THE ROLE OF TUMOR MICROENVIRONMENT IN THE PROGRESSION OF PROSTATE CANCER

A Senior Scholars Thesis

by

MOZHDEH MAHDAVI

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Genetics

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Approved by:

Research Advisor: Suma Datta
Associate Dean of Undergraduate Research: Robert Webb

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ABSTRACT

The Role of Tumor Microenvironment in the Progression of Prostate Cancer.
(April 2010)

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My research aims to investigate how reactive oxygen species(ROS) induced transcription factors NFKB, ATF-2 and RUNX-2 affect expression of 2OST. 2OST is a sulfotransferase responsible for sulfation of the heparan sugar chain . Previous unpublished research by our lab has made evident 2OST's essential role in the progression of prostate cancer. My lab noted a four –fold increase in the transcription of 2OST between benign LNCAP cells and highly metastatic C42B cells; knocking out 2OST made cells less metastatic. Furthermore, the research noted increased transcription of 2OST when the cell encountered hypoxic conditions. As prostate cancer progresses dividing epithelial cells form a solid tumor, leaving the cells in the innermost region of the tumor with a lack of oxygen and nutrients. HIF1a is a transcription factor induced in response to a lack of oxygen. We have shown that in conjunction with its heterodimer HIF1b,that HIF1abinds to the 2OST promoter and turns on transcription of 2OST. This suggests that 2OST is turned on in response to

hypoxic stress. ROS are a type of oxidative stress that we hypothesize will induce a similar 2OST response. Functioning on the assumption that the cell will react similarly to an equally stressful condition, I am investigating the effect of ROS induced transcription factors on 2OST. Since 2OST is essential for the progression of prostate cancer, it is now key to understand the factors which contribute to its over expression. This understanding will help halt the progression of the disease as well as similar cancers.

DEDICATION

To Sam Mahdavi, Mariam Mahdavi and Bahareh Mahdavi for all their encouragement, love and support.

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Thank you to Dr. Suma Datta for her mentorship, guidance and the opportunity to be a member of the lab. I would also like to thank Mr. Brent Ferguson for his help and patience and advice.

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CHAPTER I

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men. The American Cancer Society estimates that192,280 new cases of prostate cancer will be diagnosed and 27,360 deaths will occur from prostate cancer in the United States in 2009.One man in 6 will get prostate cancer during his lifetime. And one man in 35 will die of this

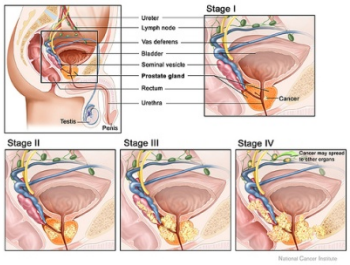


Figure 1- Progression of Prostate Cancer

disease. The role of tumor microenvironment is essential to the progression of prostate cancer (see Figure 1).

As prostate cancer progresses, dividing epithelial cells form a solid tumor, leaving the cells in the innermost region of the tumor with a lack of oxygen and nutrients. HIF1a is a transcription factor induced in response to a lack of oxygen and, in conjunction with its heterodimerHIF1b, it binds to the 2OST promoter and turns on transcription of 2OST [1]. 2OST is a sulfotransferase responsible for sulfation of the heparin sugar chain.

This thesis follows the style of the Journal of the American Medical Association.

Research by the Datta lab noted increased transcription of 2OST when the cell encountered hypoxic conditions [1] (see Figure 2). This suggests that 2OST is turned on in response to hypoxic stress.

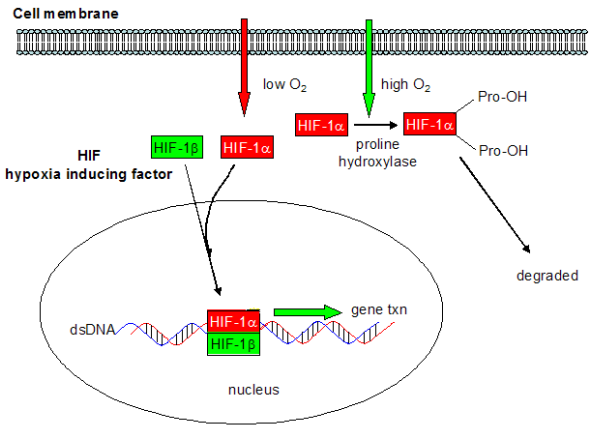


Figure 2- HIF1 Heterodimer

2OST has been shown to be an essential player in the induction of metastatic behavior in cancer cells. Previous unpublished research by the Datta lab has made evident 2OST's essential role in the progression of prostate cancer. The lab noted a four –fold increase in the transcription of 2OST between benign LNCAP cells and highly

metastatic C42B cells; knocking out 2OST made cells less metastatic (see Figure 3).

