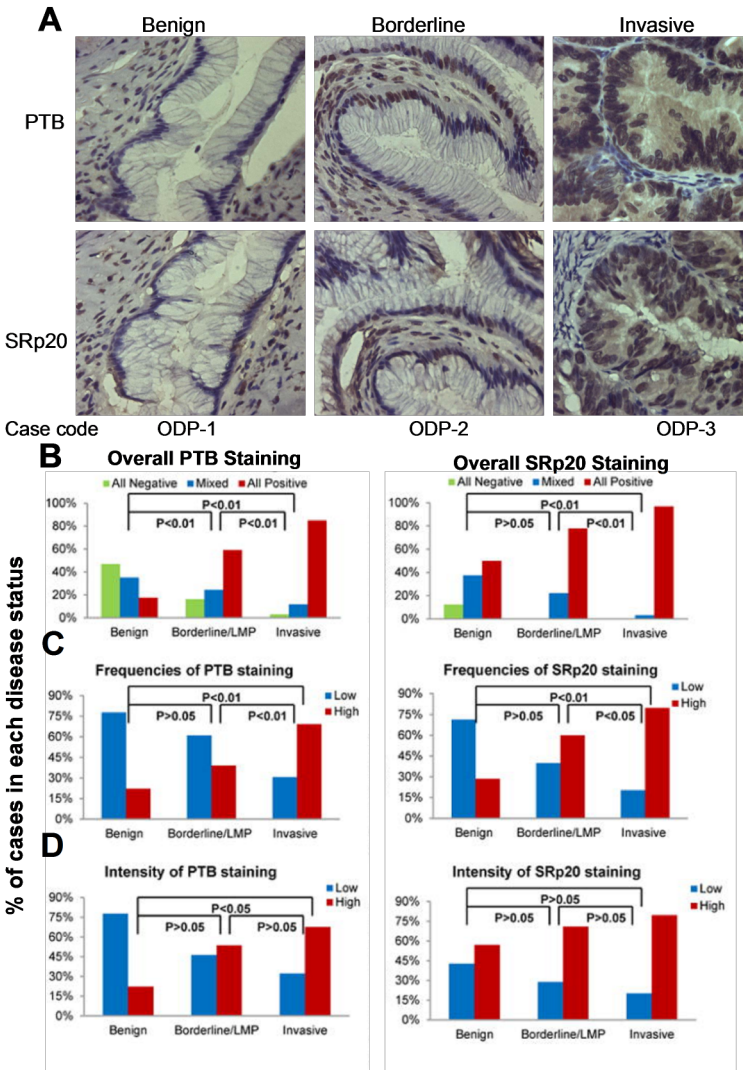

Fig 4.6 Expression of PTB and SRp20 is associated with malignancy of human ovarian tumors. **A.** Sample micrographs of staining for PTB or SRp20 of ovarian disease status TMA. Shown are cases of mucinous ovarian tumors. Magnification: 400×. **B.** Summary of overall PTB and SRp20 staining categorized into all negative, mixed and all positive. For PTB staining, $p<0.01$ for all pair-wise comparisons; For SRp20 staining, $p<0.01$ for benign vs invasive tumors and borderline/LMP vs invasive tumors; $P>0.05$ for benign vs borderline/LMP tumors. **C.** Frequencies of PTB and SRp20 staining in Ovarian Disease Status TMAs. For PTB staining, $p<0.01$ for benign vs invasive tumors and borderline/LMP vs invasive tumors. For SRp20 staining, $p<0.01$ for benign vs invasive tumors; $p<0.05$ for borderline/LMP vs invasive tumors. **D.** Intensity of PTB and SRp20 staining in Ovarian Disease Status TMAs. For PTB staining, $p<0.05$ for benign vs invasive tumors. Reprinted by permission from Nature Publishing Group: Oncogene [1].

4.4 Discussion

We previously reported that overexpressed PTB played an important role in maintaining ovarian tumor cell growth and transformation properties [2]. In this chapter, we provide the first evidence to show that overexpressed SRp20 is also required for ovarian tumor cell growth and survival. Moderate knockdown of SRp20 expression caused substantial inhibition of tumor cell growth and colony formation in soft agar while nearly complete suppression of this protein triggered substantial apoptosis in these cells. The mechanism(s) mediating SRp20's role in ovarian tumor cells remains to be elucidated. Based on its known molecular functions, SRp20 may exert its role directly by itself and/or indirectly via other molecules regulated by its activities. A recent report has demonstrated that SRp20, as well as SF2/ASF, associates with chromatin before and after mitosis but is excluded from chromatin during mitosis [277].This newly identified interaction between SR proteins and the chromosome implies that such proteins may be involved in the regulation of chromatin structure and function and thus may play a role in the control of cell cycle progression. Therefore, the depletion of SRp20 could directly disrupt the cell cycle process, which subsequently causes growth inhibition and apoptosis. Nonetheless, it is still possible that the effects of SRp20 knockdown are mediated by other proteins under its regulation. For example, we showed that SRp20 knockdown-triggered apoptosis was mediated by down-regulation of Bcl-2 (Fig 4.5C), which subsequently activates the intrinsic apoptotic pathway.

Aberrant splicing is a common phenomenon found in human tumors [161]. Results presented in this chapter and those of others [206] suggest that abnormal regulation of splicing factor expression could be responsible, in part, for this phenomenon. Given the importance of alternative splicing in the generation of proteomic complexity [132], it is conceivable that up- or down-regulation of splicing factors may be an indispensable component of the process of tumorigenesis that is involved in mediating the effects of transformation. Thus, controlling splicing factor expression may become a novel and effective way to inhibit tumor cell growth; that is, certain splicing factors may be good therapeutic targets. Our data reported here showing the effects of SRp20 knockdown as well as our previous studies [2] support this idea.

In the present chapter, we also examined the expression of PTB and SRp20 in ovarian tumors by immunohistochemical staining of two specialized ovarian tumor TMAs - one focusing on tumor progression and the other focusing on cancer stages. Our results reveal that PTB and SRp20 are differentially expressed among benign ovarian tumors, borderline/LMP ovarian tumors and invasive EOC, with benign tumors having the lowest percentage of all positive cases and the highest percentage of all negative cases and invasive EOC having the highest percentage of all positive cases and the lowest percentage of all negative cases. By contrast, we found no significant differences among invasive EOC and both splicing factors were highly expressed in ovarian tumors of early stages as well as late stages. Together, these results suggest that the

expression of PTB and SRp20 is associated with malignancy of ovarian tumors. This observation is consistent with our previous finding that overexpression of PTB and SRp20 is an early event in the ovarian tumorigenesis [2]. Because we did not have access to follow-up information of the cases on the TMAs, we could not assess whether there was a correlation between the expression of these two splicing factors and patient clinical outcome. It merits further investigation to determine whether there are differences in clinical outcome between negatively stained cases and positively stained cases.

Overexpression of PTB and SRp20 in invasive EOC also raises a question of how other splicing factors are changed in this disease. Our previous and current studies revealed that the overexpression occurred only in certain splicing factors, because we found that two other splicing factors, SF2/ASF and U2AF65, were expressed at similar levels in both normal ovarian epithelia and tumor ovarian cells [2]. Therefore, to have a better understanding of the roles of alternative splicing and splicing factors in ovarian tumorigenesis, we will need to examine the expression profile of all splicing factors in normal and transformed ovarian epithelial cells as well as in ovarian tumor cells and correlate these changes with ovarian tumor progression and response to therapy. According to some proteomic analyses [34] , there are approximately 336 proteins identified as components of splicing machinery. It will be clinically important to know whether there exist any splicing factor signatures for ovarian tumors or their subtypes.

Chapter 5

A high throughput assay to identify small molecule modulators of alternative pre-mRNA splicing

5.1. Background

Alternative splicing (AS) is an efficient mechanism that involves the generation of transcriptome and protein diversity from a single gene. Defects in pre-mRNA splicing are an important cause of numerous diseases, including cancer. AS of pre-mRNA as a target for cancer therapy has not been well studied. We have reported previously that a splicing factor, polypyrimidine tract-binding protein (PTB) is overexpressed in ovarian tumors, compared to matched normal controls, and knockdown (KD) of PTB expression by shRNA impairs ovarian tumor cell growth, colony formation and invasiveness. Given the complexity of PTB's molecular functions, a chemical method for controlling PTB activity might provide a therapeutic and experimental tool. However, no commercially available PTB inhibitors have yet been described. To expand our ability to find novel inhibitors, we developed a robust, fluorometric, cell-based high throughput screening HTS assay in 96-well plates that reports on the splicing activity of PTB.

This chapter describes the development and validation of a cell-based reporter HTS assay for the discovery of small molecule modulators of PTB activity. In an attempt to use the cells for large-scale chemical screens to identify PTB

modulators, we established cell lines stably expressing the reporter gene. Our results suggest that this high throughput assay could be used to identify small molecule modulators of PTB activity. Based on these findings and the role that upregulated PTB has on cell proliferation and malignant properties of tumors targeting PTB for inhibition with small molecules offers a promising strategy for cancer therapy.

5.2 Methods

5.2.1 Cell lines, culture conditions and chemicals

Human ovarian cancer cell line A2780 was received as a generous gift from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. Lenti-X 293T was obtained from Clontech (Mountain View, CA). All cell culture reagents were purchased from Mediatech (Manassas, VA). All cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated Fetal Bovine Serum and 2 mM L-Glutamine. Assay and optimization steps were performed in phenol red-free DMEM.

All compounds used in this study were dissolved into DMSO prior to treatment of cells. Compounds used for pilot screening were added 24 h after cell plating by pipetting 1μL containing either vehicle or small molecule compound at a final DMSO concentration of 1% and incubated for 48 h in a humidified incubator at 37ºC with 5% CO_2.

5.2.2 Microplates, Cell Seeding and Plate Reader.

Stock subcultures of parental A2780 and stable sublines were grown to ~70-80% confluency on standard cell culture-treated growth surfaces for 4 d, and were then harvested using trypsin. Harvested cells were diluted in complete growth medium and counted. All cells were then seeded into microplates (CELLSTAR 96 well, Greiner Bio-One, Longwood, FL) microplates at a density of 15K cells/well. All plates were seeded using the PerkinElmer Janus automated liquid handling system (PerkinElmer, Boston, MA). The seeded plates were incubated at room temperature for 45 min to allow the cells to settle uniformly on the growth surface of the well, and then incubated overnight in a humidified incubator at 37ºC with 5% CO_2 prior to beginning the assay read out. Following 48 h incubation with the compounds, the plates were analyzed with either the PerkinElmer EnVision (PerkinElmer, Boston, MA)

5.2.3 Microarray analysis

The splicing-sensitive microarray was purchased from Jivan Biologics (Greenbrae, CA); this specific product has been discontinued but updated, and similar products can be obtained from Jivan Biologics or ExonHit Therapeutics (Gaithersburg, MD). The array contains 116,205 unique probes representing 36,397 splice events (goo.gl/vGSRj). Array was used to discriminate between spliced, un-spliced and alternatively spliced RNAs of PTB-depleted and -undepleted samples. A2780 cells with and without PTB depletion were seeded in 100-mm dishes, and incubated to 70-80% confluency. Total RNA was harvested in TRIzol reagent (Invitrogen) following the manufacturer's protocol. The probe

labeling, array hybridization, washing, scanning, and data retrieval steps were performed by Jivan Biologics. The raw data obtained from Jivan Biologics was analyzed using SpliceFold software (goo.gl/TEPMX). The software generates a splice analysis report based on the pre-set filtering thresholds for signal probe intensity and splice-fold difference. The cut off value for the significance of the signal probe intensity difference was set as p=0.01. To identify differently expressed splice variants, we used the absolute splice score value cut-off of ≥ 0.3, which corresponds to a 2-fold difference for $Expression_{Short\text{-}form}/Expression_{LongForm}$ ratio. Results were confirmed by standard RT-PCR.

