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The Role of SRSF3 in Control of Alternative Splicing of CPEB2 in Triple Negative Breast Cancer

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THE ROLE OF SRSF3 IN CONTROL OF ALTERNATIVE SPLICING OF CPEB2 IN
TRIPLE NEGATIVE BREAST CANCER

A Thesis submitted in partial fulfillment of the requirements for the degree of Master's of
Science at Virginia Commonwealth University.

by

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DEDICATION

*To my family,
for your support, faith in me,
and providing that push I needed to make it through to the very end*

Acknowledgements

Without the efforts and care of many people, I would not be in the position that I am today. These people have been there for me when I needed them and given me the support and motivation to push on.

I feel that it's only fitting to acknowledge my first support network first, My family has always been there for me, encouraging me and pushing me to be the best that I can be. Through all of the work, you guys have always been positive and supportive, despite the ups and downs of the process.

Next, I want acknowledge all of the people in my science life who have helped and supported me throughout the rocky and challenging process that has been my thesis project research. Everyone who has been there to help me, from either class or lab, has been invaluable and critical in putting me in the position that I am now.

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List of Abbreviations

7-AAD	7-aminoactinomycin D
CDC2	Cyclin-dependent kinase 2
EGFR	Epidermal growth factor receptor
eIF4F	Eukaryotic initiation factor 4F
EMT	Epithelial-mesenchymal transition
GSK3	Glycogen synthase kinase 3
HIF1 α	Hypoxia-inducible factor 1-alpha
hnRNP	Heterogenous ribonucleoprotein particle
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
mRNA	Messenger RNA
mTOR	Mitogenic target of rapamycin
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PVDF	Polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute medium
RRM	RNA recognition motif
RS	Arginine/serine rich region
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
STAT3	Signal transducer and activator of transcription 3
TNBC	Triple negative breast cancer

Abstract

ROLE OF SRSF3 IN CONTROL OF ALTERNATIVE SPLICING OF CPEB2 IN TRIPLE
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by

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In the presented study, we identified that SRSF3 controls the alternative splicing of CPEB2 and consequently promotes a metastatic phenotype in triple negative breast cancer (TNBC). TNBC causes thousands of deaths annually, frequently due to a lack of effective treatments and a high rate of metastasis in patients. Alternative splicing has been found to be dysregulated in numerous cancers, while splicing factors such as SRSF3 are variably expressed. In this study we performed a siRNA panel to screen potential splicing factors, then used specific siRNA to study the effect of its knockdown on cellular function. These results showed that SRSF3 encourages the production of the pro-metastatic isoform of CPEB2, which contributes the aggressive phenotype of the

tumor. We utilized numerous methods to measure the metastatic function of cultured TNBC cells to determine if SRSF3 strongly promoted the metastatic function. These data showed that siRNA reduction of SRSF3 was able to reduce the metastatic potential of cancer cells. These findings suggest that SRSF3 has great potential as a therapeutic measure to reduce and minimize the aggressiveness of TNBC tumors.

CHAPTER 1

BACKGROUND

1.1 Cancer

Cancer is a terrible disease that affects millions of Americans every year. In 2014, 1,665,540 Americans were diagnosed with some form of cancer [1]. Second in numbers to only heart disease, cancer kills numerous Americans annually. According to the National Cancer Institute, 35.2% of patients diagnosed with cancer die annually [1]. In 2014, cancer accounted for 585,720 deaths in the United States [1]. Cancer's high mortality rate is due to the heterogeneity of tumors and the lack of effective treatment methods.

Breast cancer is the most commonly diagnosed form of cancer, with 235,030 diagnoses in 2014 [1] (Figure 1-1). Additionally, breast cancer is the third most deadly type of cancer, causing 40,430 deaths in the US in 2014 [1] (Figure 1-1). As medical treatments have improved, both the mortality rates and the recurrence rates for breast cancer have dropped significantly. Yet, until we find a cure, there is still room for further progress. As of 2011, the breast cancer mortality rate is 17.2%, representing the percentage of diagnosed patients that died during treatment [1]. Similarly, the 5-year survival rate is only 91.8%, demonstrating that breast cancer tumors tend to readily metastasize or develop resistance to current therapies [1]. The cases in which current treatments are most ineffective belong to one of two particular subtypes of breast cancer: triple negative breast cancer (TNBC) and HER2 positive breast cancer. Both of these cancers tend to be particularly metastatic and lack targeted treatment options [2].

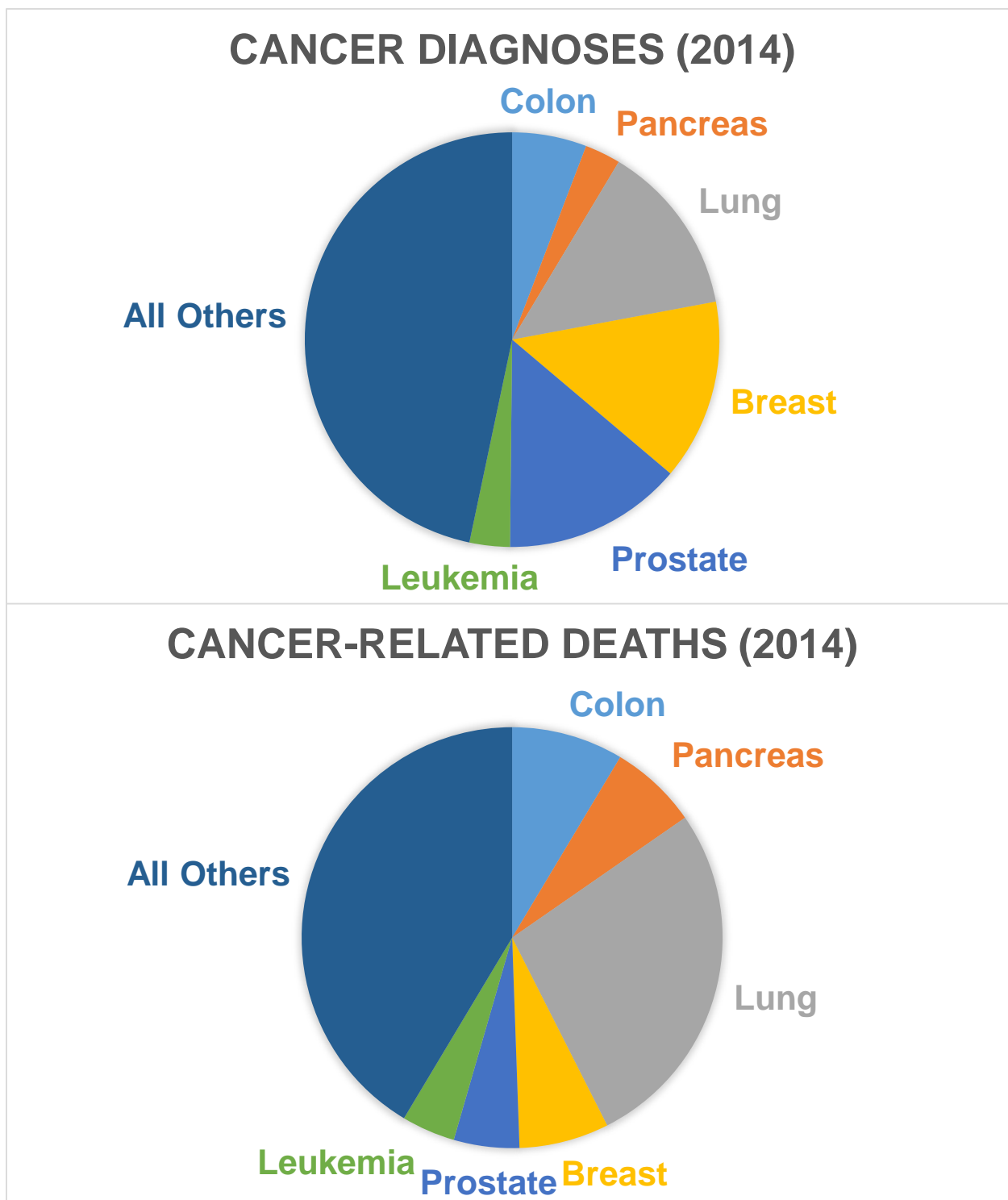


Figure 1-1. Cancer diagnoses and deaths in 2014. According to the National Cancer Institute, breast cancer is the most commonly diagnosed cancer subtype. 235,030 new cases of breast cancer were reported in 2014. Additionally, breast cancer is the third leading cause of cancer deaths. 40,430 deaths in 2014 were attributed to breast cancer. Source: SEER 2014 [1]

