PROSTATE CANCER EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR SPLICE FORMS IN HYPOXIA

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by

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Chapter 1: Introduction

I. Angiogenesis

Angiogenesis is the process by which new blood vessels are formed within the body from those that already exist (Klagsbrun and Moses, 1999; Ferrara, 1999). It is known to play significant roles in normal development and embryogenesis, as well as everyday bodily processes including wound healing and those associated with reproduction, especially in females (reviewed in Ferrara 1999; Klagsbrun and Moses, 1999). The creation of new blood vessels has also been shown to be an important part of the induction and function of various pathologies, such as diabetes, rheumatoid arthritis, and excessive vascularization of the eye (reviewed in Ferrara 1999 and Carmeliet 2003). One area of study that has become of more and more interest to researchers is the link between cancer and angiogenesis (Chen et al, 2000; Ferrara, 1999). Blood vessel formation is necessary in promoting the growth of a tumor by supplying it with adequate oxygen and nutrients (Chen et al, 2000; Russo et al, 2012; Bhat and Singh, 2007). It not only aids in the survival of the tumor cells, but also provides the opportunity for cells to infiltrate the blood system and metastasize to other organs in the body (Chen et al, 2000; Josko et al, 2000). This increase in tumor vasculature is believed to be the result of the activation of what is known as the "angiogenic switch" (Ranieri et al, 2006; Russo et al, 2012; Folkman, 2002). This can be accomplished through the upregulation of factors which promote angiogenesis or the inhibition, or downregulation, of those that inhibit

angiogenesis (Ranieri et al, 2006; Russo et al, 2012; Folkman, 2002). One of the main players in both normal and pathological angiogenesis is vascular endothelial growth factor (VEGF). VEGF helps increase the proliferation of tumor cells and the number of blood vessels that vascularize the tumor (Figure 1) by functioning in pathways which enhance existing blood vessel permeability, invasion of the stroma, and the formation of new vessels from preexisting blood vessels within and around the tumor (Whitlock et al, 2003; Cressey et al, 2005; Chen et al, 2010; Josko et al, 2000). Angiogenesis is believed to be regulated by a variety of growth factors including the fibroblastic growth factors (aFGF and bFGF), transforming growth factors (TGFα and TGFβ), and tumor necrosis factor alpha (TNFα), as well as the angiopoietins (reviewed in Ferrara, 1999 and Josko et al, 2000). Because an increase in angiogenesis within a tumor has been associated with an increased risk of metastasis, studies have shown that an increase in VEGF in cancer patients may correlate with negative outcomes and a lower probability of survival (Shen et al, 2000; Josko et al, 2000; Chen et al, 2000).

