EPIGENETIC REGULATION OF ALTERNATIVE SPLICING IN CANCER

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ABSTRACT

Prostate cancer is the most frequently diagnosed cancer among men and the second leading cause of cancer death in the United States. Major cause of mortality and morbidity is due to the metastasis of this cancer to the secondary organs. Identification of potential molecular targets and pathways is essential to devise effective therapeutic strategies to cure metastatic prostate cancer. A closer look at molecular processes such as alternative splicing and epigenetic regulation would provide useful insight into the mechanistic controls of tumor dissemination.

Aberrant methylation and dysregulated alternative splicing play a major role in tumor progression but scientific evidence of a definite link between the two is yet to be found. In our study we report that a differential methylation of four different sites in the intragenic region of *CXCR3* regulates its alternative splicing. To showcase this proof of principle, a bichromatic reporter minigene construct with a nucleotide switch at the differentially methylated cytosines to mimic the observed methylation change was used. The unique property of this construct helps obtain a quantifiable metric of the splice ratio for a change in every cytosine moiety and compute a dose dependent effect for every extra base pair altered. It was identified that a critical methylation ratio is essential for a spice switch of *CXCR3* in prostate cancer cells. In addition, the synergistic effect of methylation and a specific micro-environment on the phenotypic expression of the cell was also tested. The functionality of this construct to provide a snapshot on

the effect of an external stimulus on the splice ratio will provide with a useful tool in obtaining quick data on factors that influence splicing in the context of the epigenome. In-vivo studies in the future will identify the propensity of invasion or arrest of tumor cells in light of its splice axis.

PUBLIC HEALTH OVERVIEW : Prostate cancer is the second leading cause of death in the United States alone. The quality of life of patients in the terminal cancer stages is poor and the survival rate is very low. Current therapeutic options do not improve the quality of life and improve survival rate only marginally. This study aims at identifying potential therapeutic targets with prognostic capabilities.

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PREFACE

First and foremost, I would like to thank my mentor, Alan Wells, for providing me with the opportunity to explore research and basic science, constantly molding my strengths and weakness as I went along.

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1.0 INTRODUCTION

Cancer is defined as the malignant growth of a subpopulation of cells characterized by uncontrolled cellular proliferation. It is the leading cause of death around the world accounting for 30% of the deaths due to cancer in 2008 (www.who.org). The total death toll in the year 2030 is predicted at 13.1 million (globocan.iarc.fr). In the United State alone, it is estimated that 580,350 Americans will die from cancer. It is the second leading cause of death only next to heart disease, making it an important public health issue.

Dysregulated gene expression is the primary cause of malignant cellular proliferation which is usually the first step in the cascade of events that lead to tumor formation. The expression of genes in a cell is an exquisitely orchestrated process that involves intricate regulation at the DNA, RNA and protein levels. A significant event in cancer progression occurs when the tumor traverses tissue barriers to invade secondary sites, a process that is termed metastasis (Hanahan and Weinberg, 2000). Metastasis occurs as a coordinated sequence of events that involves a shift in the protein expression profile of a cell. From a clinical perspective, metastasis is a threshold beyond which rates of cancer survival decrease dramatically. Thus it is imperative to understand the molecular mechanisms that regulate metastatic transition.

Prostate cancer is a highly invasive cancer with pronounced metastatic capacity. Previously published reports have suggested that dysregulated protein expression in prostate cancer can occur as a result of an alteration in the splice axis, the ratio of splice isoform

expression, of the cell. This leads to the expression of alternative splice isoforms of proteins that promote tumor progression. Additionally, several studies that focus on the molecular mechanisms of metastatic transition have identified a role for epigenetic changes in the cell in this process. Epigenetic alterations are reversible marks in DNA that regulate gene expression levels by modifying the three dimensional structure of DNA. In this study, we use prostate cancer as a model to investigate the regulation of alternative splicing through epigenetic alterations and the impact on tumor progression.

1.1 PROSTATE CANCER

Prostate Cancer (PCa) is the most aggressive form of cancer in men and is described as a highly multifocal infiltrative tumor originating in the prostates. In 2013 alone a total of 238590 new prostate cancer cases will be diagnosed in the United States and the total number of deaths will reach 29,720 (www.cancer.gov) It is the second most vicious of all cancers in men with the second highest death rate.

1.1.1 Public Health Overview

A worldwide estimate of men with new PCa diagnosis is close to 900,000 new cases and 258000 deaths in 2008 (McNeal 1988, Ferlay, Shin et al. 2010, Jemal, Bray et al. 2011). A twenty-fold variation in the incidence of PCa around the world has been observed with developed countries sharing the major load of the incidence. The rates are highest in Australia and New Zealand with 104 cases per 100,000 and Western Europe with 93 cases per 100,000 in 2008. It is lowest in South-Central Asia at 4 cases per 100,000 (Jemal, Center et al. 2010). In the United States alone it is estimated that 1 in 6 men will be diagnosed with PCa and of that 1 in 36 will die from PCa (American Cancer Society 2013). Among Americans, the disease is more prevalent in African Americans with an age adjusted incidence of 233.8/100,000 for African Americans and an age adjusted incidence of 149.5/100,000 for European Americans (Howlader 2011). Thus there is an approximately two fold increase in risk of prostate cancer for men of African American descent. It is predicted that the five year survival rate of prostate cancer patients is 90% for patients with

localized tumors and 28% for patients having tumors with distant metastases. In an attempt to facilitate a decrease in PCa death rate, a prostate screening program was initiated with the underlying thought that an early detection would enable early treatment hence preventing the progression to the invasive metastatic state.

The PCa screening process began as a precautionary measure to identify PCa at a very early stage to have a better prognostic effect. It was based on quantifying prostate specific antigen (PSA) secreted by prostatic epithelial cells. PSA helps maintain the semen in the liquid state and some PSA escapes into the blood stream which provides a quantifiable measure produced by the prostate at a given time point in a man's life (Webber, Waghray et al. 1995). The PSA test is minimally invasive method of testing the levels of PSA in the serum. Various research studies established a significant association of high PSA blood levels to PCa which made it an efficient screening method for men at potential risk of PCa (Barry 2001). With the advent of PCa screening, the incidence of PCa increased in the early 1990s because of the increased awareness leading to more men being diagnosed. Consequently, there was a sharp decline in the rates of detection of late stage PCa along with deaths due to PCa. As with any diagnostic test, the PSA test also produces a lot of false positives that require further invasive testing procedures which are accompanied with an increased risk of infection, urinary incontinence, and impotency. It has been determined that for every 1000 men screened, one death due to PCa is prevented. However, since 44% of African-American males (population at a higher risk) and 29% of Caucasian males are over-diagnosed (Carter, Albertsen et al. 2013) the PSA test recommendations are being reconsidered. The current changed recommendation is that men between 40 and 50 years of age need not get tested unless they are at a higher risk because of their race or family history; men between that fall in the 50-69 year age bracket can be

screened periodically, and men over 70 years with an average life expectancy of less than 10-15 years need not get tested (Force 2012). Over-diagnosis and the inaccuracy of the PSA test have led to the necessity to identify of more accurate biomarkers to stratify patients in continuum with the screening process. Studying the molecular genetics and protein expression profile of neoplastic tissue during the tumor progression sequence should lead to effective identification of markers that provide valuable information of the invasiveness of the tumor. One of the popular targets for biomarker identification is the aging prostate tissue that represents the first step in the cancer progression cascade.

1.1.2 Prostate gland

The prostate is a walnut-sized exocrine gland that is present in males under the urinary bladder, in front of the rectum. The function of the prostate gland is to produce a fluid that is part of the semen which protects and nourishes the sperm (Mydlo 2003). At the tissue level the prostate is chiefly glandular. The cellular architecture consists of two major cell types: epithelial and stromal. The epithelial cells form the luminal secretory and basal cells of the prostate gland. A small population of neuro-endocrine cells is known to exist that is thought to regulate growth and differentiation of the epithelial cells (Abrahamsson 1999). The stroma surrounding the prostate gland consists of fibroblasts and smooth muscle cells. The outer edge of the prostate is primarily connective tissue and is called the capsule beyond which are the looser connective tissue with nerves and the blood vessels (McNeal 1988). Androgens which are the chief male reproductive hormones, play a critical role in every aspect of the prostate gland from its embryonic development to its maintenance and function. Their effects on the prostate are mediated by the

Androgen Receptor (AR) (Yadav and Heemers 2012). Prostate carcinoma arises in the glandular epithelium and is intimately associated with aging of prostate and androgen function.

1.1.3 Prostate cancer progression

The different subtypes of prostate cancer include acinar adenocarcinoma, ductal adenocarcinoma, squamous and adenosquamous carcinoma, urothelial carcinoma, small-cell carcinoma, basal cell carcinoma and clear cell adenocarcinoma. Acinar adenocarcinoma, the most common type of PCa (Randolph, Amin et al. 1997) originates from the epithelial cells in the glandular epithelium (Taylor, Toivanen et al. 2012).

As shown in Figure 1, prostatic intraepithelial neoplasia (PIN) is a precursor to invasive carcinoma (Bostwick and Brawer 1987) and has been found to be present in young men at the age of 20 even though PCa has been associated with aging. It is indolent and clinically undetectable, characterized by cancer like cytogenetic changes and nucleolar enlargements, luminal epithelial hyperplasia, reduction in basal cells and high expression of cellular proliferation at specific focus points that can be identified by histological analysis (Bostwick and Qian 2004, Shen and Abate-Shen 2010). PIN has a 23% predictive value for cancer. A scoring system known as the Gleason Score (GS) was established which assigns a number to a histological sample based on the number of PIN and other neoplastic foci (Gleason 1992). A Gleason score of 3+3 is regarded as the lowest grade of clinically detectible PCa (Gleason and Mellinger 1974). Low grade foci represent an increased risk of cancer with advancing age, growing out into tumors that become clinically detectible with a high Gleason score (GS). Men diagnosed with local prostate cancer, PIN and BPH have a 100% 5 year survival rate (American Cancer Society 2013).

With time, the tumor cells from these foci breach the basal membrane of the glandular epithelium to invade the surrounding seminal vesicle. This stage is marked as local invasion or seminal vesicle invasion (SVI) where the carcinoma has invaded the muscular walls of the seminal vesicle. At this point, the 7-year patient survival rate drops drastically to 32% (Potter, Epstein et al. 2000). Lesions in the seminal vesicle observed with PCa are only moderately differentiated with a luminal phenotype and are mostly non-glandular in appearance. The final stage in the neoplastic cascade is metastasis to distant organs where the tumor cells intravasate into the blood stream to reach secondary organs where they form micrometastases in conducive micro-environments.

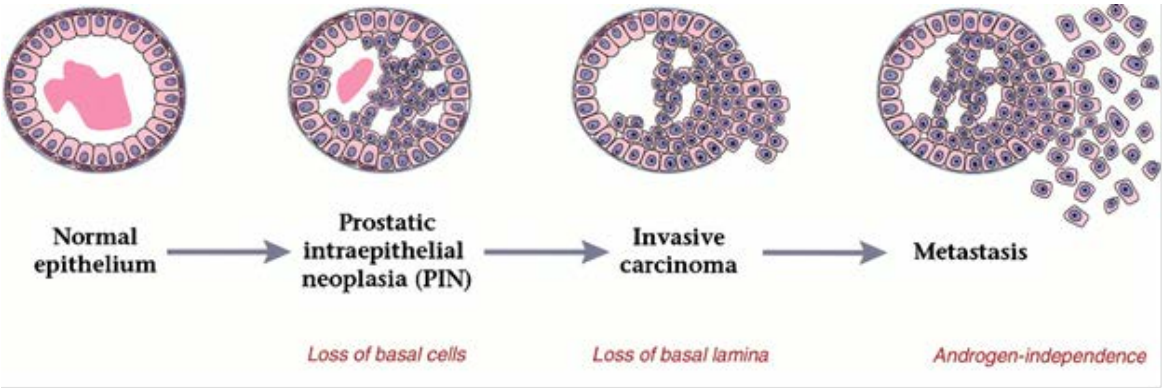


Figure 1. Tumor progression cascade

This figure represents the neoplastic transformation sequence of prostate cancer from normal epithelium to the invasive metastatic tumor (Adapted from Shen and Abate-Shen 2010).

As seen by the drop of the 5-year survival rate, the major reason of mortality and morbidity of PCa is due to the metastasis of the tumor form the primary site to the secondary site. The major sites of metastasis are to bone, lung and liver with bone being most frequently

metastasized to as observed from an autopsy study of PCa patients (Bubendorf, Schopfer et al. 2000) (Figure 2).

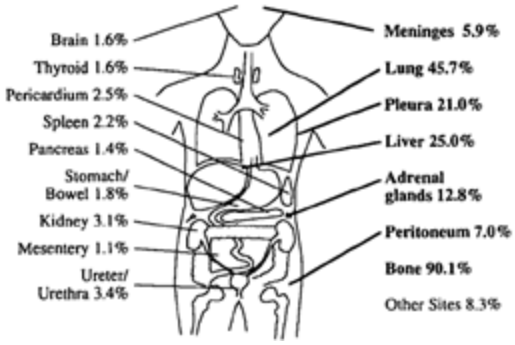


Figure 2. Metastatic sites of prostate caner

The frequency of prostate cancer metastasis to various secondary organs in the body. Bone, lung and liver are the most frequent sites of metastasis (adapted from Bubendorf et al., 2010) .

1.1.4 Current therapies

PCa depends on androgens for tumor growth, maintenance and survival (Huang and Tindall 2002). In 1940, it was found that depriving the prostate of androgen through castration shrunk the tumor and caused regression (Huggins and Clark 1940). A therapeutic strategy to treat local and metastatic tumor cells is depriving the cells of androgen, which reduces survival signals for tumor cells. Treatment options at the moment include surgical removal of the prostate gland with the tumor, radiation and androgen depravation through surgery and chemotherapy (Adamo, Noto et al. 2012). In the past 12 years, although there has been an increase in the incidence of prostate cancer with the advent of PSA screening, the mortality rate has remained constant. This indicates that early detection facilitated early treatment, thus validating the effectiveness of the

aforementioned therapeutic options. However, current treatments for PCa that either block androgen secretion or antagonize AR function are not completely effective. Chemical castration that blocks androgen secretion leads to the occurrence of a castration-resistant PCa (CRPCa) after initial remission (Feldman and Feldman 2001, Kohli and Tindall 2010). CRPCa is resistant to androgen deprivation as these tumors progress in an androgen-indepent manner leading to a high probabilitiy of metastasis and as a result, a low 5-year survival rate.

Until 2010, all treatment options were valuable only to cure localized tumor with not many options for treatment once the cancer has metastasized (Dittrich, Dittrich et al. 1991, Vogiatzi, Cassone et al. 2009, Stavridi, Karapanagiotou et al. 2010). Recently, four different clinical trials were approved to treat castration-resistant cancer with distinct therapeutic strategies: targeting androgen-receptor signaling, cytotoxic chemotherapy (cabazitaxel), immunotherapy (sipuleucel-T), and bone-targeting radiation (Omlin and de Bono 2012). From the clinical trials it is evident that these treatment options only prolong the survival marginally but do not improve the quality of life. The effectiveness of these studies emphasize the fact that a clear understanding of the molecular underpinnings of prostate cancer progression is required to effectively treat the disease. Analyzing the molecular and genetic changes in the neoplastic sequence of prostate cancer is critical to identify good starting points for prostate cancer diagnostics and therapeutics.

1.1.5 Molecular genetics

A familial inheritance of prostate cancer has been known to contribute 5 – 10% of the prostate cancer load. To date, genes that are associated with this inheritance have not been identified but an association with genetic loci on the X chromosome and chromosome arm 1q has been

observed (Smith et al. 1996; Xu et al. 1998). A mutation in the *BRCA-1* gene in men has also been implicated with susceptibly to prostate cancer (www.mskg.org). A genetic change in the prostate is one of the hallmarks of prostate cancer that is initiated with aging. PCa is a highly multifocal cancer with a heterogeneous genetic basis at different focal points which has been associated with genetic instability, mutations and epigenetic alterations (Ibeawuchi, Schmidt et al. 2013). For example, loss of *PTEN* (Phosphatase and tensin homolog), a tumor suppressor gene, is linked to the initiation of PIN and a faster progression of PIN to carcinoma (Luchman, Benediktsson et al. 2008). A *TP53* mutation has been associated with metastasis of prostate cancer (Eastham, Stapleton et al. 1995) and aneusomy of chromosomes 4, 6, 20 and X indicative of lymph node metastasis (Braun, Stomper et al. 2013). Kang et al. tested the differential methylation of 11 genes in PIN, PCa and normal prostate cells and found that methylation signatures were very similar in PIN and PCa except that there was an increased frequency in PCa showcasing a change of epigenetic signature with cancer progression (Kang, Lee et al. 2004).

At the molecular level, the proliferation of PCa cells is dependent upon the action of androgens through the androgen receptor (AR) pathway (Feldman and Feldman 2001). However, subsequent to relapse after androgen deprivation therapy, the cancer is androgen-independent. However, androgen independent CRPCa cells still require AR function to proliferate. Recent studies have identified alternative splicing (AS) of the AR as a mechanism to circumvent the requirement of androgen for AR activation (Knudsen and Scher 2009, Dehm and Tindall 2011). A genome-wide study of regulation of AS in PCa cell lines and primary tumor tissues identified specific splicing signatures associated with PCa cells compared to cancer cell lines from other tissues. The same study also differentiated normal and neoplastic prostate tissues based on their splicing signature, as some of the alternatively spliced genes had altered gene expression levels

as well, showcasing an intimate association between transcription and splicing (Zhang, Li et al. 2006, Munoz, Perez Santangelo et al. 2009). These studies identify splicing in prostate cancer cells as a molecular mechanism to acquire a more invasive phenotype with the capability to survive and metastasize.

1.2 TUMOR INVASION AND METASTASIS

Tumor metastasis is a complex process that begins with the dissemination of tumor cells from the primary tumor to invade the surrounding tissue. This is followed by intravasation into the blood vessels coupled with a delicate balance between survival and arrest finally, extravasation into secondary organ ectopic sites to form micro metastases (micromets) (Liotta and Stetler-Stevenson 1991, Cairns, Khokha et al. 2003). These micromets form tumor nodules at the metastatic site and proliferate in the secondary organ similar to the primary site to form neoplastic lesions. A host of molecular events occurring in a sequential manner contribute to invasiveness of the tumor, the study of which would provide us with potential points of intervention for improving tumor prognosis. One of the key events in the metastatic cascade is the epithelial to mesenchymal transition (EMT) of tumor cells. This transition is identical to one that occurs during embryonic development, when cells de-differentiate from an epithelial state to a more motile mesenchymal phenotype (Micalizzi, Farabaugh et al. 2010). A change from the apical-basal polarity of epithelial cells to front-rear mesenchymal polarity enables directional migration of the cells (Nelson 2009). E-cadherin, an epithelial marker, is an important intercellular junction molecule is down regulated in EMT thus reducing the number of contact points for the cell (Wendt, Taylor et al. 2011). Loss of cell-cell adhesion and apical junction

contacts facilitates the disengagement of the cells from the primary tumor mass. A reduced expression of E-cadherin at the primary tumor site is implicated in the local invasion and distant metastasis of prostate and breast carcinoma (Kowalski, Rubin et al. 2003, Pontes, Srougi et al. 2010, Behnsawy, Miyake et al. 2013). The hypothesis of epithelial de-differentiation to a more motile mesenchymal phenotype is supported by data from numerous research groups. A marked reduction in the expression of epithelial markers such as occludin, claudin and cytokeratin as the tumor progresses to a more invasive phenotype along with increased expression of mesenchymal markers such as vimentin, TWIST and N-cadherin has been reported (Royer and Lu 2011). At a clinical level, the expression of mesenchymal markers in prostate, breast, colon and renal carcinoma has been associated with poor patient survival with a high rate of metastasis to multiple organs (Tarin 2011). Preventing EMT and thus localizing the tumor to the primary site is an effective approach to curtail metastasis.

The epithelial to mesenchymal transition is accompanied by a paradigm shift in the protein expression profile of the cancerous cell to increase cell motility and proliferative ability; re-organization of the cytoskeleton and matrix re-modeling for higher levels of de-adhesion proteins that enable disengagement from the primary site (Chambers, Groom et al. 2002, Friedl and Wolf 2003). For example, increased proteolytic activity acquired from higher expression levels of matrix metalloproteases (MMPs) such as MMP9, MMP 10, MMP11 degrades the matrix to facilitate invasion of the basement membrane (Schmalfeldt, Prechtel et al. 2001). Similarly, signaling through growth factors such as EGF (Epidermal Growth Factor) and TGF-β (Transforming Growth Factor β) present in the tumor microenvironment induce motility and enhance proliferation, conferring a survival advantage to the cancerous cells. Autocrine and paracrine signaling in the tumor and its microenvironment has been known to promote motility,

chemotactic directed migration but also inhibit angiogenesis and promote endothelial cell death. These signaling cascades are very important from a therapeutic standpoint to subvert the pro migratory stimulus to an inhibitory stimulus.

Chemokines, a family of cytokines that induce chemotaxis play a major role in the directed migration of cancer cells. They exert their effects by binding to chemokine receptors present on the target cell surface and initiating chemotaxis. The CXC family of chemokines are implicated in the inhibition of angiogenesis and increased motility of cancer cells though the CXC ligand receptor function (Bodnar, Yates et al. 2006). The pleiotropic function of these chemokines that recruit immune cells, inhibit motility and angiogenesis make them and their receptors very good targets to shift the protein balance of the cell to reinstate the epitheloid characteristics. In a recent study, a chemokine ligand-receptor axis has been implicated in the invasion and metastasis of breast cancer to the lymph nodes where the breast cancer cells had membrane expression of chemokine receptors associating them with organ specific metastasis (Zlotnik, Burkhardt et al. 2011). This places emphasis on the importance of chemokine signaling in metastasis. The present study focusses on the chemokine receptor *CXCR3* and its role in prostate cancer progression.

1.2.1 *CXCR3*

CXCR3 is a G-protein coupled receptor that is activated by the CXC chemokines, IP-9, IP-10, and PF-4 to facilitate chemotactic migration, cellular proliferation, endothelial cell death and inhibition of motility (Lasagni, Francalanci et al. 2003, Dagan-Berger, Feniger-Barish et al. 2006). *CXCR3* has seven trans-membrane helices and was first discovered on the surface of natural killer (NK) cells and later found to be present on lymphocytes, endothelial cells, T-cells,

B-cells and tumor cells (Yao, Sgadari et al. 1999, Pertl, Luster et al. 2001, Kruizinga, Bestebroer et al. 2009). *CXCR3* has been posited to play a significant role in wound healing, with its ability to inhibit motility and induce apoptosis, thus acting as a physiological 'STOP' signal to prevent the neoplastic transition during wound healing. In 2006, it was discovered that this receptor has an alternative isoform with a longer N terminal end. The longer isoform was named CXCR3B and the shorter isoform CXCR3A. The two isoforms are sequentially identical except for the longer amino tail of CXCR3B that contains an extra 50 amino acids. Though otherwise structurally identical, the isoforms activate distinct signaling pathways though differential G-protein coupling to produce reciprocal effects. CXCR3B inhibits motility and promotes apoptosis while CXCR3A induces proliferation and motility (Lasagni, Francalanci et al. 2003). Some evidence points toward an overlap in the signal transduction of these two isoforms, which brings to notice that there is cell type specific expression of these isoforms, leading to differing cellular phenotypes (Satish, Blair et al. 2005, Mueller, Meiser et al. 2008). Conversely, the phenotypic effect observed in a cell is a representation of the cellular proteome.