5.2.4 Construction of Minigene Reporter Plasmid

Human genomic DNA was extracted from A2780 cell lines and amplified by Platinum *Pfx* DNA Polymerase (Invitrogen) for 35 cycles with an annealing temperature of 58°C and a 2 min extension time, using PTBP2 (NCBI Gene ID: 58155) specific primers E9F and E11R , with *EcoR* I and *BamH* I overhangs. The resulting 2,019 bp PCR product was selectively excised from the 1% agarose gel, purified using Qiagen gel extraction kit (Qiagen, Valencia, CA). The resulting fragment was digested with the restriction enzymes *EcoR*I and *BamH*I (NEB, Beverly, MA) and cloned into pEGFP-N1 (Clontech, Mountain View, CA), upstream of the reporter gene, to create pGreen. DNA sequence of the ligation product was confirmed by sequencing on an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA). Minigene containing plasmid pGreen was transformed into DH5α-competent cells. Plasmid DNA was prepared with

NucleoBond Xtra plasmid DNA purification kit (Macherey-Nagel, Clontech, Mountain View, CA) and the resulting plasmid was further confirmed by restriction digestion. The plasmid pEGFP-N1, encoding Enhanced Green Fluorescent Protein (EGFP) controlled by the CMV promoter (Clontech, Mountain View, CA) was used as a source of the coding sequence of the pGreen minigene. Later, the pGreen minigene was amplified using minigene-specific primers pGF and pGR, with *Mlu*I and *Spe*I overhangs, and the 2,914bp PCR product was subcloned into pLV-tTR/Krab lentiviral vector that results in LV-pGreen. DNA sequence of LV-pGreen plasmid was confirmed by restriction digestion and sequencing. The lentiviral vector was a generous gift of Dr. Didier Trono (University of Geneva, Switzerland).

5.2.5 Preparation of lentiviruses carrying reporter plasmid

The resultant lentiviral vector LV-pGreen was packaged to generate viral particles. Lentivirus preparation and establishment of sublines of ovarian cancer cells were done as described in Chapter 3. LV-tTR harbors EF-1α promoter within 3' LTR/SIN region and pGreen minigene as a reporter driven by this promoter [278]. Lentiviruses were generated by cotransfection of Lenti-X 293T (Clontech, Mountain View, CA) cells with three plasmids: a lentiviral vector plasmid plus pMD2.G (expressing envelop protein VSV-G), psPAX2 (expressing packaging proteins). Media ware changed 16 h after transfection and the supernatants were harvested 48 h after transfection. Cell debris in the media was removed by 0.45μm filtration following centrifugation at 1500g for 10 min. The titers of lentiviruses in the media were determined by flow cytometry that ranged

from 2 to 6 x 10^7 transducing units/ml. Packaging plasmids were also gifts from Dr. Didier Trono (University of Geneva, Switzerland).

5.2.6 Preparation of lentiviruses carrying tetracycline-inducible expression cassette of shRNA

To manipulate PTB protein expression (positive controls in the assay) we used tetracycline-inducible expression cassette of shRNA. The DNA fragments coding for PTB shRNA were generated by annealing of two pairs of complementary oligonucleotides. The procedures for for preparation of lentiviruses are detailed in the methods of Chapter 3 and 4.

5.2.7 The establishment of stable cell lines

Two new sublines were established using lentiviral particles; A2780/pGreen, A2780/pGreen/Test. The former expresses a doxycycline-inducible PTB shRNA and pGreen reporter gene and was used as either a positive control (with doxycycline added) or negative control (without doxycycline added); the latter, expressing the pGreen reporter alone was used as compound test cell line and/or negative control. The establishment of stable cell lines expressing reporter pGreen alone, and pGreen and PTBshRNA were accomplished in multiple steps. To establish the A2780/pGreen/Test subline, parental cells (A2780) were transduced by lentiviruses carrying an expression cassette of the reporter minigene pGreen. Positive clones expressing pGreen were picked and enriched using flow sorting (Beckman Coulter MoFlo, Miami, FL).

To establish the A2780/pGreen subline, we first established cell lines transduced by lentiviruses LV-tTR/KRAB, and then re-infected them with lentiviruses LV-TH/PTBshRNA. Clones expressing both KRAB protein and PTBshRNA were selected and expanded. The regulation by doxycycline of shRNA expression and KRAB protein expression in these clones was verified by measuring PTB expression by Western Blotting. Later picked clones transduced by lentiviruses carrying an expression cassette of the reporter minigene pGreen. The isolated cell colonies were picked from the wells, transferred to 24-well plates, and grown in the presence or absence of 1μg doxycycline/mL. Positive clones were identified by measuring both PTB expression by Western Blotting and increased expression of green fluorescent protein in doxycycline treated wells (data not shown).

5.2.8 Splicing reporter assay

This assay was designed to identify compounds that can modulate the splicing activity of the PTB protein. *The assay uses fluorescence detection* to monitor splicing of a green fluorescence protein-fused minigene, pGreen (derived from the PTBP2 genomic sequence, described above), to short or long splice variants (SV). Cellular PTB levels control the exclusion or inclusion of exon 10 in this minigene. Screening positives are compounds that block the splicing activity of PTB protein, therefore increasing the long SV. Induction of PTB shRNA by doxycycline completely blocks the splicing activity of PTB in this assay and is used as a positive control. DMSO alone is the best negative control for this assay

during the HTS campaign. In detail, without any sequence modification, EGFP is in the reading frame when exons 9, 10, and 11 are spliced together (long-form) in response to lower PTB activity, but it is out of the reading frame when exon 10 is skipped or repressed (short-form) in response to higher PTB activity. While the former translated into more EGFP, the latter expressed low levels EGFP (Fig. 1A). We then developed a fluorescence cell-based assay *that exploits this* mechanism to allow HTS of small molecules for their ability to inhibit PTB activity. To perform the screening assay, experimental conditions were optimized to a 96-well plate format. To prepare the plates, A2780, A2780/pGreen$_{(+)doxycycline}$, A2780/pGreen$_{(-)doxycycline}$, and A2780/pGreen/Test cells were trypsinized, placed in each well of the 96-well plates, and allowed to attach overnight ($1.5x10^4$ cells per well [final]). The next day, DMSO was added to wells through columns 2 through 10 at a single concentration of 1% for HTS optimization; columns 1 and 12 were contained positive controls (A2780/pGreen$_{(+)doxycycline}$), negative controls (A2780/pGreen$_{(-)doxycycline}$), and a background control (A2780). All wells had a final volume of 100 µl and 1% DMSO (nontoxic at this level). The plates were then placed back in the incubator for 48 h, after which they were removed and rinsed twice with phosphate-buffered saline. Fluorescence measurements were carried out on a PerkinElmer EnVision plate reader (PerkinElmer, Boston, MA), using filters at 485/14 nm for excitation and 520/8 nm for emission. All liquid handling was carried out using an automated liquid handling system PerkinElmer Janus (PerkinElmer, Boston, MA). A more detailed HTS protocol is present in Table 1. In this study, an inducible PTB shRNA was used as an inhibitor to

demonstrate the ability of the splicing reporter assay to discern between low and high PTB activity, since there is no known small molecule inhibitor that targets PTB.

5.2.9 Data Analysis

We used positive (plus doxycycline) and negative (no doxycycline) signal controls in the plate wells to calculate Z'-values during the assay optimization in order to monitor assay robustness [279]. Assays with Z' factor of 0.5 indicate that the assay is robust enough to identify inhibitors of PTB activity reliably [279]. Moreover we used signal window (SW), Coefficient of variance (CV), and assay variability ratio (AVR) values when distinguishing signals from positive control cell line activity from parantel and negative control cell lines [280].

5.3 Results:

5.3.1 PTB regulates the alternative splicing of PTBP2 pre-mRNA

Given the observation that PTB knockdown enhances the suppression of tumor cell proliferation, suppression of anchorage-independent growth, and suppression of invasiveness [2], we performed global exon array studies to identify potential regulatory targets responsible for these changes. The effect of PTB depletion on alternative pre-mRNA splicing and abundance was measured for 36,397 unique splice events in A2780 ovarian cancer cells using the Jivan Biologics' splicing-sensitive microarray. PTB depletion identified 317 genes whose splicing patterns were consistently altered more than 2-fold after PTB depleted in two separate experiments (Fig. 5.1B). We validated differentially

expressed splice variants identified by microarrays by conventional RT-PCR. Based on the confirmation data, high levels of PTB expression were associated with increased skipping of exon 10 in the PTBP2 gene (a PTB homolog, also known as brPTB or nPTB [281]) in these A2780 epithelial ovarian cancer cells. Moreover, PTB depletion by shRNA knockdown introduces a premature stop codon (PTC), which causes nonsense mediated decay (NMD) of the PTBP2 mRNA. This splicing event shown in Fig. 5.1C and Fig. 5.2A both creates the short- and long-form gene products of PTBP2.

Table 5.1 Protocol table for the splicing reporter assay

Step	*Parameter*	*Value*	*Description*
1	Induction of the (+)control cells	96 h	20ng Doxy/µL
2	Plate cells	100 µL	$1.5x10^4$/well
3	Incubation time	24 h	37°C, 5% CO_2
4	Compound/DMSO	1 µL	10µM or 1% Final
5	Incubation time	48 h	37°C, 5% CO_2
6	Assay Readout-1	485/510nm	Plate reader, fluorescence mode
7	Remove Media	10 µL/sec	Plate Washer
8	Wash 2 times	100 µL	PBS w/Mg^{2+} Ca^{2+}
9	Assay Readout-2	485/510nm	Plate reader, fluorescence mode
Step Notes			
1	T-75 tissue culture flasks, A2780/pGreen cells		
2	PerkinElmer 96-well clear bottom black ViewPlates. Wells 1A-C & 12G-H positive controls, 1G-H & 12A-C negative controls, 1D,1E, 12D, 12E background controls.		
3	Incubators with extra water tray to maximize humidity.		
4	PerkinElmer Janus liquid transfer system, p20 tip, (sequence: aspirate 1µL compound: aspirate 15µL additional from cell media: dispense 16µL)		
5	Incubators with extra water tray to maximize humidity.		
6	PerkinElmer EnVision Plate reader, top read, 20 flashes/well		
7	PerkinElmer Janus, p100 tips, aspiration speed: 10 µL/sec, dispense speed: 10 µL/sec		
8	PerkinElmer Janus, p100 tips, aspiration speed: 15 µL/sec, dispense speed: 10 µL/sec		
9	PerkinElmer EnVision Plate reader, top read, 20 flashes/well		