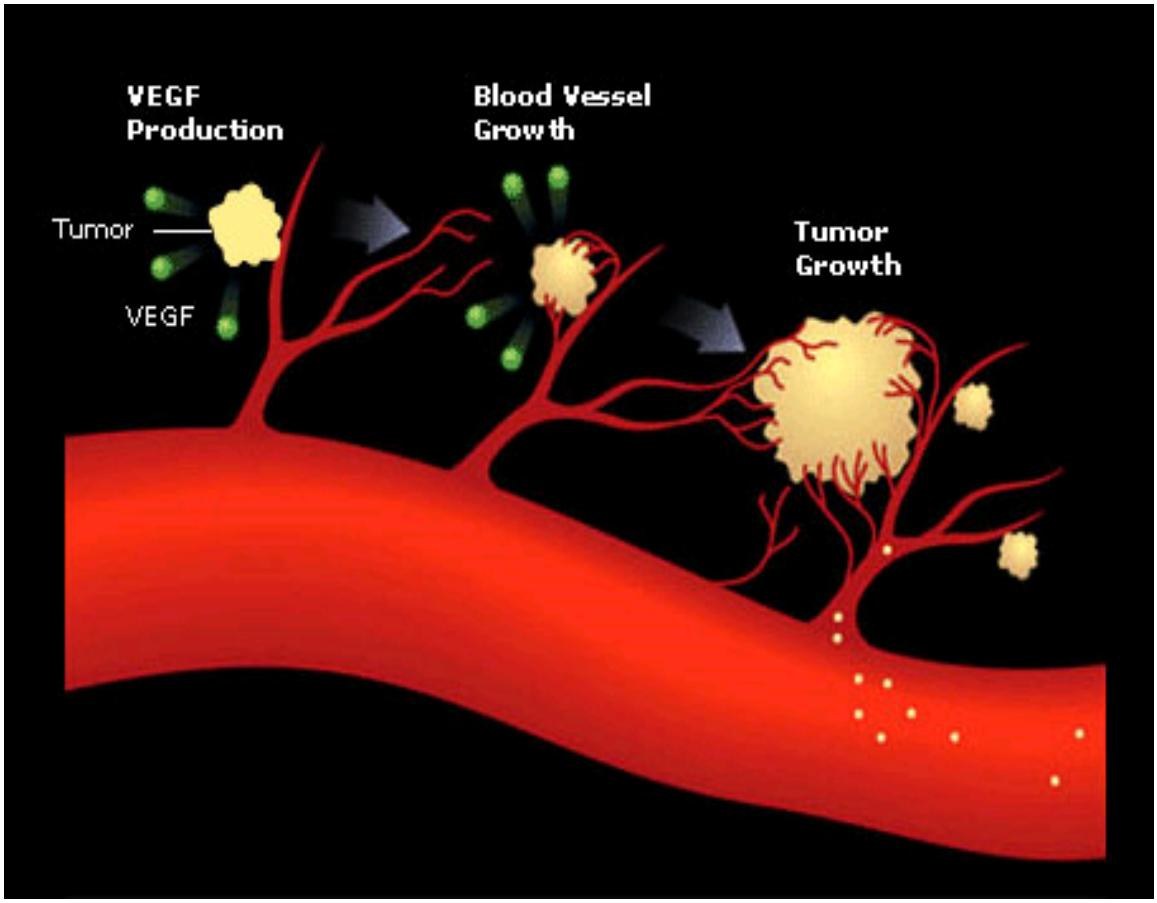

Figure 1: VEGF production and its effect on tumor growth. VEGF secretion by tumor cells and cells surrounding the tumor induces blood vessel growth within and around the tumor which in turn promotes tumor growth and increases the likelihood of metastasis. http://www.genebyte.firm.in/p53/index.php?option=com_content&view=article&id=57&Itemid=63

Angiogenesis is not only induced by the growth factors mentioned above. The tumor microenvironment also has a significant impact on the induction of VEGF, specifically through hypoxia inducible factor (HIF) mediated transcription (Dai et al, 2011; Russo et al, 2012; Tsai and Wu, 2012). Due to the abnormal and inconsistent vascularization that occurs in cancer, some parts of the tumor are hypoxic, existing under low oxygen levels that do not support the survival and proliferation of the tumor cells (Russo et al, 2012). Under these hypoxic conditions, the tumor's response is to activate the hypoxia inducible factor family of transcription factors to increase or restore oxygen and nutrient flow to the cells (Alqawi et al, 2007; Yuan et al, 2003). Under normoxic conditions, HIF1α is hydroxylated (Figure 2), thereby marking it for ubiquitylation by the von Hippel-Lindau protein (pVHL) and degradation by proteasomes (Yuan et al, 2003). Under hypoxia, however, this hydroxylation does not occur due to the lack of oxygen, allowing HIF1α and HIF2α to translocate to the nucleus and function as transcription factors of hypoxia-regulated genes (Yuan et al, 2003). These hypoxia inducible factors upregulate a variety of genes that are beneficial to the tumor cells but also have the potential to enhance tumor angiogenesis and metastasis under hypoxic conditions, including genes encoding receptors such as CXCR4, a variety of metalloproteinases, and the angiogenic factor, VEGF (Tsai and Wu, 2012).

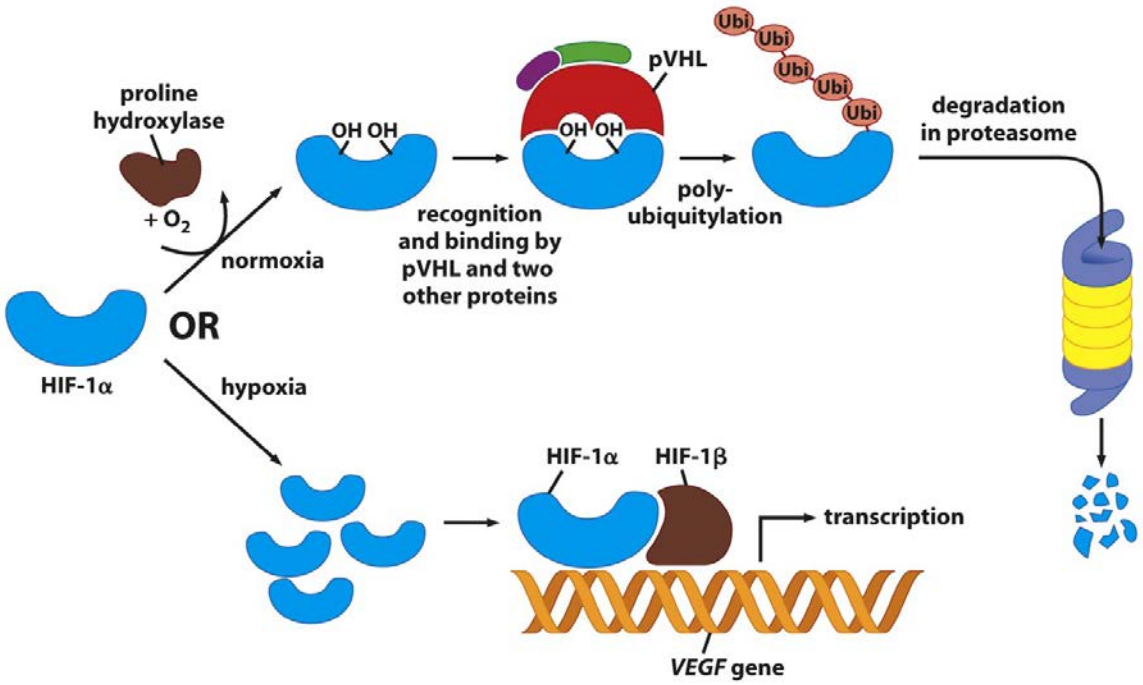

Figure 2: HIF1α activity under normoxic and hypoxic conditions. In the presence of oxygen, HIF1α is hydroxylated which allows it to bind and be ubiquitylated by the von Hippel-Lindau (VHL) protein. This ubiquitylation marks HIF for proteasomal degradation. Under hypoxic conditions, HIF does not get hydroxylated and therefore does not undergo degradation, which allows it to translocate to the nucleus and commence transcription of its target genes, including VEGF. (Weinberg, 2013)