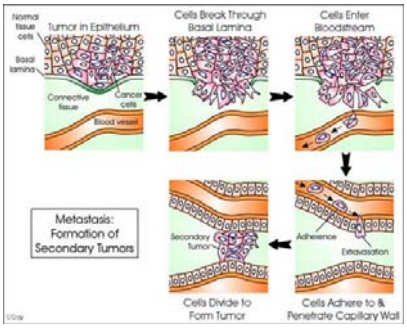


Figure 3- Onset of Metastasis

Reactive Oxygen Species(ROS) are a type of oxidative stress that I hypothesize will induce a similar 2OST response. Reactive oxygen species (ROS) and the coupled oxidative stress have been associated with tumor formation. [2] It has been suggested that elimination of excessive ROS by chemical or antioxidants may decrease the metastasis of various types of cancer…there is a growing body of evidence suggesting a role for oxidative stress in the pathogenesis of prostate cancer. Prostate may be particularly vulnerable to oxidative stress because androgen activity may alter the pro-oxidant–antioxidant balance of prostate cells. [2] Functioning on the assumption that the cell will react similarly to an equally stressful condition, I am investigating the effect of ROS induced transcription factors Runx-2, NF-KB and AT-F2 on 2OST. Research by Gasparian et. al notes NF-KB is constitutively activated in human androgen-independent Prostate cancer cells and suggests blockage of NF-KB activity in carcinoma cell lines results in arrested growth .[3] It is important that NF-KBalso plays a

key role in cell protection against diverse apoptoticstimuli including chemotherapeutic drugs and -irradiation throughactivation of the anti-apoptotic gene program in cells. [4] Results indicate that NF-KB isconstitutively activated in human androgen-independent PC cells. [3] Barnes et al. reports metastatic breast cancer cells, which are similar in nature to prostate cancer cells, express the transcription factor Runx2, which may regulate multiple factors in metastatic breast cancer cells involved in tumor-bone cell interactions.Runx2 expression in breast cancer cells provides a molecular phenotype that enables the interactions between tumor cells and the bone microenvironment that lead to osteolytic disease. Runx2 activity is required for both the response of tumor cells to the bone environment and the influence of tumor cells on bone cell differentiation .[4]ATF-2 is another transcription factor that has been shown to turn on transcription of 2OST, and whose links to the progression of prostate cancer look promising. [1]Ultimately I aim to uncover what causes up regulation of 2OST and the progression of prostate cancer. My research aims to address two specific issues. Firstly, what are the effects of ROS induced transcription factors, NFKB, ATF-2 and RUNX-2, on transcription of 2OST? How will knockdown of these specific transcription factors affect 2OST levels in the highly metastatic C4-2B cells? And secondly, do these three transcription factors bind directly to the 2OST promoter? Does knockout of each respective binding site change expression of 2OST? Since 2OST is essential for the progression of prostate cancer, it is now key to understand the factors which contribute to its over expression. This understanding will help halt the progression of the disease as well as similar cancers.

CHAPTER II

MATERIALS AND METHODS

The procedure is based around two distinct experiments, the RNAi procedure and the site-directed mutagenesis experiment. Both experiments were done simultaneously.

RNAi experiment

Highly metastatic C4-2B cells were plated and allowed to grow for 2-3 days and then split equally into 3 wells. The RNAi mixture was prepared by mixing 50 microliters of lipofectamine to 1 mL of optimem and letting it sit for 5 min. That mixture was then added to a mixture consisting of 40 microliters of RNAi and 1mL of optimem and allowed to sit for 20 minutes. 600 microliters of the RNAi solution is added to each of the three wells. The solution is left to incubate for 24 hours. After24 hours, the RNAi solution is removed from and the cells are harvested and spun down. The RNA is isolated and collected by using the RNAeasy mini-kit. The RNA was then reverse transcribed and a real time PCR was set up.