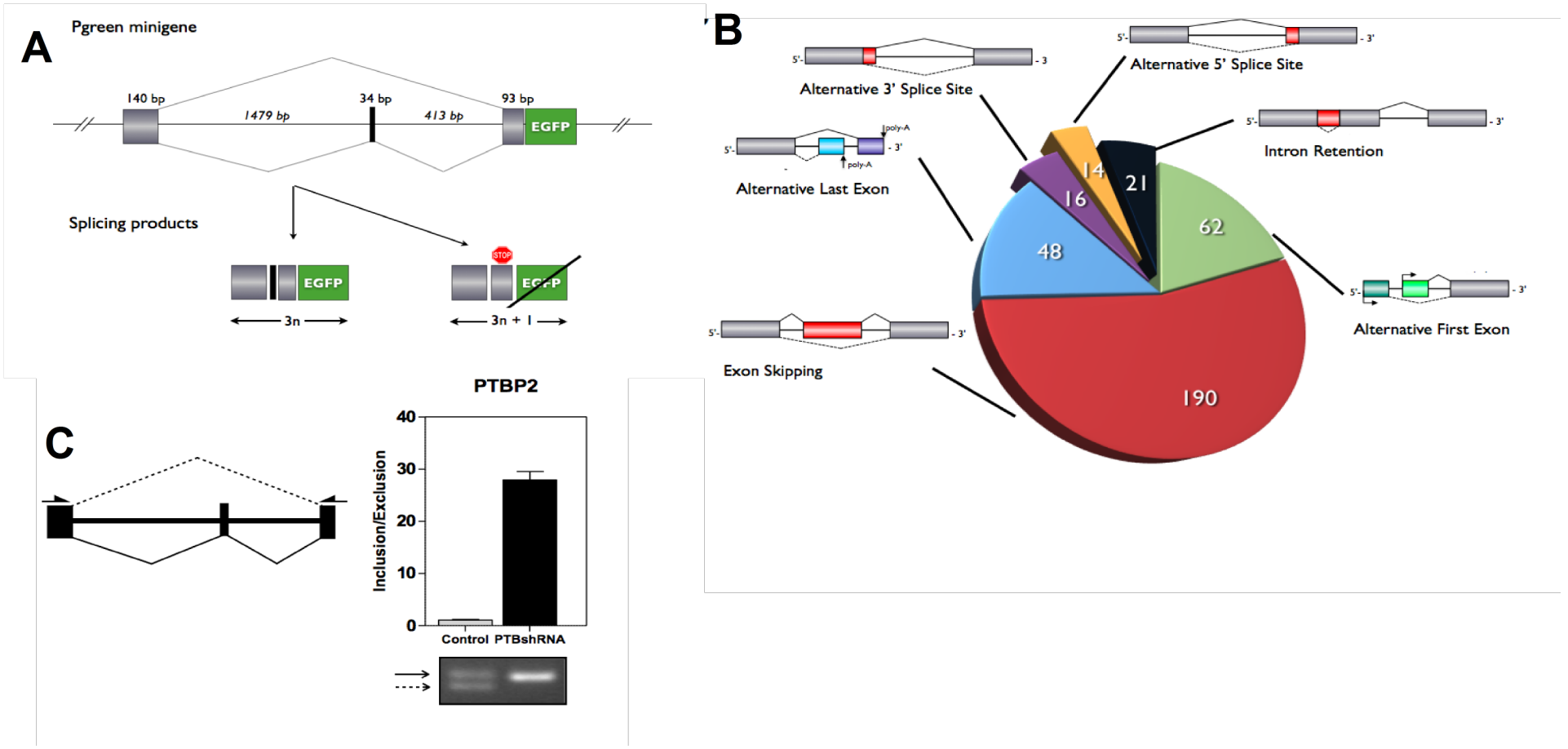

Fig. 5.1 A PTB responsive splicing FL reporter system. **A.** Design of the minigene reporter system used for monitoring PTB activity in live cells. The reason for getting varying EGFP expression that are proportional to PTB levels in a given cell is the length of the exon 10(34bp) and the frame shift that is caused by skipping of the exon10 at PTB depleted conditions. **B.** Validation of microarray analysis results. PTB regulated alternative splicing events. **C.**Validation of PTB-regulated exon skipping (PTBP2) pattern by RT-PCR. PTBP2's alternate structure is diagramed. Long dashed and solid arrows represent splice variants. Small black arrows located on the gene structure schematics represent the PCR primers pair.

5.3.2 Cell-based EGFP reporter assay detects PTB activity

Our previous findings [2, 282] support the idea that manipulation of AS by targeting PTB may have therapeutic potential for ovarian cancer treatment. To test this, we developed a cell-based fluorescent reporter assay to monitor PTB-mediated RNA splicing using an EGFP reporter as an indicator of PTB activity in

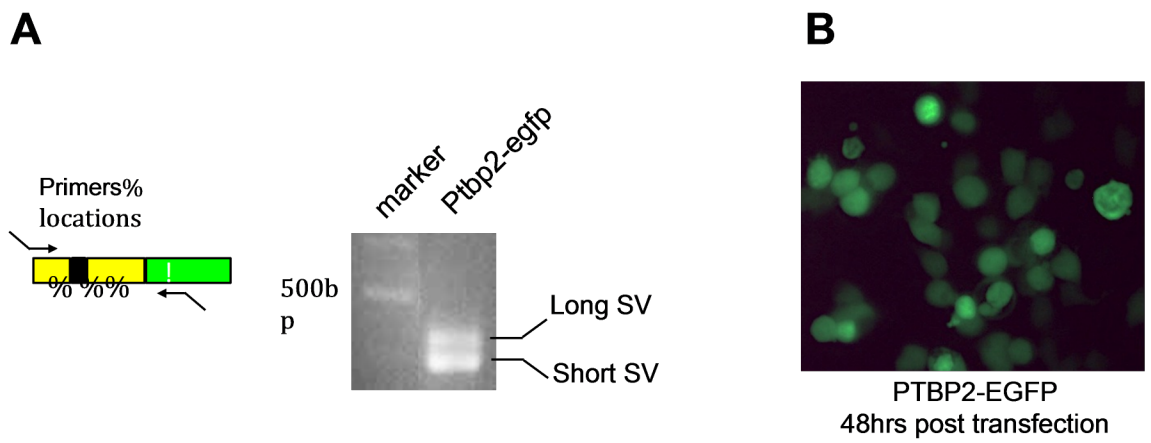

Fig. 5.2 PTB responsive splicing FL reporter system transfected into the cells to test its functionality. **A.** RT-PCR of the PTBP2-EGFP SVs in transfected 293T cells and the location of the primers. **B.** FL micrograph of PTBP2-EGFP transfected 293T cells expressing EGFP.

live cells (Fig. 5.1A, 5.2B). This EGFP fused minigene reporter was designed based on differential splicing of a PTB target gene, identified with microarray analysis. This reporter also depends on the PTB levels in cells that were visualized by changes in reporter activity in terms of EGFP expression.

The microarray analysis and validation results (Fig. 5.1B-C**)** show that splicing of exon 10 of PTBP2 is regulated by PTB. Depletion of PTB by shRNA completely suppressed skipping of exon 10 (long form), which led to an increase in PTB reporter activity. In contrast, high levels of PTB result in both the short and long forms being present in the transcript (Fig. 5.3A). We designed the reporter construct (called pGreen) so that the downstream EGFP will be in the reading frame when all three PTBP2 exons are spliced together (long form). When exon 10 is skipped, the distruption of the EGFP reading frame will lead to a decrease in PTB reporter activity (short form) by introduction of a PTC, which causes NMD of PTBP2 mRNA (Fig. 5.3B). Therefore, the higher reporter activity indicates the existence of the long variant with three exons included and the lower reporter activity indicates the existence of the short variant with exon 10 skipped. When this construct is introduced into cells expressing high levels of PTB, such as Lenti-X 293T cells and A2780 cells, low EGFP levels are detected because both PTBP2 mRNA splice variants are generated in such cells. Fig. 5.2B shows the experimental result of transfection of the construct into Lenti-X 293T cells. RT-PCR results indicated that the PTBP2 exons 9, 10 and 11 in the construct were coordinately spliced to form two distinct variants, the short and long forms, respectively (Fig. 5.3B), in response to altering the PTB levels. These results confirm the feasibility of using these constructs to detect the alternative splicing of PTBP2 exon 10 and PTB activity in our HTS system.

5.3.3 Minigene reporter responses to varying PTB protein levels

We performed sevreal analyses to confirm that the regulatory elements in the minigene and EGFP expression were still functional in our engineered reporter cell lines. We first assessed if this reporter gene is responsive to different PTB levels. Following total RNA purification, vector-specific primers were used to amplify only the minigene reporter by RT-PCR (Fig. 5.2A). The results, in agreement with the microarray data, showed that the splicing pattern of the minigene reporter changes in PTB-depleted cells compared to PTB-expressing controls. We then purified the amplified PCR fragments from agarose gels and sequenced them, confirming that splicing of the minigene is still functional, and that expression of EGFP can be detected (Fig. 5.3B). Next, we performed image-based analysis, using fluorescence (FL) microscopy (Fig. 5.4A). Based on the image analyses, we observed more than 2-fold increase in PTB reporter activity in PTB-depleted cells, compared to controls (Fig. 5.4B), and this was due to alternative splicing of the minigene. In addition to image-based analysis, we also performed flow cytometry experiments to validate our observations. Flow cytometry analysis resulted in similar changes in the reporter activity pattern, as observed in image-based analysis (data not shown). Overall, our results reveal that the cell-based reporter system is robust and sensitive enough to monitor PTB activity in live cells.