II. Hypoxia

Actual hypoxia is not the only inducer of the HIF-mediated transcriptional pathway. Iron chelators and metals such as cobalt have been found to stabilize the HIF protein under normal oxygen conditions, which allows HIF to transcriptionally upregulate the same genes it would under hypoxic conditions (Yuan et al, 2003). Cobalt chloride is known to mimic the effects of hypoxia in cancer cells, and has been used as a substitute when actual hypoxic conditions cannot be achieved (Liu et al, 2000; Elias and Dias, 2008; Yuan et al, 2003). Yuan et al (2003) postulated that cobalt chloride has the ability to bind to HIFα, which not only prevents the hydroxylation of the transcription factor, but also blocks its binding by VHL when it is hydroxylated, thereby preventing its degradation. More sophisticated methods of hypoxia induction involve the use of a hypoxia chamber, which lowers the oxygen content to the desired levels by flooding the incubator with nitrogen gas.

Tumor hypoxia plays a significant role in the initiation of metastasis. Studies have shown that hypoxia is capable of influencing the metastatic potential of tumor cells (Alqawi et al, 2007; Dai et al, 2011). Angiogenesis within the tumor plays a major role in determining the metastatic abilities of the tumor cells, and activation of the angiogenic switch previously discussed promotes not only tumor growth but metastasis as well (Dai et al, 2011; Ranieri et al, 2006). Because VEGF plays a significant role in angiogenesis and its mRNA production is also increased under hypoxic conditions by HIF, it seems that low oxygen levels would enhance both the vascularization of the tumor as well as its ability to metastasize.

III. Alternative splicing of VEGF in cancer

Alternative splicing of mRNA is an important post-transcriptional modification that impacts the diversity of proteins made in the body as well as their functionality. In order to generate the great amount and variety of proteins which the body requires to function, mRNA can be edited after transcription in order to slightly alter the sequence or number of amino acids within the resulting protein (Oltean and Bates, 2014). These alterations come in the form of the removal of introns and some exons, which leads to the production of proteins of different lengths and varying functions from a single gene (Oltean and Bates, 2014; Whitlock et al, 2003). Alternative splicing is important in both normal and pathological processes within the body, including cancer.

VEGF is one of the genes that can be alternatively spliced to create proteins of different amino acid lengths. These splice forms are prevalent in both normal tissue and cancer (Elias and Dias, 2008). The VEGF gene is made up of 8 exons, and when it is alternatively spliced, the majority of the splice forms retain exons 1 through 5 (Tischer et al, 1991). The major splice forms that are present in normal and abnormal human tissues are VEGF121, VEGF165, and VEGF189, and are named for the number of amino acids each form contains (Elias and Dias, 2008). The longest of these three isoforms is VEGF189 which contains all eight exons found in the VEGF gene; exon 6 is spliced out of VEGF165, and both exons 6 and 7 are missing from the VEGF121 isoform (Ranieri et al, 2006; Elias and Dias, 2008; Cressey et al, 2005; Tischer et al, 1991; Ferrara, 1999). The different mRNA splice forms can be identified using specific TaqMan probes that span the alternative exon junctions (Figure 3). The different isoforms of VEGF have all

been shown to induce angiogenesis; however their functions differ in part based on their exon content (Grunstein et al, 2000). Exons 6 and 7 are known to be capable of binding to heparin which is found within the extracellular matrix and on the surfaces of cells (Robinson and Stringer, 2001). Because VEGF189 retains both exons 6 and 7, it has the highest rate of retention in the extracellular matrix out of the three major splice forms (Ranieri et al, 2006; Cressey et al, 2005; Elias and Dias, 2008; Tischer et al, 1991; Ferrara, 1999). VEGF165, however, only contains exon 7; therefore it is still bound by the heparin in the extracellular matrix, but it can also be found in a diffusible form (Ranieri et al, 2006; Cressey et al, 2005; Tischer et al, 1991; Ferrara, 1999). VEGF121 lacks both of these exons so it is not sequestered on the cell surface or extracellular matrix and is instead easily diffusible to distant target sites (Ranieri et al, 2006; Tischer et al, 1991; Ferrara, 1999).