Real time PCR

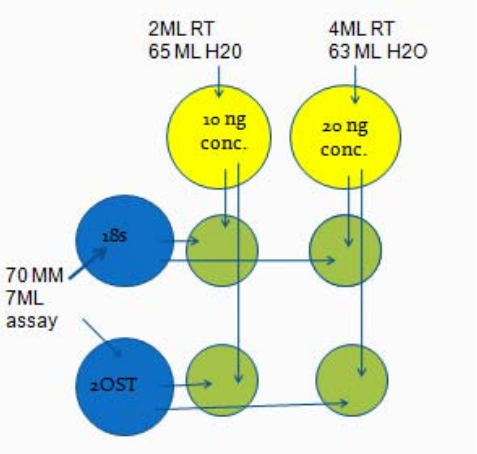


Figure 4-Real Time PCR Setup

A 10 ng and a 20 ng concentrations of the RT reaction product. concentration real time PCR was set up. The 10ng concentration contained 2 microliters of RT and 65 microliters of water. The 20 ng concentration contained 4 microliters and 63 microliters of water. The real time was set up according to Figure 4.

The criteria for analyzing the results obtained from the PCR will be explained in the Results section.

Deletion constructs of transcription factor binding sites

Primers were designed to PCR up the 2OST promoter, spanning 2830 base pairs(from - to +380).(The 2OST promoter is PCRedusing DNA extracted from Pro4 prostate cancer cells. The PCR product is run on a gel to confirm it is the correct size piece. The band is then extracted from the gel and more product is PCRed up off the band. The PCR product was also sent to be sequenced to ensure the sequence of the isolated

fragment is a match to the 2OST promoter sequence data provided by the Human Genome Project.

Cloning the promoter into a vector

The 2OST promoter is cloned into the vector containing a gene for ampicillin resistance as well as a LacZ reporter gene to track the expression of 2OST (see Figure 5).

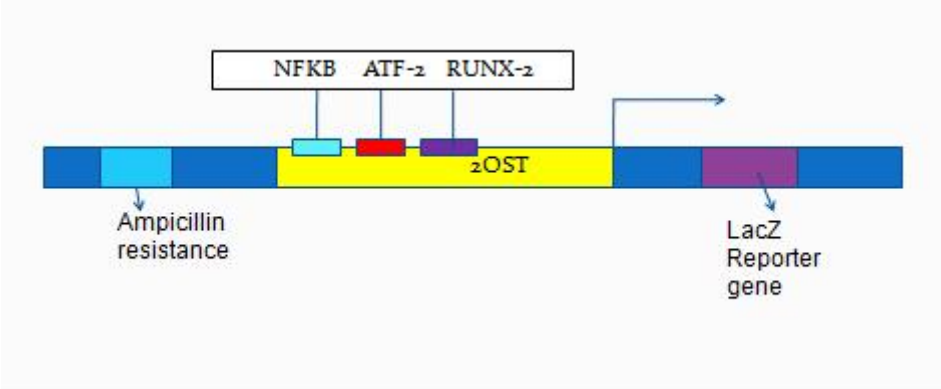


Figure 5- Representation of Vector with 2OST Promoter

Topo-cloning reaction

Three microliters of the promoter PCR product, and 1 microliter of each the Topo-cloning vector Salt Solution and dH2O were combined and left at room temperature for 10 minutes.

Transformation reaction

2 Microliters of the topo-cloning mixture were added to the One-Shot Topo Chemically competent E. Coli and the solution was left to incubate on ice for 10 minutes. The solution was then plated on LB broth plates and left for 24 hours.

Mini-prep

After 24 Hours, colonies were collected from the LB plates and placed in a liquid culture designed to lyse the cells.. The liquid culture was left for 16 hours, the DNA was collected and run on a gel to check for the insertion (see Figure 6).