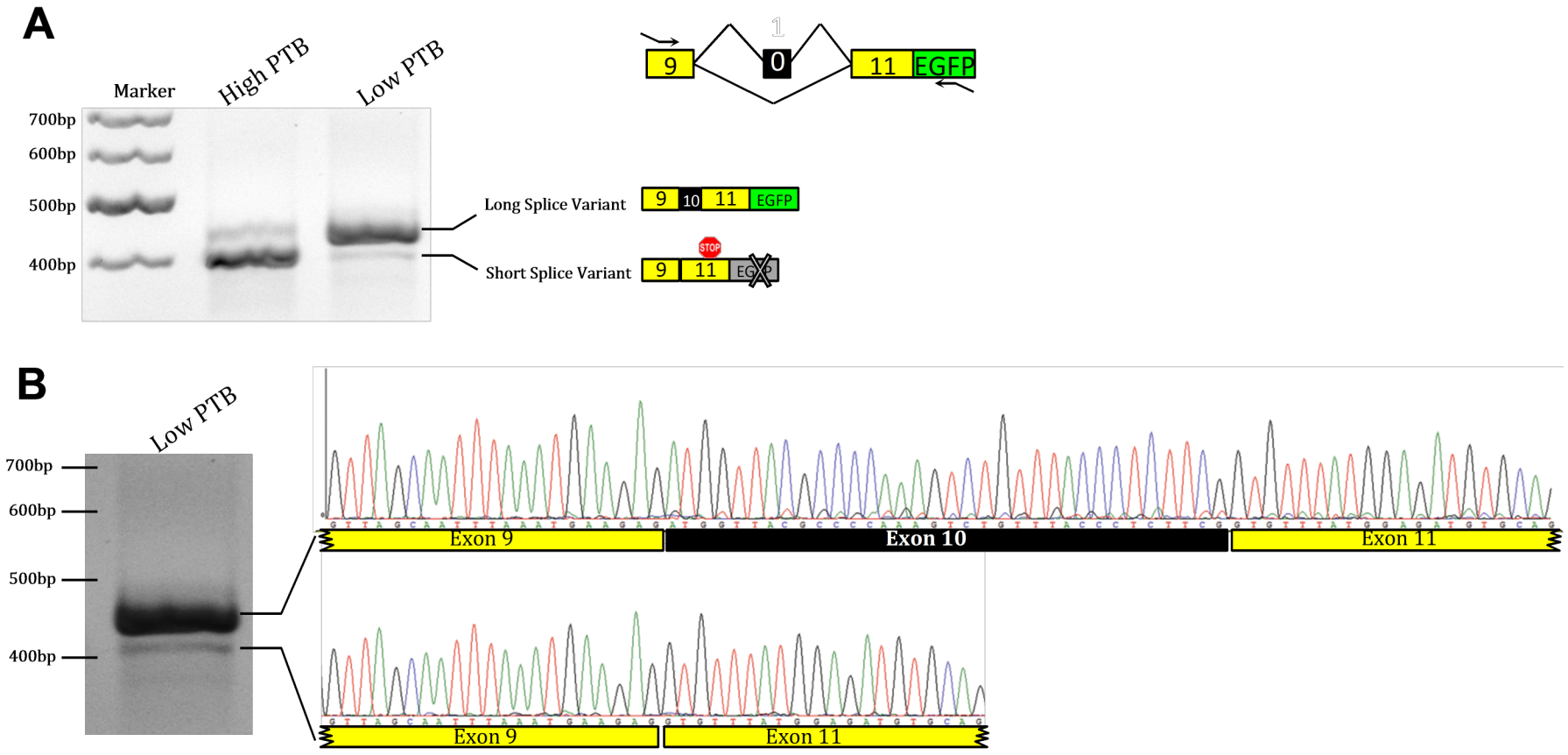

Fig. 5.3 Functional minigene splicing analysis. **A.** Agarose gel of the RT-PCR products. Schematic representation of the PTBP2 minigene structure. Exon 11 is alternatively spliced. Small arrows indicate the position of the primers used for PCR amplification. The structure of the PCR products is shown on the right to the gel. PTBshRNA repressed the inclusion of exon 11. **B.** Change of splicing pattern of the PTBP2 minigene by knocking down PTB protein using shRNA. The resulting PCR products were separated on an agarose gel and sequenced to validate their specificity.

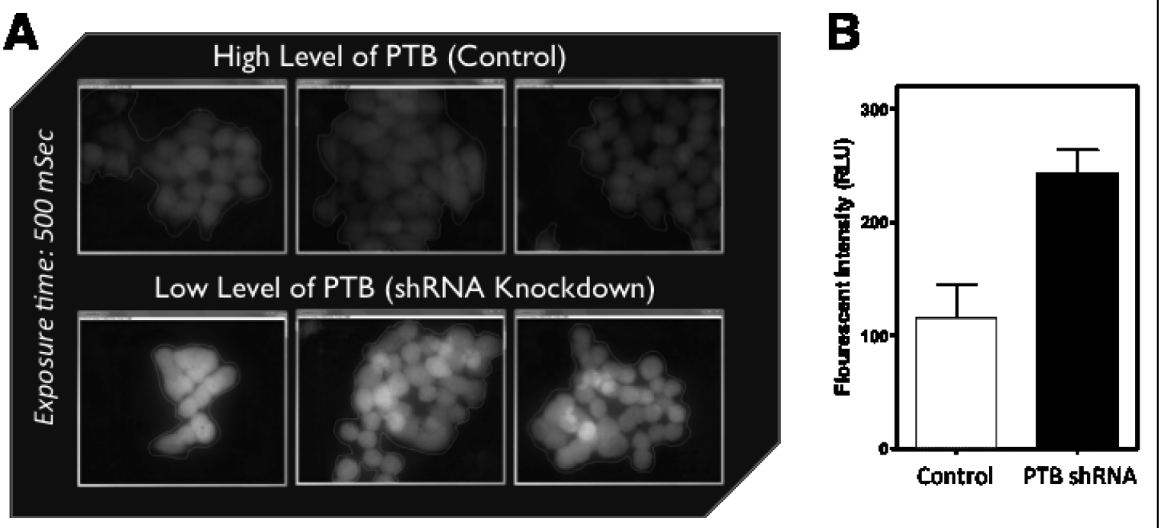

Fig. 5.4 Quantitation of FL intensity change in engineered cells after PTB KD. **A.** Acquired FL images analyzed with NIH ImageJ software. All images were acquired on gray scale channel for a better dynamic range. Exposure time was set to 500 mSec for all the images. **B.** Measured FL intensity difference was ~3 fold between control and PTB shRNA treated samples.

5.3.4 Establishing a reliable high-throughput assay in 96-well plates

In order to establish maximum signal strength, and minimize intraplate and interplate variability, we prepared several plates containing 1% DMSO, as described in the methods. This validation was completed using all three engineered cell lines (A2780/pGreen$_{(+)doxycycline}$), A2780/pGreen$_{(-)doxycycline}$, and A2780). The assay plates showed a coefficient of variation (CV) of 6.2% for the inducible positive controls and 4.5% for the negative controls. We next addressed the assay performance measures (APMs) for our cell-based assay in terms of signal window (SW); Z-factor; and assay variability ratio (AVR) values to assess whether our assay is suitable for high-throughput screening [280]. We

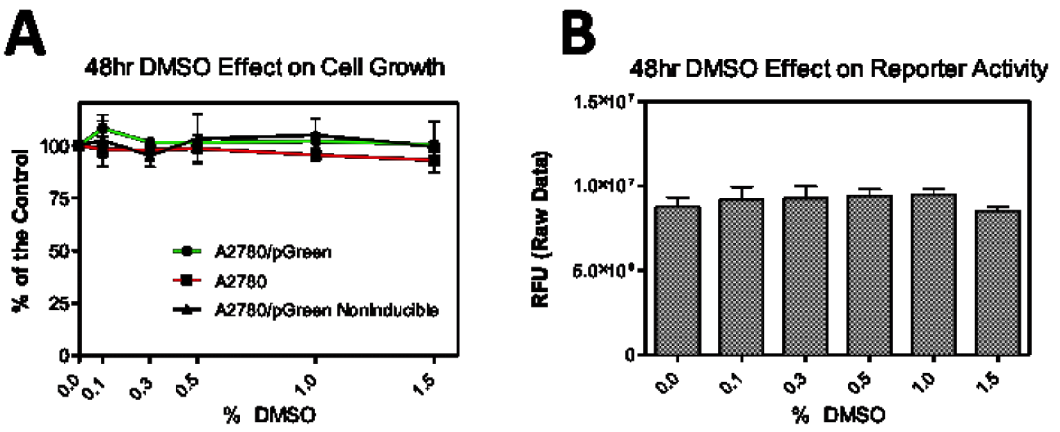

Fig. 5.5 The DMSO tolerance of the assay was examined on reporter cell lines. **A.** Viability of the cells was evaluated in cells treated with increasing DMSO concentrations by MTT assay. No significant change was observed. **B.** Effect of DMSO on reporter activity assessed using FL microplate reader after 48 hr incubation. DMSO did not cause any significant activity change on the reporter carrying cells.

conducted multiple test runs to examine the SW, AVR and Z' of our HTS assay. We used a fixed number of controls to mimic a real run during the evaluation of the assay (6 samples of positive and negative controls per plate). We determined the Z′-factor [279], which measures the dynamic window between total signal and background, taking into account the errors associated with each, 0.63 ± 0.09 (n=9). Overall, we assessed 9 different reading conditions during these test runs, and calculated means and standard deviation of positive and negative controls, SW, AVR and Z' as shown in Table 2. Furthermore, we could not detect any significant edge effects in our assay.

The common way of preparing stock libraries of compounds in 100% DMSO solution raises the toxicity concerns in a cell-based assay. Using the proper DMSO controls in the chemical screen minimizes the possible DMSO effect on the compound screens. The degree of chemical cytotoxicity depends upon the nature of the cell background, the concentration of the chemical, and the length of exposure [283]. In two experiments, we assessed the effects of DMSO on assay cell growth and reporter activity to optimize the final chemical concentration to use in our HTS assay. To optimize final the DMSO concentration, medium with DMSO concentrations ranging from 0% to 1.5% was applied to test cell lines and control cell lines for 48 h. We found that cell viability and reporter activity were not affected significantly with increasing DMSO concentrations (Fig. 5.5A-B). The assay tolerated up to 1.5% of DMSO without significant impact on the signal window and cell viability. Based on the results, we used 1% DMSO as the final concentration in both positive and negative control wells.

The kinetics of PTBP2 pre-mRNA processing (splicing) in cancer cells is not known, and this prevented us from estimating the exposure time to the compounds needed in our HTS assay. To better understand the kinetics of PTBP2 mRNA splicing, we used a simple time-course knockdown approach. We employed PTB-targeting synthetic oligonucleotides. After transfecting the siRNA oligos into the A2780 cells, RNA samples were collected at 0, 2, 5, 9, 18, and 48 h to analyze splicing products of the PTBP2 reporter minigene, and also mRNA

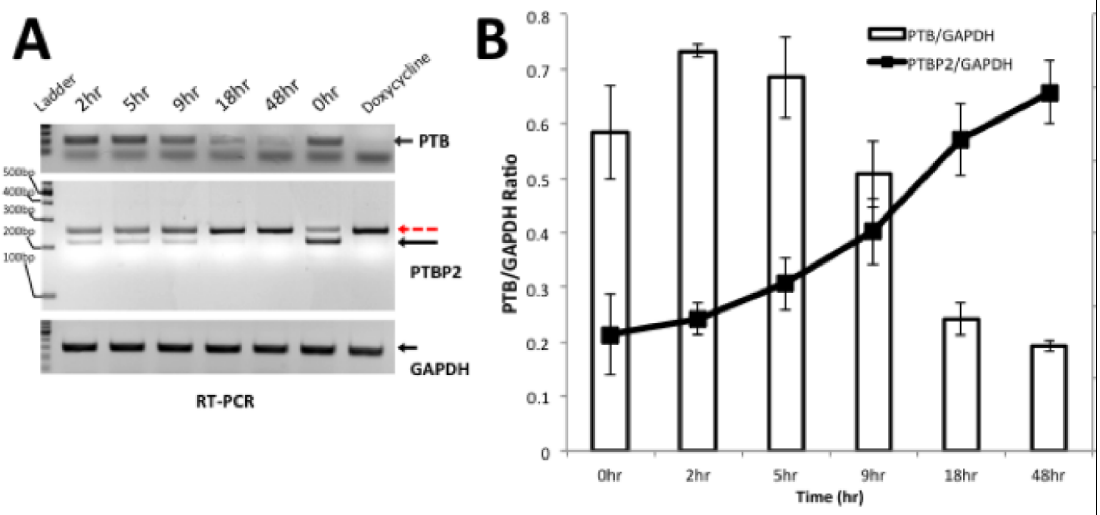

Fig. 5.6 Time-course study of PTB knockdown effect on PTBP2 mRNA long splice form formation. **A.** RNA samples were converted to cDNA and resulted PCR products separated on agarose gel. GAPDH gene was used as a loading control. Mean intensity of each band was quantified by Photoshop CS4 (www.adobe.com) **B.** Separated bands were quantified and plotted on an Excel graph. PTBP2 mRNA long form formation reaches the highest level at 48 hr time point.

levels of PTB (Fig. 5.6A). RNA samples were reverse transcribed into cDNAs, and these cDNAs were used as templates in PCR reaction mix. Resultant PCR products were separated by agarose gel electrophoresis (Fig. 5.6B). The PTB siRNA time course study of the minigene splicing activity showed an increase in the long form expression of the PTBP2 while PTB mRNA was downregulated (Fig. 5.6A). Based on this time-course study, an incubation time of 48 h was selected for further experiments that produced sufficient signal for fluorescence readout.