Figure 3: The major mRNA splice forms of vascular endothelial growth factor. This figure also shows the location of TaqMan probes used to identify VEGF121 and VEGF165. The probe for VEGF121 spans exons 5 and 8 while the probe for VEGF165 spans exons 5 and 7. The probe for total VEGF (not pictured) spans exon 3 and 4 and thus detects all VEGF isoforms. *Figure adapted from S. Alomair, unpublished.*

IV. Model systems and prostate cancer cell lines

Angiogenesis and metastasis in prostate cancer have a profound impact on the prognostic outcome of cancer treatment and survival rate (Catena et al, 2007; Chen et al, 2004). If the prostate cancer remains confined to the original tumor site, the survival rate after five years is 100%; however, if it has metastasized, the chance of five-year survival drops down to 31% (Chen et al, 2004). In some studies, prostate cancer has been found to contain increased levels of VEGF relative to normal prostate tissue (Stefanou et al, 2004), and metastatic prostate cancer patients have shown an even greater increase in VEGF within the plasma versus those whose cancer has not metastasized (Ranieri et al, 2006). The increase in VEGF may be a significant cause of increased tumor vasculature within prostate cancer, and contribute to an increased proclivity for metastasis when angiogenesis is upregulated (Ranieri et al, 2006; Chen et al, 2004). Studies have shown that VEGF can increase the metastatic potential of prostate cancer cells, and that there is a relationship between increased angiogenesis and metastasis, progression, and an aggressive phenotype in prostate cancer (Catena et al, 2007; Ranieri et al, 2006). Studying the regulation of VEGF splice forms in different prostate cancer cell lines which are known to have different metastatic potentials is one way to better understand the relationship between differential blood vessel formation and metastatic potential.

LNCaPs and PC3s are two of the more well-known, better-established human prostate cancer cell lines that exhibit distinct characteristics which have an impact on how they function (Table I). PC3 cells were initially characterized in 1978 and are a human prostate cancer cell line derived from a bone metastasis (Chen et al, 2000; van

Bokhoven et al, 2003; Kaighn et al, 1979). LNCaPs are also human prostate cancer cells but were derived from a lymph node metastasis (van Bokhoven et al, 2003; Thalman et al, 2000; Horoszewicz, 1983). These two cell lines have many different characteristics but one of the more notable is the presence or absence of androgen receptors on the cells. PC3s are androgen-independent cells which lack androgen receptors, meaning they are not responsive to treatment involving androgen depletion (van Bokhoven et al, 2003). LNCaPs, on the other hand, require androgens to grow *in vivo*, which means they respond better to androgen deprivation therapy (van Bokhoven et al, 2003). It is believed that there may be a relationship between VEGF production and the presence of androgens in normal prostate tissue, so it may be possible that a similar mechanism functions in malignant prostate tissue (Ranieri et al, 2006; Aalinkeel et al, 2004). Work in our lab has previously identified hormone responsive regions in the VEGF promoter (Eisermann et al, 2013). Prostate cancers that have progressed to an androgen-independent state are harder to treat because most therapies initially involve some sort of androgen blockade (van Bokhoven et al, 2003; Dozmorov et al, 2009). PC3s are believed to be a more aggressive tumor cell line and exhibit a greater metastatic potential than LNCaPs (Brett et al, 2013; Aalinkeel et al, 2004; Dozmorov et al, 2009), which may also indicate a difference in the production of VEGF and its splice forms between the cell lines.

Table I: Comparison of Prostate Cancer Cell Lines

Cell Line	**Origin** [a]	**Androgen Receptor** [a]	**VEGF levels** [b]	**Migratory potential** [c]	**Metastatic potential** [d]
LNCaP	lymph node metastasis	+	low	low	low
PC3	bone metastasis	-	high	high	high

a: van Bokhoven et al, 2003; Horoszewicz et al, 1983; Kaighn et al, 1979

b: Aalinkeel et al, 2004

c: Brett et al, 2013; Dozmorov et al, 2009

d: Dozmorov et al, 2009; Aalinkeel et al, 2004

Hypoxia has also been associated with a poor prognosis in prostate cancers, since the inner tumor environment generally lacks oxygen and therefore will allow HIF to upregulate its many diverse transcriptional targets (Dai et al, 2011; Tsai and Wu, 2012). VEGF has been implicated in angiogenesis, cell migration, metastasis, and proliferation of prostate cancer cells (Chen et al, 2004; Aalinkeel et al, 2004; Ferrara, 1999; Robinson and Stringer, 2001). Based on this connection, VEGF has the potential to be a good target for anti-cancer and anti-metastatic therapy (Chen et al, 2004; Bhat and Singh, 2007; Folkman, 2002). If the various splice forms were found to be regulated differently under hypoxic conditions in cell lines with different metastatic capabilities, these splice forms may also act as more precise targets of anti-cancer therapy. To our knowledge, this is the first study that has compared the production of the different VEGF splice forms in LNCaP and PC3 cells exposed to hypoxic conditions.

V. Aims and Goals of the Study

Aim 1: Determine if treatment of prostate cancer cell lines with cobalt chloride induces increased expression of VEGF splice forms

Before we were able to obtain a hypoxia chamber, we examined the effect of cobalt chloride on the regulation of the VEGF isoforms in the LNCaP prostate cancer cell line. Cobalt chloride has been shown to mimic the effects of hypoxia by binding to HIFα, thereby preventing its degradation by proteasomes in the cell and allowing it to commence transcription of the genes which it regulates (Yuan et al, 2003). Because VEGF is one of the transcriptional products of HIF1α-mediated transcription in hypoxia,

we hypothesized that cobalt chloride treatment of prostate cancer cells would induce VEGF splice form production. We treated LNCaPs with cobalt chloride or control diluent for 48 hours, then isolated and reverse transcribed the RNA, and performed quantitative real-time PCR to determine the mRNA levels of each isoform present in the cultured cell lines, comparing $CoCl_2$-treated ("hypoxic") to untreated ("normoxic") cells. Based on these results which showed that the splice forms of VEGF are upregulated by treatment with cobalt chloride, it was expected that they would also be upregulated when the cells were exposed to actual hypoxic conditions (tested in Aim 2 below).