With respect to cancer and tumors, *CXCR3* has been associated with poor survival of patients with breast carcinoma (Ma, Norsworthy et al. 2009) and associated with prostate cancer, renal carcinoma, osteosarcoma, colon cancer and melanoma (Kawada, Sonoshita et al. 2004, Datta, Contreras et al. 2008, Pradelli, Karimdjee-Soilihi et al. 2009, Wu, Han et al. 2012). *CXCR3* is a key factor in promoting the metastasis of colon cancer and melanoma to lymph nodules and specifically in melanoma, it was demonstrated that a knockdown of *CXCR3* using antisense RNA markedly reduced metastatic frequency (Kawada, Sonoshita et al. 2004, Kawada, Hosogi et al. 2007) . Interestingly, it has been discovered in prostate cancer that there is a switch in expression between these two isoforms wherein prostate cancer cells express higher levels of

CXCR3A in comparison to normal prostate cells which express higher levels of CXCR3B although the total receptor levels being the same in both types of cell lines. In light of their expression in cancer cells, the two isoforms CXCR3A and CXCR3B link differentially to pro- and anti- invasion signals with CXCR3B acting as the physiological 'STOP' signal which decreases the invasive phenotype. This switch in the expression of the isoforms with a higher levels of CXCR3A is directly associated with its pro-invasive properties, which confer cancer cells with a survival advantage. *In-vitro* experiments have demonstrated that increasing the levels of CXCR3B in these cancer cells through exogenous transfection plasmids results in a decrease in cell motility and invasiveness. In other cancer types it has been shown that CXCR3B prevents or blocks angiogenesis and *CXCR3* medicated chemotactic migration (Lasagni, Francalanci et al. 2003). Current evidence thus points to a critical angiostatic - angiogenic balance being maintained in the cells by the receptor isoforms. This suggests that reverting the splice shift in the cancer cells to re-express CXCR3B would be a significant step towards curtailing the tumor at the primary site thus preventing its metastasis. Knowledge of the alternative splicing mechanism that underlies this switch is essential to identify therapeutic targets that will allow us to re-instate this 'STOP' signal.

1.3 ALTERNATIVE SPLICING

Alternative splicing is a mechanism that generates proteome complexity in eukaryotes. It is a process by which two different mRNA transcripts are produced from the same gene. Proteins with different or even opposing functions are generated from alternatively spliced mRNA. The complexity, plasticity and functional significance of different cell types and tissues of

multicellular eukaryotic organisms are a consequence of alternative splicing during embryonic development (Graveley 2001). As an orchestrated process, it can shift the protein expression axis of a cell between two states at a given time.

Splicing is a process by which introns in the genes are spliced out and exons which are the coding regions are conjoined to form the mRNA that is later translated to protein (Wahl, Will et al. 2009). Excision of introns is carried out with the help of the splicing apparatus, called spliceosome, which is a complex assembly of proteins and RNAs that enable splicing. The small nuclear RNA present in the spliceosome exists as rinonucleoproteins (RNP) and are called snRNPs. The group of snRNPs consisting of U1, U2, U4/U6 AND U5 scan the pre-mRNA for the consensus splice recognition sites GU/AG at the intron-exon junction (Kramer 1996, Smith and Valcarcel 2000). Typically, introns begin with a GU dinucleotide known as the splice donor site at the 5' end, with the AG dinucleotide known as the splice acceptor site at the 3' end (Burset, Seledtsov et al. 2000). Through a series of trans-esterification reactions, the introns are excised from the RNA at donor and acceptor sites which conjoin to form the exon-exon boundaries. Non-consensus splice recognition sites, such as GC/AG pairs and AT/AC pairs are deemed weaker splice sites (Garg and Green 2007) that occur naturally in the gene or could be acquired due to single nucleotide substitutions, deletions or insertions could, interfere with the splice site selection (Faustino and Cooper 2003). Other elements that are part of the genetic architecture that influence splicing are exonic splice enhancers (ESE) and Intronic splice suppressors (ISS). ESEs are consensus sequences that are six bases long to which the SR proteins bind enabling splicing at the adjacent intron exon junction (Blencowe 2000, Wang, Smith et al. 2005). An ISS can be hundreds of base pairs in length, which aids the binding of splicing repressors which suppress splicing at the flanking junctions (Carstens, Wagner et al. 2000). The

choice of the splice site to be used depends on the sequences spanning the intron-exon junction that plays a role in the secondary structure generated. Thus, a small change even in a single base pair in the exon, though deemed a translationally silent mutation can affect the choice of splice site if the protein is alternatively spliced (Maquat 2001, Cartegni, Chew et al. 2002). The functional importance of this regulatory machinery places a strong emphasis on the factors influencing and affecting it.

Alternative splicing specifically denotes the process where exons are alternatively selected for inclusion during the splice event to produce different splice isoforms from the same mRNA (Luco, Allo et al. 2011). The patterns of alternative splicing include (i) exon skipping (ii) 3' alternative splicing (iii) 5' alternative splicing and (iv) intron retention. Figure 3 is a depiction of the alternative exons that are included to form the mRNA (Huang, Horng et al. 2005).

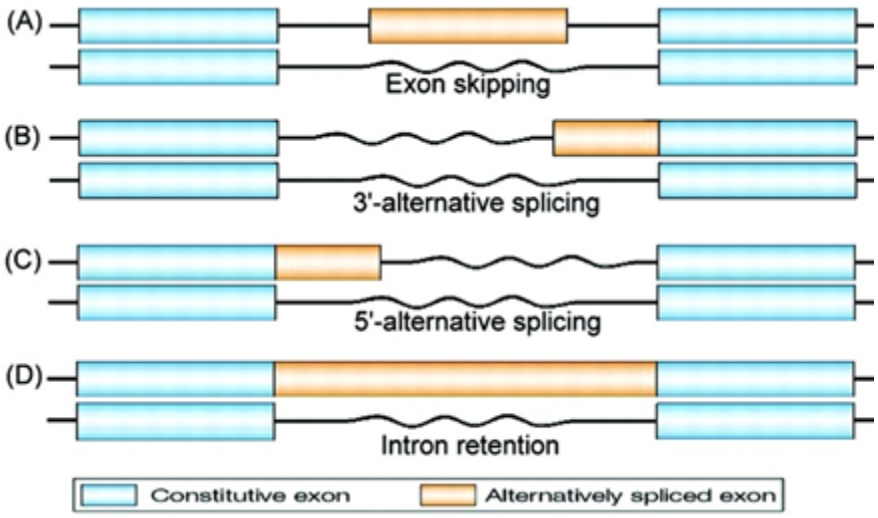


Figure 3. Mechanisms of alternative splicing

The different modes of alternative splicing. Of particular interest to this study is the 3'-alternative splicing mechanism (adapted from Huang et al., 2005).

Specific to 3' alternate splicing, the 3' alternate acceptor site lies embedded in the exon-exon junction between the alternative exon and constitutive exon. Within this model of splicing, there are two different modes that are defined as leaky splicing and regulated splicing. Leaky splicing is governed by the positioning of non-consensus acceptor sites at the alternative sites where selection is based on utilization of the alternative and constitutive site. Whereas in regulated splicing, the gene architecture plays a critical role. The loading of SR proteins to certain consensus sequences such as splicing enhancers and splicing silencers play a critical role, as will be explained later.

Growing evidence is being published stating that the AS of genes occurs co-transcriptionally in the mammalian genome indicaties an intimate association between gene and mRNA in time and space. In a recent study by the ENCODE (Encyclopedia Of DNA Elements) consortium, subcellular fractions of RNA were isolated which included chromatin-associated RNA. These RNA fractions were tested for intronic presence and it was found that mRNA associated with these fractions did not contain any introns. This demonstrated definitive proof of co-transcriptional splicing and the nature of association of the gene at the DNA level with its splicing at the RNA level (Djebali, Davis et al. 2012). As RNA polymerase II (RNA pol 2) spans the transcribing gene with a growing tail of pre-mRNA, splice proteins, such as the SR proteins are recruited to the intron-exon junctions. The carboxy-terminal domain of the RNA pol 2 recruits the SR proteins (RNA splicing protein) to the transcriptome which ties the functional association of the spliceosome with RNA pol 2 (McCracken, Fong et al. 1997, Misteli, Caceres et al. 1997). An intimate association between RNA pol 2 elongation in space and time directly involved in exon skipping or inclusion. A slow elongation rate allows for more time in exon recognition thus increasing the probability of exon skipping where the exon is spliced out while

on the contrary a faster elongation rate decreases the time for exon recognition increasing the exon inclusion rate (Hirose and Manley 2000, Proudfoot 2000, Das, Dufu et al. 2006). The complexity of the process and delicacy that a change even at a single base pair causes a splice shift has made alternative splicing a major target for prognosis of human diseases and cancer.

1.3.1 Alternative splicing in cancer

One of the most intriguing factors about the proteome expressed in cancer is that, most of the genes that transcribe proteins implicated in cancer metastasis, angiogenesis, local invasion, uncontrolled proliferation and apoptosis are alternatively spliced (Venables 2004, Ghigna, Valacca et al. 2008). The cell uses alternative splicing to subvert the splice axis to express splice isoforms that facilitate tumor progression and metastasis . A few genes whose splicing regulation was found to be shifted in different type of cancer are *BCL-X*, *FGFR2*, *CCND1* and caspases among others. For example, BCL-X has two isoforms BCL-XL and BCL-Xs where the former is a pro-survival isoform and the latter pro-apoptotic. Compared to normal cells , breast cancer cells express more of the BCL-XL isoform that confers pro-survival ability (Boise, Gonzalez-Garcia et al. 1993). Similarly in, prostate cancer it was observed that there was a tip in the balance of expression of cyclin D1 (CCND1) isoforms – CCND1a which promotes androgen receptor (AR) dependent transcription and CCND1b which represses transcriptional activity of AR. Shifts in the balance to favor CCND1b initiate androgen-independent growth of prostate cancers which makes them refractory to hormone depravation therapy (Lu, Gladden et al. 2003, Burd, Petre et al. 2006, Comstock, Augello et al. 2009). In light of this evidence, it is thought to be ideal to classify cancer stages according to their splice signatures. Extensive studies of the transcriptome were carried out to evaluate the splice shift in prostate cancer, lung cancer and ovarian cancer

progression (Zhang, Li et al. 2006, Misquitta-Ali, Cheng et al. 2011). The study was performed by isolating RNA from human prostate cancer tissue samples and a microarray was used to determine the splice patterns. It was found that over 200 genes that are alternatively spliced were mis-regulated in prostate cancer. (Zhang, Li et al. 2006). This important study provided a clear snapshot of dysregulated alternative splicing penetration in cancer.

With the advent of next generation sequencing techniques, it was possible to conduct whole genome transcriptome sequencing to study the differences at a single cell level. A recent study using next generation sequencing techniques found that transcriptomes of individual bone marrow derived dendritic cells from a seemingly homogenous population had a different splice pattern (Shalek, Satija et al. 2013). This is extremely relevant to prostate cancer which has a reputation for cellular heterogeneity and unpredicted patient response to therapies, which may be attributed to potential intercellular splice differences. The splice states of cancer cells disseminated from the primary tumor determine its fate of quiescence & dormancy; micrometastases formation or apoptosis (Aguirre-Ghiso 2007). Not only is alternative splicing important in this situation, but analysis at a single cell level is critical to understand cell fate. In our study we target the epigenome of *CXCR3* that is differentially spliced in prostate cancer with a splice switch that favors the expression of the pro-survival CXCR3A isoform (Wu, Dhir et al. 2012). Specifically we use a bichromatic reporter minigene that identifies the splice shift at a single cell level to discern if the splice switch is a homogenous or a heterogeneous change.

1.3.2 *CXCR3* gene architecture

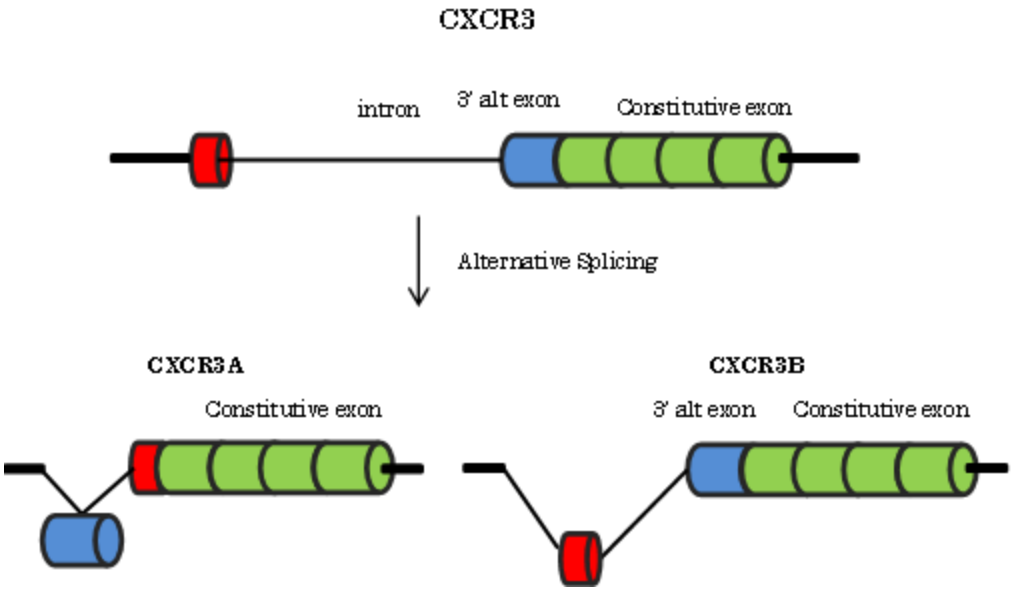


Figure 4. Alternative splicing of *CXCR3*

The figure is a pictorial representation of the exon and intron positioning in the CXCR3 gene and the mRNA transcripts. Different colored cylinders represent the exons while the line represents the introns. Black rectangular boxes are the 5' and 3' untranslated regions (UTR)

As described before, *CXCR3* is an alternatively spliced G-protein coupled receptor with two isoforms CXCR3A and CXCR3B. The two isoforms are identical except for a longer amino terminal in CXCR3B. A close look at the architecture of this gene is essential in devising tools to unravel the enigma of the *CXCR3* splice switch in cancer. *CXCR3* contains one intron, one constitutive exon and one alternative exon. It essentially abides the rule of 3' alternative splicing where the constitutive exon codes for the sequentially identical fragments of the isoform. Figure 4 gives a pictorial representation of the exon positioning. The green represents the constitutive

exon, blue represents the alternate exon that codes for the longer N terminal exclusive CXCR3B and the red represents the small fragment that codes for a tri peptide unique to the amino terminal of CXCR3A. The 5' heterogeneity of the mRNA arises from the fact that both isoforms have the same 5' donor while an alternative 3' acceptor site. The 'ATG' start codon for CXCR3B lies in the alternative exon (Lasagni, Francalanci et al. 2003). As determined from the sequence analysis of splice junctions, the 3' splice donor site for CXCR3B is a weak non-consensus site AG/**GG** while the constitutive splice site contains the consensus AG/**GT**. Thus the inclusion or exclusion of this intron and alternate exon is of primary importance in studying the splicing of this gene.

At the gene level, SNPs in the intronic region of *CXCR3* have been associated with an increased risk of asthma and functionally with reduced gene expression (Cheong, Park et al. 2005). Though the SNPs did not alter the splice protein expression profiles, this presents evidence of the importance of *CXCR3* intron in its expression. With respect to other syndromes, a *CXCR3* mutation has been named a risk allele for inflammatory bowel syndrome with this SNP associated with an altered *CXCR3* axis (Choi, Park et al. 2008). With respect to cancer, to unravel the mechanism behind the aforementioned switch in splicing variants, we analyzed the *CXCR3* gene. The gene was scanned for insertions, deletions or substitutions that create an intronic splice silencer or exonic splice enhancer that would enhance the inclusion or skipping of the alternate exon. Interestingly it was found that the cancer cells and the normal epithelial cells that were studied were identical. Both receptor isoforms are fully functional in the cells which eliminate the possibility of a mutation in the coding region (Wu, Dhir et al. 2012).

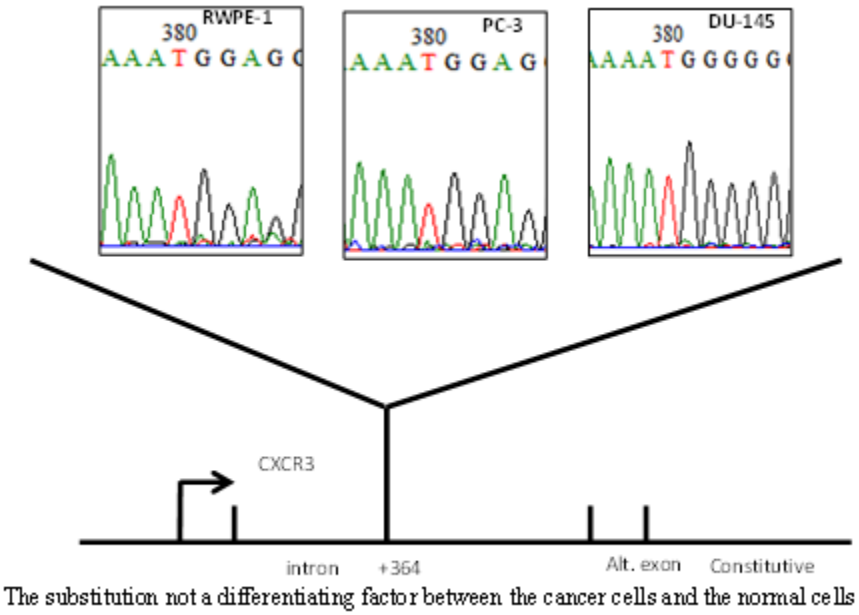


Figure 5. Intronic variant in the cancer cell lines

The intronic variant found in the DU-145 cells but not in the PC-3 cells or RWPE-1.

A mutation free intron suggests that there is a different mechanism that alters this gene expression profile at the DNA level. A mutation is a permanent change in the gene whereas an epigenetic alteration is a reversible change that confers the ability of the cells to alter the state at a given point. The ability of the cells to metastasize from the primary site to the secondary site is incumbent on their ability to display plasticity. Epigenetics allows for this proficiency in cells where they switch between epitheliod and mesenchymal states. This is further supported by evidence that tissue specific differentially methylated regions are preferentially located in exons and introns laying further emphasis on DNA methylation dependent alternative splicing (Wan, Oliver et al. 2013)

1.4 EPIGENETIC REGULATION

Epigenetics is the study of changes in the DNA without an alteration in the sequence template. This is accomplished by either post-translational histone modification or DNA methylations, which are the two forms of epigenetic regulation of gene expression (Holliday and Pugh 1975, Riggs 1975, Hebbes, Thorne et al. 1988, Landsberger and Wolffe 1997). It is thought that both mechanisms are inter-dependent, acting in concert to one another to actively alter the proteome (Nan, Campoy et al. 1997, Jones, Veenstra et al. 1998). The epigenome is a tissue specific ratio of methylated cytosines and acetylated histones that result in a characteristic protein expression profile specific to the imprinting. Evolutionarily epigenetics has occupied central importance in determining gene-environment interactions, often times mediating the response of a cell to its micro-environment (Jaenisch and Bird 2003). It is a dynamic process that is reversible (Liang, Kanduri et al. 2000, Reik, Dean et al. 2001), conferring the cell with the ability to wield expressional control over the gene. In the aging prostate, for example, the hypoxic environment in the prostate tissue evokes an epigenetic response in the tumor cells. At the cellular level, chronic hypoxia alters the epigenetic signature of DNA and in prostate cancer it has been demonstrated to increase the global methylation agenda accompanied by gene specific alterations (Watson, Watson et al. 2009). Aberrant DNA methylation has been implicated in prostate tumor heterogeneity (Aryee, Liu et al. 2013), which is a product of its multifocal infiltrative nature (Aihara, Wheeler et al. 1994, Macintosh, Stower et al. 1998). Along with hypoxia, genetic instability, telomere attrition, accumulation of mutations over a lifetime [reviewed in (Strehler 1986, Aguilera and Garcia-Muse 2013, Lopez-Otin, Blasco et al. 2013)] , and passive loss/gain of methylation (Maegawa, Hinkal et al. 2010, Thompson, Atzmon et al. 2010) are a few hallmarks of tissue aging that are implicated in tumor progression as well. A balanced

therapeutic approach towards prostate cancer that deals with the heterogeneity and plasticity of the tumor would be achieved by looking at the epigenetic mechanisms that render the tumors invasive.

1.4.1 Histone marks

Histones are subunits of the 'molecular clips' or nucleosomes that coil DNA and hold the double helix in place to form chromatin structures of higher organization. These proteins contain a longer N-terminal tail that is susceptible to post-translation modifications such as methylation, acetylation and phosphorylation (Allfrey, Faulkner et al. 1964, Chen, Ma et al. 1999, Thomson, Mahadevan et al. 1999). The proximity between histones and the DNA confers the ability to alter the openness of the DNA conformation on the histones. A post-translational modification on histone proteins will thus differentially alters the affinity of the protein with the chemical subunits of the DNA. Acetylation of the histone tail decreases the affinity to the DNA thus making the helix more loosely wound or more open, and the DNA becomes more accessible to polymerases enabling efficient transcription whereas a deacetylation closes the DNA conformation making it inaccessible to the to the transcriptional machinery (Krajewski and Luchnik 1991). Specific to prostate cancer, global patterns of histone modification have been associated with cancer recurrence (Seligson, Horvath et al. 2005). Histone modifications in synergy with the DNA methylation differences alter gene expression patterns. It is hypothesized that a difference in histone modifications implies a methylation difference in the DNA as well.

One of the important transcriptional effects of an epigenetic alteration is the genetic architecture of the chromatin at that genetic locus. Regulatory sequences such as promoters are CpG rich regions that are susceptible to manipulation by methylation to turn a gene on or off.

Other elements of the regulatory sequences aside of the promoters are the sequences that bind transcription activators, repressors, enhancers and silencer. If altered, the CpG islands in these regions have a greater effect on the proteome. Thus the gene structure and location of the CpG island plays an important role. In our study we will focus on identifying methylation differences in the DNA that would alter the splicing.

1.4.2 DNA methylation

DNA methylation is the process by which a methyl group is transferred to the cytosine moiety of DNA in a reaction catalyzed by DNA methyl transferases (DNMTs). However, not all cytosines in DNA are methylated. Cytosines that occur as dinucleotides paired with a guanine (called a CpG dinucleotide) are methylated in mammals and eukaryotes. The CpGs are present in clusters of dinucleotides 1-2kb long called CpG islands that are present in the promoters and first exons. 1% of genes in human DNA consist of CpG dinucleotides, 60 – 90% of which is methylated (Bird 1986, Takai and Jones 2002). An error rate of 4 ~ 5% in the maintenance of methylation has been observed for every cell replication cycle (Riggs, Xiong et al. 1998). Several types of DNMTs are expressed in cells that execute specific methylation events. *De-novo* methylation marks are established by the DNMT3a and 3b enzymes while genomic imprinting, a process in which methylation marks are maintained during replication is achieved by DNMT1. (Bestor 1992, Okano, Xie et al. 1998, Hsieh 1999, Pradhan, Bacolla et al. 1999).