Cancer Subtype	New Cases in 2014	Deaths in 2014	5 Year Survival Rate (%)
Breast	235,030	40,430	91.8
Prostate	233,000	29,480	99.8
Lung	224,210	159,260	18.2
Colon	96,830	50,310	66.5
Pancreas	46,420	39,590	7.2

Table 1-1. Cancer Statistics. Breast cancer is the most commonly diagnosed form of cancer in the United States. It is also responsible for the third highest number of cancer-related deaths per year. Despite that, the 5 year survival rate for breast cancer is high (91.8%), but still provides room for improvement. This table compares diagnoses, deaths, and survival rates to four other prominent forms of cancer. Source: SEER 2014 [1]

1.1.1 Process of Cancer Metastasis

One of the most dangerous aspects of cancer is its ability to metastasize to other areas in the body. This process makes cancers very challenging to treat, even if surgery effectively resects the tumor.

To better understand metastasis, scientists have broken down the process into five general stages (Figure 1-2). The first of these stages is initial tumor proliferation and local invasion (growth). During this stage, the tumor grows into local tissues while accumulating mutations that promote growth and cell-cycle progression. The next stage is intravasation, when individual tumor cells slough off into the circulating blood system. At this point, most tumor cells undergo epithelial to mesenchymal transition (EMT) to encourage anchorage independent growth [3]. In general, when the cells are no longer attached to the extracellular matrix or other epithelial cells, the cells will experience detachment-induced cell death (anoikis) signaling. In the case of cancer, if the tumor cells have not acquired sufficient mutations to resist this apoptotic signaling, the tumor cells will not survive intravasation. After the third stage, migration, the tumor cells will reach the site of metastasis and undergo extravasation. During extravasation, the tumor cells invade the walls of the capillaries and surrounding tissue in the new location. The cells will now undergo mesenchymal to epithelial transition (MET) to encourage accelerated cellular growth in an anchorage-dependent manner [3]. This final stage is referred to as colonization and is where the new tumor gains size and tumor cells multiply.

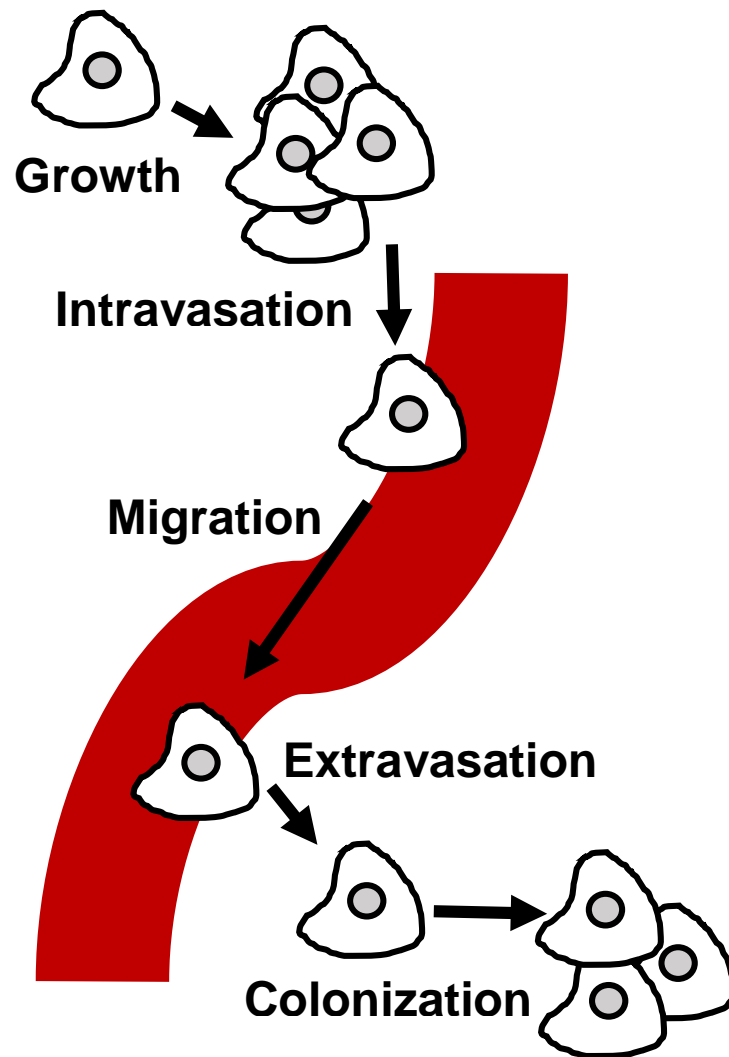


Figure 1-2. Schematic Representation of the Stages of Cancer Metastasis. There are five steps that occur during tumor metastasis. 1) Growth: tumor grows in size in initial location until it acquires sufficient mutations to metastasize. 2) Intravasation: cells from the initial tumor detach from the surrounding tissue, undergo EMT, and enter the circulatory system. 3) Migration: cells travel through the body's vasculature. 4) Extravasation: cells invade site of distant metastasis. 5) Colonization: cells undergo MET to promote anchored cell growth.

1.1.2 Triple Negative Breast Cancer

There is a significant clinical need to improve treatments for the triple negative subtype of breast cancer (TNBC) due to the high mortality rates and high rates of recurrence associated with this cancer. The term triple negative refers to a subtype of breast cancer tumors that do not express the estrogen, progesterone, and HER2 receptors [2,4]. While only 15-20% of all breast cancers are triple negative, they account for almost 70% of the eventual deaths, either due to recurrence or resistance to standard therapies [1,2].

Data analyses have shown that TNBC has a hazard ratio (relative mortality rate between TNBC and standard breast cancer) of 4.35 [1]. Due to a lack of a specific or unique drug target, less effective broad spectrum treatments must be used [4]. Common treatment strategies include the use of anthracyclines, taxanes, ixabepilone, platinum agents, select biological agents, and anti-EGFR drugs [4-7].

Initially, patients with TNBC will appear to respond well to treatment, but will ultimately suffer a worse long term prognosis [4]. It appears that tumor cells in TNBC patients are able to develop metastatic characteristics that allow them to avoid complete eradication by standard treatment methods. Despite breast cancer having a 91.8% 5-year survival rate, patients with TNBC only exhibit a 30.0% 5-year survival rate, demonstrating a dramatic need for clinical improvement [1,2].

Currently, there are a few commonly used *in vitro* models of TNBC that utilize human tumor cells cultured for study (e.g. MDA-MB-231, MDA-MB-468, BT549, SUM1315) [8]. These cell lines were acquired from patient tumors and cultured to ensure accurate representation of the initial tumor. Researchers frequently use these models in

conjunction with mouse models to observe the effects of various treatments on the metastatic functionality of the TNBC cells [4]. Mouse models provide *in vivo* models of TNBC that allow system wide observation of metastasis in a controlled setting. *In vivo* models can be used to test potential drug treatments that could potentially be translated to human clinical trials in the future.

1.1.3 HER2/ErbB2 Positive Breast Cancer

Like TNBC, HER2 positive breast cancer lacks effective clinical treatments due to its aggressive and metastatic nature. HER2 positive breast cancers overexpress the membrane protein ErbB2/HER2 and tend to yield poor patient prognoses [2]. The National Cancer Institute determined that patients with HER2 positive breast cancer have a hazard ratio of 3.60, rating as the second most dangerous subtype of breast cancer [1].

Overexpression of ErbB2/HER2 accumulates at the plasma membrane and leads to chronic activation of the ErbB2 survival intracellular signaling pathways [2]. These pathways include Src, STAT3, PI3K, and MAPK, and all promote metastatic characteristics such as proliferation, survival, motility, and tissue invasion [2]. Enhanced signaling may occur because activated ErbB2 is recycled to the plasma membrane of the cell instead of undergoing lysosomal proteolysis [2]. Lack of precise understanding of this phenomenon presents a challenge in designing targeted treatments.