Aim 2: Determine if hypoxia affects the production of the VEGF121 and VEGF165 splice forms in LNCaP prostate cancer cells

We next wanted to study the effect that hypoxia had on the VEGF121 and VEGF165 splice form production in the LNCaP prostate cancer cell line. LNCaPs are an androgen sensitive prostate cancer line that has a low metastatic potential compared to other prostate cancer cell lines such as PC3 and DU145 (Brett et al, 2013; Dozmorov et al, 2009; Aalinkeel et al, 2004). LNCaPs were cultured under either normoxic (control) or 1% O_2 (hypoxic) conditions. We then isolated and reverse transcribed the mRNA as in the cobalt chloride experiments and performed quantitative real-time PCR to determine the extent of VEGF splice form upregulation. These results were compared to the cobalt chloride experimental data. We hypothesized that under the control conditions, VEGF165 would be the more abundant isoform because it has been shown to have an effect on both the formation of inner tumor microvasculature and the recruitment of

previously existing systemic blood vessels. However, under hypoxic conditions, we predicted that VEGF121 would be upregulated to a greater extent than VEGF165 because of its diffusible nature and ability to send an angiogenic signal to sites more distant from the tumor.

Aim 3: Determine if hypoxia affects the production of the VEGF121 and VEGF165 splice forms in PC3 prostate cancer cells

In order to determine whether or not there would be a difference in the upregulation of the VEGF splice forms in cell lines of differing metastatic potentials in hypoxia, we decided to investigate the regulation of the splice forms in the PC3 cell line, which has a greater potential for metastatic growth than LNCaPs. PC3 cells were given the same treatment and mRNA assessment as the LNCaPs. We hypothesized that, due to its already elevated metastatic potential over the LNCaPs, VEGF121 would be the more abundant isoform under normoxic conditions. We expected that when exposed to hypoxia, this difference between the VEGF165 and 121 isoforms would become even greater in order to elicit a stronger angiogenic response than in the controls.

Chapter 2: Materials and Methods

Cell lines and routine culture conditions

PC3 and LNCaP prostate cancer cells were cultured in 100mm tissue culture dishes in 10mL of a media solution consisting of RPMI-1640, 10% FBS (Fetal Bovine Serum), and 1% Pen-strep (Penicillin streptomycin). Plates were kept under conditions of 5% CO_2 and ~20% O_2 at 37˚C. Cells were routinely split by removing the existing media, rinsing with PBS, and treating with 2mL of trypsin. When passaging LNCaPs, the trypsin was removed prior to incubation (dry trypsinization) whereas, when passaging PC3s, the trypsin was left in the plate during incubation (wet trypsinization). After 3-7 minutes of incubation, the dishes were taken out of the incubator and enough fresh media was added to the dish in order to bring the total amount of liquid to 5mL (5mL media added to LNCaP plates; 3mL added to PC3s). The plates were usually split at a 1:10 ratio for LNCaPs and a 1:20 ratio for PC3s with some variation based on the confluency of the original plate.

Routine experimental culture conditions

Prior to the introduction of any treatment, PC3s and LNCaPs had to be seeded into six-well plates in order to adhere to the wells and form a confluent monolayer. In order to do this, the media was removed from the 100mm tissue culture plate, and the cells were rinsed with PBS. The cells were then trypsinized according to the method

attributed to the specific cell line, as previously described. They were then placed in an incubator at 37˚C for approximately 3-7 minutes, or until the majority of the cells had detached themselves from the culture dish. Fresh media was then added to the dish to bring the total volume to 5mL. Depending on the plate confluency, number of wells needed, and cell type, 200-500µL of the cells were removed from the dish and put in either a 15mL or 50mL tube. Enough fresh media was then added to the tube so that each well to be seeded would contain a total of 2mL of media. Cells were then placed in an incubator at 37˚C under 5% CO_2 (normoxic conditions) and remained in normoxia until 60-80% confluent (usually 3-5 days).

Cobalt chloride treatment

After the cell monolayers had reached confluency, the media was removed from the wells and then replaced with 2mL of fresh media supplemented with either 0µM (control), 100µM, or 300µM $CoCl_2$ which had been created from a stock solution of 200mM $CoCl_2$. Then the cells were placed in the incubator under the previously described normoxic conditions for approximately 48 hours.

Hypoxia

LNCaPs and PC3 cells were seeded into six-well plates or groups of three individual plates and kept in normoxia to become 60-80% confluency as per the previously described methods. Each hypoxia-treated experiment was seeded at the same time as its controls. The media was then removed and replaced with 2mL of fresh media

per well, and the cells were placed in either normoxia at ~20% O_2 and 37˚C or in a hypoxia chamber set at 1% O_2, 5% CO_2, and 37˚C for 48 hours.