A change in the methylation state of cytosines in the gene alters the coiled state or the DNA conformation by increasing or decreasing the DNA compaction. The concerted action of the methyl binding enzyme and histone acetylating enzymes on the methylated cytosine condenses the chromatin structure, rendering it repressive for protein expression (El-Osta 2003,

Clouaire and Stancheva 2008). Hyper-methylation, a state of increased methylation, at a specific locus creates torsion making the DNA tightly coiled thus decreasing the accessibility of the gene to transcription factors and polymerases. The openness of the DNA conformation in three-dimensional space determines the bound or unbound state of transcription factors or repressors to the DNA. A lack of transcription of a specific gene due to its methylation state decreases or abrogates its protein levels in the cell leading to a paradigm shift in the expression profiles (Ochs, Fensterer et al. 2003). Conversely, hypomethylation, a state of decreased methylation decreases torsion and changes the DNA to an open conformation which increases transcription at that locus.

Loss of imprinting at a specific locus could activate a particular gene that was silent or silence a gene that is essential for cellular maintenance. If the location of this aberrant methylation coincides with a proto-oncogene or a tumor-suppressor gene it proves to be fatal to the cell increasing the probability of transition from the normal state to the neoplastic state. Hypomethylation cause the genetic instability and inappropriate expression of proto-oncogenes whereas a hypermethylation silences the tumor suppressors and apoptotic genes (Rodriguez, Frigola et al. 2006). The first evidence of differential methylation implicated in cancer was observed in 1983 (Feinberg and Vogelstein 1983). Since then, promoters of a variety of genes have been found to be hyper methylated or hypo methylated with altered expression of the respective protein observed. Aberrant methylation has been associated not only with transcriptional regulation, but also with chromosomal instability, a hallmark of many cancers (Vilain, Vogt et al. 1999, Vilain, Bernardino et al. 2000).

Altered methylation states have been linked to prostate cancer as well. In prostate cancer cells it has been observed that there is global hypo methylation but a gene specific

hypermethylation (Yang, Sun et al. 2013). Specifically, 29 genes have been found to be aberrantly methylated when compared to the normal prostate tissue and all of which have been confirmed to be present in the tissue specimen obtained from patients [reviewed in (Li, Okino et al. 2004, Cooper and Foster 2009)]. For example, *WNT5a*, *CRIP1* and *S100P* are hypo-methylated (Wang, Williamson et al. 2007); while *APC* and *GSTP1* are hyper-methylated. An overexpression of methylating enzymes in the prostate cancer cells could explain hyper-methylated CpGs associated with prostate cancer progression. Methylation state of a gene and the chromatin structure in a diseased state is a strong indicator of the extent of change on the protein axis that alters the phenotypic expression of the cell.

An invaluable aspect of the epigenetic approach is the stability of the imprinted cytosine that can be detected in the body fluids such as serum and urine ejaculate (Goessl, Muller et al. 2001, Ellinger, Haan et al. 2008). This makes it a versatile tool to be used as a biomarker for the onset of cancer and track its progression. One of the most important examples is the *GSTP1* gene in prostate cancer cells where the promoter has been found to be methylated and is accompanied by a decreased expression of the protein in 90% of prostate cancers (Lee, Morton et al. 1994). It is thought of as suitable candidate to differentiate false positives in patients that tested positive for the PSA analysis because the penetration of differential methylation at the gene level is an effective indicator of disease progression and prognosis. A Methylation Specific PCR (MSP) assay and quantitative PCR demonstrated that the *GSTP1* gene was 6% methylated in proliferative inflammatory atrophy (PIA) of prostate, a precursor to lesion; 69% methylated in prostatic intraepithelial neoplastic cells and 90% methylated in prostate cancer cells that are metastatic (Nakayama, Bennett et al. 2003). Detection of the precursor to lesion at the PIA stage is the most effective intervention point for preventive care. The staging of the cancer and

detection at very early stages facilitates control of the tumor, preventing it from metastasizing to distant secondary organs leading to mortality. A balanced approach incorporating establishing a critical balance between gene panels used for CpG island testing to identify aberrant methylation and protein expression profiles that correlate with the staging of prostate cancer will yield potential biomarkers and therapeutic targets.

1.4.3 Promoter methylation in cancer metastasis

The promoter is that region of the gene where RNA polymerases and transcription factors bind to transcribe the gene producing mRNA transcript that is exported to the cytoplasm and translated into the protein. Methylation of the promoter is the first step in the chain of protein production that alters the expression levels of the protein. Methylation at the promoter or putative promoter site will repress the expression of the gene which translates to phenotypic outcomes at the cellular level and clinical outcomes at the macro-level. A number of these aberrant methylation patterns in the promoter present themselves at sites of tumor-suppressor genes thus silencing the gene and increasing tumor invasiveness. Epigenetics research in the past 20 years has identified a number of genes in different types of cancer that have aberrantly methylated promoters. In the process of EMT where cells differentiate to become more invasive and motile, there is a paradigm shift in the protein expression profile some of which is the outcome of silencing active housekeeping genes and activating oncogenes. This shift in the protein expression profile is a continuous process that happens through the course of tumor progression and metastasis. The promoter of the E-cadherin gene *CDH1* contains 22 CpG dinucleotides six of which have been found to be methylated in various cancers such as leukemia, breast cancer, prostate cancer and ovarian cancer (Corn, Smith et al. 2000, Li, Zhao et al. 2001). Corn et al. proved that a

methylation at these six positions represses the expression of the E-cadherin gene and shuts down protein expression, which reduces the number of points of cell to cell contact. This increases the possibility of a tumor cell escaping from the primary tumor site into the blood stream, leading to metastasis. In prostate cancer, several studies have identified a number of genes whose promoters have been methylated during stages of cancer progression (Maruyama, Toyooka et al. 2002). Of note are *RARβ* (Retinoic acid receptor β), which suppresses oncogene-induced focus formation (Lee, Si et al. 1995); *RASSF1A* (Ras association domain family 1 isoform A), a tumor suppressor gene, and *GSTP1* (Glutathione S Transferase P). These genes are increasingly methylated with decreased protein expression as cancer progresses. Additionally, Ras association domain family 2 (*RASSF2*), a tumor suppressor gene has been found to be methylated in PCA with decreased expression (Liu, Yin et al. 2013). Another study has shown that the use of sulforaphane, an isothicyanate derived from vegetables causes an activation of the cyclin D2 with a demethylation of its promoter leading to increased transcript and protein levels (Hsu, Wong et al. 2011). The evidence presented above places emphasis on the methylation state of the promoter as a very important focus point in cancer progression and a viable target for developing therapeutics. Prostate cancer is thus a very good model to study the effect of external stimuli on the protein expression profiles that are modulated via the epigenetic pathways. The inherent advantage that is conferred on a cell with an epigenetic alteration is the fact that the change is reversible (Liang, Kanduri et al. 2000, Reik, Dean et al. 2001) and transient while the protein that is transcribed is fully functional but only its molarity in the cell is changed. Thus, chemical reversion of the methylome will revert the associated proteome which making it a popular drug target (Daskalakis, Nguyen et al. 2002).

1.4.4 Intragenic methylation: a new theory

A genome-wide study to identify DNA methylation loci across 20 eukaryotic species identified intragenic methylation to be highly conserved compared to promoter region (Hellman and Chess 2007, Zemach, McDaniel et al. 2010, Hsu, Wong et al. 2011). It is known that intragenic methylation is present in highly transcribed genes, with higher methylation specifically in exons that are transcribed (Ball, Li et al. 2009, Lyko, Foret et al. 2010). Previous studies have published that an intronic point mutation either exerts expressional control via the promoter or alters the splice ratio of the protein (Choi, Park et al. 2008, Nordin, Larsson et al. 2012) implicating introns in splicing. Recently it has been observed that intragenic methylation, specifically in the exon, is associated with alternative splicing events though the biological mechanism is still under scrutiny.

One of the interesting aspects of the genetic architecture of intragenic regions is the stark difference in the CpG content between the exons and the introns. The exonic regions are known to be CpG rich when compared to the introns and this high difference between the two regions inspires a great shift in the expression levels of splice proteins upon methylation of a single nucleotide in the gene (Choi 2010). In mammals, splicing of the mRNA happens co-transcriptionally as the DNA is being transcribed (Beyer and Osheim 1988, Tennyson, Klamut et al. 1995). A change in the chromatin structure alters the elongation rate of Pol II which in turn affects the spliceosome loading onto the growing pre mRNA (de la Mata, Alonso et al. 2003, Nogues, Kadener et al. 2003). A methylation at a particular CpG coils the DNA tighter around the nucleosome which slows down or stalls the Pol II elongation. At the methylated fork, the polymerase has to uncoil the DNA for further transcription which causes pausing, giving the splicing machinery time for exon recognition and protein recruitment for splicing (Shukla,

Kavak et al. 2011, Maunakea, Chepelev et al. 2013). Thus aberrant methylation affects co-transcriptional alternate splicing by altering Pol II elongation rates. Supporting evidence was published in 2010 when depletion of DNA methylation in the *Hox* genes was proved to promote Pol II elongation facilitating efficient splicing (Tao, Xi et al. 2010).

One of the hypotheses proposed to explain the effect of intragenic methylation on splicing is that an aberrant methylation in the intron, first exon or alternatively spliced exons affects the loading of the spliceosome on to the DNA which affects the rate of splicing. Bioinformatics analyses of the published methylome studies have proved that the methylation of the CpG in splice sites and exon splice enhancers (ESE) have been associated with altered splice ratios. In light of this new information about the intronic regions, substantial new data in the past six years have linked intronic alteration to a splice change or gene silencing (Anastasiadou, Malousi et al. 2011). For example, a methylation in the intron of *EGFR2* was found to cause higher levels of the protein in the tumor tissues found in patients. Though the mechanism was not clear, it was proposed to support enhancer like activity (Unoki and Nakamura 2003). On the contrary, in prostate cancer cells, methylation in the intron at one position in the *PMP24* gene was found to have shut down the expression of the gene (Zhang, Wu et al. 2010). A number of correlative studies have been published associating intronic methylation to tumor progression and malignancies. Micro RNA 199a (miR199a) regulation was found to be associated with the methylation of the intronic locus in testicular malignancies (Cheung, Davis et al. 2011) while the expression of *PTPRG* was indirectly regulated by a methylation in its first intron of a single nucleotide (van Roon, de Miranda et al. 2011). Thus, intragenic methylation is an exciting new branch of study in the field of epigenetic regulation. In our study, we used a novel tool to demonstrate the role of intragenic differential methylation in protein expression

1.5 SPECIFIC AIMS AND HYPOTHESIS

Prostate cancer is the second most common cause of cancer deaths among men, with metastatic disease being the main reason for the high rates of mortality and morbidity. The initial escape of a tumor cell from the primary mass to disseminate shares many of the same cell behaviors as noted during wound healing; a main difference being signals in the wound bed that stop this process. A key 'stop' signal is via the *CXCR3* receptor, which modulates cell migration and proliferation. *CXCR3* is a G protein coupled receptor which has 2 splice variants CXCR3A and CXCR3B; dysregulation of this signaling axis has been implicated in the poor survival of breast, renal, ovarian, and prostate cancer patients. In our lab, we have observed that there is a switch in the expression between the *CXCR3* splice variants and an over-expression of CXCR3A in disseminated prostate cancer with this alteration being present at both the RNA and protein levels. The mechanism behind the switch is currently unknown. Since the switch is present at the RNA and protein level, the trigger causing the change is present at the DNA.

To better understand the genomic signature of nontransformed (RWPE-1) and tumorous (DU-145 and PC-3) prostate cells under study, we screened the *CXCR3* gene for mutations and found that the gene is intact except for a single nucleotide substitution in the intron which is not a differentiating factor between the normal cells and cancer cells. While a mutation in the gene causes a permanent change in the genetic profile of the cancer cells, an epigenetic change is a transient change that would alter the protein expression without changing the template. This reversible change enables the cancer cell to exercise control over the expression of the splice variants at different stages of cancer progression, thus exploiting the functionality of both splice variants. Since we found no mutation in the gene and a reversible change that would confer an advantage to the cancer cells for the critical period, **I hypothesize that epigenetic changes at**

the DNA level alter the splicing of *CXCR3* in prostate cancer cells to favor a higher CXCR3A : CXCR3B ratio.

Epigenetic regulation includes methylation at the gene level and acetylation at histone level that maintains the conformation of the DNA, making it open or closed for the access of transcription factors. A change in the methylation in the promoter or intragenic regions would affect the gene expression levels and the alternative splicing. I tested my hypotheses using the following aims.

Aim1. To determine if there is an epigenetic change at the DNA in the cancer cells that would alter the splice isoforms levels

Aim2. To determine if the differential methylation in the *CXCR3* intragenic region alters its splicing

2a. To determine if the cancer and normal endogenous methyl marks differentially alter the *CXCR3* splice isoform ratio, and

2b. To dissect the effect of single nucleotide differential methylation on alternative splicing.

Successful completion of these experiments would shed light on the molecular mechanism behind the switch in the expression of the *CXCR3* splice variants in prostate cancer. The knowledge of the switch mechanism would provide us with possible points of interjection to prevent metastasis and the epigenetic information would open new avenues for diagnostic tests and drug development.

2.0 MATERIALS AND METHODS

2.1.1 Cell lines used

The following cell lines were used for the puposes of the study. The previously published which demonstates a switch in the expression of CXCR3 isoforms in the prostate cancer cells were carried out in this cell line system where the DU-145 and PC-3 cell lines are metastatic prostate cancer cell lines and RWPE-1 is the immortalized normal prostate epithelial cell line. As part of good lab practices , DNA fingerprinting of cell lines is carried out every two years to validate the cell lines.

DU145: Human immortalized prostate cancer cell line of epithelial origin derived from the metastatic tumor in the brain. The cells were maintained in DMEM (4.5 g/L glucose) with fetal bovine serum (FBS) to a final concentration of 10%, 1mM sodium pyruvate, nonessential amino acids, 2mM glutamine and pen-strep.

PC-3: Human immortalized prostate cancer cell line of epithelial origin derived from the metastatic tumor in the bone. The cells were maintained in F-12K medium with FBS to a final concentration of 10% and pen-strep.

RWPE-1: Human immortalized normal prostate epithelial cell line. The cells were maintained in keratinocyte serum free Medium (K-SFM) with 0-05 mg/mL PE and 5ng/mL EGF and FBS to a final concentration of 10%.

2.2 SEQUENCING

2.2.1 DNA extraction and sequencing

The cells from a 100 mm dish were collected for DNA isolation. The cells were lysed and the DNA was extracted using phenol chloroform isolation. 200 ng of DNA was used to perform PCR to amplify the *CXCR3* gene for sequencing. The *CXCR3* gene was sequenced in 4 pieces with 4 different primers. The primers were designed to overlap to ensure sequencing of the ends. The following table provides with a list of primers used for sequencing the *CXCR3* gene. The PCR was carried out using platinum PCR mix purchased form life technologies in the Techne TC-312 and TC-5000 thermal cycler.

Table 1. *CXCR3* gene sequencing primers

Forward primers	Reverse primers
5'GTGGCCTGAGGTTTAGGGAGGTC 3'	5' TCAGTGCCCCACCCCCAAC 3'
5'GGCGTGGTGACTTGTAGAGAGGC 3'	5' CCCAGAGCGGCAGTGTCAGC 3'
5'GTGGCAGCCGTGCTGCTGAG 3'	5' TGGCTGCCTCTGGAGCCCTC 3'
5'TGCACTGCTGCCTCAACCCG 3'	5'TGTAATGATGCACGAACATGCCCTG 3'

The PCR products were Sanger sequenced at the Genomic and Proteomic Core Laboratories, University of Pittsburgh. The cDNA of *CXCR3* was sequenced with the primers below.

Table 2. cDNA sequencing primers

Forward primers	Reverse primers
5' CAGAGCACCAGCCCAGCCAT 3'	5' CTACGCAGGAGCCCTCCT 3'
5'TGAGGTGAGTGACCACCAAGTGCT3'	5' ACAGCCAGGCAGGTGAGGGT 3'
5'TCAGCTTTGACCGCTACCTGAACA 3'	5'TACAAAGGCATAGAGCAGCGGGTT3'
5'ACTGCTGCCTCAACCCGCTG 3'	5' TGCTGAGCTGGAGGCCTGGG 3'

2.2.2 Bisulfite sequencing

As outlined before, the DNA was isolated from the cells and 1µg of DNA was bisulfite treated with the Qiagen Epitect bisulfite kit. 200ng of the bisulphite treated DNA was used to amplify the intragenic regions of *CXCR3* to determine the methylation pattern using nested PCR. The *CXCR3* intron was sequenced in two fragments and the 3' alternate exon was sequenced as a single fragment. The following table provides a list of primers that were used to amplify *CXCR3* gene for bisulfite sequencing.

Table 3. Bisulfite sequencing primers

Forward primer	Reverse primer
Primers intron fragment 1	
5’GTTTAGTTATGGTTTTTGAGGTAAG 3’	5’CATACAATCTACCCAATCCTT 3’
Nested primer	
5’ATGGTTTTTGAGGTAAGTGTTGTTGTTTAG3’	5’CCAATCCTTCCCCCTCCCTACAC 3’
Primers intron fragment 2	
5’ATTGGGGAGATTTGTAGGTTTATAGTGTTGGG3’	5’CCCTCCAATACCCAAAACCCTCTCTACCC3’
Nested primer	
5’AGGTTTATAGTGTTGGGGGTGAGGGGATAAAG3’	5’TCTCTACCCACTATCCTCTCTCTCCCTACT3’
3’ alternate exon primers	
5’AGGTAAGTTTGAAGGGAGAGTA3’	5’TAAGTTTGAAGGGAGAGTAGGG3’
Nested primers	
5’CTA ACA AAAAAACCCGATCGAAAT3’	5’CAAAAAAACCCGATCGAAATTCAAAC

2.3 CLONING

2.3.1 Bichromatic reporter

The bichromatic reporter minigene construction was done in two parts where the bichromatic reporter was first engineered and the reporter frames were validated after which the *CXCR3* minigene was cloned in. The reporter consisted of two pieces: dsRED and EGP that were cloned into the pFLAG-CMV-3 vector that was purchased from SIGMA-ALDRICH. The two fluorochromes were sequentially cloned in with EGFP first followed by dsRED. EGFP was amplified from the pEGFP-N1 vector in the lab and the forward and reverse primers were tagged with the bgl2 and bamh1 restriction enzyme sequences respectively. The PCR product obtained was agarose gel purified using the Qiagen gel purification kit after which the clean PCR product was cloned into the TOPO-TA cloning vector purchased from life technologies. TOP 10 one shot cells from life technologies were transformed with the TA vector with the purified PCR product. The pFLAG-CMV-3 vector and the TA vector with the insert were restrict digested with the blg2 and bamh1enzymes to facilitate the ligation of the EGP insert in the vector. Since bgl2 and bamh1 are compatible cohesive ends, the restriction digested vector was treated with TSAP to remove the phosphate group form the 5' end of the DNA to prevent recircularization. The insert excised form the TA vector was ligated into the open pFLAG-CMV vector using the quick ligation kit purchased from Promega. The top 10 one shot cells were transformed with the ligation mix after which mini cultures were set up and plasmid was extracted using the Qiagen miniprep kit. Following the same sequence of steps, dsRED was cloned in the following primers where the 5' and 3' were tagged with Ecor-1 and bgl2 respectively. Two different primer pairs

were used to generate pMGR and pMGR-FS. The dsRED was amplified from the pdsRED2 plasmid available in the lab stock.

Table 4. Bichromatic reporter primers

Forward primer	Reverse primer
pMGR – Bichromatic reporter producing dsRED	
5'GAATTCAATTGTGGCCTCCTCCGAGG ACG3' – Ecor1RE	5'GAATTCAATTGCCTCCTCCGAGAACGT CATCACC3' – bgl2 RE
pMGR-FS – Bichromatic reporter producing EGFP	
5' GAATTCAGTatggtggcct cctccgaggacg 3' - Ecor1RE	5'GAATTCAGTATTGCCTCCTCCGAGAAC GTCATCACC3'- bgl2RE

The highlighted nucleotides are the frame shifts that introduce the +2 ORF that codes for the non-fluor dsRED without stop codons.

2.3.2 Bichromatic reporter minigene

After validation of the reporter frames, the minigene was cloned in to the reporter pMGR. The minigene was amplified as two fragments and sequentially cloned in to the pMGR reporter. The primers used were tagged with restriction enzymes to enable cloning. The following table provides with the primer information with the respective restriction enzymes. The inserts were amplified from the RWPE-1 DNA to be ligated into the bichromatic reporter minigene.

Table 5. Minigene primers

Forward primer	Reverse primer
Amplicon 1 for intron	
5'AAGCTTATTGTCCTTGAGGTAAGTGCT 3'- Hind3 RE	5'GCGGCCGCGTACTTCCTCAACTCCA TC3' : Not1 RE
Amplicon 2 for 3' alternate exon	
5'GCGGCCGCGGCCCTGGAAGACTGGCGGG GAC3' : NOT1 RE	5'TGAATTCCACGAGTCACTCTCGTTT TC3' – EcoR1 RE

Upon completion of ligation and building the entire plasmid, maxi cultures were set up to obtain clean plasmids and the Hurricane maxiprep kit was used to extract plasmid DNA with high concentrations.

Mutants

The mutants were generated using the Agilent Quick change directional mutagenesis kit 2. The following primers for the four different methylation sites were purchased from idtDNA for use.

Table 6. Mutant primers

Forward primer	Reverse primer
Mutant 1	
5'AGCCAGGCCAGGCCAGAATTGGAAGTG GAAAACCCGGGGCAT 3'	5'- ATGCCCCGGGTTTTCCACTTCCAA TTCTGGCCTGGCCTGGCT -3'

Table 6 continued

Mutant 2	
5’AATTGTGGAAAACCCGAAGGGGGCATC AGGGGAATGCCA 3’	5’AATTCGAAGTGGAAAACCGGGGGC ATCAGGGGAATGCCA 3’
Mutant 3	
5'GGGCGGGGGGCAAGTAGACTGGAA GGCTCCCTTCAGGAGACT 3'	5'AGTCTCCTGAAGGGAGCCTTCCAGT CTACTTGCCCCCCGCCC -3'
Mutant 4	
5' GGCCCTGGAAGACTGGGGGGGACAGTT ATAGGA -3'	5'TCCTATAACTGTCCCCGCCAGTCTT CCAGGGCC 3'

2.3.3 Transfection

The cells cultured in a 100 mm dish were seeded on coverslips inside a 6 well plate. The cells were allowed to attach and reach 70% confluence after which they were transfected with the plasmid. 2 µg of pMGR-*CXCR3* and mutants were transfected into cells using Lipofectamine 2000 and serum free Optimem. The transfection was performed for 5 hours and then medium was changed to the regular medium described above.

2.4 MOLECULAR ANALYSIS

2.4.1 Immunoblot

The cells that were transfected with the plasmid were collected after 48hours and lysed with the sample buffer to prepare for western blotting. The prepared protein samples were loaded on 10% gel and migrated at constant current of 300 amps. The protein was transferred to the nitrocellulose membrane after which they were blocked for an hour with 5% BSA. The blots were incubated in primary rabbit anti Flag antibody purchased from Sigma-Aldrich overnight. The following day the blots were washed and incubated with the secondary antibody for an hour. The blots were then treated with ECL and blots were exposed.