To gain greater understanding of HER2 positive breast cancer, researchers have developed a number of models to study the disease. Numerous patient samples have been acquired and repurposed to function as cellular models of HER2 positive breast cancer models (e.g SKBR3, MDA-MB 453) [8]. These cell lines do not express the other

two receptors (estrogen and progesterone) and consequently only highly express ErbB2/HER2. Similar to TNBC models, HER2 positive cell lines are commonly used in conjunction with animal models to study systemic metastasis. This can be performed through the use of tumor xenografts or tissue specific injections to act as an *in vivo* model [8].

1.2 Alternative mRNA Splicing

Alternative RNA splicing is a mechanism that contributes to the incredible diversity of protein messages produced by a single cell. Through this mechanism, RNA sequences can be altered through the inclusion or exclusion of certain RNA exons at specific splice sites [9,10]. Variants of the same protein that differ by selective inclusion of exons are referred to as isoforms of the protein and can have a wide range of cellular functions, despite sequence similarities (Figure 1-3). Splice sites are binding sites for the spliceosome, a protein complex that produces the newly spliced mRNA sequence.

The actions and interactions between cellular splicing factors and RNA sequences control the selection of the splicing sites. [10,11]. Splicing factors are RNA-binding proteins that recognize particular RNA motifs to either guide (enhancers) or block (silencers) the spliceosome [9,10]. The action of the splicing factors allows for the vast genetic diversity of an organism, while only using a small portion of the genome. According to Guttmacher and Collins, the mechanism of alternative splicing gives cells the ability to encode over 100,000 proteins using only 30,000 genomic bases [12].

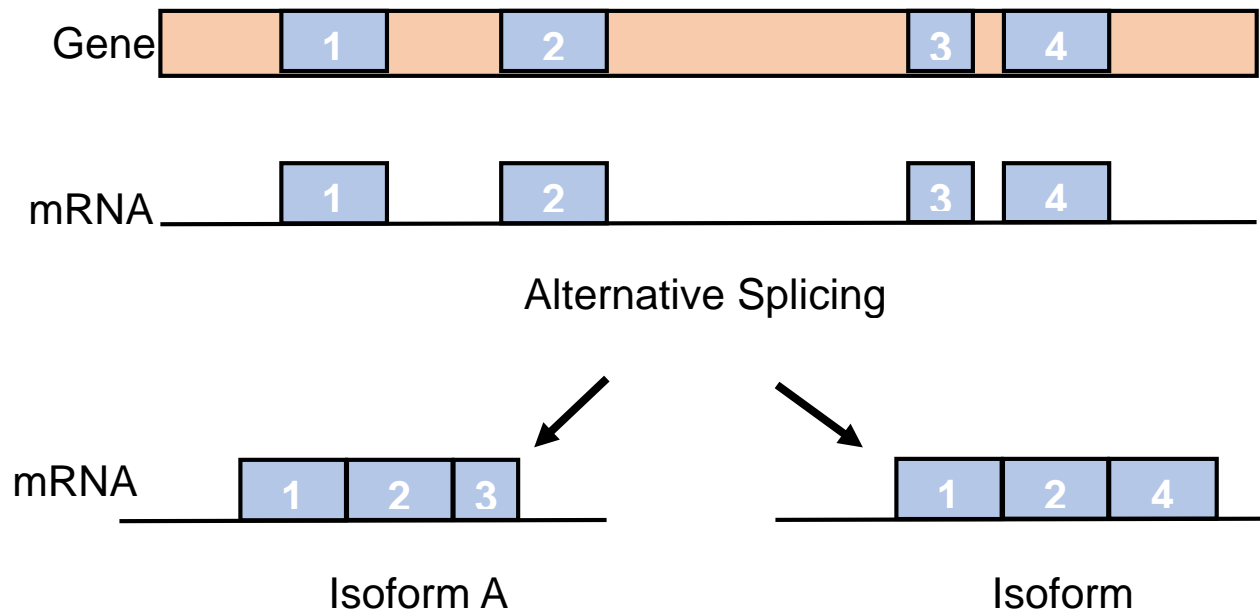


Figure 1-3. Mechanism of Alternative Splicing. During alternative splicing, gene exons are selectively incorporated to produce mature mRNA for translation into proteins.

1.2.1 SR Splicing Factor Family

The cellular function of the SR family of exonic splicing enhancers is to regulate selection of alternative splice sites to promote inclusion of exons into the finalized sequence [10,11]. The SR family of splicing factors antagonizes the activity of the hnRNP family of exonic splicing silencers, preventing them from promoting the removal of mRNA exons [11]. SR proteins are made of one or two N-terminal RNA recognition motifs (RRM) followed by a downstream arginine/serine-rich (RS) domain characterized by consecutive RS or SR repeats [11]. The RRM provides substrate specificity of the particular SR protein with its target short mRNA splicer enhancer sequence [11] (Figure 1-4). As a result, each SR protein interacts with a very specific set of proteins, typically to promote a particular function. SR proteins are involved in many processes of gene regulation, including RNA maturation, transport, and translation [10,13]. Thus, the SR family of proteins plays important roles in alternative splicing and subsequent cellular signaling.

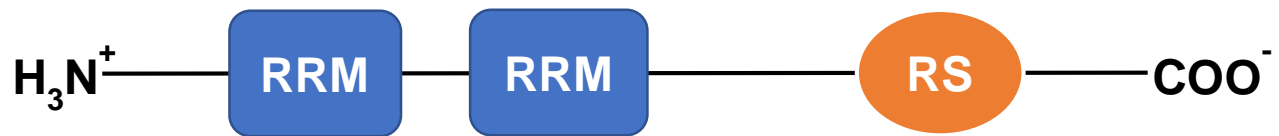


Figure 1-4. General Structure of SR Protein Family. All SR protein family members contain N-terminal RNA-binding domains (RRM's) and C-terminal arginine/serine-rich domains (RS's). The number and length of these domains vary between SR family members.

1.2.2 SRSF3 / SRp20

SRSF3, also known as SRp20, is a member of the SR family of splicing factors. Multiple studies implicate SRSF3 to have a role in protein translation, mRNA polyadenylation, as well as numerous other cellular pathways important for cellular growth, EMT transition, and RNA processing [10,11,14,15]. Cancers show overexpression of SRSF3, suggesting that it might exhibit an important role in growth control [15]. Jia et al. also found that increased levels of SRSF3 are a critical step for tumor initiation, progression, and maintenance [15].

Stickeler et al. showed that there was a positive correlation between high levels of SRSF3 and a higher severity of mammary tumorigenesis [16]. He et al. showed that cells with reduced SRSF3 expression grow slowly, are not resistant to anoikis, and will readily undergo apoptosis proportional to the reduction in SRSF3 [17]. Other studies have implicated dysregulation of SRSF3 with alternative splicing of p53, a known tumor suppressor commonly mutated in all cancer types [18]. While research connects SRSF3 and cancer, the mechanism has not been conclusively determined.

1.3 Cytoplasmic Polyadenylation Element Binding Protein 2 (CPEB2)

CPEB2 is a member of the CPE family of cytoplasmic polyadenylation proteins responsible for control of protein translation [19-21]. This family of proteins has distinctive RNA recognition motifs and C-terminal zinc finger domains to allow specific interaction with U-rich mRNA elements [19-21]. Through binding of mRNA, CPEB2 stimulates the complete polyadenylation of immature mRNA and promotes the formation of the ribosome

by specifically recruiting eIF4F, one of the first components needed to initiate protein translation [19-21].

Research shows that CPEB2 exhibits substrate specificity, interacting with TWIST1 directly and HIF1 α via eEF2 [19,22,23]. Signaling factors TWIST1 and HIF1 α are frequently mutated in cancers, suggesting a correlation between these two observations. In addition, CPEB mRNAs appear to be downregulated in numerous tumor samples, suggesting that cancer cells lose their ability to regulate translation [19]. Hagele et al. researched the interconnectedness between CPEB2 and HIF1 α and determined that CPEB2 has an inverse relationship with the activation of HIF1 α [21]. Through its control of HIF1 α mRNA, CPEB2 may be involved in cancer malignancy, but this has not been experimentally confirmed [19]. Studies have shown organ specific patterns of CPE activation, suggesting tissue specific functionality of CPEB2 [21].