RNA isolation and PCR analysis of cDNA

After 48 hours, RNA was then isolated using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) as per the manufacturer's instructions. Briefly, the cells were lysed with a mixture of GenElute™ lysis buffer and 10µL/mL of 2-mercaptoethanol and RNA was recovered from lysates using a silica-based column. RNA purity and quantities were measured using the NanoDrop ND1000 system. The mRNA was then reverse transcribed in order to obtain cDNA from 1µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was then reduced to a concentration of 5ng/µL, and quantitative real-time PCR (qPCR) was performed using 10ng of cDNA per well. Each sample was run in triplicate. The VEGF121 TaqMan primer contained a probe which spanned exons 5 and 8, the VEGF165 TaqMan primer contained a probe which spanned exons 6 and 8, and the total VEGF Taqman primer probe spanned exons 3 and 4 (see Figure 3 and Table 2). The 18S TaqMan primer was used to create baseline values against which to normalize the raw VEGF isoform data.

Table 2: TaqMan PCR Primers and ABI Assay Identifications

Target Gene	Probe Span[a]	ABI Assay Identification
VEGFA: total VEGF	exons 3-4	Hs00900055m_1
VEGFA: isoform 121	exons 5-8	Hs03929005m_1
VEGFA: isoform 165	exons 5-7	Hs00900057m_1
18S		Hs99999901s_1

a. See Figure 3 for locations of exon-spanning TaqMan probes.

PCR quantitation and statistical analysis

The CT (cycle threshold) value for each sample was normalized to the 18S value by taking the average 18S CT value for each sample and subtracting it from the average VEGF CT value for the same sample (ΔCT). To account for the two-fold increase in DNA concentration produced during each cycle of amplification, each ΔCT value was then transformed to a $2^{(-\Delta CT)}$ value. The $2^{(-\Delta CT)}$ averages of each control and treatment sample were calculated and used to determine the relative changes due to treatment; that is, the fold changes in the treatment samples were calculated relative to the primer-specific $2^{(-\Delta CT)}$ control average by taking the average $2^{(-\Delta CT)}$ of the treated samples and dividing it by the average $2^{(-\Delta CT)}$ of the control sample. The ratio of $2^{(-\Delta CT)}$ to the sample-specific average $2^{(-\Delta CT)}$ was calculated for each well and then standard error about the mean was calculated for each control or treated group. Statistical significance was determined by means of a one-tailed t-test using the $2^{(-\Delta CT)}$ values of the controls and treated cells as the compared data sets; p-values of less than 0.05 were determined to be statistically significant.

Similar methods were used to calculate the ratio of each isoform to the total. However, we believed that the total VEGF primer used had a different efficiency than those of the specific VEGF isoforms, so that data was not included in these calculations. This approach was adopted because we observed that the CT values obtained with the total or “pan” VEGF TaqMan primers were larger than those of the VEGF 165 isoform-specific TaqMan primers, indicating that for our samples the “pan” VEGF TaqMan primers amplified cDNA less efficiently than did the VEGF 165-specific TaqMan

primers. For this reason an alternative analysis was used to measure the relative contribution of the VEGF121 and 165 isoforms to the "total," a method similar to Catena et al (2007). The $2^{(-\Delta CT)}$ and average $2^{(-\Delta CT)}$ values for all groups were calculated as before. The average $2^{(-\Delta CT)}$ values of the VEGF121 and VEGF165 isoforms were then added together to create an additive "total" VEGF estimation, since these are the most prevalent isoforms of VEGF. The average $2^{(-\Delta CT)}$ of each isoform was then divided by the additive "total" VEGF in order to obtain a proportion. The standard error about the mean was then calculated by taking each individual $2^{(-\Delta CT)}$ value and dividing it by its respective average $2^{(-\Delta CT)}$ value. A one-tailed t-test was then performed to determine the statistical significance of these proportions, using the same well-to-total proportions used in the calculation of the SEM as the compared data sets. To be determined statistically significant, p-values should be less than 0.05.

Chapter 3: Results

Aim 1:

Treatment of prostate cancer cells with cobalt chloride induces increased expression of VEGF splice forms

Cobalt chloride ($CoCl_2$) treatment has been shown to mimic hypoxia in its upregulation of VEGF in cancer cells (Liu et al, 2000; Yuan et al, 2003). Initially we lacked a hypoxia chamber and thus studied the effect of $CoCl_2$ on the expression of total VEGF, VEGF121, and VEGF165 mRNA in LNCaPs. LNCaPs were cultured as per the methods described in the prior chapter and seeded into six well plates in either normal media, 100μM $CoCl_2$ treated media, or 300μM $CoCl_2$ treated media. The cells were then cultured in normal oxygen conditions (20% O_2) at 37˚C for 24 hours. RNA was isolated and reverse transcribed, and quantitative real-time PCR was performed to determine the amount of VEGF121, VEGF165, and total VEGF mRNA present in each group of cells. Statistical analysis was performed as described in the previous chapter and statistical significance was assessed by performing a one-tailed t-test ($p<0.05$).