CXCR3 antibody was purchased from R&D sciences to probe for whole *CXCR3* and CXCR3B purchased from Protein Tech antibody purchased from the blots were visualized using chemiluminescent substrate for HRP (Thermo Scientific, IL) and X-ray film processor (AFP imaging, NY).

2.4.2 Microscopy

The cells seeded on coverslips were fixed in 4% paraformaldehyde 48 hours after transfection. The fixed cells were permeablised with 0.5 % Triton X 100, stained with DAPI and then mounted on slides with gelvatol. Microscopy was performed with the slides. Thresholding for the signal generated from the construct pMGR-*CXCR3* was done using the positive controls: cells expressing dsRED alone and EGFP alone.

2.4.3 qPCR

The cells transfected with the plasmids were trypsinized after 48 hours and pelleted for RNA extraction. The Qiagen RNeasy kit was used to extract whole RNA from cells. 1µg of RNA was reverse transcribed using the Quantitect RT-PCR kit to produce cDNA. 1µL of cDNA was used to perform qPCR using SYBR green master mix from Agilent technologies. Construct specific primers that would amplify CXCR3A and CXCR3B generated from the minigene in the plasmid. The levels of test RNA were expressed relative to the levels of GAPDH.

Pan *CXCR3* Forward: 5' GAGAACTTCAGCTCTTCCTATGACTAT 3'

CXCR3A - Reverse: 5' CTC GGG GAA GGA CAG CTT CT 3'

CXCR3B-Reverse: 5' CCT TCA GCT CGA TGC GGT TC 3'

2.4.4 Flow cytometry

A 100 cm dish plate split into a six cell plate and equal number of cells were seeded in each well. The cells were transfected with 2 µg of the plasmid and 36 hours after transfection the cells were collected. Flow cytometry was carried out with Cyan at the Hillman Cancer Center flow lab, University of Pittsburgh. The cells expressing dsRED only and EGFP only were used as controls to compensate and gate for the expression levels from constructs. The unstained cells were used to control for cell size and auto-fluorescence.

2.4.5 Hypoxia

The cells were cultured and maintained in the Biospherix hypoxia chamber at 5% oxygen and 1% oxygen and were maintained in the system for 1 day, 3 days and 5 days respectively for experimental purposes.

3.0 RESULTS

The *CXCR3* promoter and intron were bisulfite sequenced to analyze for a differential methylation in the cancer cells that possess the switch in the expression of the isoforms.

3.1 EPIGENETIC REGULATION OF ALTERNATIVE SPLICING

3.1.1 *CXCR3* Promoter methylation analysis

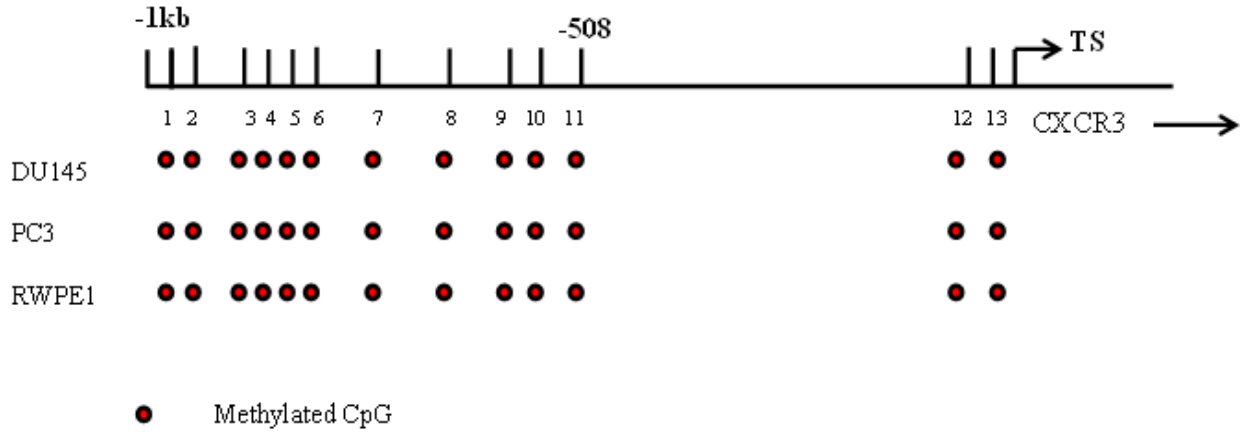


Figure 6.Methylation analysis of the *CXCR3* promoter

This is a schematic representation of the CXCR3 promoter where 13 CpGs were analyzed using bisulfite sequencing. The red circles represent methylated CpGs. It was found that the promoter

was free of methylation in the promoter region. TS represents the transcription start site of CXCR3. The analysis was performed two cancer cell lines DU145; PC3and one normal prostate epithelial cell line RWPE-1

The *CXCR3* gene contains one promoter that regulates the expression of the gene, and both the splice variants are transcribed from the same promoter. An intronic variant of the *CXCR3* gene was known to alter the differential levels of *CXCR3* isoforms through the promoter (Choi, Park et al. 2008). This emphasizes the role of the promoter in gene expression control of CXCR3 In an attempt to uncover the mechanism behind the switch of splice variants in prostate cancer, the promoter was analyzed for differential methylation. 1 kb upstream of the transcription start site was bisulfite sequenced to analyze a total of 13 CpGs of which 11 CpGs that were arranged in clusters and two that were very close to the transcription start site. The promoter was heavily methylated but it was found that there is no difference in the methylation pattern of the promoter in the prostate cancer and normal prostate cells. The promoter is a transcriptional regulator and a methylation in the promoter shuts off the expression of the gene. A difference in the methylation could also be a reason for binding of transcriptional repressors or activators. But in our case, we did not observe a change in the expression levels of the gene but only a change in the splice isoform expression. Thus an absence of a differential methylation in the promoter is well- justified in the specific splicing case of *CXCR3*.

3.1.2 Differential methylation of intron and first exon in prostate cancer cells.

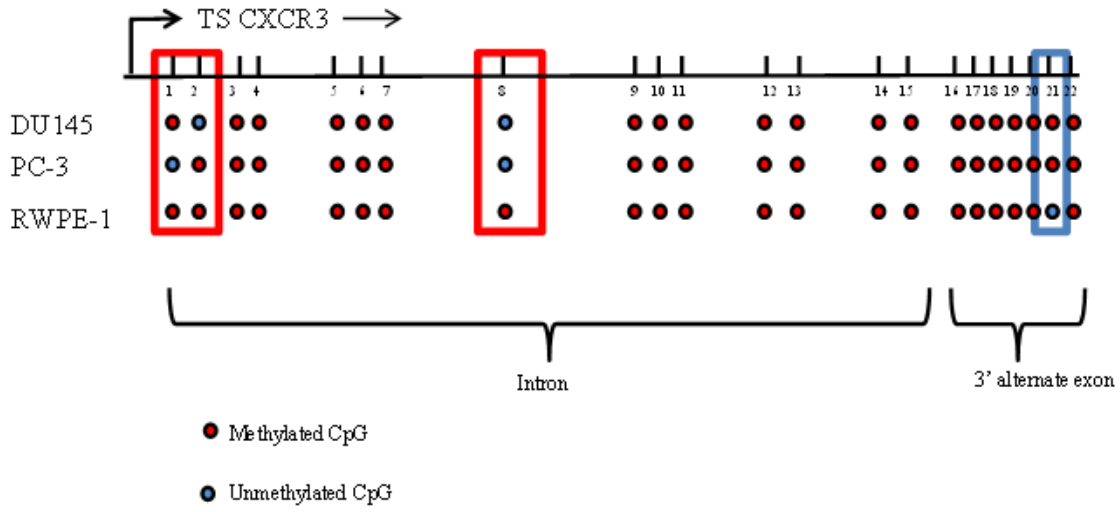


Figure 7. Differential methylation of *CXCR3* intron

This is a schematic representation of the CpG islands in the intron and 3' alternate exon of the CXCR3 gene. The red circles indicate methylated CpG and blue circles indicate unmethylated CpG. The differentially methylated sites are indicated in rectangles outlining them. DU-145 and PC-3 cells are the prostate cancer cells and RWPE-1 is the normal prostate cell line.

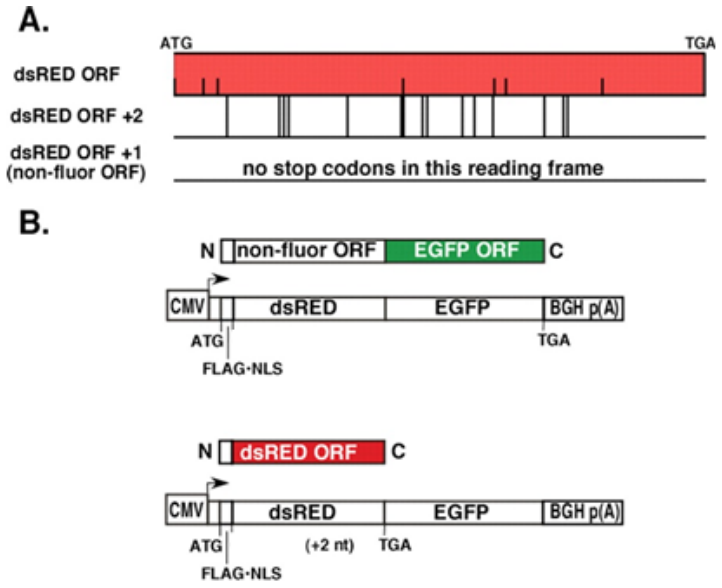
The *CXCR3* gene was scanned for differential methylation sites in the intron and 3' alternate exon. The intron of the gene was found to be heavily methylated with differential methylation at four different sites, one of which lies in the alternate exon that contains the translation start site for CXCR3B which is unique for this isoform. A high rate of methylation in the intron suggests that even a small change in the status of a single CpG would alter the conformation of the DNA leading to a change in the RNA pol 2 elongation rates. Recent studies have identified that intragenic methylation alters alternative splicing via MeCP2 recruitment at these sites. This emphasizes the fact that these differentially methylated sites could possibly play

a role in the alternative splicing of the pre-mRNA. The four differentially methylated cytosines for the sake of simplicity were names in increasing order of their proximity from the translation start codon 'ATG' of the *CXCR3* gene. Differential methylated CpG 1 and 2 lie near the 5' splice donor site flanking the splice junction; 3 lies in the intron and 4 lies proximal to the alternate splice junction in the 3' alternate exon. A differential methylation at these sites present in or near the alternate exon would affect the inclusion and skipping of the respective exons. Intragenic methylation has been associated with an increased inclusion of the alternate exon via the recruitment of MeCP2 to the DNA (Maunakea, Chepelev et al. 2013).

Interestingly, the sites 1 and 2 flanking the 5' splice junction of *CXCR3* were methylated in the DU-145 and PC-3 cells respectively. CpG 4 in the 3' alternate exon skipped in CXCR3A was methylated in the cancer cells and unmethylated in the normal prostate calls. This opposite pattern of differential methylation in the intron and exon suggests a possible splicing program change that favors the expression of the A isoform in the prostate cancer cells. This differential methylation in the *CXCR3* gene of prostate cancer cells provides with a good model to study the effect of intragenic methylation on splicing. Prostate cancer with its plasticity and ability to incorporate environmental cues in its epigenome is a perfect system to study the mechanism behind a proteome shift mediated via epigenetic pathways. To test if a change at these sites alters splicing, we used a *CXCR3* minigene with bichromatic reporters. The differentially methylated sites were mutated to guanines eliminating the methylation factor from the splicing of the gene under study. A difference in the splice read out was used as a phenotypic measure of splicing.

3.1.3 Bichromatic reporter minigene

In order to tie together the concepts of alternative splicing of CXCR3 and its intragenic differential splicing , we used a bichromatic reporter minigene. A bichromatic reporter minigene is a single plasmid that reports alternative splice variants with unique fluorochromes. A bichromatic reporter is a single plasmid that contains both dsRED and EGFP but produces both fluorochromes from mutually exclusive reading frames.



Modified from Orengo J P et al. Nucl. Acids Res. 2006;34:e148

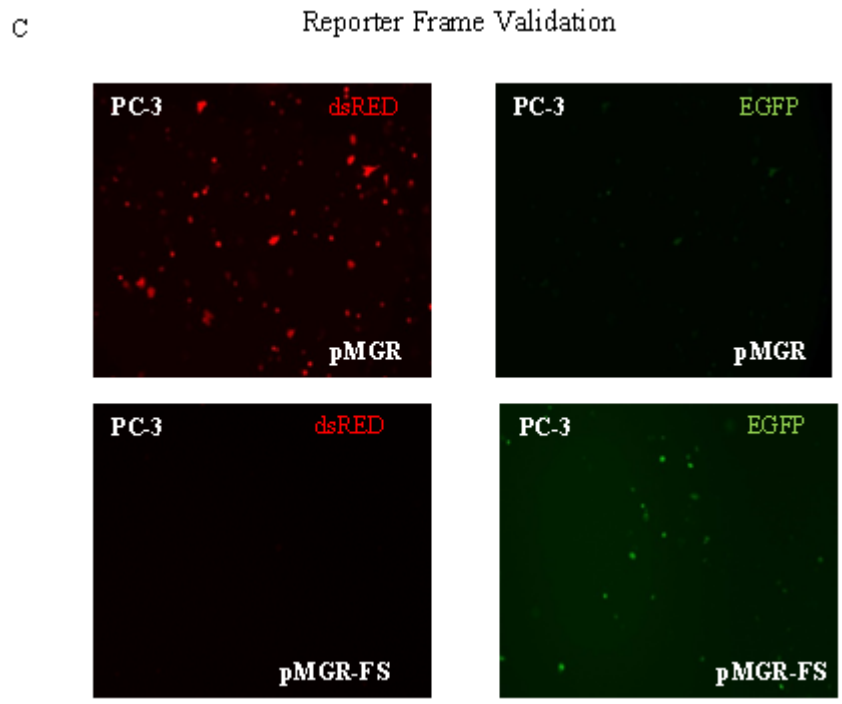


Figure 8. Bichromatic reporter: ORF and mechanism of action

A: Distinct reading frames of dsRED with the first dsRED ORF translating functional dsRED and the third +1 ORF translating a non-fluor ORF with no stop codons; B: Expression of dsRED and EGFP from the same construct but unique frames where pMGR expresses dsRED and pMGR-FS expresses EGFP tagged to non-fluor dsREd; C: Cellular expression of the fluorochromes in the PC-3 cells. pMGR expresses only dsRED with pMGR-FS expressing only EGFP, thus validating the frame shifts successfully.

The bichromatic reporter was engineered as explained in materials and methods. It is a single construct with a CMV promoter that expresses both dsRED and EGFP from mutually exclusive reading frames. The unique property of dsRED was exploited in developing this construct. dsRED contains two alternate reading frames, one which produces a functional dsRED and a +1 reading frame that produces a dysfunctional dsRED but removes all stop codons from

that specific reading frame (as shown in figure 8A) . Fusing EGFP ORF with the alternate +1 dsRED ORF produces a functional EGFP tagged with a non-fluor dsRED. Thus this bichromatic reporter expresses two different fluorochromes from mutually exclusive alternate reading making it a perfect candidate for alternative splicing analysis. Figure 8B demonstrates the expression of the fluorochromes from different reading frames. The dsRED expressing construct was named pMGR and EGFP expressing construct was named pMGR-FS. Figure 8c shows the expression of the individual fluorochromes from the pMGR and pMGR-FS in the PC-3 cells thus validating the working frame shifts and mutually exclusive reading frames. In our study, we used this property of the construct to tag CXCR3A and CXCR3B to dsRED and EGFP respectively to study the splice difference in cancer.

3.1.4 *CXCR3* bichromatic reporter map

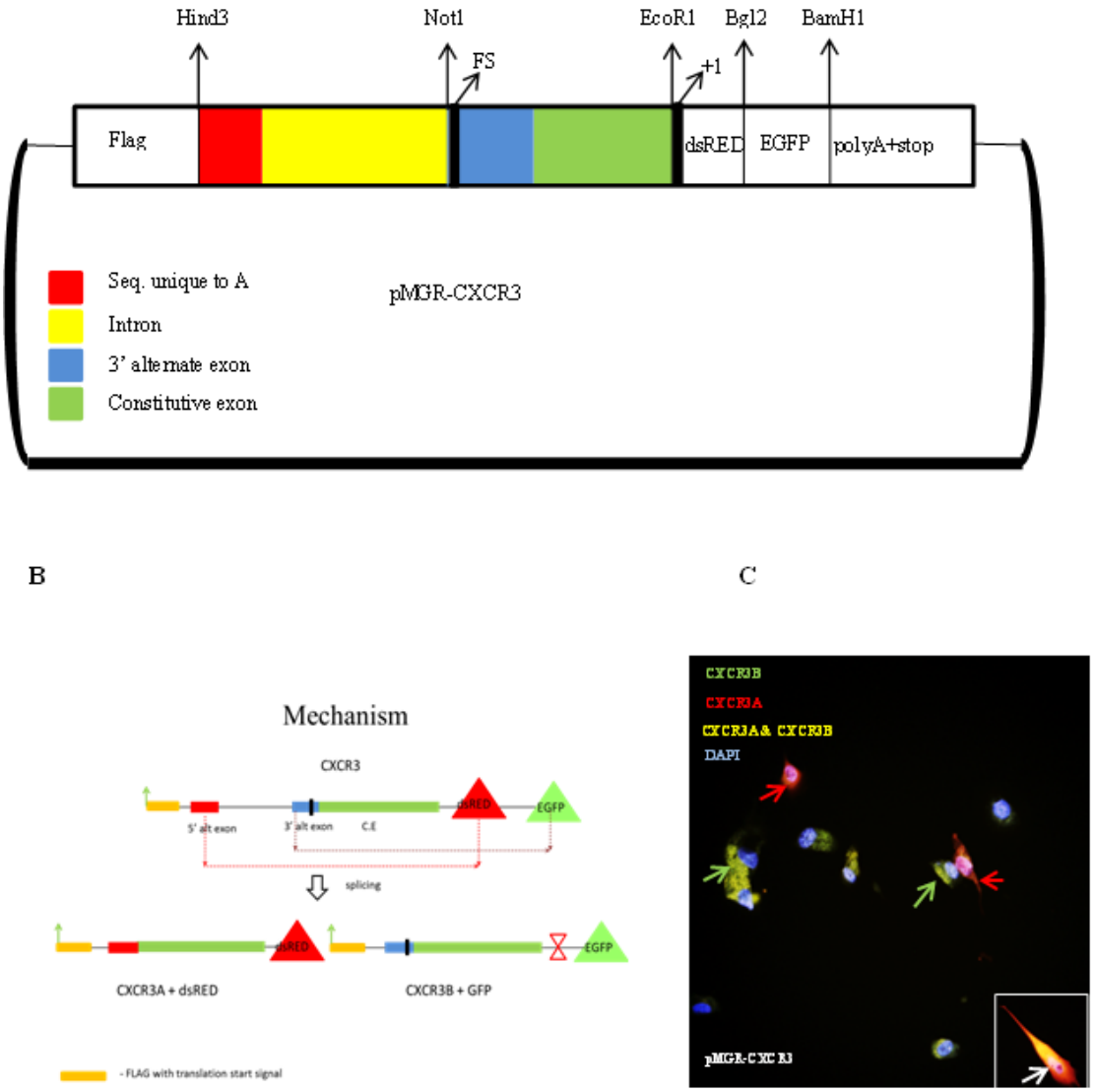


Figure 9. Bichromatic reporter characterization

A: pMGR-CXCR3 vector map with the positioning of the exons and the frame shifts ; B : Mechanism of Action of the bichromatic reporter minigene where CXCR3A *is in frame with dsRED and* CXCR3B *is in frame with EGFP; C : Cellular expression of the construct with the construct transfected into PC-3 cells. The cells express both* CXCR3A *and* CXCR3B *where the*

red arrows indicate only CXCR3A *expression, green arrows indicate* CXCR3B *expression and yellow arrows indicate the expression of both the isoforms that appears as yellow, a merge of green and red*.

The *CXCR3* gene with its intron, 3' alternate exon and part of constitutive exon were cloned into the bichromatic reporter, pMGR explained in the previous section. The 5' splice site with the intron, 3' alternate exon and the constitutive exon were amplified from the DNA of the RWPE-1 cells as two different amplicon and ligated into the construct. The splice junctions were conserved to ensure effective splicing. A +1 frame shift in the 3' alternate exon was introduced by adding two nucleotides which places it out of frame with dsRED and in frame with EGFP.

Mechanism

As depicted in the figure 9B, CXCR3A is in frame with dsRED whereas CXCR3B translated from the 3'alternate exon is in frame with EGFP. The CXCR3A isoform contains the constitutive exon; 5' end unique to CXCR3A and is tagged with dsRED, flag. Translation of this protein produces a protein of molecular weight 31 kDA. The CXCR3B isoform contains the constitutive exon; 3' alternate exon and is tagged with EGFP, flag. Translation of this alternate isoform results in a protein of molecular weight 67 kDa Immunoblot probing for FLAG in the cells transfected with pMGR-*CXCR3* would generate bands of molecular weight 31kDa and 67 KDa for CXCR3A and CXCR3B respectively. Once the construct was engineered, the expression of the isoforms was tested by transfecting into 3 different cell types each of which show a specific pattern of isoforms expression. The unique property of this construct is that it allows for testing of the splice ratio at the single cell level. Figure 9C shows that the cells are a heterogeneous

population where a few cells express only CXCR3A; some only CXCR3B while some express both the isoforms. The co-expression of both the splice variants is visible as the merge of red and green, a bright yellow indicated with a white arrow in figures 9C. This unique property of the constructs enables to test if a change of the splice variants is a homogenous intercellular or a heterogeneous intracellular change while it allows for observing the splice change at a single cell level.