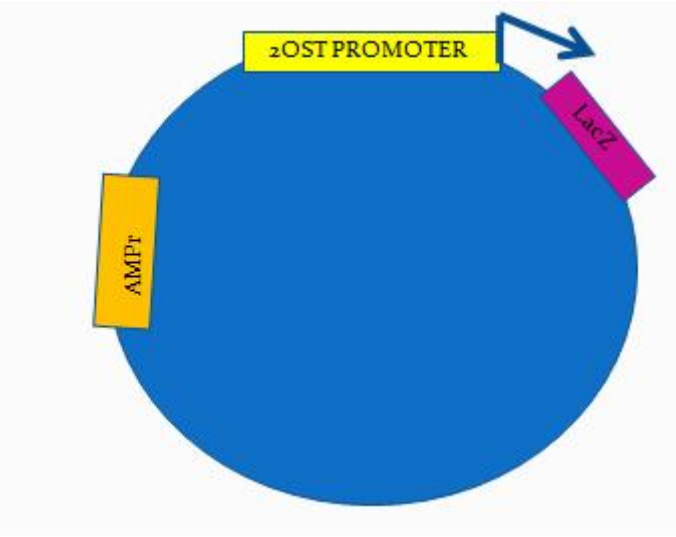


Figure 6- Vector with Insert

Enzyme digest

Once we were certain that the vector contained the promoter insert an enzyme digest was carried out to ensure the insert was in the correct orientation. The 2OST promoter needed to be in the correct orientation to drive expression of the LacZ reporter gene. Using NotI, an enzyme with sites in both the promoter and the vector we were able to predict a clear band pattern to be expected from the vector we needed. Digest with NotI should yield bands of 3258 and 6965 base pairs (see Figure 7).

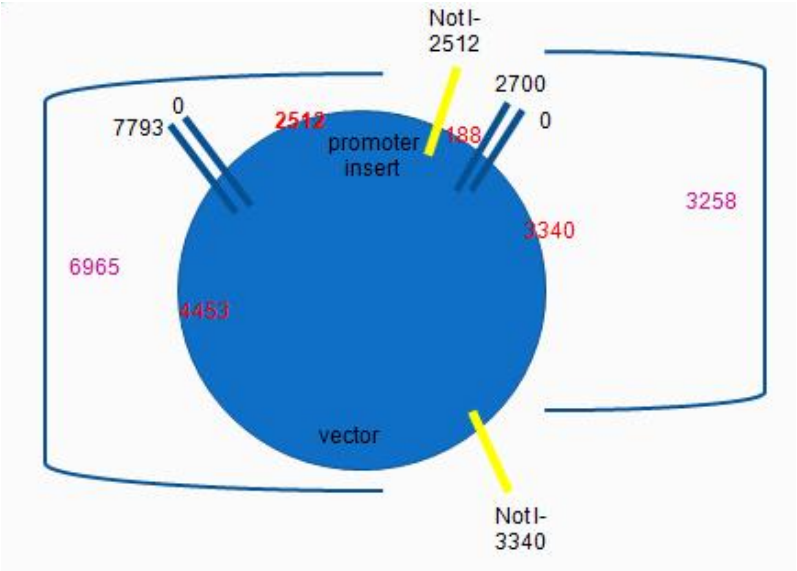


Figure 7- Not I Enzyme Digest Banding Pattern

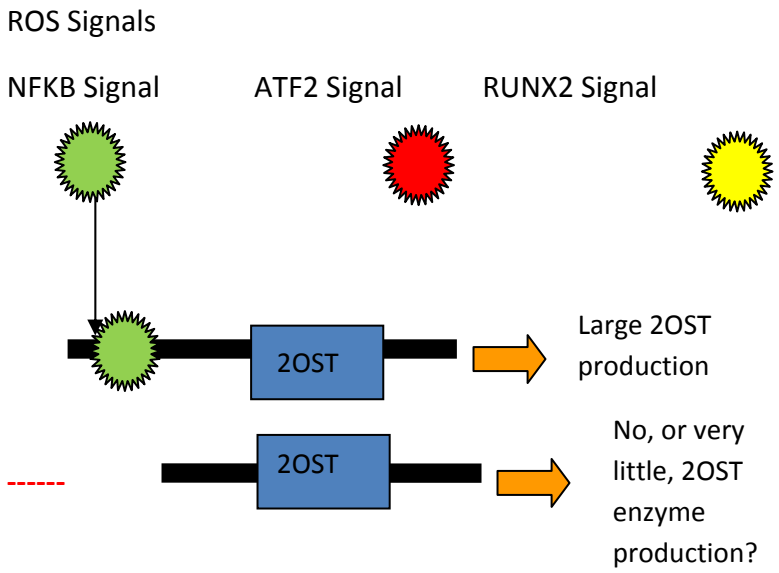
Sequencing of the construct confirmed insertion of the promoter.