In addition to its downstream action, numerous groups have looked into alternative roles of methods of CPE activation and consequent effects. Research by Di Nardo et al. suggests that CPEB2 may also promote a polyadenylation function after stimulation from the mechanistic target of rapamycin (mTOR) pathway [24]. The mTOR pathway is naturally activated by cellular stresses such as hypoxia or insulin [21]. Other studies show that different phosphorylation pathways, including phosphoinositide 3-kinase GSK3, the aurora A kinase, and the CDC2 pathways are associated with activation of CPEB [25].

Wang et al. shows that CPEB2 has multiple isoforms due to the inclusion of particular exons in the mature mRNA [26]. These various isoforms have different functions based on their substrate specificity and complete 3-dimensional structure. The most common isoforms of CPEB2 are the A and B isoforms. As shown in Figure 1-5, they

differ only through the inclusion of exon 4, which is retained in CPEB2B. Preliminary data suggest that the B isoform of CPEB2 promotes metastatic activity in cells, which commonly produces an extremely aggressive cancerous tumor [27]. Through greater understanding of the alternative splicing action between the isoforms of CPEB2, we hope to be able to develop more effective patient treatments for the future.

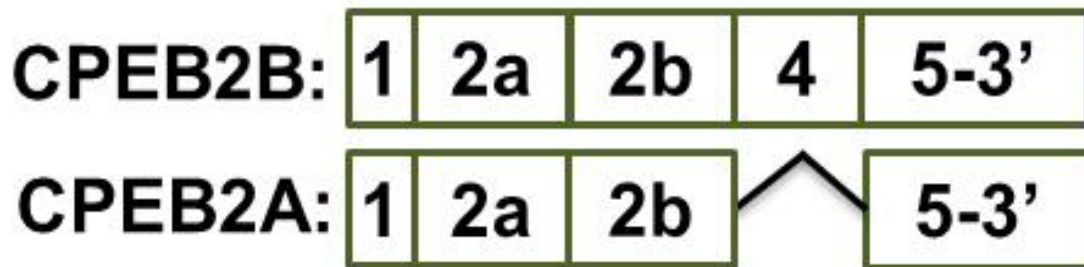


Figure 1-5. Alternative Splicing of CPEB2. The two most common isoforms of CPEB2 differ only by the inclusion of exon 4. The splicing factor that controls this interaction is not yet known.

CHAPTER 2

DETERMINATION OF CPEB2 SPLICING FACTOR

2.1 Introduction

While much research has gone into understanding the mechanisms that cause cancer and cancer metastasis, there are still many gaps in our knowledge. One of the stages in cancer metastasis is the development of anoikis resistance, which allows cells to travel through the vasculature of the body to colonize in other locations of the body. This is one of the events that make many cancers very difficult to treat via traditional surgical techniques. As a result, there is great clinical interest for any methods that reduce the ability of a tumor to develop anoikis resistance. One of the less explored ways to study metastasis is through investigating alternative splicing of signaling molecules. By gaining an understanding of how these upstream pathways act in cancer, one could alter splicing to prevent the development of cancer.

Studies by our laboratory have investigated the role of cytoplasmic polyadenylation element binding protein 2 (CPEB2) in promoting a metastatic phenotype in triple negative breast cancer. We have shown that the splicing of CPEB2 is altered in triple negative breast cancer patients to produce a higher abundance of the larger isoform (B) of CPEB2 [28]. Further research has shown that CPEB2B seems to promote cell growth when endogenously expressed in MDA MB 231 Par cells, while CPEB2A does not promote the same growth [28]. With further study, preventing CPEB2B splicing could be used as a very promising anti-metastatic cancer therapy. Due to the fact that CPEB2 has low basal expression in non-tumorigenic cells, it could be used as a targeted therapy to only affect

cancerous cells. This has great therapeutic potential and is therefore a worthwhile strategy for research.

2.2 Materials and Methods

2.2.1 Cell Culture

The lab acquired MDA MB 231 parental TNBC cells (231 Par) from ATCC. They were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (BioWhittaker) at 5% CO₂ and at 37°C. When the 231 Par cells reached 70% confluence, they were passaged to a maximum passage number of 9. MDA MB 231 anoikis resistant TNBC cells (231 AnR) were acquired by plating 231 Par cells on 10cm² culture dishes coated with 20 mg/mL poly(2-hydroxyethyl methacrylate) (polyhema) (Sigma-Aldrich) for at least 3 passages on polyhema-coated plates.

2.2.2 Proteomics Study

In order to identify splicing factors that regulate alternative splicing of CPEB2, we chose to investigate exon 4 interactions (the exon included in CPEB2B). First, we collected nuclear extracts from 231 AnR cells in standard culture conditions. Next, we added either specific or non-specific competitor sequences (Dharmacon) to the samples to act as loading controls. After that, we combined the samples with FITC-conjugated exon 4 of CPEB2 (Dharmacon). Next, the samples were run on a DNA polyacrylamide gel until bands were visible. We performed selective excision of the visible bands and

sent these samples to The Ohio State University proteomics core for analysis to determine interactions with known splicing factors. After analysis, we received a list of interacting proteins to the query region. Using online protein databases, we were able to determine which results were potential splicing factors to experimentally test.

2.2.3 siRNA Panel of Candidates

Candidate splicing factors as determined above were experimentally tested to observe their effect on CPEB2 alternative splicing. The lab purchased commercially available *Silencer* siRNA (Ambion by Life Technologies) for SRFS3, hnRNPA2B1, hnRNPF, hnRNPH1 and reconstituted to 20 μ M. 231 Par (2×10^5) were plated on 6 well tissue culture plates (Costar) in appropriate media as described previously. We added siRNA at a concentration of 25 nM in accordance to Dharmafect protocol (GE Healthcare). After 6 hours, cell media was replaced. We harvested cells after 48 hours to ensure maximal siRNA effect.

2.2.4 Western Blot Analysis

Quick-Start Bradford Reagent (Bio-Rad) was used to determine the protein concentration of the samples for consistent protein gel loading. We added 10 μ g of each sample to a 7.5% polyacrylamide gel (Bio-Rad) and run at 60 mV for 3 hours in 1X Tris/Glycine/SDS buffer (Bio-Rad). The gel was transferred to a PVDF membrane for 2 hours in transfer buffer (70 H₂O : 20 MeOH : 10 10X Tris/Glycine Buffer (Bio-Rad)). After transfer, the membranes were blocked in 5% milk for 30 minutes at room temperature and washed 3 times in wash buffer (1X PBS + 0.1% Tween-20) for 5 minutes each. Next,

the membranes were incubated in primary antibody diluted in 5% milk (Actin 1:8000, SRSF3 1:1000, CPEB2 1:1000) overnight at 4°C. The following morning, we washed the membranes 3 times in wash buffer (1X PBS + 0.1% Tween-20) for 5 minutes each and then incubated in the appropriate secondary antibody (Actin-Mouse 1:8000, SRSF3-Rabbit 1:1000, CPEB2-Rabbit 1:1000) for 1 hour at room temperature. Following secondary antibody incubation, the membranes were washed 3 times in wash buffer (1X PBS + 0.1% Tween-20) for 5 minutes each and then developed the membranes using SuperSignal Pico Developing Solution Reagents (Thermo) and imaged using a film developer.

2.3 Results

2.3.1 Proteomics Panel

After determination that alternative splicing of CPEB2 affects the metastatic potential of triple negative breast cancer cells, the next logical step was to determine what splicing factor(s) control(s) this action [27]. In order to do this, we ran samples on a DNA polyacrylamide gel as described in 2.2.2. We selectively excised distinct gel bands as shown in Figure 2-1. Results from The Ohio State University provided a list of proteins that bound to exon 4 of CPEB2 (the exon alternatively spliced between the A and B isoforms of CPEB2). This list provided numerous candidates that interacted with the region of interest. (Figure 2-1). We recognized many of these candidates as artifacts of the proteomics screen and discounted them. As a result, the remaining candidate factors to examine were SRSF3, hnRNP A2B1, hnRNP A0, hnRNP F, and hnRNP H1. All are

known splicing factors that have been shown to be dysregulated in cancer. These results directed our studies to investigate the specific roles of those factors in MDA MB 231 cell metastasis.