VEGF121, VEGF165, and the total VEGF mRNA was found to be slightly upregulated in LNCaPs treated with 100μM $CoCl_2$, but 300μM $CoCl_2$ was most effective at mediating a statistically significant upregulation of the both of the targeted genes. Treatment of LNCaP cells with 100μM $CoCl_2$ caused a slight upregulation of both VEGF splice forms and total VEGF mRNA levels. VEGF121 was upregulated not quite four-

fold compared to control levels, while VEGF165 was increased by slightly more than two-fold, and the total VEGF mRNA was increased by less than two-fold (Figure 4). In contrast, treatment of the LNCaPs with 300µM $CoCl_2$ caused a significant increase in VEGF121, VEGF165, and total VEGF ($p<0.05$). VEGF121 was upregulated approximately fourteen times versus control levels, VEGF165 was upregulated by seven, and total VEGF mRNA was increased by eight (Figure 4). In both100µM and 300µM $CoCl_2$ treatments, the increase in VEGF121 was statistically significant and greater than the increase in VEGF165. Similar results were observed in PC3 prostate cancer cells treated with 300µM $CoCl_2$ (data not shown). Since we observed a significant increase of both VEGF isoforms, we hypothesized that the total amount of VEGF mRNA as well as the splice forms would be upregulated in response to actual hypoxic conditions once we obtained a hypoxia chamber.

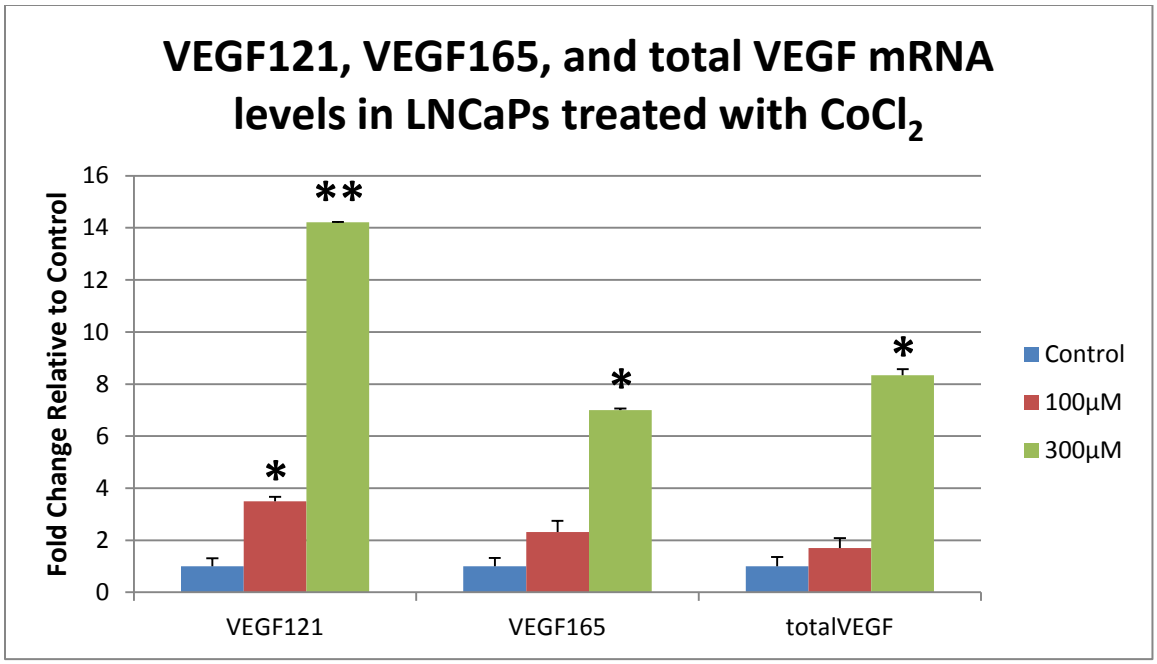

Figure 4: Cobalt chloride treatment of LNCaP prostate cancer cells increases production of total VEGF and individual splice forms. LNCaPs were treated with 0µM $CoCl_2$ (control), 100µM $CoCl_2$, or 300µM $CoCl_2$ as described in text. The cells were lysed after 24 hours of treatment and then RNA was isolated and reverse transcribed. Quantitative real-time PCR (qPCR) was then performed using the TaqMan primers as described in the methods. Shown is the fold-change increase of expression of VEGF121, VEGF165, and the total VEGF normalized to 18S transcript levels; data are presented relative to the untreated control cells. Experiments were done in triplicate and reproduced twice. A one-tailed t-test was performed and significance was determined by comparing expression of treated with untreated cells (*$p<0.05$; **$p\leq0.01$).