Deletion constructs

Once the clone construct was complete, various combinations of deletion constructs were created by either mutating transcription factor binding sites or deleting them entirely (see Figure 8).

6 Total Deletion Constructs.

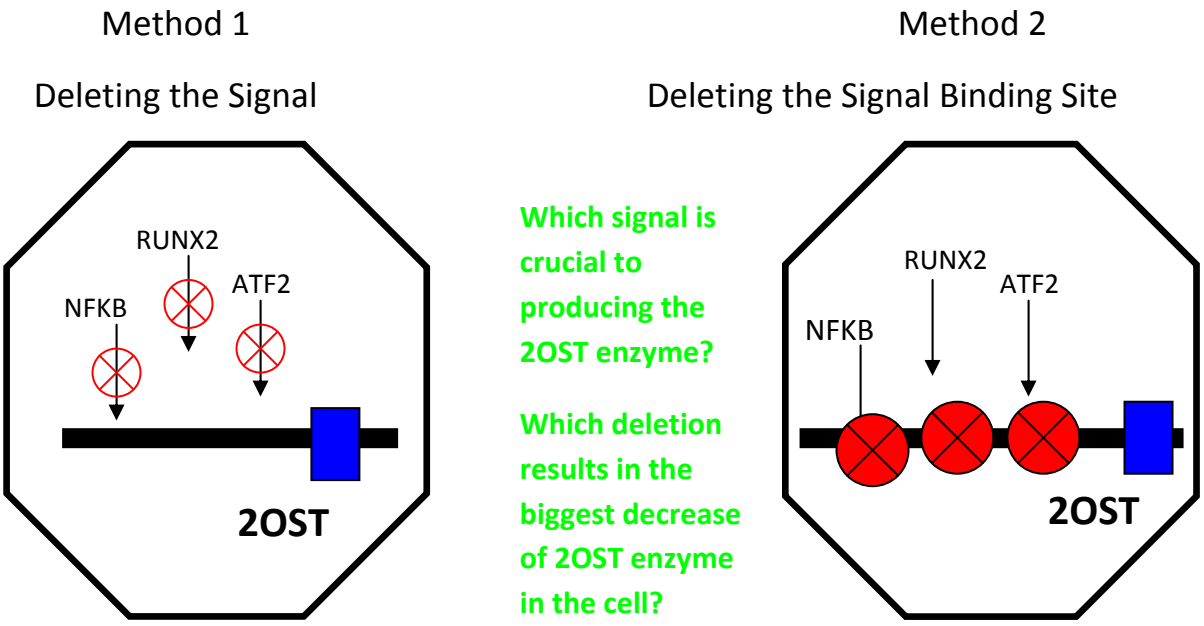
1. 2830 bp (full length promoter)
2. 2427 bp
3. 1930 bp
4. 1439 bp
5. 916 bp
6. 380 bp



If the signal is able to bind, a large amount of the enzyme should be present in the cells but if the signal is unable to bind because the binding site is either deleted or destroyed or the signal is simply not in the cell there will be a distinct decrease in the amount of enzyme present. So the question is, which of these signals plays the biggest part? **Which of these binding site deletions results in the biggest decrease in the amount of 2OST enzyme?**

Figure 8- Deletion Constructs

My research aims to investigate at the molecular level how hypoxic stress promotes production of 2OST, ultimately initiating metastasis and driving prostate cancer to the point of lethality. This was done in two ways, by eliminating candidate transcription factors by RNA interference and secondly by eliminating the transcription factor binding sites in the 2OST promoter (see Figure 9).



After deleting each of the signals the amount of 2OST enzyme in the cell is measured to determine whether the deletion had a significant effect. Is this signal absolutely necessary to the production of 2OST?

Figure 9- Overview of the Experiment

CHAPTER III

RESULTS

Deletion constructs

Before various deletions could be designed the 2OST promoter had to be isolated from Pro4 cell lines.

Figure 10 shows the gel picture of the isolated 2OST promoter.