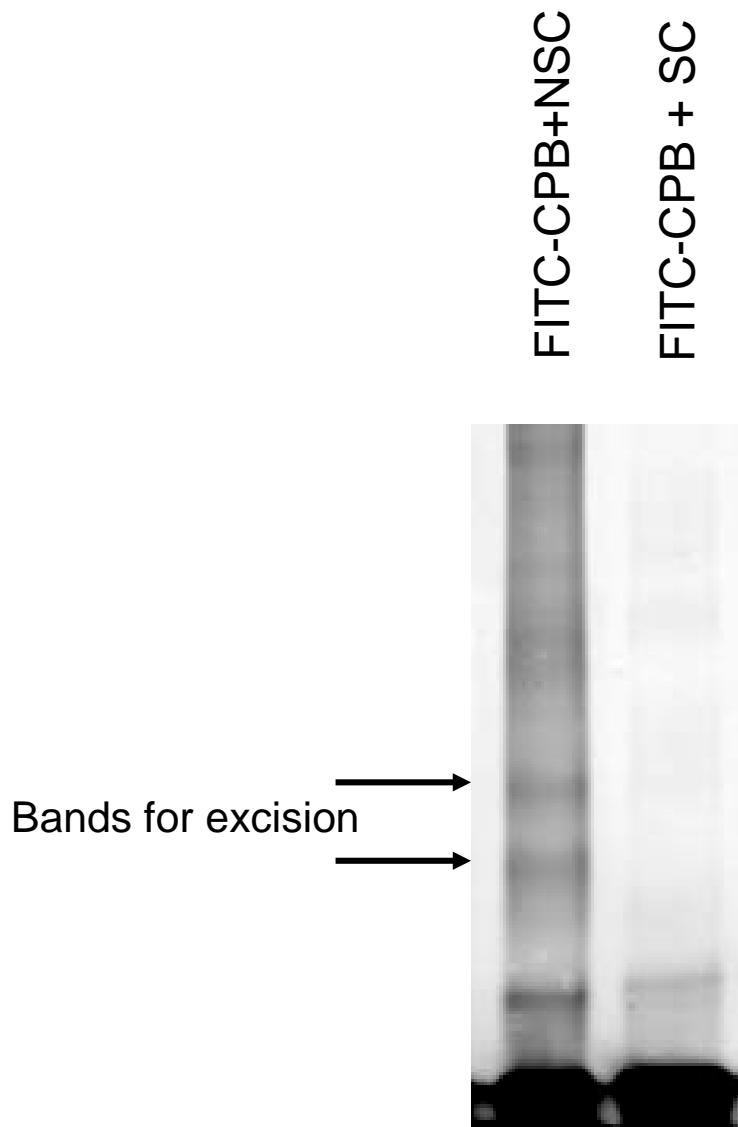


Figure 2-1. EMSA Allows Selective Excision of Exon 4 Binding Factors. Nuclear extracts from 231 AnR cells were added to FITC-conjugated exon 4 of CPEB2. The mixture was run on a DNA polyacrylamide gel until distinct bands were visible. Individual bands were excised and sent to The Ohio State University for proteomics analysis.

2.3.2 siRNA Panel of Candidates

To investigate the role of a particular splicing factor in alternative splicing of CPEB2, we chose to use siRNA to knockdown each of the potential splicing factors and observe the effect on the relative abundance of the splice variants of CPEB2. We used a siRNA cocktail specific to each candidate splicing factor as well as combining the siRNAs for hnRNP H1 and hnRNP F as they have been shown to react cooperatively in the literature. The results show a marked decrease in the B isoform of CPEB2 when treated with siRNA specific to SRSF3 while other siRNAs did not show a significant effect on CPEB2 splicing (Figure 2-2). Comparing the ratios of the two splice variants allows clear visualization of the effect of knocking down SRSF3 on CPEB2 alternative splicing. Use of the CPEB2 A:B ratio provides a means of distinguishing the alternative splicing of CPEB2 and allows quantification of the observation. As the relative abundance of the pro-growth isoform (B) decreases, the A:B ratio will increase. Samples with a high A:B ratio are less metastatic and easier to treat. These results suggest that SRSF3 mediates CPEB2 alternative splicing and that it causes an increase in the pro-survival B isoform of CPEB2.

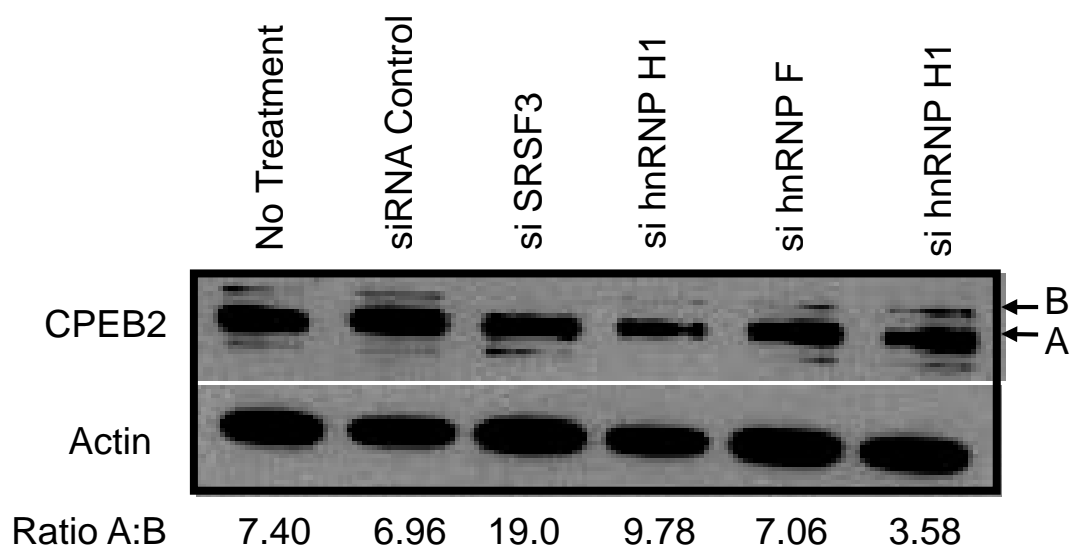


Figure 2-2. siRNA Panel of Alternative Splicing of CPEB2. 231 Par cells were treated with siRNA (25 nM) for 6 hours, then media was replaced. After 48 hours, the cells were collected and analyzed via western blot for isoforms of CPEB2. Densitometry was performed on the A and B isoforms of CPEB2 as indicated with arrows. The ratio of the densitometry results were calculated for each sample.

2.3.3 Western Blot Analysis

After completion of the siRNA screen, we chose to verify that reduction of SRSF3 causes a change in alternative splicing in both 231 Par and 231 AnR cells. As shown in Figure 2-3, treatment with siRNA for SRSF3 reduces the amount of the B isoform and consequently increases the A:B ratio of those samples. One can also see that the 231 Par cells basally have a higher A:B ratio than 231 AnR cells. The differences are more pronounced in the 231 Par because of their naturally lower levels of the B isoform. This reflects the metastatic nature of the 231 AnR cell line.

We also chose to examine the levels of SRSF3 expressed in 231 Par and 231 AnR cells to see if the more metastatic nature of 231 AnR cells correlated with SRSF3 levels. Figure 2-4 shows that 231 AnR cells overexpress SRSF3 compared to 231 Par. Additionally, treatment with siSRSF3 causes significant reduction in SRSF3 in both cell lines, restoring 231 AnR SRSF3 levels to that of basal 231 Par cells. This suggests that alteration of SRSF3 occurred during the acquisition of anoikis resistance in these cell lines.

In order to more effectively study the effects of SRSF3 knockdown, we looked to optimize knockdown of SRSF3 to provide the most dramatic effects to the cells. We took the components of the purchased siRNA cocktail and tested each of the 3 siRNAs individually to determine which provided the greatest effect on reducing SRSF3 expression. We were able to quantify this knockdown using densitometry to compare the relative intensity of SRSF3 to Actin. As shown in Figure 2-5, the second and third siRNA components provided the greatest reduction in SRSF3 expression and consequently the

lowest SRSF3:Actin ratio. As a result, future experiments used a combination of siRNAs 2 and 3 to knockdown SRSF3.