3.1.5 Cell type specific expression of the construct

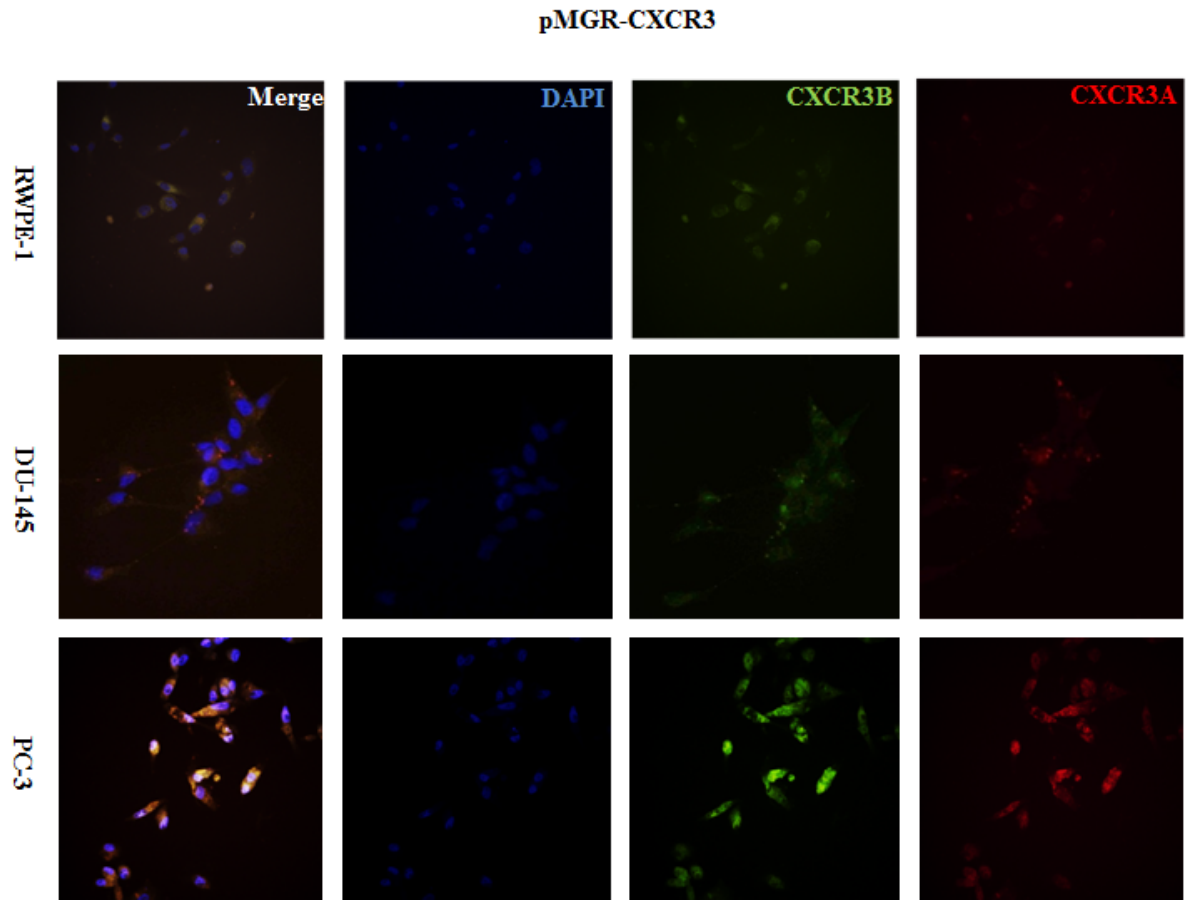


Figure 10. Cell type specific expression of the reporter

The cells were transfected into the cancer cells line and the normal prostate cell lines. Cell type specific expression was observed. PC-3 cells show a higher expression of CXCR3A *while RWPE-1 cells show* CXCR3B *only.*

The construct was transfected into the two cancer cell lines: DU-145 and PC-3, one normal prostate epithelial cell line: RWPE-1, to compare the expression pattern generated from the construct with the published endogenous *CXCR3* receptor isoform levels in the cell lines. The RWPE-1 cells show higher levels of CXCR3 B; the two cancer cells lines DU-145 and PC-3 show higher CXCR3A (Wu, Dhir et al. 2012). Upon transfection of the construct into the respective cell lines, cell type specific expression was observed as expected with PC-3 showing a high expression CXCR3A isoform and the RWPE-1 normal prostate cells showing a higher expression of the CXCR3B isoform. The expression of the receptor from the construct is a demonstration of the splice isoform expression at the native state of the cell which concurs with the endogenous expression patterns. Thus an alteration in a single base pair in the intron or the alternative exons would reflect the shift in the receptor expression pattern of the cell at a given time. From the cell type specific expression displayed by the construct, it can be said that this construct is a best model to predict the endogenous splice shift upon a mutation or a differential methylation in the intragenic region. The transfection efficiency of the plasmids between the two cancer cell lines were the best in PC-3 allowing room for analysis that is quantifiable using flow cytometry and qPCR. All the future analyses were carried out using PC-3, a highly metastatic androgen independent cell line derived from the bone.

3.1.6 RNA and protein expression from construct

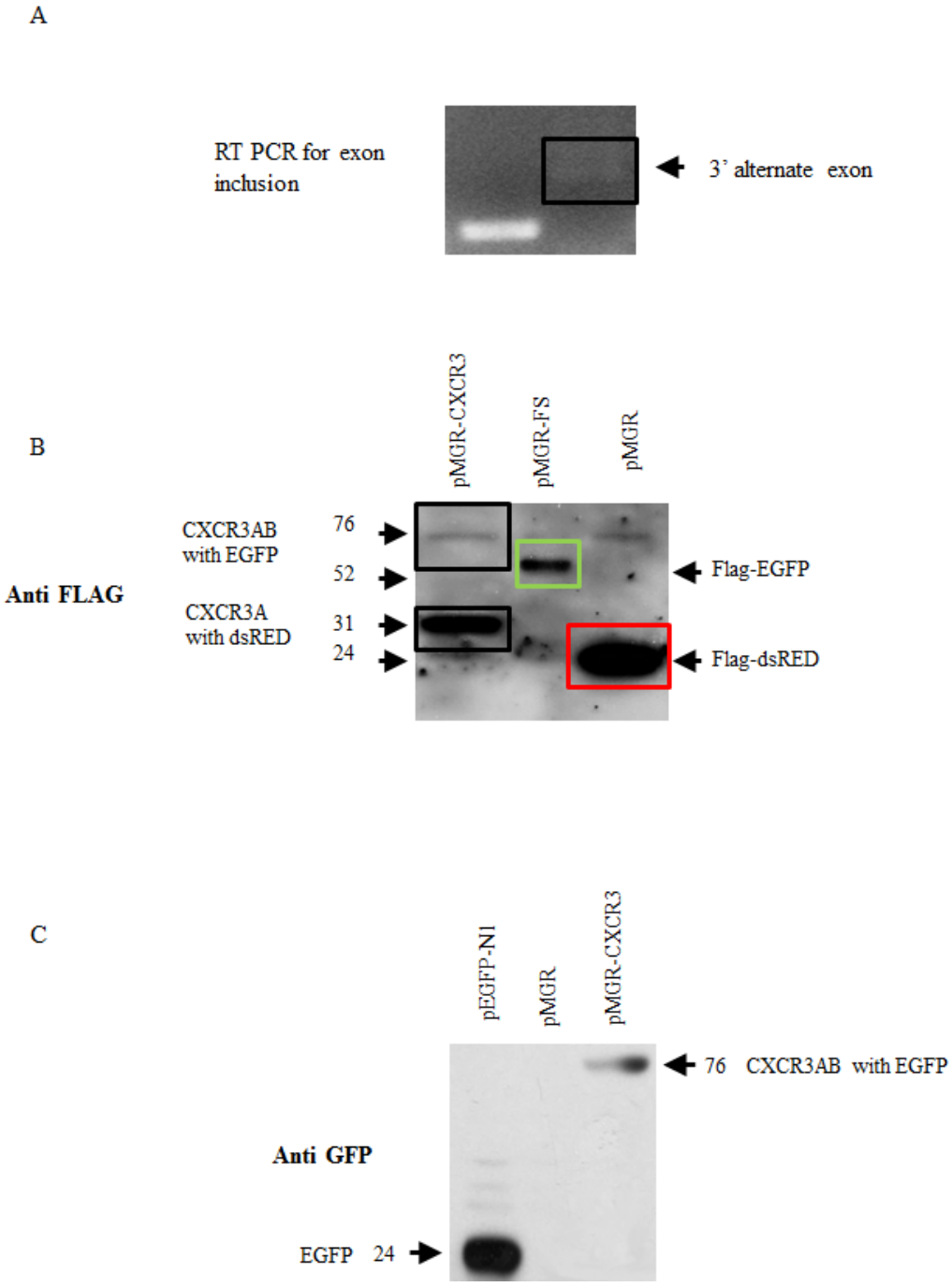


Figure 11. RNA and protein expression of the isoforms from the pMGR-*CXCR3* plasmid

A: RT PCR performed with RNA extracted from PC-3 cells that was transfected with pMGR-CXCR3. Construct specific primers were used to amplify the exons. The black rectangle highlights the 3' alternate exon. B: Immunoblot against flag antibody. pMGR with dsRED and flag was identified at 24 kDa (red box); pMGR-FS was identified at 53 kDa with EGFP, flag and non-fluor dsRED (green box); pMGR-CXCR3 with two bands, CXCR3A *at 31 KDa and* CXCR3B *at 76KDa.*

In order to verify the expression of the *CXCR3* splice isoform expression with their unique tags, immunoblotting against FLAG was performed. This splicing switching was additionally verified with qPCR using construct specific primers. Figure 11A shows the expression of the 3' alternate at 870 bp where the forward primer was targeted to the 3' alternate exon and the reverse primer to the EGFP to obtain a band of size 870 bp. The CXCR3A transcript was amplified with primers unique to the 5' end with a dsRED reverse primer to obtain a band 300 bp in length. The western blot was probed with anti-flag for flag fusion protein in pMGR: dsRED flagged with tag (31 kDa), pMGR-FS: flag with non-fluor dsRED and EGFP (52 kDa) and pMGR-*CXCR3*: CXCR3A-dsRED (31kDa) & CXCR3B-EGFP (76 kDa). The two bands highlighted with a red box and green box represent the two isoforms tagged with unique fluorochromes. To further confirm the validity of the 76 kDa band, the pMGR-*CXCR3* lysates were probed with GFP that is shown in panel 2. A 76 kDa band was obtained and a positive control of cells expressing EGFP was used.

3.1.7 Exogenous DNA methylation signature

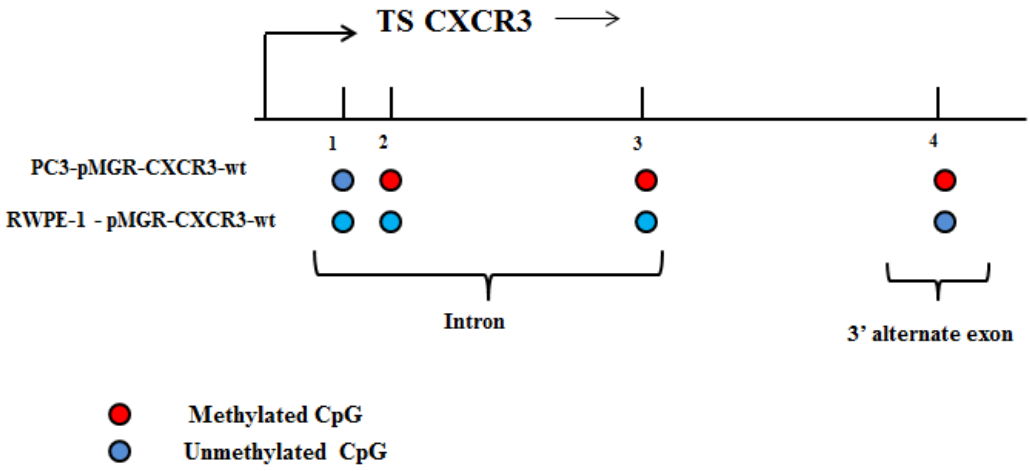


Figure 12. Exogenous DNA methylation in normal and cancer cell types

The constructs were transfected into the PC-3 and RWPE-1 cells and the methylation difference of the cells were observed using bisulfite sequencing with primers that are construct specific. Red circles indicate complete methylation blue indicates lack of methylation and orange indicates partial methylation at the CpGs. It was found that the RWPE-1 cells were unmethylated while the PC-3 cells were partially methylated at position 2 and 4. It was found that a CpG site 1 was consistently unmethylated in all cell lines while site 3 was completely methylated in the cancer cells, but not in the normal epithelial cells.

The differential imprinting of the *CXCR3* gene in the normal and cancer cells was tested. The cells were transiently and stably transfected with the construct to draw a metric on the quantum of differential methylation in the two different cell types to dissect if the loss or gain is an active or passive process. The transient transfection shows that the cancer cells were methylated with 30% penetration of the methylation whereas, surprisingly the RWPE-1 cells

were found to be unmethylated. A cell type specific CpG processivity was observed with the PC-3 containing methylated CpGs in the exogenous *CXCR3* gene. A higher DNMT enzyme levels in the PC-3 cells could be a reason for this differential pattern of imprinting. The differentially methylated cytosines were mutated to guanines to remove the methylation factor out of the transcriptional equation. Single mutants, double mutants and triple mutants were generated to analyze if the removal of a single methylation site alters the splice ratio and if the splice shift increases in a dose –dependent fashion with an increase in the number of intragenic sites that are altered. The mutants that were generated were transfected into the PC-3 cells to analyze the change in splice ratio when the methylation status of a single nucleotide is altered.

3.1.8 Rationale

In order to study the effect of methylation on splicing, it is essential to remove the methylation at certain cytosines of the CpG to observe a change. An efficient method to prevent methylation at this site was to mutate these cytosines to guanines. Upon transfection this mutation prevents prevent methylation. The cytosines at the site 1 and 3 were mutated to guanines. in the wild type to generate mutant 13. This mimics the differential imprinting of the endogenous CXCR3 gene found in cancer cells. Site 4 was mutated in the WT plasmid to generate mutant 4, where the methylation at CpG 4 would be removed to mimic the endogenous CXCR3 gene imprints in the normal prostate cell. The genetic effect of absence of methylation will be reflected as a change in the CXCR3A:CXCR3B expression ratio.

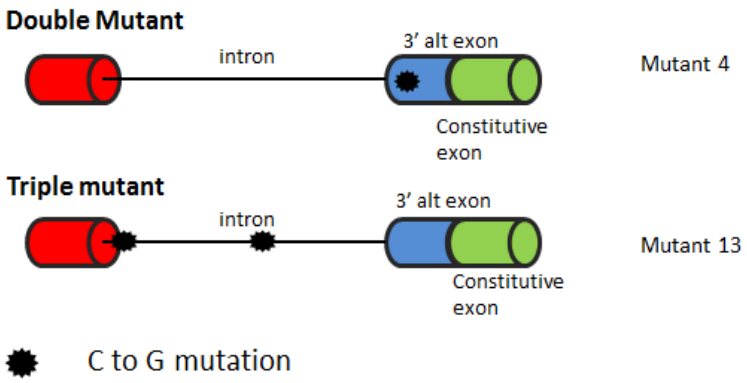


Figure 13. Mutants mimicking endogenous gene

This is a pictorial representation of mutants that were generated from the bichromatic reported minigene plasmid. The differentially methylated sites were mutated to guanines to prevent methylation at that site. Two specific plasmids were created. ***Mutant 13:*** *The cells were mutated at position 1 & 3 to generate a construct similar to the methylation pattern of PC-3 cells.* ***Mutant 4:*** *The cells were mutated at position 4 to generate a construct similar to the methylation pattern of PC-3 cells. The black star indicates a mutation.*

3.1.9 Endogenous CXCR3 specific mutant methylation ratios with their splice axis

In order to observe the effect of epigenetic marks on splicing, we transfected the WT and mutants plasmids into PC-3 cells and the splice readout was measured using flow cytometry. To draw a parallel between the endogenous pattern of methylation and that of the exogenous, construct specific primers were used to determine the methylation state of the CpGs under study in the minigene. It was found that unmutated CpGs under study were methylated as expected. In figure 14A , the methylation profile form the mutants and the cancer CXCR3 are mapped out. To

analyze the effect of the methylation change on splicing ratio, the protein expression profile was quantified using flow cytometry and the transcript levels were measured with qPCR.

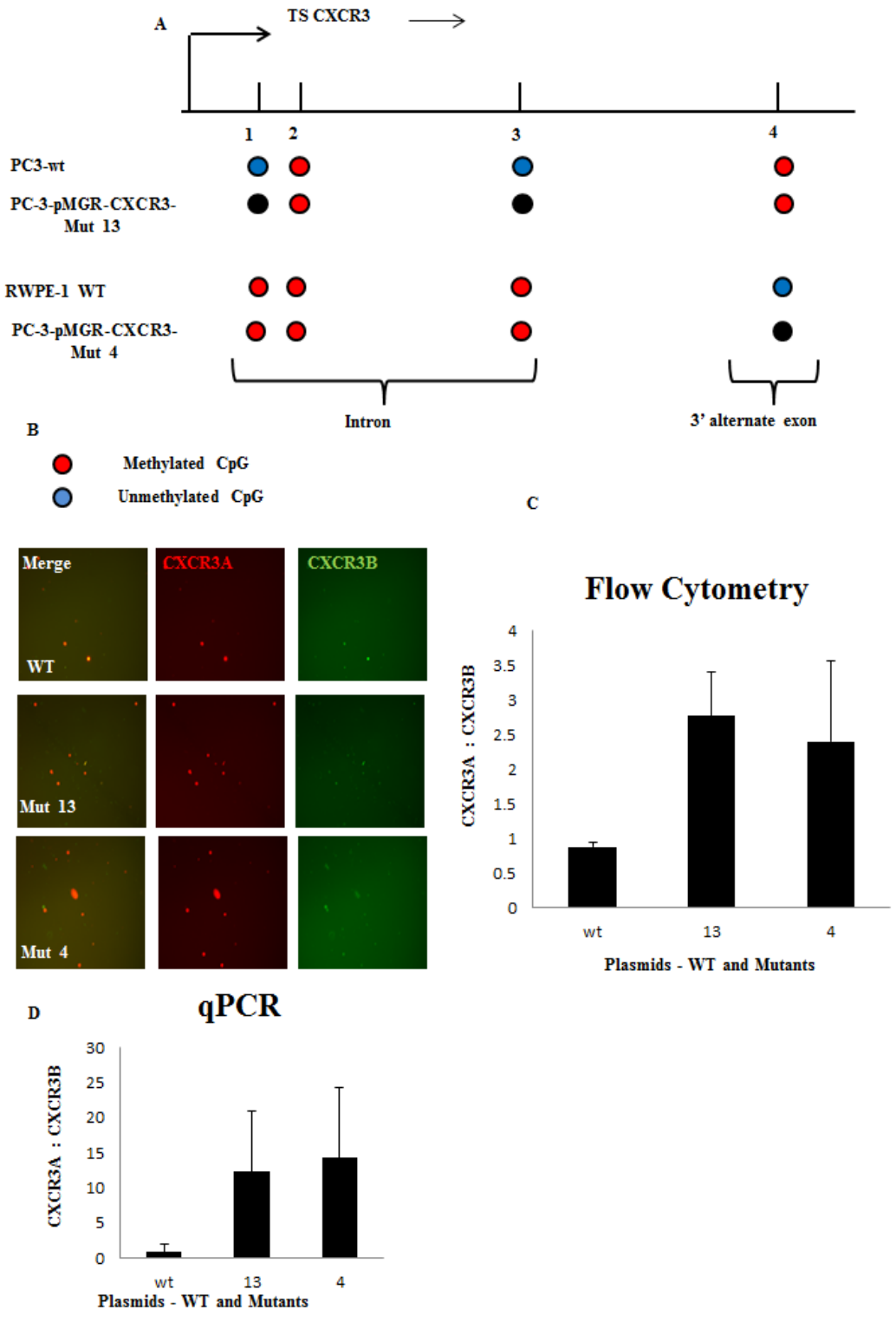


Figure 14. Endogenous CXCR3 specific CpG methylation ratio and it splice axis

A: The pMGR-CXCR3 wt and mutants 13 and, 4 were transfected into the PC-3. The methylation state of the CpG of interest was tested. The circles represent the CpG of interest in the CXCR3 minigene from the plasmid with red denoting methylation, blue, a lack of methylation and black denoting a C to G mutation in the plasmids. The methylation pattern of the endogenous CXCR3 gene is added for a frame of reference. They are labelled as PC-3 wt and RWPE-1 wt.

B: Microscopy analysis of the fluorochromes expression when the plasmids were transfected into PC-3 cells

C: Flow cytometry quantifying the flurochromes to obtain the CXCR3A: CXCR3B ratio for comparison between the wildtype and mutant plasmid.

D: qPCR quantifying the transcripts generated from the plasmids

It was found that the mutants 13 and 4 increase the ratio of the isoforms to favor the expression of CXCR3A when compared to the wt. It is expected that mutant 13 which resembles the differential methylation of the cancer cells increases the ratio to favor CXCR3A and the ratio is also higher than mutant 4 that is similar to the normal cells. Surprisingly the mutant 4 that resembles the normal cells also seemed to shift the splice axis to favor CXCR3A. Since the cancer cells express a higher A:B isoform ratio, it is expected that mutant 13 which resembles the cancer cells would also shift the ratio to favor CXCR3A. Intriguingly, it was found that even mutant 4 that resembles the normal cells favors a higher A:B ratio. It could be that methylation in conjunction with DNA binding proteins or a differential methylation at other sites play a role. Taking a closer look at the methylation pattern it is seen that in Mutant 4 only one CpG is altered. A change/alteration even in one nucleotide would alter the splice ratio. Next we wanted

to analyze how a change at other sites would alter the splice ratio. We mutated the differentially methylated sites individually to analyze its effect on splicing.

4.0 DISSECTING THE EFFECT OF SINGLE NUCLEOTIDE METHYLATION ON SPLICING: A NOVEL APPROACH

Mutants were generated to study the effect of single nucleotide methylation difference on splicing. Single , double and triple mutants were made to study the dose dependent effect , effective methylation ratio required for splicing and the frequency of methylation of individual sites to analyze the importance of each site on splicing.