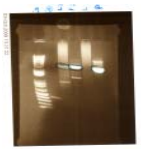


Figure 10- Isolated 2OST Promoter from LNCAp and Pro4 Cells

The 2OST PCR product was sent for sequencing and the results returned a 96% match to the 2OST promoter sequence.

Primers were then designed to create various 500 base pair deletions from the 2OST promoter. The deletion constructs were of the following lengths:

2830 bp(full length promoter)

2427 bp

1930 bp

1439 bp

916 bp

380 bp

Figure 11 is a PCR of check of each of the deletion constructs.

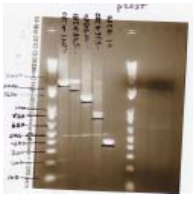


Figure 11- Gel Picture of the PCR Check of Deletion Constructs

Beta-Gal assays were performed on the full length promoter as well as the 1439bp deletion construct (see Figure 11). Absorbencies were measured at 625nm and activity was graphed (see Table 1) and RNAi real time results are shown in Table 2.

TABLE 1: Beta Gal Assay

Control			Full-length (1B)			4C		
1ul	5ul	10ul	1ul	5ul	10ul	1ul	5ul	10ul
0	0.105	0.257	0.031	0.292	0.526	0	0.042	0.484
0	0.215	0.302	0.028	0.389	0.525	0	0.286	0.513
0	0.16	0.2795	0.0295	0.3405	0.5255	0	0.164	0.4985
	0.077782	0.03182	0.002121	0.068589	0.000707		0.172534	0.020506