During the assay performance evaluation phase, the calculated SW, AVR and Z' values met our quality control criteria as seen in Table 2. Z' factors for all but one of the assays were >0.5, indicating that they were high quality assays that were compatible with HTS. In addition to Z', all assays were below the 20% threshold for the maximum acceptable dispersion of the probability distribution, CV% (coefficient of variation) (data not shown). Taken together, these results demonstrate that our assay using our engineered cell lines is very likely to be suitable and reliable for use in a full-scale screening of random compound libraries.

	Positive Controls		*Negative Controls*				
Condition:	***Mean***	***SD***	***Mean***	***SD***	***Signal Window***	***Z'***	***Fold Increase***
Read 1	3.5E+07	1.3E+06	1.8E+07	4.6E+05	9.3	0.69	1.95
Read 2	1.2E+07	9.4E+05	5.7E+06	1.9E+05	3.6	0.49	2.18
Read 3	1.1E+07	4.3E+05	4.3E+06	1.3E+05	11.2	0.74	2.50
Read 4	1.7E+07	6.3E+05	7.1E+06	2.8E+05	12.2	0.73	2.47
Read 5	2.5E+07	1.6E+06	1.2E+07	4.4E+05	4.4	0.53	2.14
Read 6	1.5E+07	1.4E+06	1.8E+06	9.1E+04	6.2	0.66	8.13
Read 7	1.5E+07	1.7E+06	1.8E+06	1.0E+05	4.3	0.57	8.10
Read 8	1.3E+07	6.9E+05	3.2E+06	1.7E+05	10.4	0.73	4.08
Read 9	1.5E+07	1.4E+06	4.4E+06	2.9E+05	4.0	0.53	3.48

Table 5.2 Signal window and Z' Factor comparison for HTS assay. Table represents data from 9 different readout with 12 control wells per plate (positive and negative combined).

5.3.5 Definition of the controls and the plate layout

As described above, the HTS assay we developed utilizes 3 different cell lines . These are: (i) A2780/pGreen, a positive control cell line that carries the minigene reporter construct and an inducible shRNA against PTB; PTBshRNA is induced by DOX. Because no specific PTB inhibitors have yet been described, we decided to use the shRNA technology as a positive control (i.e. complete inhibition of the PTB) in our HTS assay. We previously showed that at its steady-state, this cell line expresses high-levels of PTB, and therefore represses recognition of Exon 11 within the reporter construct. This causes an increase in the transcription of the short form in which EGFP's reading frame is shifted and low or no EGFP expression is observed. Upon induction of PTBshRNA, the long form is produced predominantly and a significant increase in EGFP is detected. (ii) A2780/pGreenNI, a test cell line and also negative control cell line; PTB expression is high in these cells, leading to the low or no EGFP expression (iii) A2780 parental cell line carries no transgene, and is necessary to eliminate the cell autofluorescence factor from the assay that would otherwise result in a higher signal-to-background ratio: the general scheme for the assay is shown in Fig. 5.7A and B. Our primary screens in the 96-well format have 16 control wells: 6 positive, 6 negative and 4 background controls. In a typical 96-well format screening, wells are left empty at the outer rows (1A-H and 12A-H) of the plates. The plate layout we used for optimization and screening is represented in Fig.5.7C.

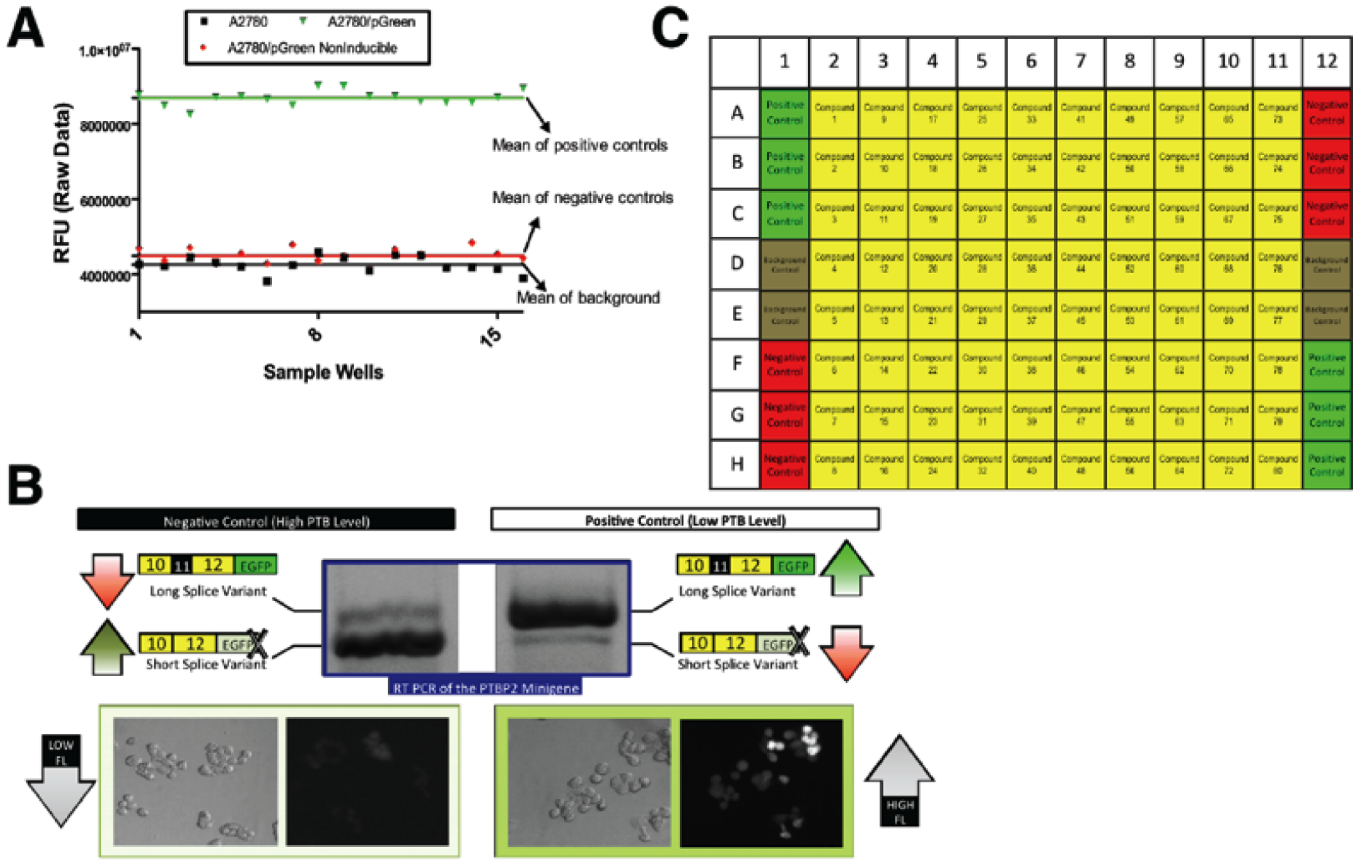

Fig 5.7 Control definition, assay principle and planned plate layout. **A**. Scatter plot of the raw data from PerkinElmer Envision fluorescence microplate reader of positive controls (green), negative controls (red) and background (black) cells. Positive control cells were induced with 20ng Doxycycline/uL to express PTBshRNA for 96 hr before seeding. **B.** RT-PCR result shows the assay principle that is based on the PTB levels in test cell lines. PTB regulates the exon 11 inclusion/exclusion that can be translated into long or short form of the reporter construct. With the long form we get an in-frame EGFP expression whereas short form generates the out-of-frame (i.e. non-functional) EGFP product. **C.** A typical 96-well screening plate has 16 control wells: 6 positive (3 on one side, 3 on other side), 6 negative (3 on one side, 3 on the other side), and 4 background (2 on one side, 2 on the other side).

5.3.6 Measuring fluorescence intensity in high-throughput manner

A critical factor in taking the advantage of the fluorescence cell-based HTS assays is the correct initial setup of the microplate reader used for data readout. Proper selection of filters, plate dimensions, measurement heights, gain and flashes can impact the instrument's sensitivity for a fluorescence assay. We identified the key instrument parameters for our HTS assay performance by using the positive control cell line, A2780/pGreen, as this cell line mimics 100% reporter activation. The EnVision microplate reader (www.perkinelmer.com) is a fluorometric, fluorescence polarization and luminometric assay technologies. To determine the optimum filter/mirror set for our assay, we performed spectral scans on the positive control cell line (A2780/pGreen) using an EnVision reader equipped with monochromator. A2780/pGreen cells ($1.5x10^4$/well) were seeded into a black 96-well plate for measurement of spectral properties. Measurement time was set to 50 flashes with 1 nm step increments. First, the emission wavelength was set to 525 nm while scanning the excitation spectra lying between 350 to 500nm. Then, the excitation wavelength was set to 425 nm while scanning the emission spectra lying between 500-550nm. We found that the excitation peak of the PTBP2-EGFP fusion product is 485 nm, and the peak emission is 520nm (Fig 5.8).

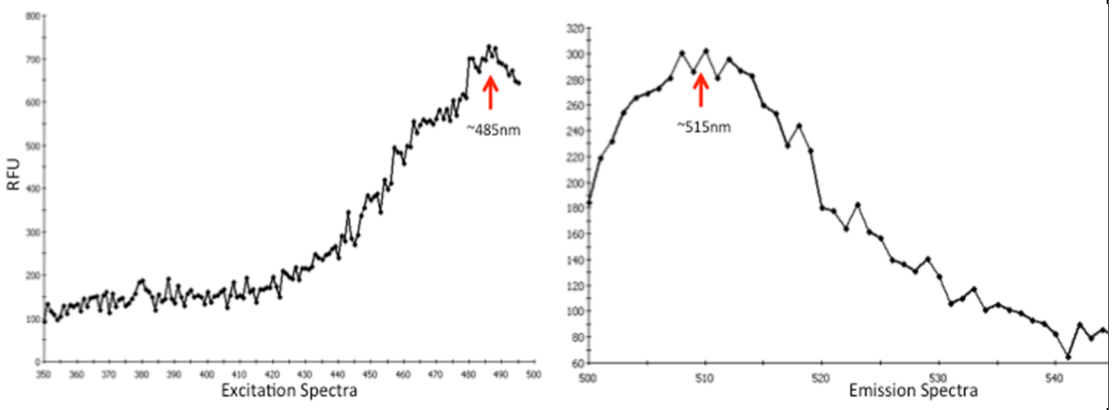

Fig 5.8 Clear excitation and emission spectra were obtained for PTBP2-EGFP fusion gene expressing cells. Peak excitation is 485nm, while the peak emission is 520nm. Therefore, we used excitation filter of 485/20nm and emission filter of 520/15nm in our assay readouts.