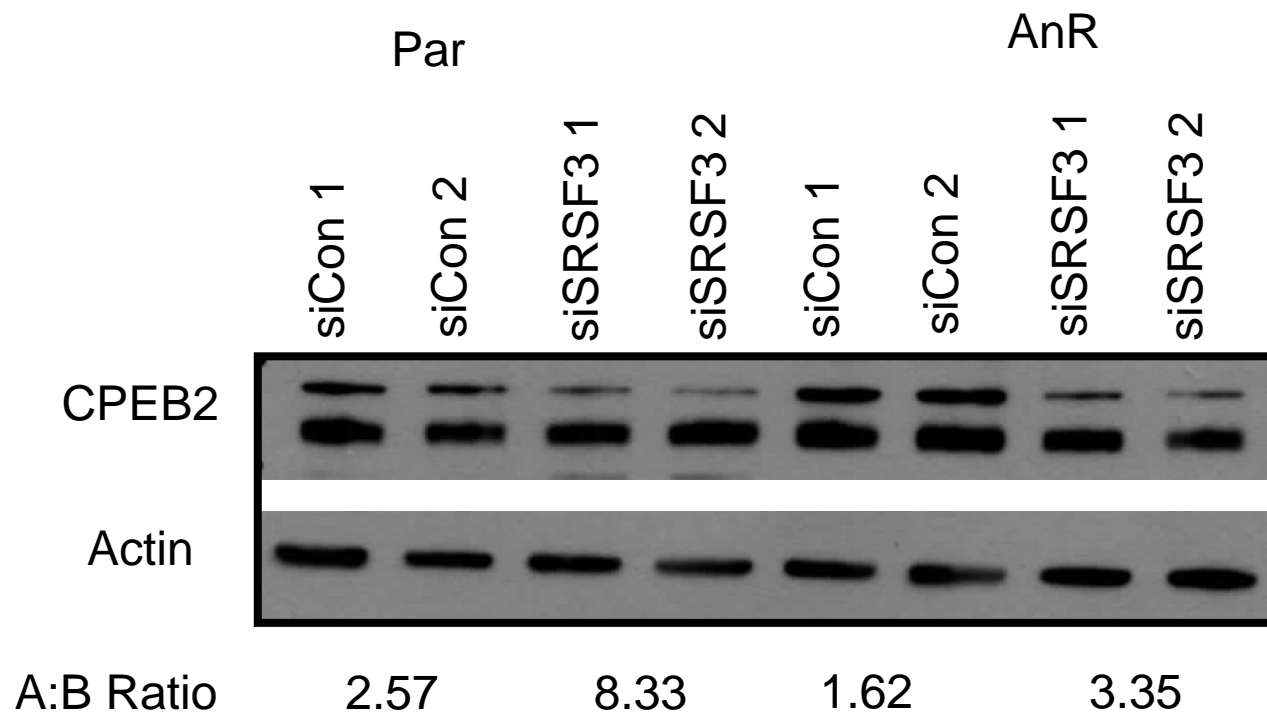


Figure 2-3. Knockdown of SRSF3 Causes Decrease in CPEB2B. 231 Par and 231 AnR cells were treated with siRNA (25 nM) for 6 hours, then media was replaced. After 48 hours, the cells were collected and analyzed via western blot for isoforms of CPEB2. Densitometry was performed on the A and B isoforms of CPEB2. A:B ratios were performed for each sample and averaged within treatment groups.

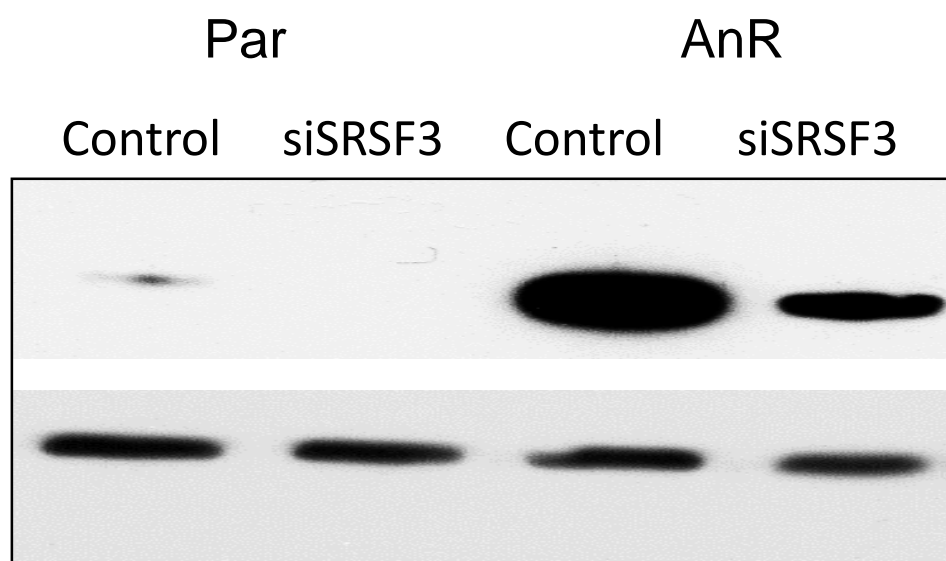


Figure 2-4. SRSF3 is Upregulated in Anoikis Resistant Cells. 231 Par and 231 AnR cells were treated with siRNA (25 nM) for 6 hours, then media was replaced. After 48 hours, the cells were collected and analyzed via western blot for knockdown of SRSF3.

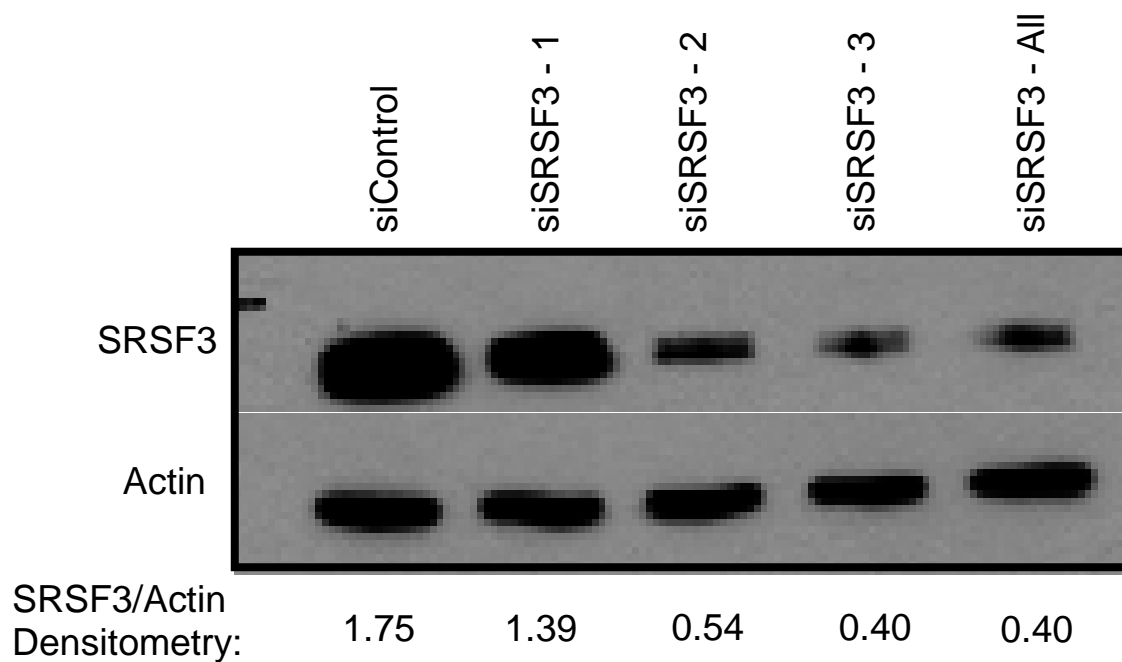


Figure 2-5. Selective Components of siSRSF3 Promote Greatest SRSF3 Reduction. 231 Par cells were treated with individual siRNA components (25 nM) for 6 hours, then media was replaced. After 48 hours, the cells were collected and analyzed via western blot for knockdown of SRSF3.