specific activity = nmoles of ONPG/mg protein

CONTROL	FULL-LENGTH 1B	4C
1.817886	8.758333	11.55942

Control	Full-length	4C
1.817886	8.758333	11.55942
0.03182	0.000707	0.020506

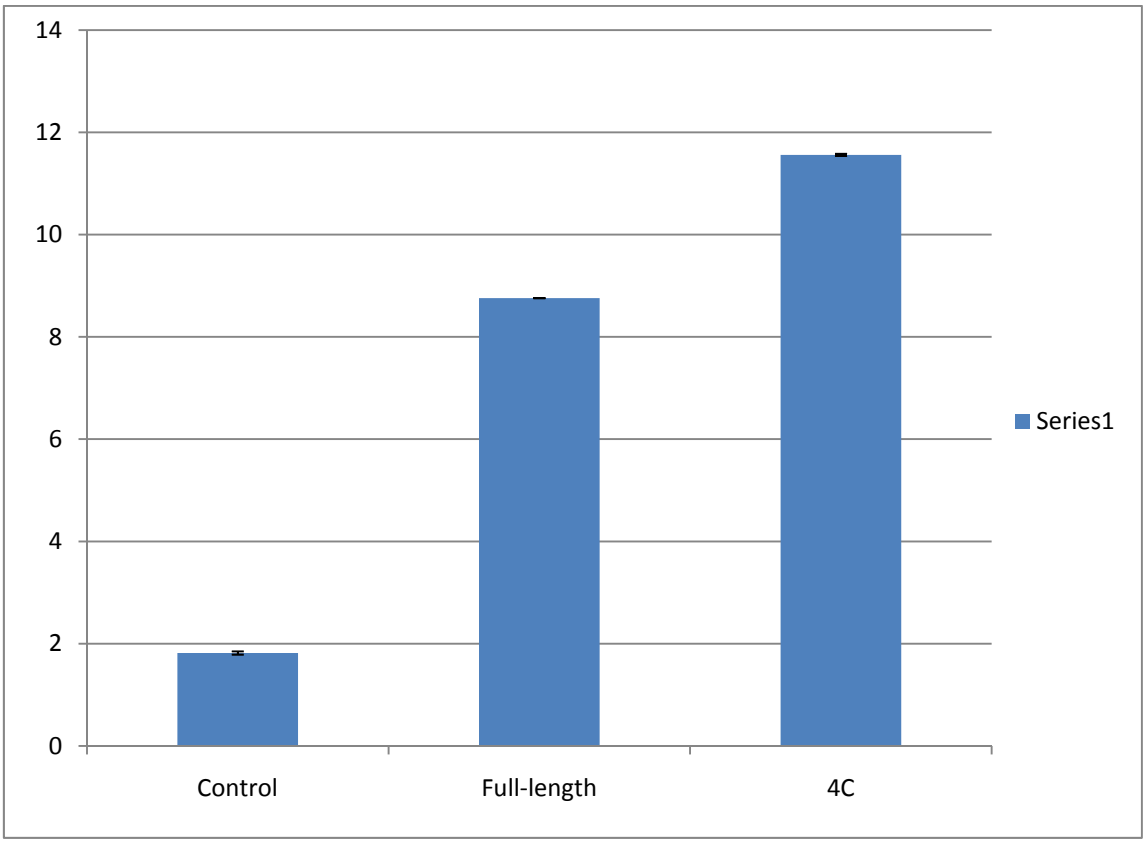


Figure 12- Beta gal Assay. Lac Z Expression in Full Length vs.1439bp(4C) Construct

RNAi real tme results

TABLE 2: RNAi Real Time Results

Control

	Average Ct Value	Standard Deviation	DeltaCt
18S	8.16	0.12	
2OST	24.50	0.11	16.34

NFkB RNAi

	Average Ct Value	Standard Deviation	DeltaCt
18S	6.24	0.22	
2OST	25.20	0.03	18.96

ATF2 RNAi

	Average Ct Value	Standard Deviation	DeltaCt
18S	6.66	0.15	
2OST	24.95	0.19	18.29

Runx2

	Average Ct Value	Standard Deviation	Delta Ct

TABLE 2
CONTINUED

18S	8.58	0.34	
2OST	26.48	0.10	17.90

Normalization of 2OST Ct Values to Control

	control	NFkB RNAi	stdev1	stdev2
2OST	1	0.16	0.08	0.00
	control	ATF2 RNAi	stdev1	stdev2
2OST	1	0.26	0.08	0.04
	control	Runx2 RNAi	stdev1	stdev2
2OST	1	0.34	0.08	0.02