4.1.1 Mutants

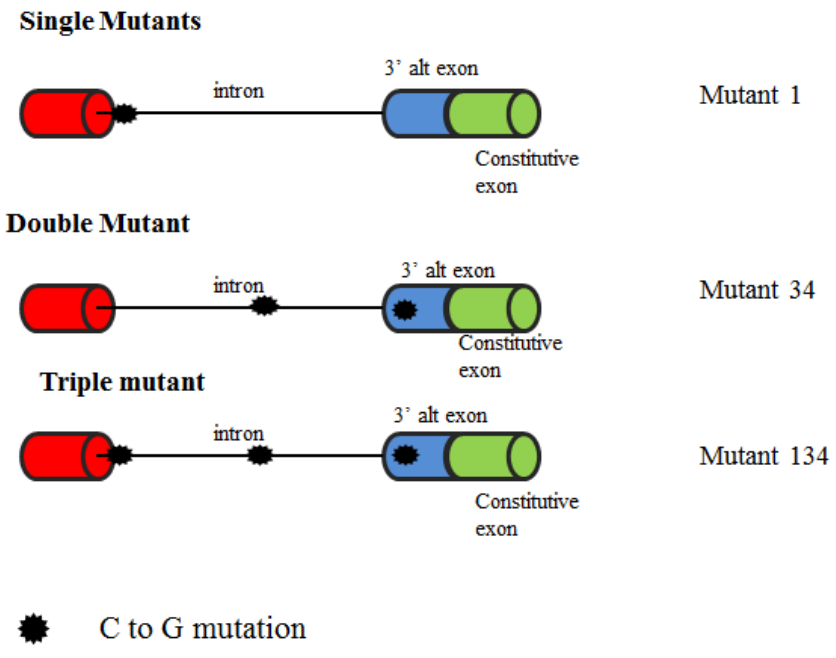


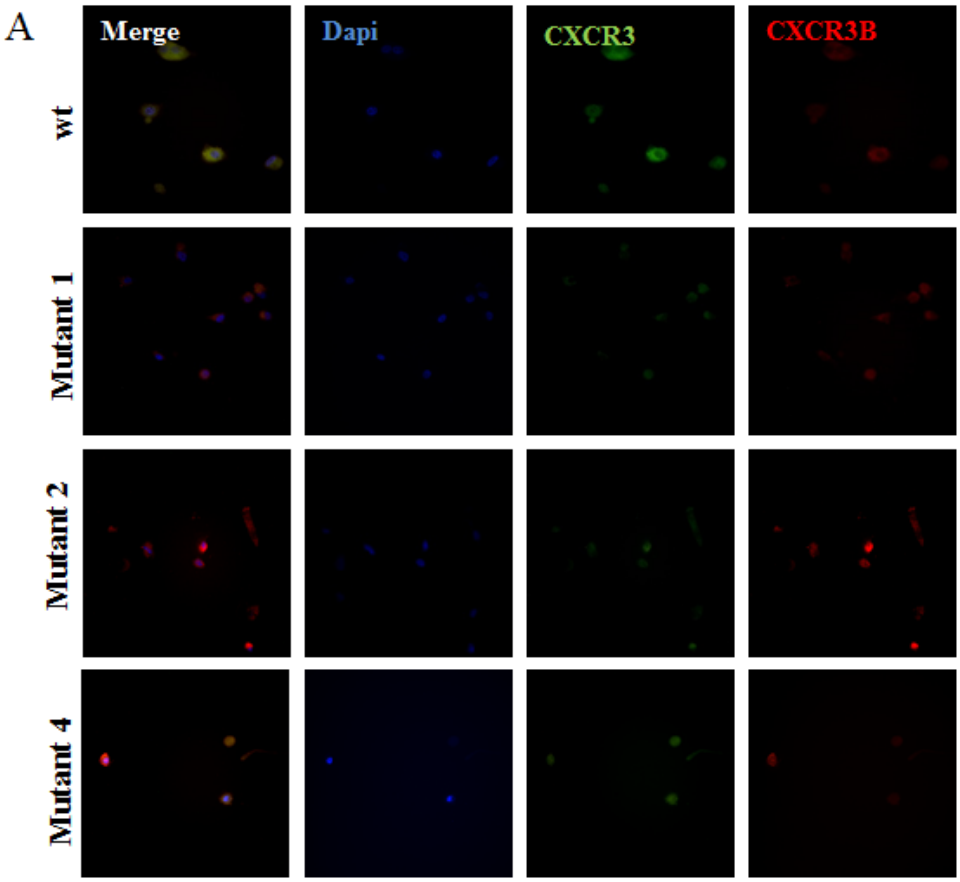
Figure 15. Single, double and triple mutants

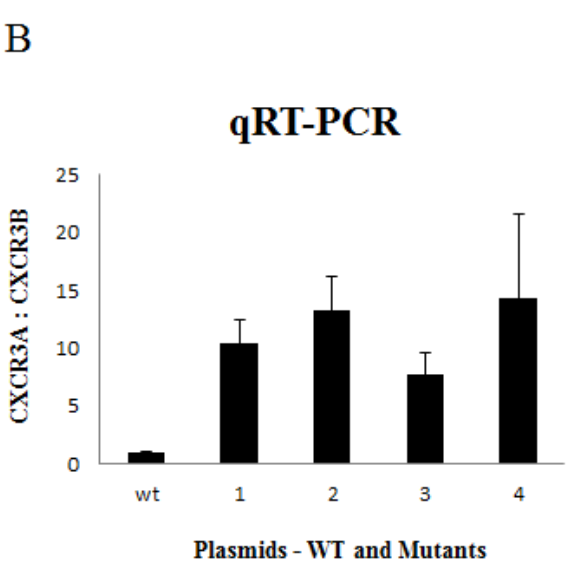
Pictorial representation of the mutants with an example of the nomenclature for single mutants, double mutants and triple mutants. The plasmids were named after the CpG site that was mutated.

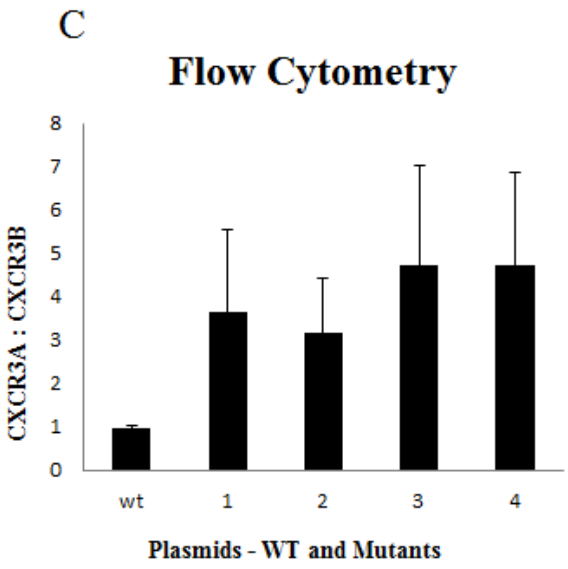
4.1.2 Mutants shift the splice axis to favor CXCR3A

The pMGR-CXCR3-wildtype and mutants were transfected into the cells and the splice ratio was computed (explained in materials and methods) using flow cytometry. It was observed that the mutants shifted the CXCR3 splicing to favor higher expressions of CXCR3A. The RNA was extracted from the cells to validate the expression profile quantified using flow cytometry. As described previously PC-3 cells that showed a quantum of methylation, these cells were used to analyze the difference in the splicing ratio.

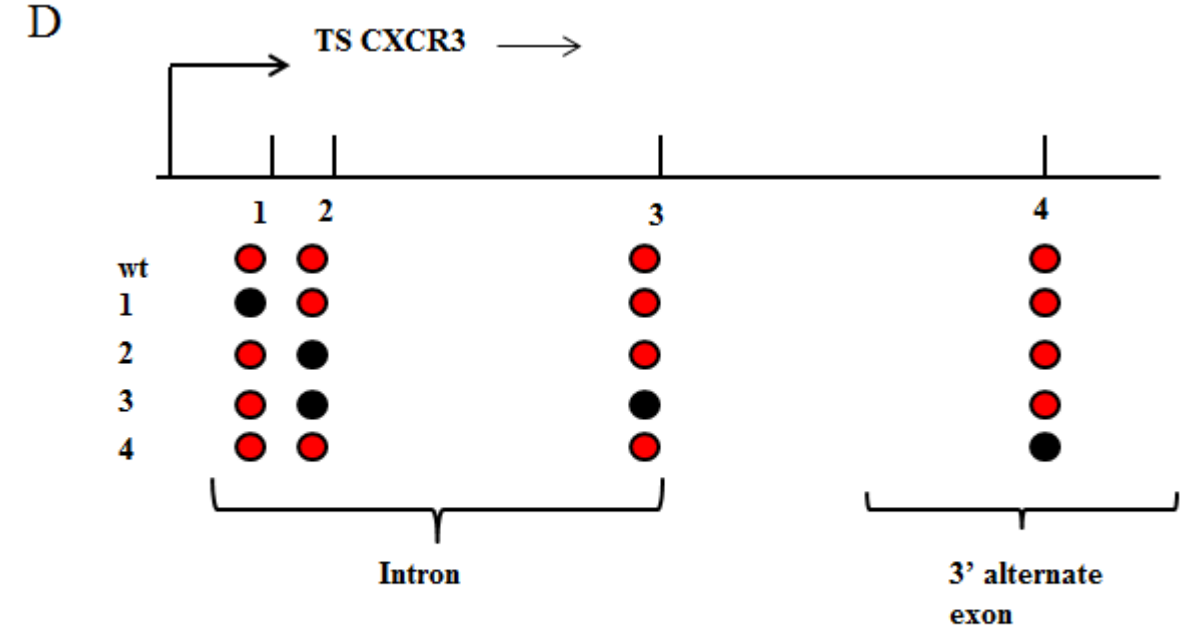
16.1 Single Mutants







D

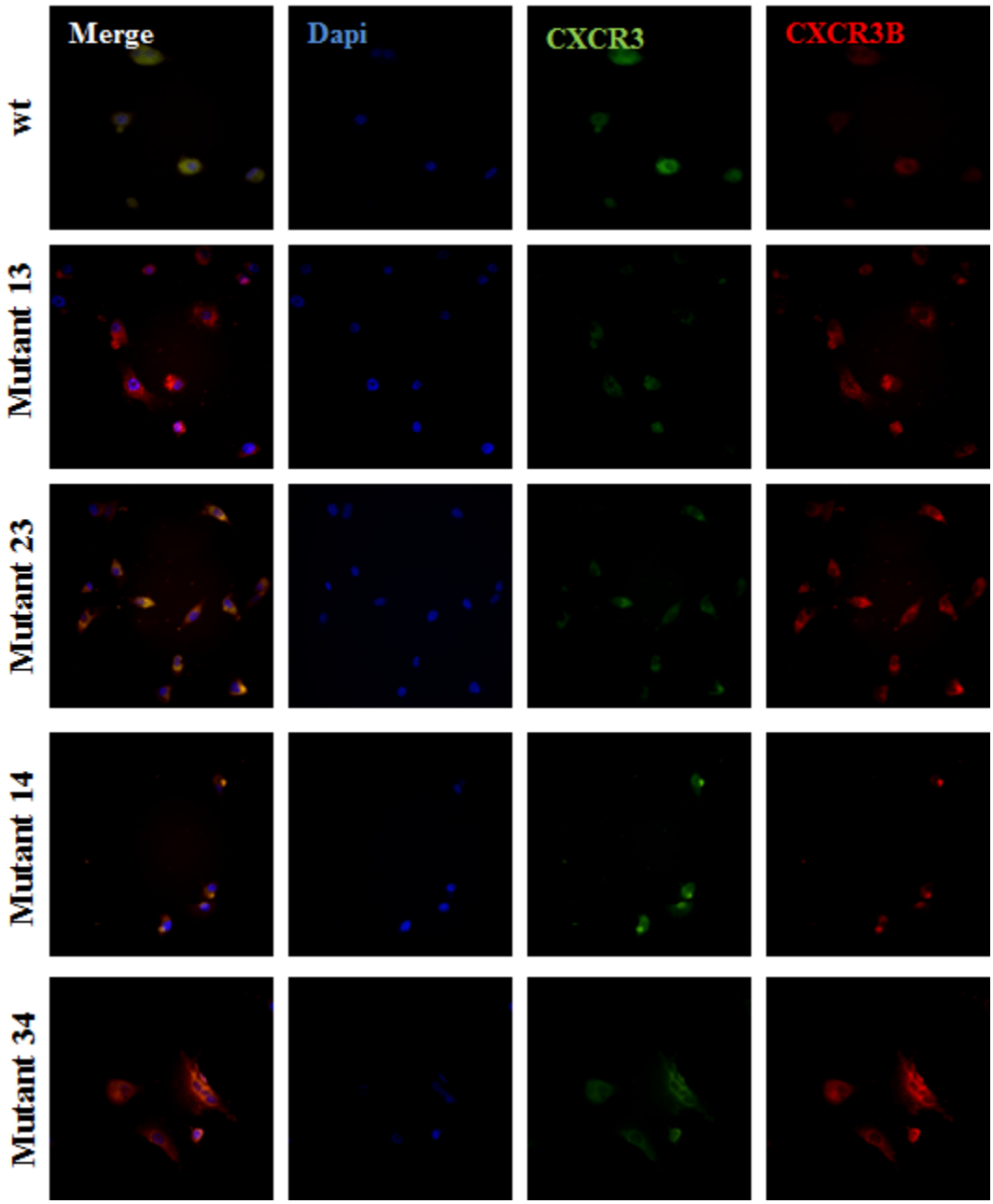


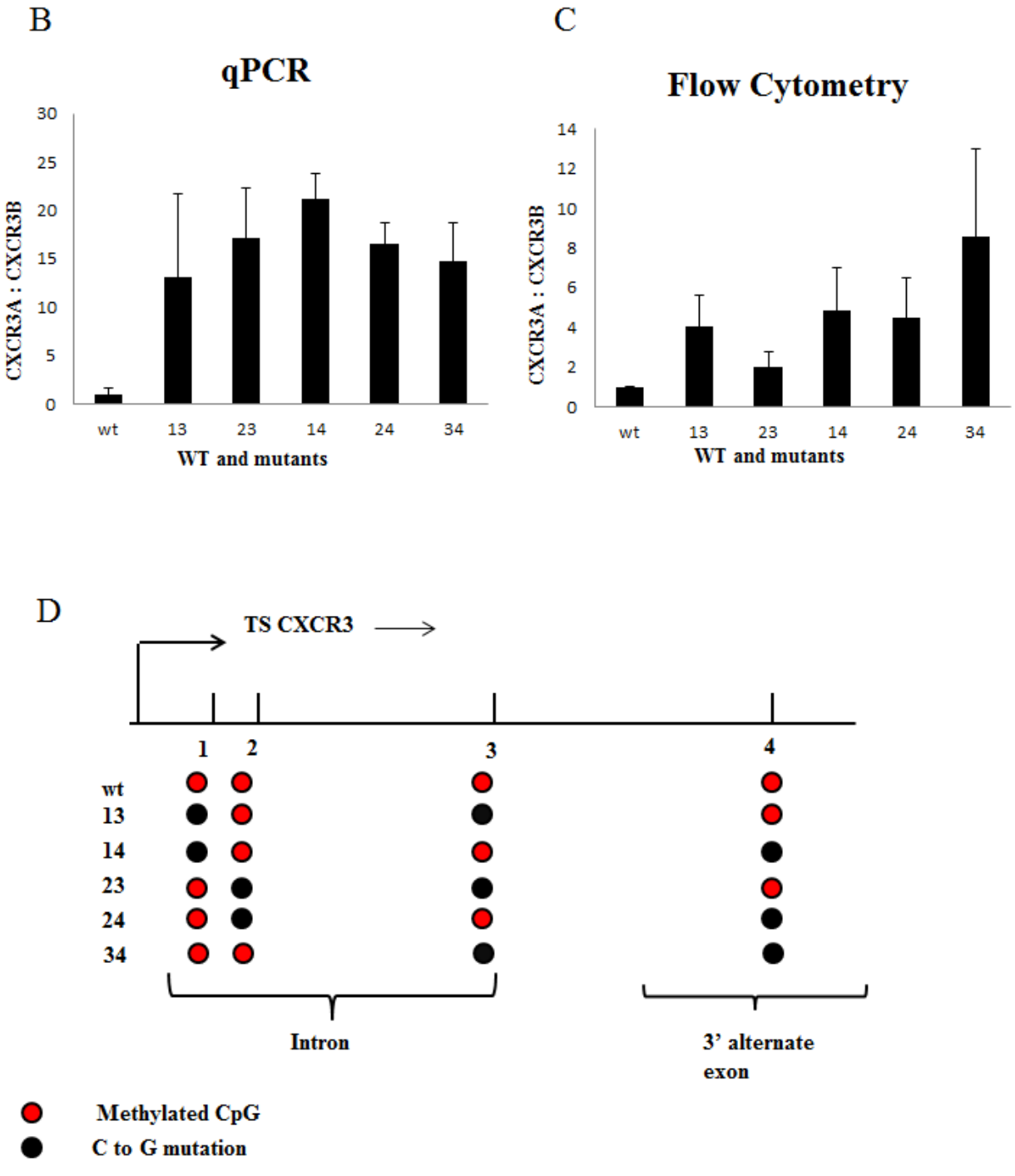
Methylated CpG

C to G mutation

16.2 Double Mutants

A




B
qPCR
CXCR3A : CXCR3B
30
25
20
15
10
5
0
wt
13
23
14
24
34
WT and mutants
C
Flow Cytometry
CXCR3A : CXCR3B
14
12
10
8
6
4
2
0
wt
13
23
14
24
34
WT and mutants
D
TS CXCR3
1
2
3
4
wt
13
14
23
24
34
Intron
3' alternate exon
Methylated CpG
C to G mutation

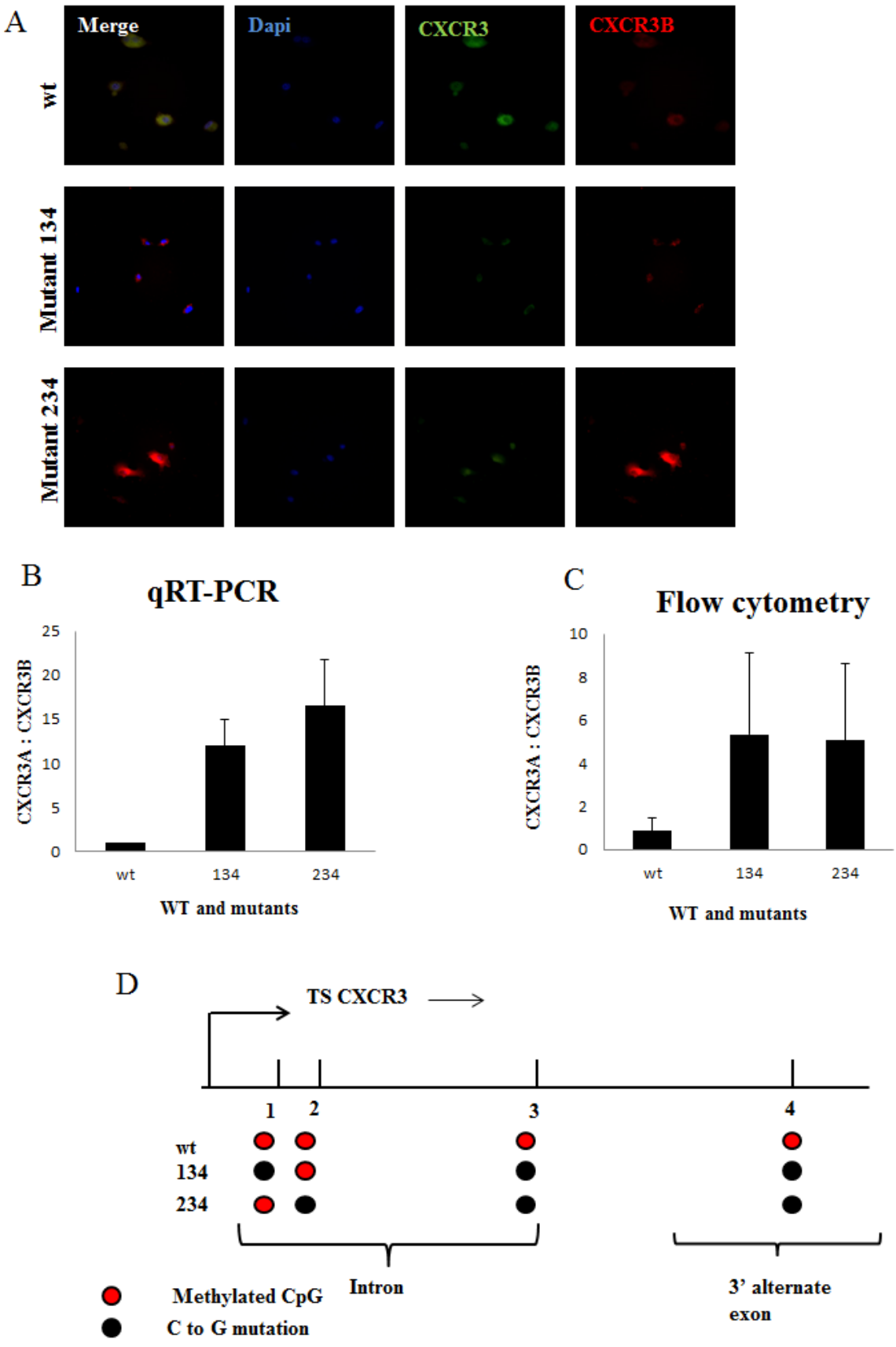

A
Merge
Dapi
CXCR3
CXCR3B
wt
Mutant 134
Mutant 234
B
qRT-PCR
CXCR3A : CXCR3B
25
20
15
10
5
0
wt
134
234
WT and mutants
C
Flow cytometry
CXCR3A : CXCR3B
10
8
6
4
2
0
wt
134
234
WT and mutants
D
TS CXCR3
1
2
3
4
wt
134
234
Intron
3' alternate exon
Methylated CpG
C to G mutation

Figure 16. Quantitative expression profile of WT and mutants

16.1 represents single mutants, 16.2 represents double mutants and 16.3 represents triple mutants.16.1, 16.2, and 16.3: The wild type and mutant minigenes were transfected into the PC-3 cells to observe the changes in the methylation pattern. A: Microscopic images of the PC-3 cells that were transfected with the plasmids, the experiments will be repeated to obtain more representative images; B: qPCR analysis of the isoforms that were generated using construct specific primers for CXCR3A and CXCR3B, the graph represents the CXCR3A: CXCR3B ratio as determined relative to GAPDH, 14.2B , needs to confirmed with more data points. C: Quantification using flow cytometry. The graph is a ratio of the cells that express dsRED to EGFP normalized to 30,000 events.

In Figure 16.1 it is seen that the single, double and triple mutants show a trend that favors an increase in the A:B ratio. The expression of the fluorochromes was quantified using flow cytometry and qPCR respectively. In short, removal of methylation at any specific site increases the levels of CXCR3A.

Results similar to the single mutants were observed when the double and triple mutants were transfected in the cell lines but the magnitude of change differed. The double mutant 34 shows the maximum switch in the expression followed by the triple mutant 134. Microscopy data showcases these results at a cellular level. Thus these results taken together suggest that the splicing of a gene in the context of epigenetics is a delicate ratio of methylation and the lack of it to create the perfect torsion that regulates the elongation rate of the RNA polymerase and thus co-transcriptional splicing. The high expression levels of the double mutant 34 but yet low

expression generated by mutant 23 signifies that the location of the splice altering cytosine and the genetic distance between the two could be highly critical in determining the splice ratio. The shift in splice ratios is thus a delicate balance between the methylated: unmethylated cytosines and a change in even a single nucleotide is sufficient to drive the splice ratio a certain direction. This emphasizes the importance of the intra-genic non-coding elements in splicing and the need to test for the epigenetic signature when assessing the protein expression profile in disease states. The single, double and triple mutants provide a wealth of data and from this an interesting pattern was observed which are outlined below.