2.4 Discussion

Analysis of our results provides some important insights into the mechanisms that are altered in triple negative breast cancer. From a panel of potential candidate splicing factors, we determined that SRSF3 not only affected the alternative splicing of CPEB2, but also promoted the expression of CPEB2B, the more metastatic isoform of the protein. This was further supported by the observation that anoikis resistant cells express higher levels of SRSF3. These findings suggest that SRSF3 plays an important role in metastatic behavior of tumors. Our initial findings provide us with potential future directions to continue to investigate. If proven as a viable mechanism, therapies altering the action of SRSF3, and therefore CPEB2 could provide an effective alternative or complement to existing cancer treatments.

Initially, the observation that CPEB2 alternative splicing is altered in cancer provided a direction for investigation. Now, with the understanding that SRSF3 controls this interaction, there are even more possible directions to investigate. The most important next step is to determine if reduction in SRSF3 expression translates to a measurable difference in a metastatic phenotype. We can measure this in a number of ways, by either analyzing cell growth or resistance to apoptosis. We could measure cell growth using a proliferation assay or by measuring cell doubling rates in culture. After promoting apoptosis, methods to measure cell resistance to apoptosis include: Western Blot expression of apoptotic proteins such as caspase 3, caspase 8, cleaved PARP, and cytoplasmic cytochrome c; flow cytometry sorting via Annexin-V and 7-AAD; luciferin fluorescence assays; or post-apoptosis colonization assays.

CHAPTER 3

MEASURING METASTATIC EFFECT OF SRSF3

3.1 Introduction

After observing that SRSF3 plays a role in CPEB2 alternative splicing in TNBC, the next step is to see if its reduction causes larger scale changes in cell functionality. As described earlier, there are a number of methods to investigate cellular functionality without use of an *in vivo* model. Lack of an *in vivo* model allows one to acquire results quickly and often without the complications that can arise by using an animal model. Therefore, we chose to use flow cytometry and western blot assays to measure the metastatic function of cells with reduced SRSF3.

3.2 Materials and Methods

3.2.1 Cell culture

We acquired MDA MB 231 parental TNBC cells (231 Par) from ATCC. They were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (BioWhittaker) at 5% CO₂ and at 37°C. When the 231 Par cells reached 70% confluence, they were passaged to a maximum passage number of 9. We acquired MDA MB 231 anoikis resistant TNBC cells (231 AnR) by plating 231 Par cells on 10cm² culture dishes coated with 20 mg/mL poly(2-hydroxyethyl methacrylate) (polyhema) (Sigma-Aldrich) for at least 3 passages on polyhema-coated plates.

3.2.2 Flow cytometry anoikis resistance assay

To measure the effect of SRSF3 reduction on TNBC cell resistance to anoikis, we plated 1.5×10^5 231 Par, 231 AnR, 231 pcDNA, or 231 CPB on 6-well tissue culture plates (Costar). After 24 hours, cells were treated with siRNA targeting nonsense sequences (siCon) or SRSF3 (siSRSF3) for 6 hours, after which we replaced the media. After an additional 24 hours, we replated these cells on 24-well tissue culture plates (Costar) that were either nontreated (NT) or coated with polyhema (PH). The cells grew overnight (12 hours) and then both the cells and media were collected for analysis.

In order to analyze cell death via flow cytometry, we resuspended and washed the cells in a 1X Binding Buffer (eBioscience). Next, the pellet was resuspended in staining buffer (1X Binding Buffer, 7-AAD, and Annexin-V). We let the cells sit covered on ice for 15 minutes. Following staining, the staining reaction was neutralized by adding additional 1X Binding Buffer. While on ice, we brought the samples to the VCU Flow Cytometry Core. Samples were gated by Forward and Side Scatter detectors, then grouped them into regions based on the 7-AAD and Annexin-V signals. Samples were run in triplicate and statistically analyzed using ANOVA.

3.3 Results

3.3.1 Flow Cytometry Anoikis Resistance Assay

The first method that we used to analyze the effects of SRSF3 knockdown in TNBC cells was to determine the portions of the cell population that were undergoing apoptosis after treatment on polyhema-coated plates (Figure 3-1). This technique causes normal cells to die due to anoikis (detachment-induced cell death). Cells that have undergone mutation promoting metastasis will not appear positive for 7-AAD or Annexin-V due to their developed resistance to anoikis.

7-AAD is a fluorescent dye that binds to double-stranded DNA [28]. When cells undergo late apoptosis the plasma membrane starts to fall apart, resulting in the release of usually contained double-stranded DNA. Annexin-V is a fluorescent dye that binds to phosphatidylserine, which are usually located on the cytosolic side of the plasma membrane due to enzymatic flippase activity [28]. However, when cells undergo apoptosis, they cease flippase activity, resulting in the extracellular presentation of phosphatidylserine. Both signals are indicative of apoptotic cells and can act as a measure of cell viability. Thus, by gating for 7-AAD and Annexin-V signal, we can count the percentage of the cell population that is undergoing apoptosis (Figure 3-2).

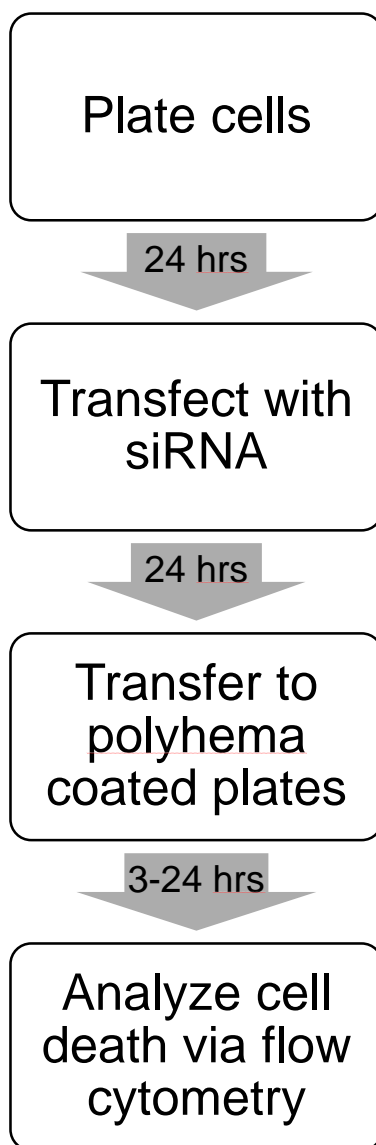


Figure 3-1. Metastatic Effect Experimental Workflow. In order to properly study cell resistance to apoptosis, we developed a method to accurately measure the influence of SRSF3 on anoikis. As shown in the schematic above, 231 Par or 231 AnR cells are plated on a 6 well plate (2×10^5 cells/well). After 24 hours, the cells are transfected with siRNA (25 nM) for 6 hours, and then media was replaced. After another 24 hours, the cells are transferred to polyhema-coated plates to stimulate apoptosis. Depending on the state of apoptosis to study, cells can be collected after 3-6 hours (early apoptosis) or after 18-24 hours (late apoptosis).