Additional optimization steps were also conducted in the A2780/pGreen control cell line: plate dimension, reading position (top/bottom), measurement height, flash number and detector gain. Briefly, plate dimension determines the exact size of the plate type and well centers; reading position (top/bottom) determines the optimal readout position for the attached cell lines; measurement height determines the optimal focal point from a range of vertical positions that provides the best FL readout; flash number determines the optimum excitation number and readout time of each well; gain determines the optimal gain to obtain the maximum possible signal without saturating the detector by a strong signal. We performed all necessary steps using the "optimization wizard" script within the EnVision software package that automatically sets readout parameters based on

Table 5.3 Evaluation of the key instrument parameters' impact on the assay performance (more + indicates bigger impact).

Optimizations and their impacts on the assay readout	
Parameters:	
Plate Dimension	**++++**
Reading Position	**++**
Meas. Height	**+++**
Flash Number	**++**
Detector Gain	**+++**

our control cell lines (positive/negative/background). The effect of each parameter was evaluated by measuring the doxycycline induced EGFP intensity in A2780/pGreen sub-line in 100µL of growth media or PBS. Table 5.3 summarizes the impact of these parameters on the assay readout. Of importance, when measuring EGFP fluorescence intensity, 10 flashes for 96-well plates were found to be sufficient. Higher flash numbers did not provide significant improvements to performance (data not shown) and reduced the readout time for the whole plate without any remarkable changes in measurement results. Overall, these optimizations enabled us to measure fluorescence intensity changes in our HTS campaign more robustly and accurately.

5.3.7 Primary pilot HTS assay: NCI-Approved Oncology Drug Set II (AODII).

This plated set (89 compounds) contains most current FDA-approved anticancer drugs (http://goo.gl/Lf1JI). This set was replated into 1 mM stock solutions in DMSO as daughter libraries and screened in cell lines using a final compound concentration of 10 µM with a final volume of 1% DMSO. Cells expressing

PTBshRNA were used as a positive control (increased FL expression), and 1% DMSO served as negative control. 1.5x10^4 cells/well/100µL were seeded in 96-well optical bottom black plates and 1 µL of DMSO controls and compounds was added to each well 24 h later. After 48 h incubation, fluorescent expression was measured by EnVision microplate reader. This drug set was screened in two replicate plates. For HTS data processing and representation, we used percent activation (% activation) which is a method based on the controls and calculated on a plate-by-plate basis [284, 285]. % activation for each well within a plate was calculated by normalizing the relative fluorescence units (RFUs) from each positive control well to the RFUs of the in-plate negative controls, which then allowed us to perform plate-to-plate comparisons. We defined it as:

$$\%\ Activation = \frac{X_i - \bar{C}_{neg}}{\left|\bar{C}_{pos} - \bar{C}_{neg}\right|} x100$$

$\bar{C}_{pos}$ and $\bar{C}_{neg}$ are the means of the FL signals of the positive and negative controls, respectively, and X_i is the raw measurement of the i[th] well of the plate we are analyzing. Calculated percent activation of fluorescent reporter for the NCI FDA-approved anticancer drug set is plotted as a 3D bar graph (Fig. 5.9B).

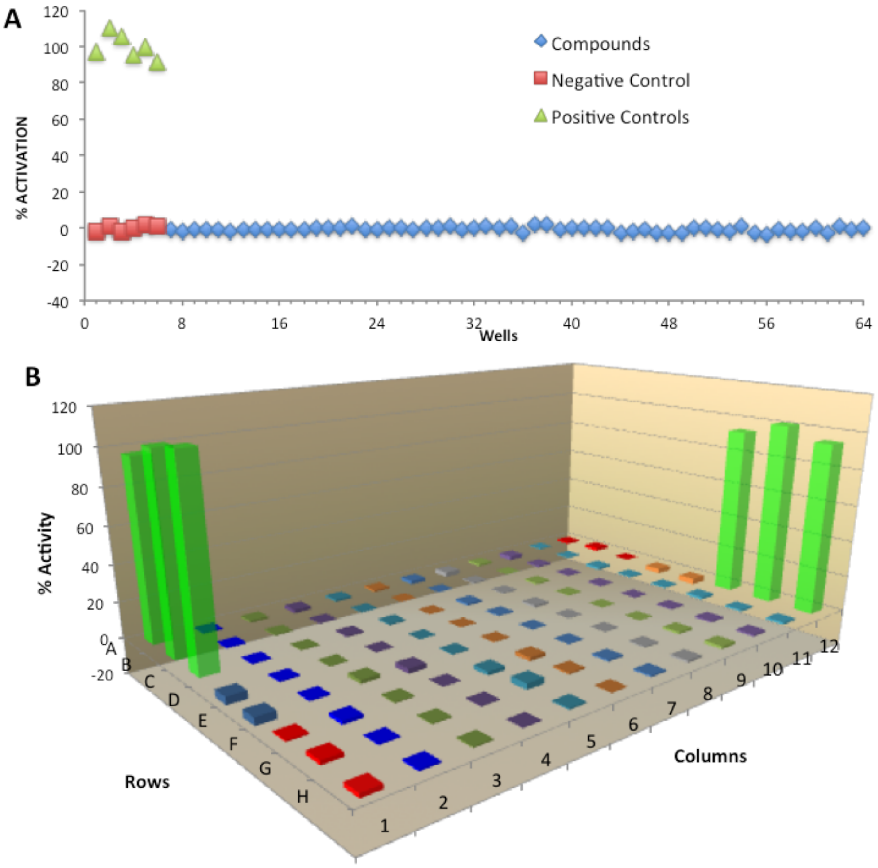

Fig. 5.9 AODII HTS data representation. **A.** Scatter plot of percent activation of fluorescent reporter gene. Each test well was normalized to positive and negative controls. **B.** 3-D bar graph representation of normalized pilot HTS screen data. Wells A1, B1, C1, F12, G12,H12 represent positive controls, F1, G1, H1, A12, B12, C12 represents negative controls and rows 2-11 represent the screened compounds.

We then examined whether our assay is suitable for a bigger run by determining the robustness and reproducibility of the results. For an HTS assay, there are multiple factors that may contribute to data variability, including non-optimized assay conditions, the precision and accuracy of each liquid handling step, uneven evaporation and/or temperature equilibration, non-normalized detection instrumentation, and lot-to-lot differences in reagents, pipette tips and microtiter plates.

We tested the reproducibility and variability of the maximum and minimum plate controls within the assay plate itself (intra-plate), from plate to plate (inter-plate) and from day to day. The inter-plate, intra-plate and day-to-day variation all generated CV values of <20%, with Z'-factors >0.5. We examined the intra-plate variability by calculating the CV based on the standard deviation and mean for each of the maximum and minimum controls on separate plates performed on a given day. For inter-plate variability, we calculated the CV based on the standard deviation and mean determined by the combined data from the multiple maximum and duplicate minimum controls on the same plates performed on a given day. For day-to-day variability, we combined the raw data points from the multiple maximum and minimum controls from plates of days 1 and 2 and calculated the mean, standard deviation and CV. We applied these pass/fail criteria to all our plate readouts.

To examine the reproducibility of the assay results, we tested the 89 compounds in two independent runs of the assay, and present the data as scatter plot of measurement 1 (%Activation Plate1) versus measurement 2 (%Activation Plate 2), along with a correlation coefficient that summarizes the relationship between the two readouts (Fig. 5.10). It is clear that the assay is reproducible and ready to screen larger libraries

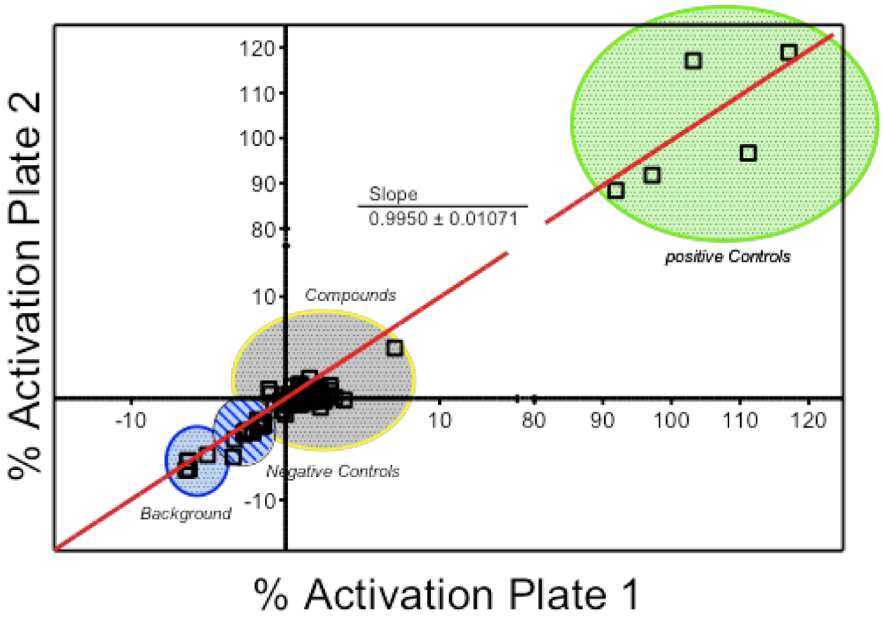

Fig 5.10 Comparison of two independent runs. HTS data were normalized to positive and negative controls on plates by calculating percent activity for each well. The data correspond to our cell based assay that showed a good reproducibility. This analysis indicated that the assay was reproducible under given conditions.

5.4 Summary

Validation and optimization screens were performed in engineered A2780 cells. Results demonstrated in this chapter that all the assay evaluation parameters were in the range considered acceptable for HTS-suitable assays.

The cell based assay we developed:

- Can identify compounds with the potential to act as mechanism-based inhibitors of PTB.
- Can discriminate between compounds causing toxicity to cells or inhibition.
- Is a highly automation-compatible assay ensuring fast, consistent and reliable data generation that allow us to use it in the academic drug discovery setting.

Chapter 6

Implementation of a cell based HTS assay to identify PTB modulators in ovarian cancer

6.1. Background

Ovarian cancer is the eighth most common cancer and the fifth leading cause of cancer death, after lung and bronchus, breast, colorectal, and pancreatic cancers, among women in the United States. In 2012, it is estimated that 23,722 women in the United States diagnosed with ovarian cancer and 16,522 women died from the disease. Despite our rapidly advancing knowledge of tumor cell genetics, potential therapeutic target pathways, as well as an ever-increasing number of gene therapy trials, the fact remains that short of prevention of tumor development, treatments still revolve around surgery, radiation, and chemotherapy. Therefore, there is a dearth of an efficacious therapy that targets a molecular therapeutic target in ovarian cancer.

Aberrations in alternative splicing of pre-mRNA splicing have been linked to many human malignancies, yet the mechanisms for these tumor-specific changes remain underexplored and represent a promising area for therapeutic intervention. We have reported the overexpression of a splicing factor, polypyrimidine tract-binding protein 1 (PTBP1), in ovarian tumor epithelial cells

compared to matched normal controls and have further shown that PTBP1 small interfering RNA–mediated down-regulation has an antitumor effect. Coordinately, the depletion of PTBP1 expression resulted in enhanced sensitivity of ovarian tumor cells to paclitaxel and cisplatin. These data support PTBP1 as a novel target for the treatment of ovarian cancer.