4.1.3 Dose dependent effect

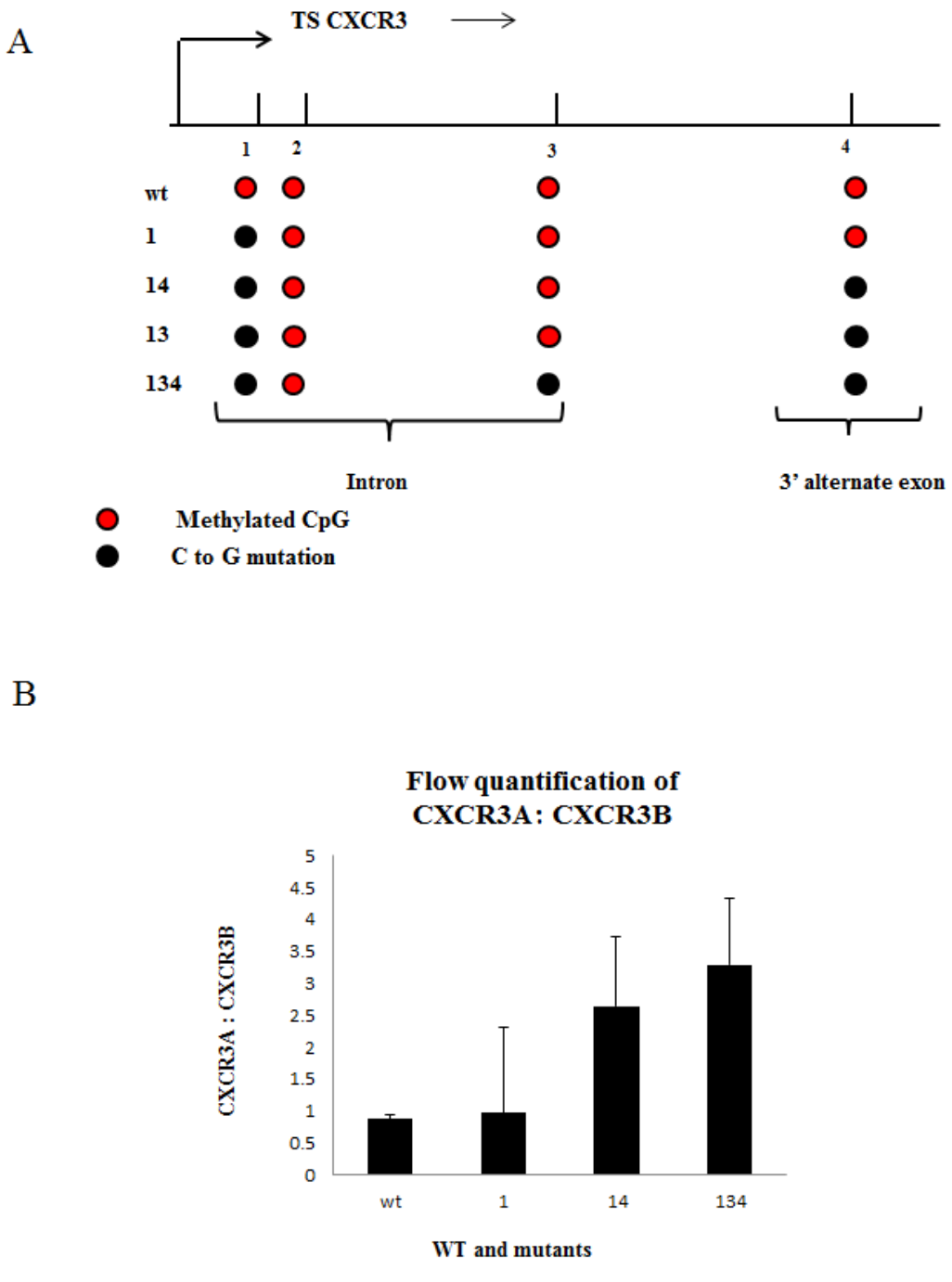


Figure 17. Dose dependent effect

A : The pMGR-CXCR3 wt and mutants 1, 14, 13, 134 plasmids were transfected into the PC-3. The methylation state of the CpG of interest was tested. The circles represent the CpG of interest in the CXCR3 minigene from the plasmid with red denoting methylation and black denoting a C to G mutation in the plasmids. The plasmids are arranged in the order of increased number of mutated sites from top to bottom

B :*Flow quantification of the CXCR3 isoforms generated from the bichromatic reporter minigenes to demonstrate the difference in the expression of the A:B ratio based on their methylation pattern*.

To test if removing the methylation from the CXCR3 minigene in an additive manner increases the splice ration in a step wise fashion, the expression profiles from the single double and triple mutants were analyzed using flow cytometry. The methylation profile and the protein expression are depicted in figure 17. It is observed that as the number of unmethylated sites increase from wt to mutant 134, the ration of CXCR3A to CXCR3B also increases with mutant 134 possessing the highest A:B ratio. One of the hypotheses that rules the regulation of protein expression via methylation at the DNA is that higher methylation causes the DNA to be inaccessible to the transcription factors and other co-activators, repressors thus altering the protein profile. The results showcased here provide multiple starting points to assess the differential loading of proteins to DNA, if any, to dissect the molecular cues associated with epigenetic alteration of protein expression.

4.1.4 Expected mutant methylation profile

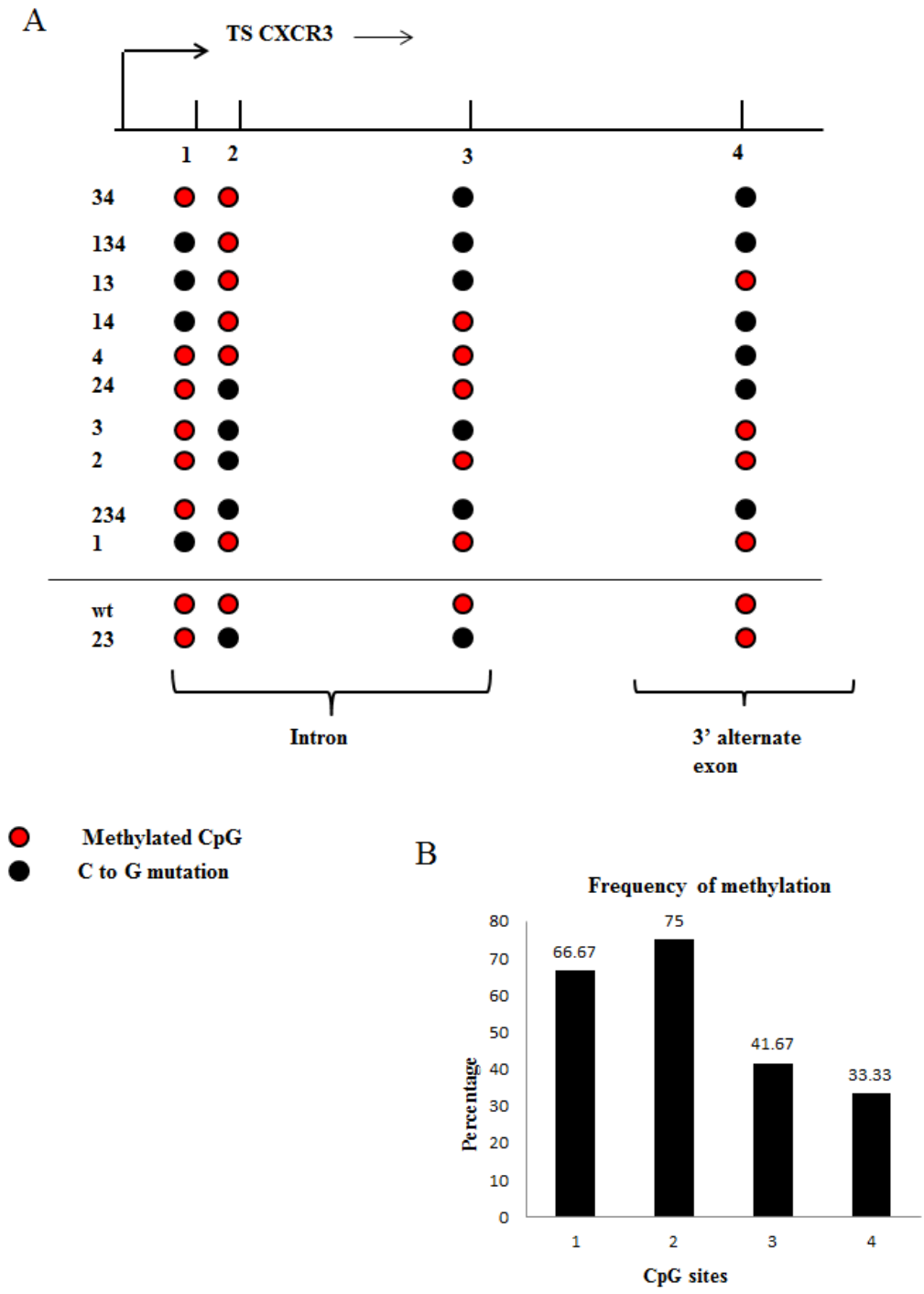


Figure 18. Predicted mutant methylation frequency

A: The mutants were bisulfite sequenced to determine the methylation pattern of the CpG sites that were not mutated. The mutants are arranged in decreasing order of their CXCR3A: CXCR3B ratio order to analyze the frequency of CpG methylation in cells that express a higher A: B ratio. The horizontal line separates the plasmids that generate a ratio higher than 1 from the plasmids with a ratio less than 1 with the higher than 1 ratio plasmids located above the line. Red circles represent methylation and black circles represent a mutation from C to G

B: The frequency of methylation of a CpG site in A: B ratios higher than 1 . It is determined as a percentage of CpGs methylated in the plasmids that express a A : B ratio of higher than 1 to the total CpGs under study.

This experiment was performed once to determine the methylation pattern of the sites 1, 2 and 3. A mutation to G is denoted as a lack of methylation. All the CpG sites except sites 4 in mutant 13, 2, 3, 1 and 23 were observed once. The methylation pattern at site 4 in these five mutants awaits confirmation with the bisulfite sequencing results. They are predicted to be methylated based on the methylation profile of the WT obtained.

The methylation profile of the CpG sites that were not mutated to guanines was analyzed in all the mutants. In order to dissect the importance of methylation at a specific CpG, the frequency of methylation at that site was determined in constructs expressing higher A:B ratio. A higher frequency of methylation in cells expressing high CXCR3A: CXCR3B would implicate its importance in driving the splice switch.. The frequency of methylation of the CpG sites was calculated as a percentage of the number of times the CpG is methylated in a specific mutant whose A:B ratio is higher than the WT. As shown in figure 17 B, the methylation frequency was obtained. It is seen that the frequency of methylation of site 1 and 2 is higher in the mutants that

express a higher A:B ratio and mutant 34 with the highest A:B ratio is methylated at both the sites. Mutant 134 and 234 where CpG 2 and 1 only are methylated respectively are associated with higher A:B. These results taken together are evidence that a methylation at site 1 and 2 increases the splice shift to favor the expression of CXCR3A. The methylation frequency of site 4 is lower in the mutants that express high A:B ratio. In other words, cells that express higher levels of CXCR3B are methylated implicating the methylation of CpG4 in 3' alternate exon inclusion.

4.1.5 Intragenic methylation ratio

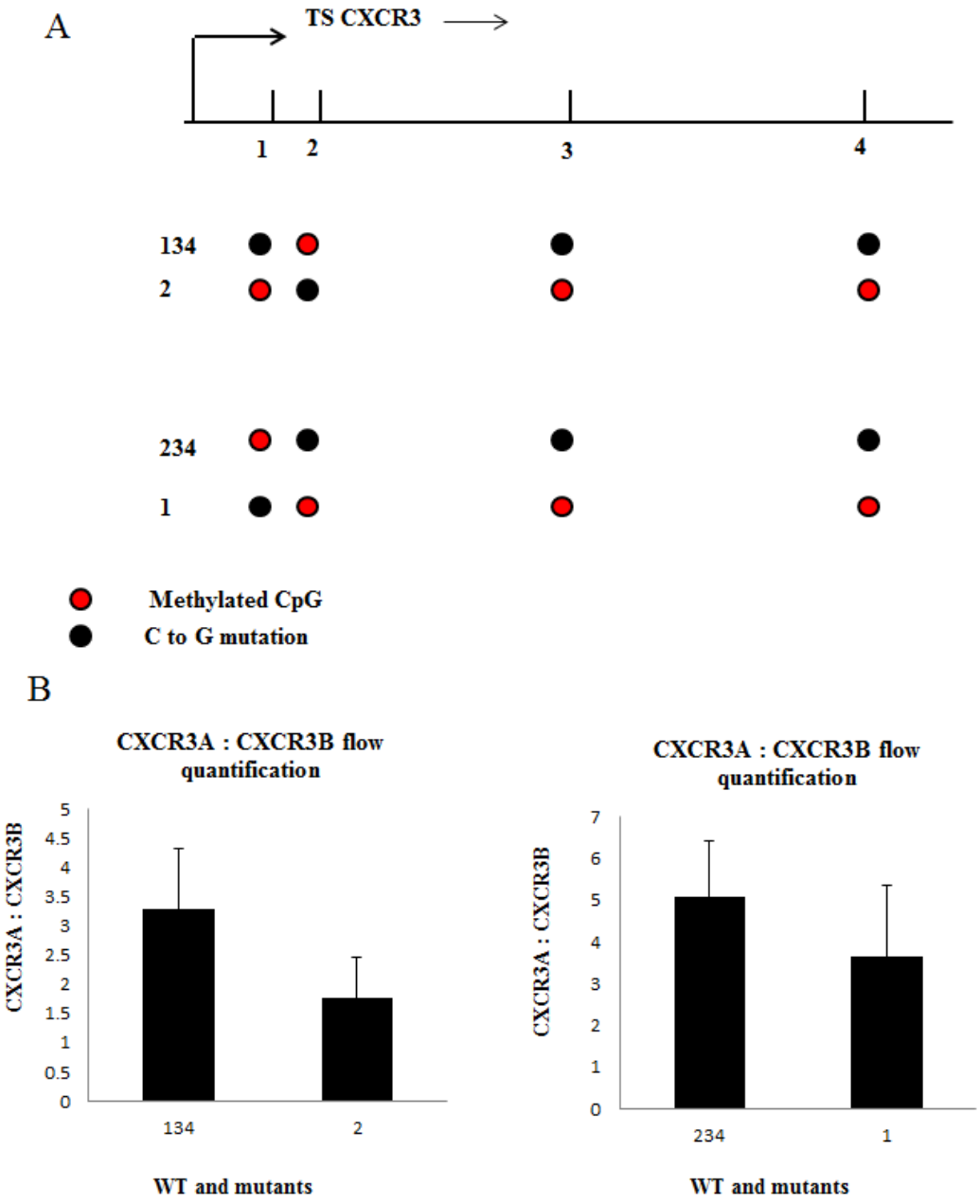


Figure 19. Methylation ratio with their expression profiles

A: The pMGR-CXCR3 wt and mutants 134, 2 , 234, 1 plasmids were transfected into the PC-3. The methylation state of the CpG of interest was tested. The circles represent the CpG of interest in the CXCR3 minigene from the plasmid with red denoting methylation and black denoting a C

to G mutation in the plasmids. It is noticed that the plasmids that are paired on the top and bottom possess an opposite methylation pattern.

B : Flow quantification of the CXCR3 isoforms generated from the bichromatic reporter to demonstrate the difference in the expression of the A:B ratio based on their methylation pattern.

The mutants 134 and 2 show an opposite methylation pattern and from the quantification of the ratios it is seen that the 134 has a higher A: B ratio. A similar trend is observed with mutant 234 and 1 where mutant 234 expresses higher A:B ratio. The critical difference between the triple and single mutants is that, the triple mutants contain one methylated and three unmethylated sites whereas it is the opposite in the single mutants. A methylated to unmethylated ratio of 1:3 is associated with higher expression of CXCR3A. When viewed in comparison with the endogenous methylation pattern it is seen that the cancer cells with a higher CXCR3A expression contain three unmethylated sits whereas the normal prostate cells with higher CXCR3B are methylated at three positions. Thus a differential methylation at these sites is a critical ratio of methylated to non-methylated CpGs and a methylation in the intragenic region favors exon inclusion.

With the splice ratio from the constructs documented, we analyzed whether the epigenetic mutations in some way confer an advantage to the cells when exposed to an extra-cellular stimulus. A significant feature of the prostate glands is that there are high levels of hypoxia and as he tumor progresses, the levels of oxygen available to the inner mass declines. We tested the behavior of the cells in hypoxia to visualize the splice difference.

4.1.6 Synergistic effect of hypoxia and differential methylation

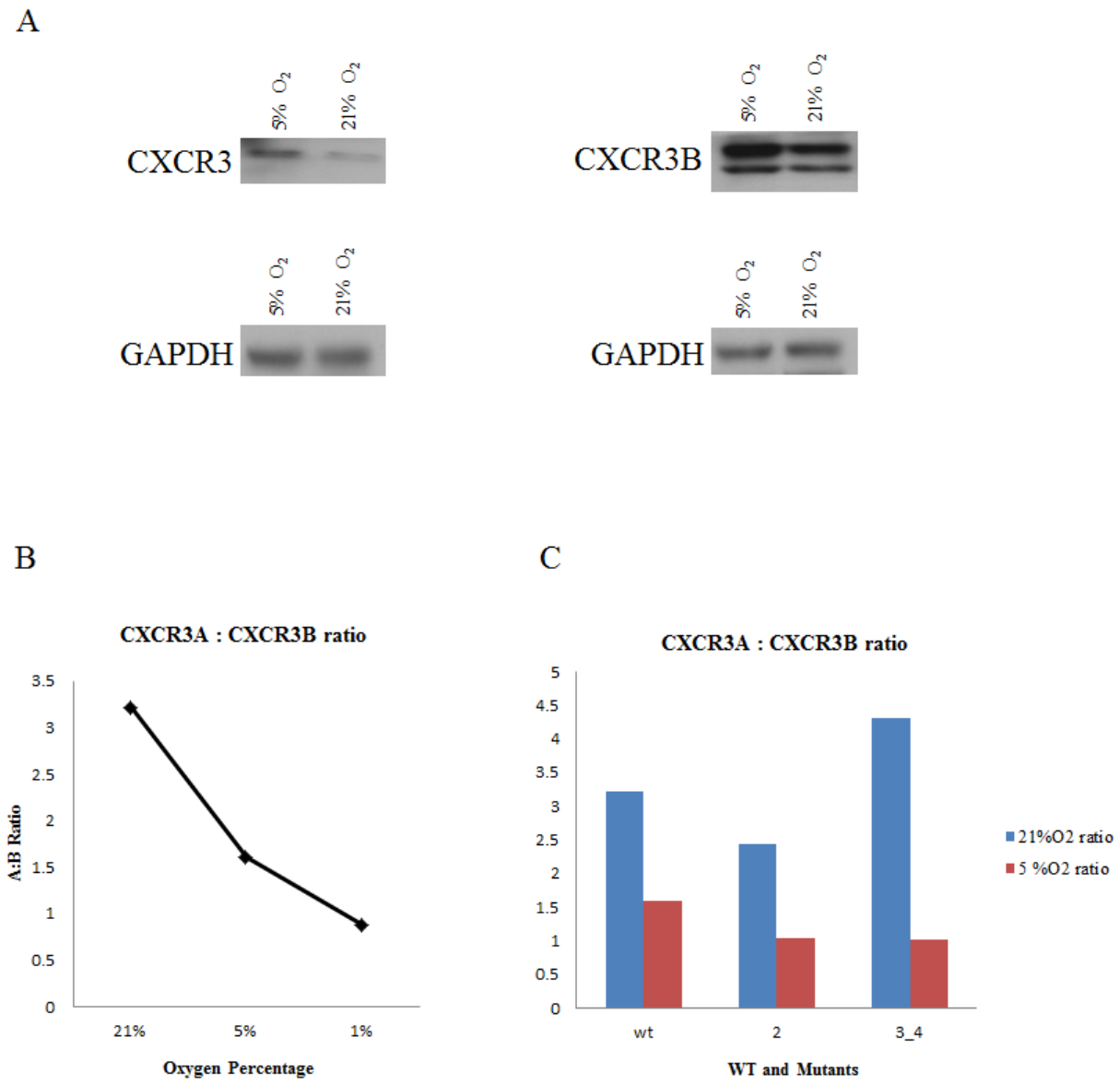


Figure 20. Synergistic effect of hypoxia

A: The PC-3 cells that were transfected with wt and mutants and the ratio was computed with flow cytometry. The cells were maintained at 21% oxygen and 4% oxygen to study the splice shift. B: The A: B ratio was computed at 21%, 4% and 1% oxygen and it is found that as the levels of oxygen decrease, the ratio also decreases indicating a higher expression of CXCR3B at

lower oxygen levels. C: The endogenous levels of CXCR3 isoforms in PC-3 were measured using immunoblots upon culturing at 4% and 21%.

One of the significant features of solid tumors and the micro-environment is hypoxia or low levels of which is a proven modulator of alternative splicing. In our study with the help of our novel construct, we tested the additive effect of the two modulators of splicing in the cancer cells. With the extent of splice change induced by intragenic methylation changes documented, we used this uni-dimensional model to study the synergistic effect of the epigenome in a hypoxic micro-environment on the cellular splice alteration of CXCR3 where the low oxygen serves as the second dimension in splicing analysis. The cells were cultured in 5% and 1% oxygen to mimic the physiological and tumor oxygen conditions respectively. The cells were maintained in low oxygen levels for two days after transfection following which the receptor levels were quantified using flow cytometry. It was found that the receptor levels shifted from dominant CXCR3A to CXCR3B progressively as the levels of oxygen were decreased. Figure 19A shows that lower the levels of oxygen, higher the levels of CXCR3B with the effect being more pronounced in the mutants. The shift to CXCR3B at very low levels of oxygen can be explained by the property of the receptor to induce apoptosis which is validated by the fact that cells at hypoxia tend to be apoptotic. Using this predictive analysis obtained from the minigene, PC-3 cells were cultured at lower oxygen levels to analyze if the same effect is produced in the endogenous receptor too. The endogenous CXCR3B receptor levels increase while the total receptor levels are unaltered suggesting a shift in the splice variants. This model is an effective predictor of how changes in the DNA at the gene level can alter the extent of proteome shift in the cells and hence the response of the cell to a given external stimulus. This is a novel tool that

would enable *in-vitro* testing of the drug efficacy on tumors that have a specific methylation signature.

5.0 SUMMARY

Taking together the experimental results described in Chapter 2.3, it is pretty evident that cells regulate their proteome expression via the promoters and intragenic regulatory regions through transcription and splicing respectively. As can be seen from the results, epigenetic regulation, methylation in particular, has the potential to cause a shift in the cellular proteome through its effects on alternative splicing.

5.1 INTRAGENIC DIFFERENTIAL METHYLATION

The structure of a gene and distribution of CpG islands in its coding and non-coding regions are major contributing factors for the phenomenon observed in our study. Usually, the coding regions possess a high CpG content when compared to the non-coding segments which introduces a high torsion (Branciamore, Chen et al. 2010, Choi 2010).A change in the methylation state of even a single CpG nucleotide alters the openness of the DNA conformation in three dimensional space, thus changing the rate of exon inclusion or skipping in co-transcriptional splicing. This places emphasis on the role of intragenic non-coding regions in studying this splice shift. As established in figure 1, though the intron forms the non-coding region, it was found to be heavily methylated except for the four different positions that show case a differential pattern. High methylation causes DNA to be more coiled in three dimensional

space in which can alter the splice ratio. In light of this theory, we wanted to test if methylation alters the splice ratio of CXCR3 and used a novel bichromatic reporter minigene to test exogenous DNA methylation and the effect of the epigenetic marks in the cancer and normal cell on its splicing.

5.2 BICHROMATIC REPORTER MINIGENE

A major drawback in using the CXCR3 system to studying splicing is that the two isoforms are so identical in their sequence that an antibody for the recognition of CXCR3A is not available. The only mechanism of identification to differentiate the two isoforms is using qPCR where the primers are designed across exon-exon boundaries wherein the longer N-terminal of CXCR3B provides it with a unique sequence. The protein expression level of CXCR3A is always deduced as a difference in the expression of pan-CXCR3 and CXCR3B. Many confounding factors play a role in the interpretation of data which including protein loading and the strength of the antibodies. Precise exposure time and protein loading are required for detection by each antibody so that the levels of CXCR3A can be calculated. Additionally, when analyzing the splice shift involved in a gene, it is required to differentiate endogenous expression from experimental exogenous gene expression. When using a CXCR3 minigene without protein tags, it is impossible to differentiate the exogenous gene from endogenous as the primers designed to distinguish CXCR3A from CXCR3B cannot be made construct specific. With the help of the bichromatic reporter construct designed for this project, we were able to circumvent this difficulty. The bichromatic reporter tags the two isoforms with unique fluorescent tags that could be visualized under a microscope and quantified using flow cytometry. The construct will allow

us to obtain high throughput data in analyzing the splicing variation of an alternatively spliced gene. The expression of individual isoforms is characterized by the expression of their fluorescent tags and the splice ratio is computed as a ratio of dsRED to EGFP.