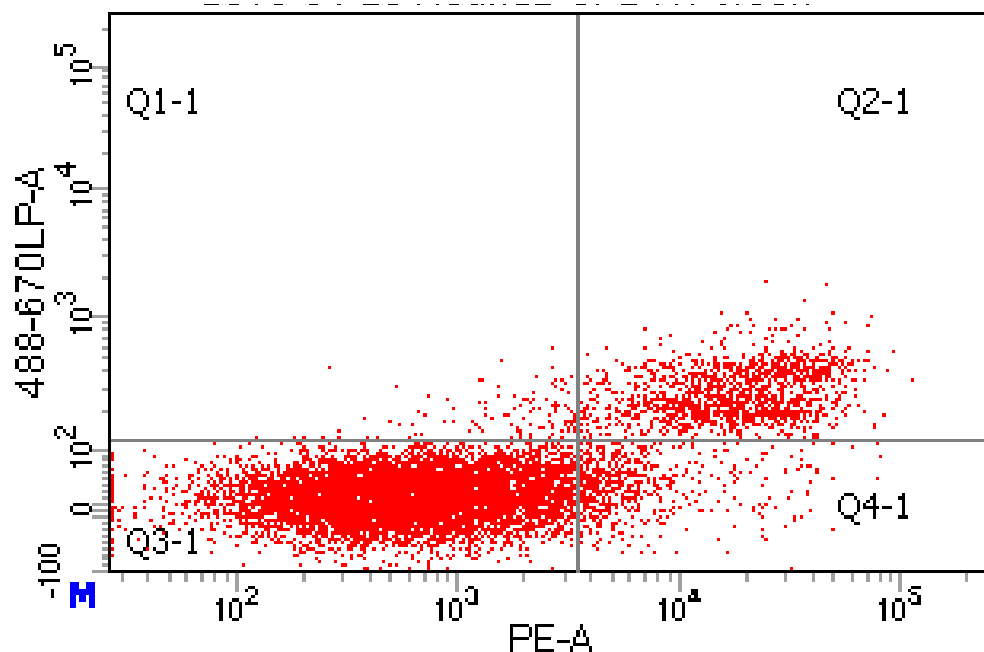


Figure 3-2. Sample Flow Cytometry Plot. After collection of a cell population, flow cytometry analysis is performed to quantify the proportion of the population that expresses particular fluorescent markers. The x-axis represents Annexin-V, an indicator of early apoptosis. The y-axis represents 7-AAD, an indicator of late apoptosis/necrosis. The gates were set to distinguish between apoptotic cells (Q2 and Q3) and living cells (Q3). Comparison of population proportions indicate resistance to apoptosis.

Following the protocol as described in section 3.2.3, we first looked at 231 Par and 231 AnR cells treated with control siRNA (siCon) or targeted siRNA (siSRSF3). Comparing the apoptotic cell populations between the treatment conditions showed a few notable things (Figure 3-3). First, 231 Par cells showed significantly higher basal levels of apoptosis when plated on polyhema-coated plates. This was expected, as part of the transformation process to create 231 AnR cells involves growth on polyhema-coated plates. Additionally, we observed that knockdown of SRSF3 increased the amount of cell death in both cell lines. This suggests that SRSF3 does account for some of the resistance to anoikis in TNBC cells. Furthermore, the 231 AnR cells anoikis sensitivity was restored to that of the pre-transformed 231 Par cell line with siSRSF3 treatment. This suggests that alteration of SRSF3 may have been one of the mutations acquire to produce the 231 AnR cell line initially.

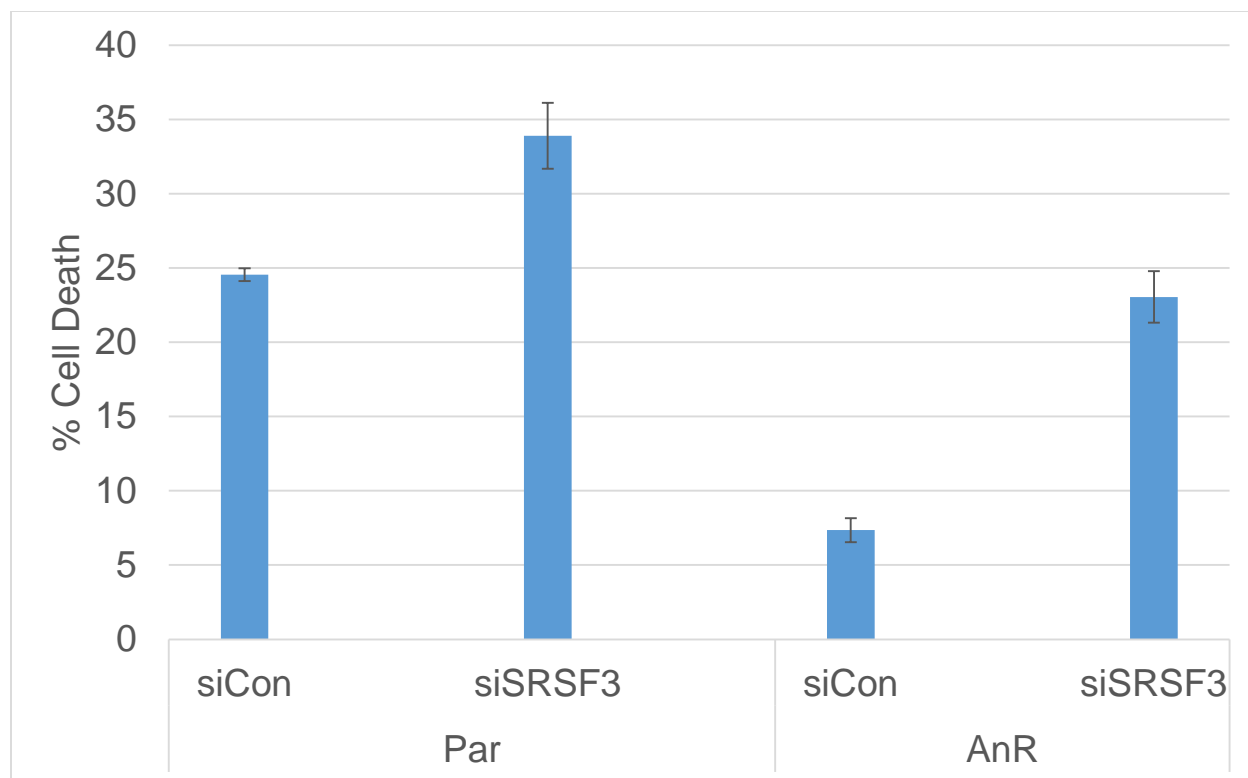


Figure 3-3. Reduction of SRSF3 Causes Increased Sensitivity to Anoikis. We graphed the cells counted via flow cytometry and compared experimental groups. As shown in Figure 3-2, we used the 7-AAD and Annexin-V markers for apoptosis to gate populations with sufficient signal. Populations above the gated threshold for Annexin-V and 7-AAD were considered apoptotic and counted for the purpose of these data. Data shown are representative of $n = 3$. Error bars indicate 1/2 standard deviation.

3.4 Discussion

Looking into the effects of knocking down SRSF3 in metastatic TNBC cells provided us with some very interesting insights into signaling pathways altered in cancer. As determined previously, SRSF3 does influence the alternative splicing of CPEB2. However, this observation is not clinically useful unless it can be utilized as a drug target for potential patient treatment. To determine that, we used siRNA to knock down SRSF3 in cellular models of TNBC. Through use of numerous methods, we measured the metastatic function of the cells when modulating levels of SRSF3. Our results showed that reduction of SRSF3 causes an increase in cellular sensitivity to apoptosis. Additionally, cells with greater resistance to apoptosis tend to overexpress SRSF3. These observations suggest that SRSF3 is critical in cellular resistance to anoikis.

Our data suggest that this anoikis resistance was developed through alternative splicing of CPEB2 into the more metastatic isoform, CPEB2B. Since endogenous expression of CPEB2B did not influence anoikis resistance in our experiments, we hypothesize that SRSF3 controls this action by promoting inclusion of exon 4 of CPEB2. This allows CPEB2B to activate cellular signaling that promotes enhanced cellular growth and inhibit apoptotic signaling. The mechanism through which this occurs is not yet known, but is a future area of investigation.

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Literature Cited

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Vita

Brian Patrick Griffin was born June 29, 1991 in Alexandria, Virginia and is an American citizen. He graduated from Thomas A. Edison High School in 2009, and subsequently moved to Charlottesville, VA to attend the University of Virginia. During his time at UVA, Brian was active in a variety of organizations including the Cavalier Marching Band, Kappa Kappa Psi (Honorary Music Service Fraternity), and the Department of Biomedical Engineering. During the summer of 2012, he participated in the Discover for the Cure internship program with Yale University. In May 2013, he graduated with a Bachelor of Science degree in Biomedical Engineering.

After graduation, Brian was admitted to the M.S. Program in Molecular Biology and Genetics at VCU in the fall of 2013. While at VCU, Brian has traveled to the ASBMB conference in Boston, MA to learn and observe peers in the field. Additionally, Brian has professionally presented his research to his professors and colleagues in the department multiple times.