Clearly, the continued emergence of multi-drug-resistant ovarian cancer cells and lack of the ability to detect ovarian cancer in the early stages demand our attention toward the discovery and development of more effective ovarian cancer therapeutics. High-throughput screening (HTS) offers an important tool in acceleration of the discovery of new antitumor leads for all cancer types. Toward this, I previously detailed the development a robust, fluorometric, cell-based high throughput screening HTS assay in 96-well plates that reports on the splicing activity of PTB in Chapter 5.

This chapter describes the implementation of the cell-based reporter HTS assay for the discovery of small molecule modulators of PTB activity.

6.2 Methods

6.2.1 Cell Culture

Human ovarian cancer cell line A2780 was received as a generous gift from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. All cell culture reagents were purchased from Mediatech (Manassas, VA). A2780

sublines were generated as described in Chapter 5. All cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated Fetal Bovine Serum and 2 mM L-Glutamine. At around 70% confluency, cells were trypsinized, washed, and resuspended in DMEM medium with a specified FBS concentration. Cells were added to a 96-well clear-bottom cell culture plates prior to assay. Cells were incubated overnight at 37 °C with 5% CO_2 and then treated with DMSO or compounds for 48h before fluorescent detection.

6.2.2 Screening Automation

High throughput cell-based assays are an important part of drug screening, and successfully dispensing viable cells and reagents are key steps in the process. For screening, cell dispensing, media aspiration, plate washing, assay reagent handling and plate handling were performed with the Thermo Scientific Multidrop Combi micro volume dispenser (ThermoLab Systems, Franklin ,MA), the Elx405 Select plate washer (BIO-TEK Instrument, Winooski, VT), PerkinElmer EP3 automated pipettor (Perkin Elmer, Boston, MA), and Thermo F3 Robotic Arm (ThermoLab Systems, Franklin ,MA). This is the largest robotic arm available from Thermo and is named F3 for its capability of carrying loads up to 3 kg. The arm is placed on 3 meter rail with access to end table as well 900 mm reach. It has 6 degrees of freedom with 360 degree range of motion and reliable placement of +/- 0.05 mm. POLARA software allows scheduling of multiple assays using the arm.

6.2.3 Compound collections and assay plate preparation

Compounds used in this study were stored, maintained, and handled by the University of Chicago Institute for Genomics & Systems Biology, Cellular Screening Center (http://www.igsb.org/services/csc/). The compound library used for screening consists of 4 commercially available collections totaling 3716 compounds. This includes 1120-compound Prestwick Chemical Library, a diverse Food and Drug Administration (FDA)–approved drugs (Prestwick Chemicals, Illkirch, France), the 1597-compound NCI Diversity Set III, 879-compound NCI Mechanistic Diversity, and 120-compound NCI Natural Products Set II. Compounds were dissolved in DMSO and stored in 96-well plates at -30°C. Included on each 96-well plate are 24 DMSO only wells. Further details on NCI compounds can be obtained at http://dtp.nci.nih.gov/index.html . Daughter plates were prepared by dispension of compounds in main stock plates into 96-well plates by the PerkinElmer EP3 (PerkinElmer, Boston, MA). Compounds from the NCI/DTP and Prestwick libraries were diluted to 1 mM daughter plates in DMSO. Compounds were arrayed in a final DMSO concentration of 1% and in 96-well plates.

6.2.4 Splicing reporter assay

In the primary screen, engineered assay cell line, A2780/pGreen plated on T-75 cell culture flasks were allowed to induce PTBshRNA for 5 days in medium supplemented with DOX as described in Chapter 5. We developed a splicing reporter assay to identify small molecule compounds that inhibit PTB activity. This

was accomplished by indirectly quantifying the activity of PTB by monitoring the splicing pattern of one of its targets. Briefly, after 5 days of DOX incubation, 100 µL of engineered A2780 cells (3×10^5 cells/mL) were dispensed into 96-well plates and incubated 24 h at 37 °C, 5% CO_2, with high humidity. The next day, compounds were added to a final compound concentration of 10µM (1% DMSO). The plates were then placed back in the incubator for 48 h, after which they were removed and rinsed twice with phosphate-buffered saline. Fluorescence measurements were carried out on Molecular Devices Analyst GT plate reader (Molecular Devices, Sunnyvale, CA), using filters at 485/14 nm for excitation and 520/8 nm for emission. All liquid handling was carried out using an automated liquid handling system PerkinElmer EP3 (Perkin Elmer, Boston, MA).

6.2.5 Data Analysis

We used positive (plus doxycycline) and negative (no doxycycline) signal controls in the plate wells to calculate Z'-values during the assay optimization in order to monitor assay robustness [279]. Assays with Z' factor of 0.5 indicate that the assay is robust enough to identify inhibitors of PTB activity reliably [279]. Moreover we used Z-scoring to normalize the data throughout the screen. We used this normalized data when distinguishing signals from "actives" from the rest of the compounds (i.e "inactives").

6.3 Results:

6.3.1 Assay quality measures

The goal of HTS is to identify a small number of compounds with desired biological activity (true positives) among the vast majority of the “null” compounds that have no or little biological activity (true negatives) from large chemical libraries. In a successful campaign the compounds with biological activity produce signals with big differences from the mean of the library sample signals and appear as outliers. Therefore monitoring assay performance is quite critical for big HTS campaigns. Z’-factor is useful in estimating assay variability in HTS. Z’-factor combines the information of both location and scale of the distributions of the sample signal and background and provides a better representation of the assay quality than signal to noise or signal to background alone.

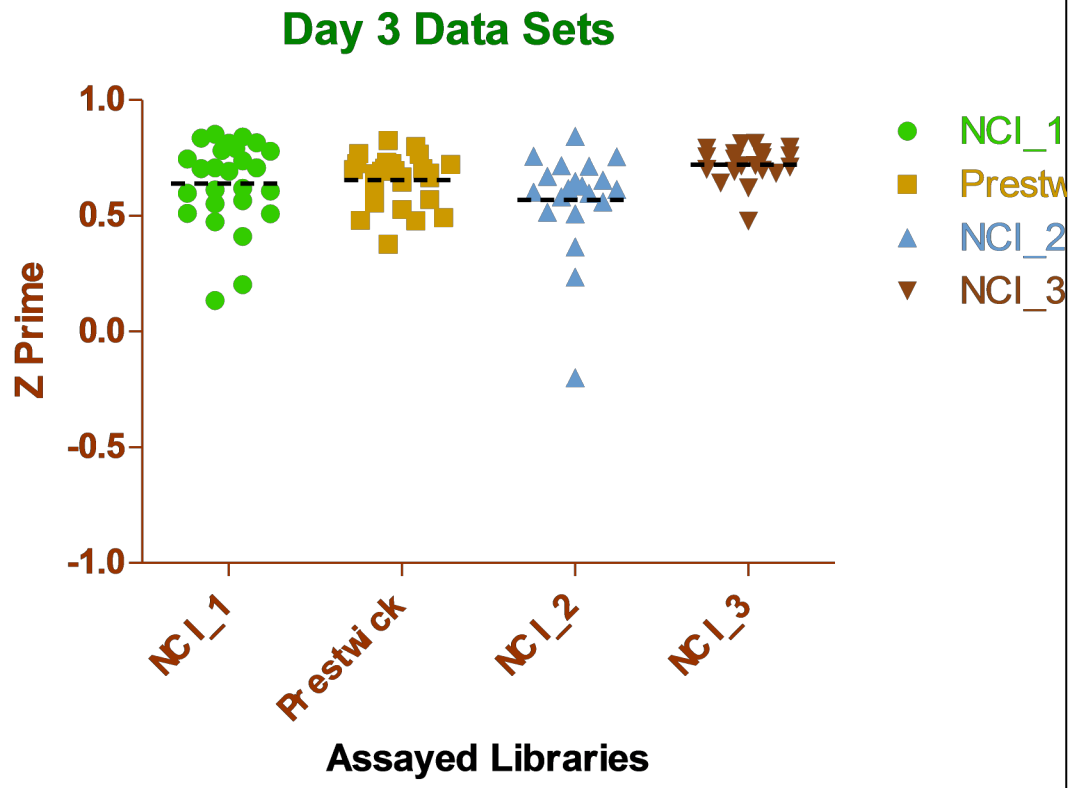

Fig 6.1 HTS campaign generated excellent Z-primes.

To assess the HTS campaign performance, we calculated the Z' factor for 4 independent HTS runs. All 4 were in the range considered acceptable for HTS-suitable assays (i.e .Z'>0.5) (Fig 6.1).

6.3.2 HTS Campaign

We used, well described in Chapter 5, in-house developed method to screen a library of 3716 known structurally diverse characterized bioactive compounds and natural products for their PTB modulating activity. After incubating cells with 10 μM of each compound or DMSO for 48 h, GFP expression was measured. In this study, we collected two points per well to reduce further auto fluorescence interference. Commonly, an assay developer observes assay interference with fluorescent protein assays in the presence of fluorescent compounds [286]. Because this common auto fluorescence interference can be a problem when using diverse compound libraries that may contain members that fluoresce in the excitation and emission spectra of EGFP, we further modified the detection format of our HTS assay to measure the progress of the splicing reaction in the kinetic mode, as opposed to collecting a single end-point read. In fact, when we performed our analyses as a single end-point read only, our hit rates were very high, primarily due to false-positive results from the fluorescent compounds. After the elimination of false positives due to auto fluorescence nature of the compounds, wells with an increase in fluorescence greater than 3 Z-score of the average data resulted in 10 initial hits (Fig. 6.1).