5.2.1 Innovation

Though a switch in the expression of CXCR3 splice isoforms has been previously established, the intra-cellular or environmental factor causing the over-expression or switch in the expression has not been established yet. While molecular studies have been identifying proteins involved in cancer progression and a possible cause for their dysregulation, genome wide association studies look at genetic mutations present in the affected population. Our study is a perfect bridge between the molecular evidence and unique epigenetic profile prevalent in the prostate cancer population. In this study a functional change associated with a specific methylation change in the cytosine of the intron was established through a novel molecular tool. Our study also identifies introns as specific gene targets to control the splice variant expression in a given cell type.

Another aspect of innovation lies in the fact that we introduced an exogenous DNA into two different cell types to study how the intra-cellular machinery process them differently. With the advent of single cell transcriptomics, emphasis is being placed on the splice signature of the circulating tumor cells that escaped from the primary tumor site. The splice state of this single cell in conjunction with its environment would determine its fate. It could either undergo apoptosis, enter the G0 phase to become quiescent or engage in the secondary site to colonize and form metastases. When studying the impact of an alternatively spliced protein on a cell, it is necessary to determine if the isoforms have the ability to induce either of the functions mentioned above and if the splice switch is a homogenous or a heterogeneous switch. The

construct used in our study enables us to study this splice difference at a single cell level and also to determine the heterogeneity of this splice switch in a population of cells. It also enabled us to test the response of the cellular isoform ratio to environmental cues generated from the methylated versus a differentially methylated genome.

5.3 SINGLE NUCLEOTIDE METHYLATION ANALYSIS

Methylation studies conducted so far include correlation the protein expression profile with the imprint of the promoter and the first exon as in the case of E-cadherin under study. A few studies involve the use of global demethylation drugs such as 5'-azacytidine to analyze the change in the protein expression profile of the cells. Few others include over-expression of DNMT enzymes to check if it cause a hypermethylation of the genome and hence the specific gene of interest.

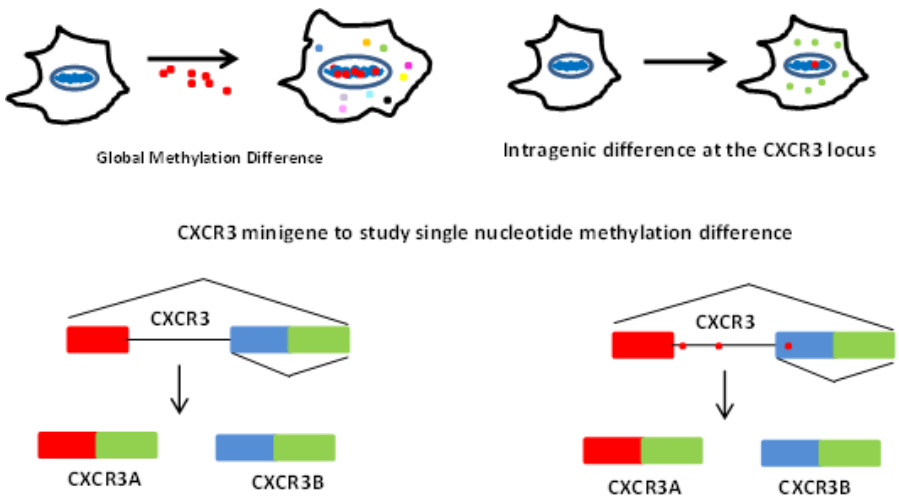


Figure 21. Single nucleotide analysis pictogram

A pictorial representation of the bichromatic reporter minigene model system to study single nucleotide methylation effects on alternative splicing of CXCR3

As shown in Figure 21, a major drawback with these studies is that, they alter the entire genome which affects several different loci causing a major shift in the transcriptome of the cell. It disrupts the native state of the cell along with altering multiple different loci in the genome accompanied by a major shift in the whole proteome. With every single molecular event in a cell being an orchestrated complex co-ordination of several proteins and pathways, a major shift in the transcriptome introduces several variables. Thus, any phenotypic effect observed in these studies could be a product of either protein-protein interaction; gene-protein interaction or gene-gene interaction. A clear cut proof of principle effect of altering a specific gene to change its transcription and splicing cannot be observed. In our case, we focused on the CXCR3 locus to study how changes to the gene affect splicing. When the wildtype plasmid was transfected into the normal and cancer cells, it was found that the exogenous DNA in the cancer cells were methylated at the CpG of interest whereas in the normal cells they were not. Thus, the cancer cells provided us with an excellent model to study if a change in the methylation of a CpG dinucleotide alters the splicing ratio.

Differential methylation was simulated as a mutation of cytosine to guanine as described above. The splice read out was measured as a quantum of dsRED to EGFP ratio which were tagged to CXCR3A and CXCR3B respectively. The splice shift in the mutants was measured relative to the wildtype as shift in the ratio between the fluorochromes.

5.4 MUTANTS ANALYSIS

Taking together results from the individual chapters, it is evident that the epigenetic marks in the cancer CXCR3 gene alter the splicing to favor the expression of CXCR3A. TO further dissect the confounding conclusion that even the normal prostate CXCR3 methyl marks shift the splicing to favor CXCR3A, we analyzed the effect of a single nucleotide methylation on splicing.

Strategic locations of these differentially methylated sites were one of the first indicators that they may play an important role in CXCR3 splicing. Sites 1 and 2 as shown in figure 7 flank the 5' splice donor sites and site 4 is present in the alternate exon that contains the translation start site for CXCR3B. Single, double and triple mutants were generated to study the intragenic methylation difference on splicing. From figure 14, it is seen that the single mutants shift the expression to favor CXCR3A demonstrating that a change even in a single nucleotide alters the splice ratio. Similar results were observed with the double and triple mutants.

To understand the effect of additive methylation in the gene mutants 1 , 13 , 14 and 134 were generated with mutating the cytosine of a methylated CpG in each step. It was observed that the A:B ratio gradually increased with the number of unmethylated sites in manner similar to a dose dependent effect, with an unmethylated site being the measure of a dose. Following this, we tested the effect of an opposite methylation pattern on the splice ratio to further confirm that a difference in the methylation does alter the splicing. Figure 19 demonstrates that two mutated sites accounting for a lack of methylation consistently showed a higher A:B ratio. This hints at a possibility that the ratio of methylated to unmethylated CpGs is important. When viewed together with the endogeneous methylation ratio in the normal and cancer wells, it is observed that the genes with two unmethylated and one methylated site have a higher A:B ratio when compared to genes with the opposite marks. This raises the question of importance of each site in

splicing. To address this, the single, double and triple mutants were taken together and the methylation frequency was calculated.

Sites 1 and 2 were found to be most frequently methylated in plasmids with a higher A:B ratio. The frequency of methylation is higher at the CpG in site 4 in plasmids expressing lower A:B ratio, in other words higher CXCR3B expression. But, a lack of methylation at site 4 has been implicated in the switch of expression to CXCR3A from the mutation analysis. When viewed in the context of the epigenetic pattern of the normal and prostate cells, it is seen that the normal cells with higher CXCR3B is unmethylated while the cancer cells are methylated at site 4, an exact opposite of the mutants' analyses. This suggests the possibility of an alternate hypothesis where regulatory proteins that might bind this site either decrease or increase the probability of this exon selection for splicing in the normal and cancer cells. It is possible that that a methyl binding protein occupying the methylated CpG in the cancer cell can alter the loading of the spliceosome, thus affecting the exon inclusion.

In summary, methylation at sites 1 and 2 favors a shift in the splice ratio to express higher CXCR3A. Frequency of methylation of the 3'alternate exon increases its inclusion to favor CXCR3B expression. Contextualising this in light of endogenous CXCR3, the epigenetic marks play a role in enhancing A: B ratio.

5.4.1 Single cell analysis

Our study devises a very unique way to study the proteome effect in tumor cells in the context of the epigenome. The biggest merit of the use of this tool is the ability to analyze the splice axis at a single cell level. As shown in figure 21, the splice axis of the cell is visible and can be analyzed at a single cell level.

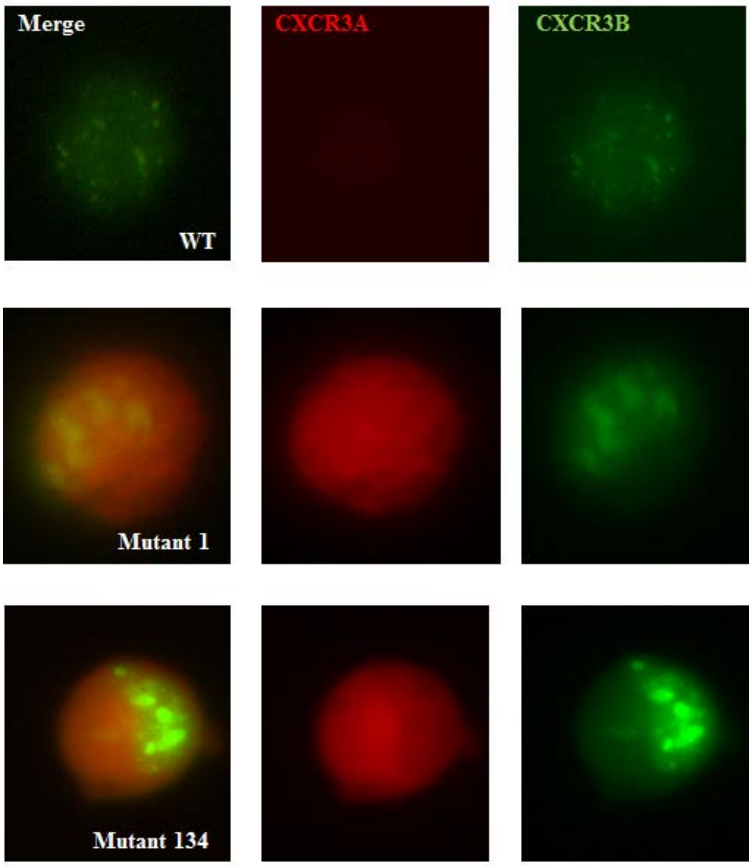


Figure 22: Single cell analysis

PC-3 cells were transfected with the WT and mutant plasmids and imaged for single cells to analyze the intracellular splice events. CXCR3B is tagged with EGFP and fluoresces green, CXCR3A is tagged with dsRED and fluoresces red. Each frame is a single cell with the isoform expression pattern.

With the advent of single cell transcriptomics, various groups have studied the splice differences at a single cells level. A seemingly homogenous population of cells was analyzed to find that the splicing axis of each cell was very different from a neighboring cell (Shalek, Satija et al. 2013). In the context of tumors, this is a very valuable discovery as the fate of tumor cells

circulating in the blood is determined by their splice signature. In-vivo studies with the plasmid constructed in this study will enable us evaluate splice changes in tumor dissemination in real time and also determine if an expression of one isoform over the other plays a role in metastasis

5.5 DISCUSSION

Epigenetic regulation of alternative splicing is a new finding with scientific evidence accumulating rapidly. One of the significant advantages of this concept is that an epigenetic change is reversible and will alter the splice axis of the cell to induce a phenotypic shift. This provides for therapeutic intervention points where the epigenetic change can be forced with an external stimulus that would reverse the phenotype of the cancer cell making it non-invasive.

In our study we used the CXCR3 gene model system in the prostate cancer cells where the prostate cancer cells express higher amounts of CXCR3A. The CXCR3 gene was screened for differential methylation in the promoter and intragenic regions. The intragenic regions – the intron and 3' alternate exon were heavily methylated and differentially methylated at four different positions. The intron and alternate exon were found to possess opposite differential methylation patterns. This specific pattern of differential methylation suggested that this could be causing the shift in the expression of the splice isoforms. In mammalian cells, where splicing is co-transcriptional, a change in the methylation state of the gene alters the elongation rate of the RNA polymerase 2 enzyme. A change in the rate of elongation of the RNA polymerase 2 , in co-transcriptional splicing, alters the rate of loading of the spliceosomal complex and thus exon recognition and splicing. Thus, in order to assess the importance of these methylation sites in alterative splicing of CXCR3, we decided to use a CXCR3 minigene where the differentially

methylated sites were altered to prevent methylation. In order to differentiate the endogenous gene from the exogenous, the two isoforms were tagged with dsRED and EGFP using a bichromatic reporter minigene. The bichromatic reporter minigene is a perfect tool that ties together the concepts of alternative splicing and epigenetics. The plasmids were transfected into the different cell types and the expression of the fluorochromes was validated using flow cytometry, immunoblotting and qPCR. Upon validation, the mutants were created to mimic the normal and cancer endogenous. It was found that the mutant mimicking the cancer endogenous CXCR3 showed a trend favoring a higher CXCR3A expression. Intriguingly, mutant mimicking the normal endogenous CXCR3 also showed a trend favoring a higher A:B ratio. As will be discussed later , a DNA methyl binding protein interface could be in play that is causing this opposite effect when transfected with the mutant that mimics the normal endogeneous CXCR3. Though the mutant 4 shifts the ration to favor CXCR3A , it is seen that altering a single nucleotide shifts the splice ratio. But , this CpG lies in the alternate exon whereas the other CpG sites lie in the intron. The intons and exon have different regulatory purposes and we wanted to test if a change in the intron alters the splice ratio in a similar or opposite fashion.

In order to assess the importance of each differentially methylated site, we created single, double, and triple mutants where the methylated cytosines were mutated to guanines to remove the methylation opportunity. It was observed that all single, double, and triple mutants showed a trend favoring a higer A:B ratio. In the single mutants, a mutation at CpG site 1 prevents its methylation. This lack of methylation favors a higher A:B ratio.

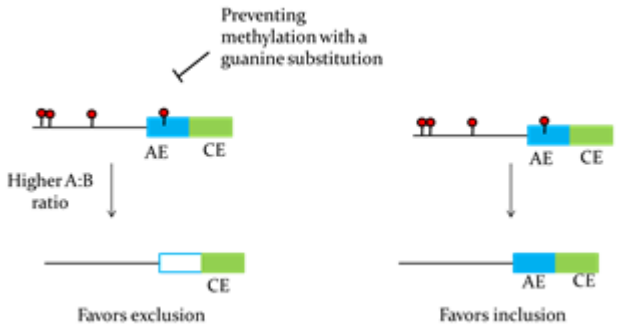


Figure 23. Methylation favors exon inclusion

Pictorial representation of the phenomenon observed. Upon inhibition of methylation through guanine substitution, there is an increased exclusion of the alternate exon. This proves that the presence of the methylation favors tis inclusion.

Essentially in our study, we have shown in our results that removal of the methylation at a specific CpG site by mutating the cytosine to a guanine shifts the splice ratio to favor a higher CXCR3A:CXCR3B ratio. A higher A:B ratio implies that the lack of methylation favors the exclusion of the alternate exon that is exclusive to the CXCR3B isoform. Since removing a methylation favors the exclusion of the alternate exon, the vice-versa – presence of methylation favors the inclusion and thus a higher expression level of CXCR3B. As shown in the figure, the presence of a methylation favors the inclusion of the alternate exon. From the single mutants results, it is evident that a methylation in all the four sites favor an inclusion of the 3' alternate exon or a higher CXCR3B expression. Recently published literature corroborates our findings and they have shown that alternate exons are methylated and this methylation facilitates the binding of the methyl binding protein, MeCp2 which facilitates exon inclusion (Maunakea, Chepelev et al. 2013)yOther meta-analyses in different species such as insects (bees), have observed a higher degree of methylation in the exons, most of which are alternates.

Visualizing these results in the context of the differential methylation of CpGs in the endogenous CXCR3, RWPE-1 which has higher CXCR3B levels has a methylation in the first two CpG sites and a lack of methylation in the fourth CpG site or a lack of methylation in a single CpG dinucleotide in the alternate exon. This is contrary to the data from the exogenous CXCR3 in the minigene which shows that a methylation in this site causes a higher CXCR3B expression. Additionally we also observed that the mutants that mimics the normal endogenous CXCR3 gene also shifts the A:B ratio to favor a higher CXCR3A expression. One of the reasons for this observed phenomenon could be that the protein architecture and native state of the normal and cancer cells are different. A DNA binding protein interface could be mediating the inclusion and exclusion of the alternate exon via binding to the methylated cytosine. We hypothesize that a DNA binding protein at the 3' alternate exon of the normal endogenous CXCR3 mediates the inclusion of the 3' alternate exon of CXCR3 that results in a higher expression of CXCR3B expression in the normal prostate cancer cells. As shown in the figure below, a DNA binding protein will bind to the 3' alternate exon to facilitate its inclusion whereas the presence of this methylation the CXCR3 gene of the cancer cells prevents the binding of this protein to favor the exclusion of the exon. A specific candidate protein that has been implicated in the alternate splicing of other genes is CTCF that binds to the DNA to mediate inclusion of the alternate exon. Binding of this protein to the alternate exon mediates the exclusion of the exon. In the event of a methylation in the alternate exon , either by virtue of a differential 3-D structure of the DNA or the mere presence of a methylation prevents the binding a protein. Identification of a potential role of a DNA binding protein would provide with starting points for therapeutics.

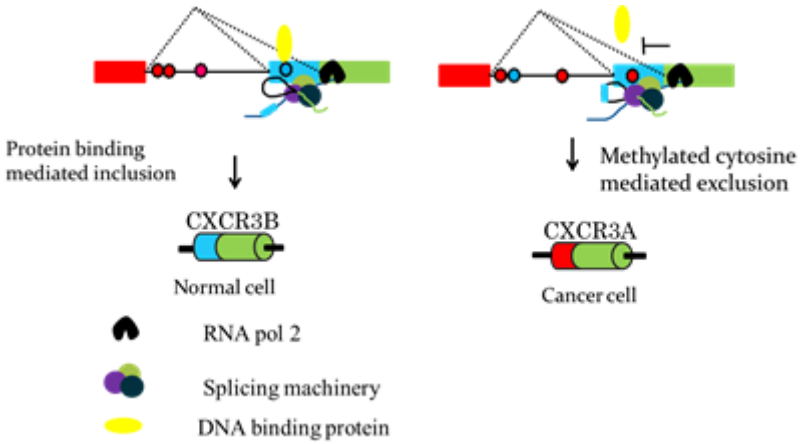


Figure 24. Proposed model

Pictorial representation of the proposed model of epigenetic regulation of alternative splicing of CXCR3.

Another clinical implication of our finding lies on the fact that we have proven that a lack of methylation mediates a higher CXCR3A:CXCR3B ratio. In previous research we have demonstrated that this higher A:B ratio is pro-invasive for the cell. With the advent of epigenomics and drugs targeting the epigenetic pathways, demethylating agents , HDAC inhibitors are common candidate drugs. This can prove to be counterproductive as an active demethylation increases the expression of CXCR3A which could lead to failure of the treatment. One way to solve this problem is to add a CXCR3 antagonist with the treatment where the invasive properties of the CXCR3A isoform would also be blocked.

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5.6 FUTURE DIRECTIONS

Some of the immediate validation studies include sequencing of the exon flanking the constitutive 3' donor site to determine the extent of methylation of the CpGs. Confirmatory studies of its effect on splicing with the available bichromatic reporter minigene tool set would validate its importance in the splicing event. Knowledge of differential methylation in the constitutive exon would enable comparison of methylation states at CpG sites flanking the alternative and constitutive exon. The effective ratio of the methylation frequency taken together in all the CpGs would provide a wholistic view of the effect of intragenic methylation on splicing. A few merits of the results obtained from this project include that it provides us with a wealth of data that can be translated to a diagnostic test through validated biomarker studies in addition to providing us with a tool to analyze the CXCR3 splice variant state of a cell in context of the epigenome in real time.

5.6.1. Single cell analysis

In our study while studying the splice switch of CXCR3 in prostate cancer cells, we found that the cells were clonal populations with a fraction of them expressing only CXCR3A, some only CXCR3B and some both. At a single cell level it was found that the cells that express both the isoforms express higher amounts of CXCR3A than CXCR3B. Upon further analysis, , we found that when transfected with the mutants, a majority of the cells homogeneously express CXCR3A.

The number of cells expressing both the constructs also increased indicating that the shift originates as an intracellular event, slowly transitioning the cell to a state of homogenous CXCR3A expression. A combination of this higher expression from homogenous and heterogeneous intracellular events contributes to the overall splice shift that leads to higher expression levels in prostate cancer cells.

5.6.2. In-vivo studies

As seen with the results from the study, the bichromatic reporter minigene provides with a mechanism to study the splice axis change that occurs in the presence of an external stimulus, making it an excellent tool to study splice changes in metastasis *in-vivo*. With the ability of this construct to demonstrate this splice difference at a single cell level, it is possible to study the intracellular splice events in the metastatic cascade after injecting the cells to initiate tumor formation in nude mice. After ascertaining the phenotypic variation of the cells transfected with the construct and their ability to spontaneously form invasive tumors, the cells can be injected into mice. The cells containing the construct when forming a tumor will display the splice pattern distribution across the cross section of the tumor. It allows observation of the change in the splice distribution during angiogenesis and initiation of metastasis. The expression of one specific splice variant at every time point can be detected using live mouse imaging. The specific events in the metastatic cascade can be detected in real time to analyze the dominant isoform at various points such as during intravasation, circulation in the blood stream and in the extravasation at the secondary site. The fate of the circulating tumor cells can be analyzed in relation to the expression of the splice isoform thus helping us determine if the expression of one isoform over another would promote the formation of organ specific micrometastases.

5.6.3. Biomarker identification

The methylation difference in prostate cancer at the intronic positions could be a passive process that is acquired through the course of tumor progression. The methylated CpG dinucleotides identified in the intron when proven to be functionally associated with alternative splicing of CXCR3 in the prostate cancer cells, would be a valuable biomarker facilitating early detection with harmless non-invasive processes. A population study with prostate cancer patients analyzing the methylation state of these CpGs through the tumor progression cascade would shed light over the importance of this gene in cancer progression. A gradual change in the neoplastic transformation sequence would facilitate its use as a diagnostic marker to identify the staging of the prostate tumors. Methylations in the DNA are very stable and can be detected even with concentrations as less as 5% in the DNA pool which makes it a non-invasive detection strategy with high sensitivity. Cancer in its primitive stages contains genetic features that are aggressive and easily detectable (Alers, Krijtenburg et al. 2001). One such genetic feature is the methylation difference in the prostate cancer genome which is detectable in bodily fluids like the serum, urine and prostatic secretions (Goessl, Muller et al. 2001, Ellinger, Haan et al. 2008). Early detection of prostate cancer gives the opportunity to treat it when it is localized (Alers, Krijtenburg et al. 2001) and prevents the metastasis which in the common cause of mortality of high risk (Labrie, Candas et al. 1999, Barratt and Coates 2004).

APPENDIX

ABBREVIATIONS

PCa – Prostate Cancer

AS – Alternative splicing

PIN – Prostatic Intraepithelial Neoplastic Lesion

A : B – CXCR3A : CXCR3B ratio

Micromets – Micrometastases

CRPCa –Castration Resistant Prostate Cancer

AR – Androgen Receptor

RNP – Ribo Nucleo Protein

ESE – Exonic Splicing Enhancer

ISS – Intronic Splicing Silencer

EMT – Epithelial to Mesenchymal Transition

FBS – Fetal Bovine Serum

K-SFM – Keratinocyte serum free medium

TSAP – Thermo Sensitive Alkaline Phosphatase

MMP – Matrix Metallo Proteases.

EGF – Epidermal Growth Factor

TGF –β – Transformation Growth Factor β

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