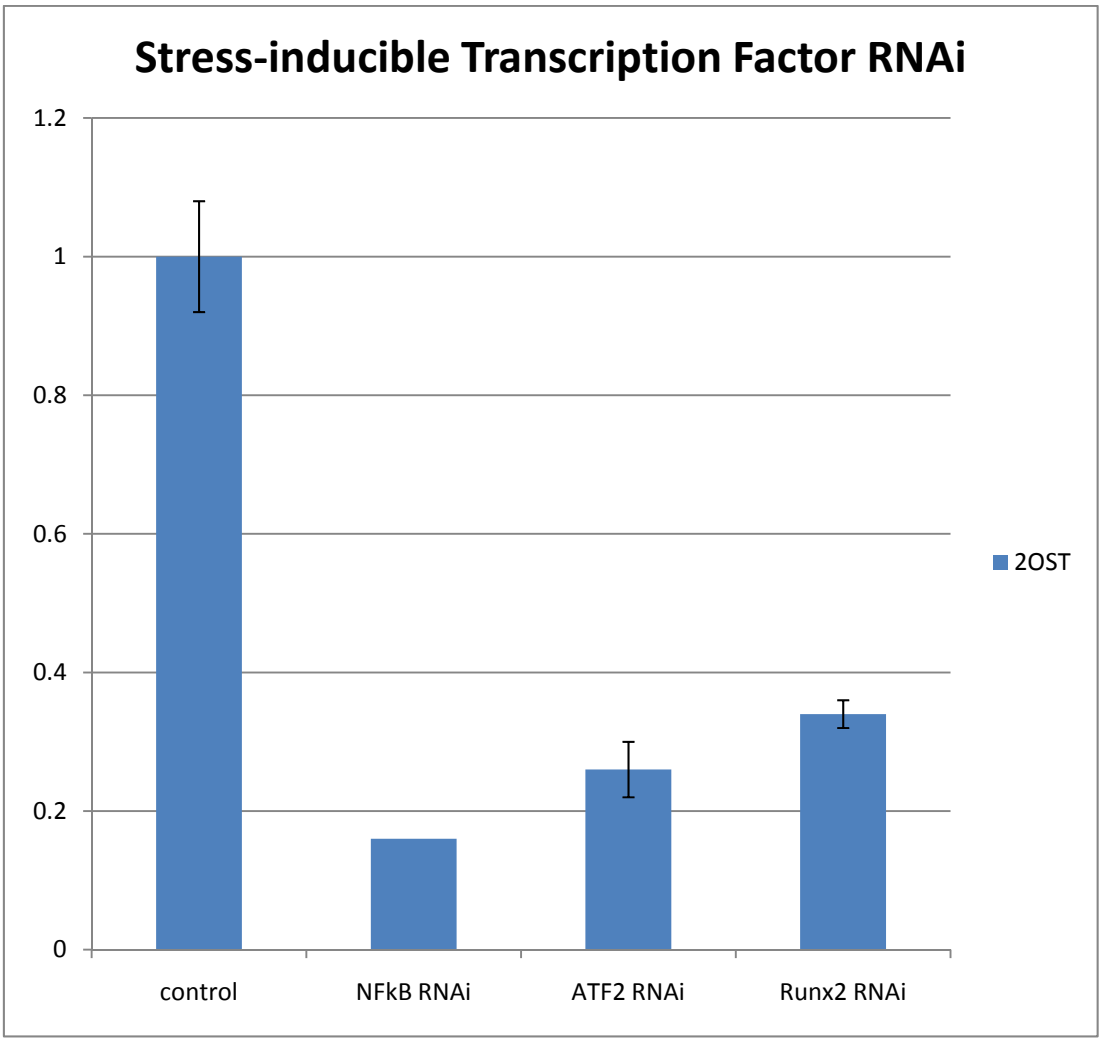


Figure 13- 2OST in C4-2B Cell Lines after RNAi Knockdown

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The Beta-Gal assay turned out somewhat unexpected results. The data shows an increase in 2OST production when the full length promoter was cut down to 1439 base pairs. This data suggests that the first 1391 base pairs of the promoter work to inhibit 2OST production. Beta-Gal assays for the remaining deletion constructs are currently underway. This information will help uncover what regions of the promoter are critical to 2OST production as well as whether each of the transcription factors bind directly to the promoter.

The RNAi experiment showed a significant decrease in 2OST levels. When NFkB was knocked out in the cell by RNAi there was an 84% decrease in the amount of 2OST mRNA produced as compared to the control metastatic C4-2B cell. Knockdown of ATF-2 resulted in a 74% decrease in 2OST mRNA and knockdown of Runx-2 resulted in a 66% decrease in 2OSTmessage levels. Such a significant decrease in 2OST levels suggests that the transcription factors NFkB, ATF-2 and Runx-2 all play an essential role in 2OST production. The next step for these experiments is to assay to what extent each transcription factor was knocked out. For example, if all transcription factors were decreased to the same extent, then NFkB appears to be the most important for normal levels of 2OST expression. But if ATF-2 was knocked down by only 50% while NFkB was knocked down by 95%, this would imply that a 95% knockdown of ATF-2 might decrease 2OST expression by much more than the 84% observed in the NFkB study.

We know 2OST plays a key role in the progression of prostate cancer. Our lab has shown 2OST to be an essential player in the induction of metastatic behavior in cancer cells. The lab noted a four –fold increase in the transcription of 2OST between benign LNCAP cells and highly metastatic C42B cells; knocking out 2OST made cells less metastatic. Since 2OST may be driving metastasis my aim was to understand what causes 2OST to accumulate in cancer cells. What factors contribute to its overexpression? The data obtained from this experiment proposes that NFkB, ATF-2 and Runx-2 play considerable roles in production of 2OST and ultimately the progression of prostate cancer. My studies have also uncovered the first evidence of a negative regulator of 2OST expression that requires the 5' most 1391 base pairs of the cloned 2OST promoter to inhibit 2OST expression.

Understanding why 2OST becomes highly expressed will help scientists develop diagnostic tools to predict the onset of metastasis. Understanding which ROS signal turns on 2OST will mean that we can use the presence of that signal in a patient's tumor as a test for whether that tumor is about to become metastatic, and thus deadly. Fully understanding the signals that regulate 2OST will enable us to develop drugs to block the positive signals or stimulate the inhibitory signals. Those drugs may enable usto prevent 2OST from accumulating and thus prevent a patient's tumor from becoming metastatic. By exploring the role of the cell stress hypoxia in prostate cancer we hope to be able to halt the progression of the disease.

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