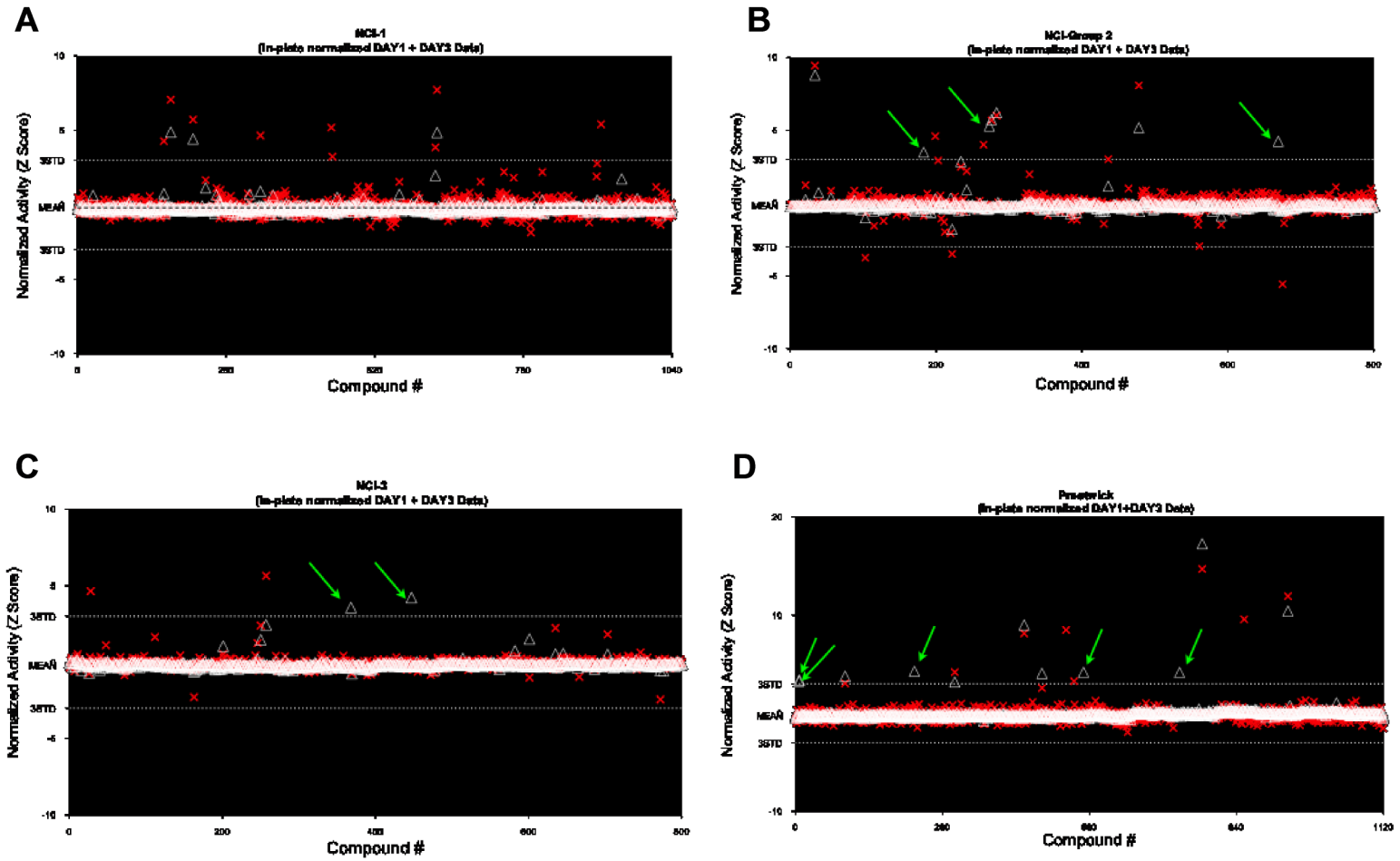

Fig 6.2 Representation of the "actives" after the data analysis. **A.** NCI mechanistic diversity and natural products set II produced "0" hits. **B.** NCI Diversity Set III (800 of 1597 compounds) produced "3" hits. **C.** NCI Diversity Set III (797 of 1597 compounds) produced "2" hits. **D.** The Prestwick Chemical Library (1120 compounds) produced "5" hits. Red star represents Day 1 readout. Open triangle represents Day 3 read out. Two-different readout performed to prevent auto fluorescence interference.

6.3.3 Conclusion

We have demonstrated that fusions of a GFP and PTBP2 (a PTB target gene) to create a reporter can be used as a sensor for monitoring PTB activity in a cellular environment. The feasibility screen with the 4 different libraries demonstrated that our HTS system is able to identify inhibitors PTB.

Data analysis provide not only a direct quantification of PTB inhibition coverage for each screening well, but also reveals compound induced cytotoxicity, which provide future opportunity for the development of antitumor compounds for studying multi-drug resistance. The development of such compounds with different modes of action is of key importance in overcoming clinical therapy resistance.

This assay method provides a suitable format for automated HTS systems with a simple fluorescent readout detection protocol using adherent cells. . It should be noted that this assay monitors the change of endogenous PTB protein in A2780 cells, and no overexpression of recombinant protein is required. The specificity, sensitivity, and ease of use of this reporter system provide the means for identification of small molecule PTB inhibitors for the treatment of ovarian and possibly other cancers. Finally, the method established in this work for PTB can be applied to the development of high throughput plate-based assays for other splicing factors.

Chapter 7

7.1 Discussion and future works

Our previous results [2, 282] that knockdown of PTB expression causes suppression of tumor cell proliferation, anchorage-independent growth, and invasiveness all strongly support the notion that PTB is important in maintaining ovarian tumor cell growth [2], and is associated with the degree of malignancy [1]. At present, it is not clear what mechanisms mediate these effects. However, current knowledge about the targets of PTB cannot entirely explain these observations. Despite these gaps in our knowledge, our results clearly support the idea that PTB has potential as a therapeutic target for the treatment of ovarian cancer, and possibly other cancers in which it is overexpressed.

Using a minigene reporter approach, we have established herein a rapid, reliable, and reproducible cell-based HTS assay system to identify bioactive small molecules capable of modulating PTB activity. To our best knowledge, ours is the first report that outlines the development of an HTS campaign that uses an engineered minigene to monitor PTB mediated splicing events in ovarian cancer cells. We first established cell lines stably expressing the reporter minigene and appropriate control vectors to identify modulators of PTB activity, and we then verified and optimized these cell lines to use in our HTS campaign. This approach represents an efficient system for the detection of novel compounds, applicable for both academic and industrial purposes. In order to successfully develop and execute an efficient, rapid, and reproducible cell-based HTS assay

in the field of anti-tumor drug discovery, one must have access to (1) automated screening technology platform, and (2) an accurate and reliable reporter system, which, in this case, was an A2780 stable cell line that expresses the reporter minigene. Traditional techniques using 12- or 24-well plates have been shown to be more problematic at screening big chemical libraries than more high-density plate formats (≥ 96 wells). In addition, small assay volumes are required for maximum efficiency, in order to minimize the cost of the assay, assay time and reagent consumption. Recent advances in HTS technologies have made it possible to execute cell-based HTS campaigns with smaller formats [287]. Use of automation systems for cell plating, compound transfer, and plate reading further maximizes efficiency [288].

A cell line carrying an accurate and reliable reporter is needed to screen large libraries of small-molecules efficiently. In our case, the selection and optimization of the most appropriate minigene splicing reporter cell line is very important. To successfully perform HTS using 96-well plates, several characteristics of the host cell line must be taken into account, such as basal PTB levels, doubling time, transduction efficiency for read-out of the reporter gene, growth characteristics in the micro-well environment and the kinetics of splicing. We chose to use the A2780 cell line because of its high level of PTB expression and high transduction efficiency [2]. The level of PTB expression appears to be very high in this cell line, although the precise comparison of expression levels between various cell lines is difficult because of differing conditions in different laboratories (e.g. the

source of the cell lines, antibodies, etc). A reporter bearing the EGFP gene was chosen because it can be measured easily, requires no additional reagents, and has a suitably long half-life [289]. Indeed, we obtained a high signal to background ratio (~1:20) in the 96-well plate format, indicating the suitability of this system for HTS assays [290].

To facilitate the detection of splicing repressors, several assays have been developed [291-294], including, a cell-based assay [294] with a luciferase reporter gene. However, this replicon model has several disadvantages: it targeted the splicesomal subunit that consists multiple associated proteins, making it difficult to predict the real target; it required additional processing to measure the reporter itself; and, because the cell lines were transiently transfected, it required extensive optimization prior to screening, makes it difficult to control the amount of the gene transfected. Stable cell lines bearing a minigene fused to an EGFP reporter gene, such as we have described herein, appear to be better suited for use in our HTS campaign to target a specific splicing factor.

Many small molecules can be cytotoxic to cells and this cytotoxicity can be mistaken for inhibitor activity by decreasing the reporter signal when only it decreases cell viability without a direct effect on reporter activity, leading to false-positive results. We therefore decided to incorporate an additional step in our assay to measure cytotoxicity in parallel in our primary HTS splicing readout. Due

to the nature of the original reporter assay we developed, we did not need to design a new assay; rather we multiplexed our assay format by measuring basal EGFP expression from the A2780/pGreen/Test cell lines, which was 5- to 10-times weaker than the induced EGFP levels in the positive control cell lines. The weak EGFP signal served as a viability marker for the cells treated with the compounds in our HTS. This additional analysis was performed in order to minimize identifying erroneous, false positive hits from increased or decreased fluorescence signals due to increased or decreased cell viability, respectively.

Despite the impressive potential of fluorescent protein based-reporter applications to measure biological interactions in a cell-based, high-throughput format, one should be aware of the difficulties encountered when using fluorescent proteins. Commonly, one observes assay interference with fluorescent protein assays in the presence of fluorescent compounds [286]. Because this common fluorescence interference can be a problem when using diverse compound libraries that may contain members that fluoresce in the excitation and emission spectra of EGFP, we further modified the detection format of our HTS assay to measure the progress of the splicing reaction in the kinetic mode, as opposed to collecting a single end-point read. While multiple measurements can increase the overall assay time, a fast-scanning reader, such as we have, in combination with an automated system, can allow rapid and repeated measurements of multi-well plates without significantly slowing the overall plate processing speed. Importantly, the collection of a two-point time

course allows the effects of fluorescent but otherwise inert library members to be eliminated to reveal the true reaction course [286]. Because the first time-point values (when the compounds are added prior to initialization of splicing) associated with each compound well are stored in the output data, a further analysis can be performed to flag interfering fluorescent library members. In our HTS study, we collected two points per well to reduce further assay interference. In fact, when we performed our analyses as a single end-point read only, our hit rates were very high, primarily due to false-positive results from the fluorescent compounds.

A cell-based HTS method alone is itself not sufficient to complete a screening campaign. In a proper design, hits are evaluated in secondary assays that test the ability of the hit to modulate a particular biological event that is distinct from the primary screening assay [295]. Therefore, an appropriate secondary assay must be incorporated to the campaign to verify hit compounds identified from primary screening. To evaluate these compounds, we will use our previously-defined gene signature that was generated by using a splicing-sensitive microarray, which is unique to PTB depleted cells as a secondary screen. As part of the screening process RT-PCR will be used to detect the effects of the compounds on other validated PTB targets.

Like other traditional cell-based HTS methods, our screening campaign was designed to be executed at a single fixed concentration per compound. The

generation of the dose-response curves for each of the compounds screened in a primary screen would be inordinately burdensome even for the most advanced screening centers. Thus, it is considerably more practical and effective to generate the dose-response data only for those compounds identified as hits in the primary screen and validated in the secondary validation screens.

Although our cell-based HTS system represents an effective screening method to identify potential modulators of PTB splicing activity, there is a potential limitation to use of this reporter as a surrogate marker of PTB activity in all cell lines because of their variable levels of PTB expression. By using cell lines expressing high levels of PTB in our study, we have addressed this particular limitation.

In conclusion, we have presented herein a PTB-specific cell-based fluorescence splicing assay that can quantitate inhibition of PTB activity by using one of its target genes, PTBP2, that we identified in a splicing sensitive microarray. We also incorporated a built-in cell viability control that exploits the steady and low level of EGFP expression at the basal state as an indicator of viability. Finally and importantly, our assay reported herein developed provides a unique way to measure the inhibited target activity, that is, the repression of PTB activity results in the increase of reporter read-out so the assay allows one to easily discern nontoxic and PTB-specific inhibitory substances.

In sum, we have developed reliable HTS methods that will accelerate the discovery of small molecules that modulate PTB splicing activity. We are currently optimizing the assay in order to extend the screening to larger libraries that are available at the Molecular Libraries Screening Centers Network. Moreover, because this protein has been linked to ovarian [2] and breast [296] tumors, as well as glioblastomas [114], this platform has potential to generate new therapeutic leads to treat these diseases.

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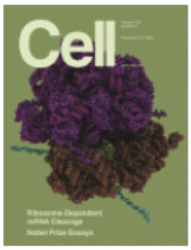

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