Aim 2:

Hypoxia affects the production of the major VEGF splice forms in LNCaPs

Once we obtained a hypoxia chamber, we then compared the $CoCl_2$ results to hypoxia-treated LNCaPs to see if the trend in the fold-change differences between the upregulation of VEGF121 and VEGF165 that occurred in both the 100µM and 300µM treated cells persisted with hypoxia exposure. As expected, Figure 5 shows that hypoxia has the ability to upregulate the total VEGF mRNA as well as the 121 and 165 isoforms of VEGF in LNCaPs. In both hypoxia and normoxia, VEGF121 was upregulated to a greater extent than VEGF165; while VEGF121 was upregulated approximately eleven times in hypoxia compared to its control level, VEGF165 was only upregulated approximately 5.5 times that of its corresponding control levels. These levels are similar to the 300µM $CoCl_2$ results shown in Figure 4, which indicate a greater upregulation of the VEGF121 splice form over the VEGF165 splice form in the control versus $CoCl_2$-treated cells. Figure 5 shows that hypoxia caused an increase in VEGF121, VEGF165, and total VEGF mRNA but it induced a more drastic upregulation of VEGF121 than VEGF165 compared to the respective control levels.

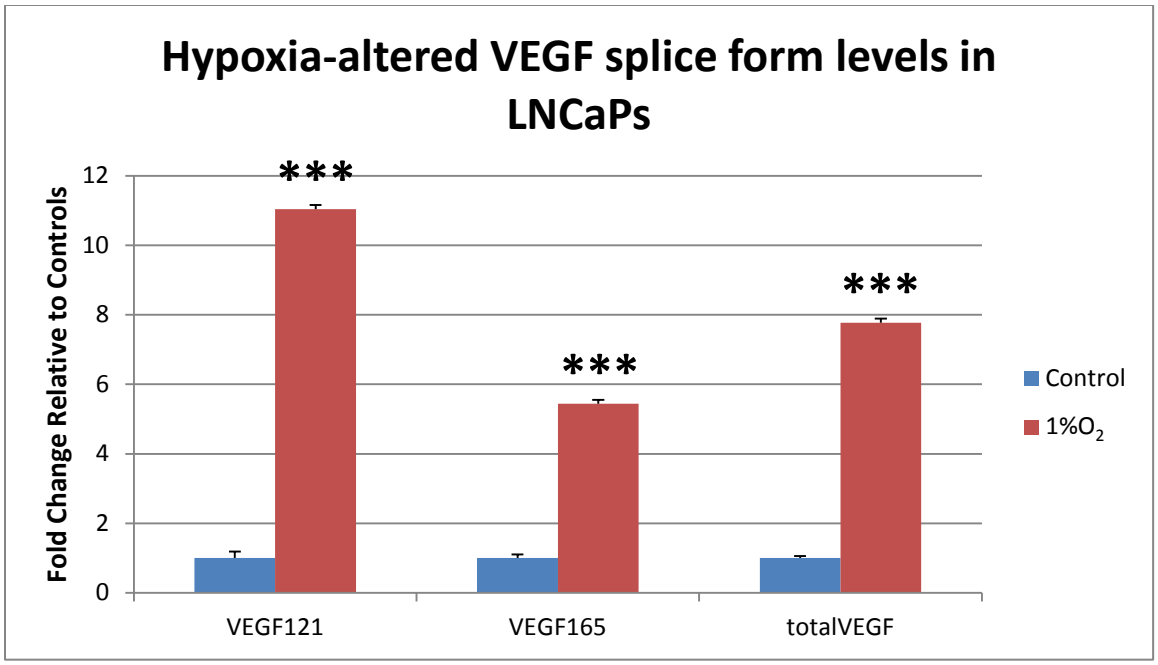

Figure 5: Treatment of the LNCaP prostate cancer cell line with hypoxia increases mRNA levels of the major VEGF splice forms. LNCaPs cells were seeded into six-well plates as described in methods and media was refreshed when cells were approximately 60-80% confluent. The cell monolayers were then exposed to either normoxia or hypoxia for approximately 48 hours, after which time the cells were lysed and RNA was isolated and reverse transcribed. Quantitative real-time PCR (qPCR) was then performed using the TaqMan primers as described in the methods. Shown is the fold-change increase of expression of VEGF121, VEGF165, and the total VEGF normalized to 18S transcript levels and data are presented relative to the untreated control cells. Experiments were done in triplicate and reproduced twice. A one-tailed t-test was performed and significance was determined by comparing expression of treated with untreated cells (***$p \leq 0.001$).

Figure 5 is indicative of an upregulation of the VEGF splice forms with exposure to hypoxia compared to their levels in normal oxygen conditions, but it does not describe how prevalent each isoform is in terms of the total VEGF levels. To address this, we have reanalyzed the data so that Figure 6 depicts the ratio of each VEGF splice form to total VEGF values cultured in either the normoxic (control) or hypoxic (treated) environment. The same method of statistical analysis was performed on the same raw data as described for the previous figure. However, the "total" VEGF levels used to calculate the ratios in this figure were found by adding together the VEGF 121 and VEGF 165 values. Of course these two isoforms do not represent all possible VEGF isoforms, but rather represent the major isoforms. To sum these two isoform values, the 18S normalized VEGF CT (cycle threshold) values (or ΔCT values) were first transformed to account for the two-fold increase in concentration produced during each cycle of amplification (see Methods for complete description). The $2^{(-\Delta CT)}$ values of both of the splice forms were added together to calculate the "total." The average $2^{(-\Delta CT)}$ of each splice form was then divided by the new "total" value to determine the splice form proportion of the additive total VEGF. This approach was used because of issues with reduced efficiency of the "pan" or total VEGF (non-isoform specific) primers (see Methods).

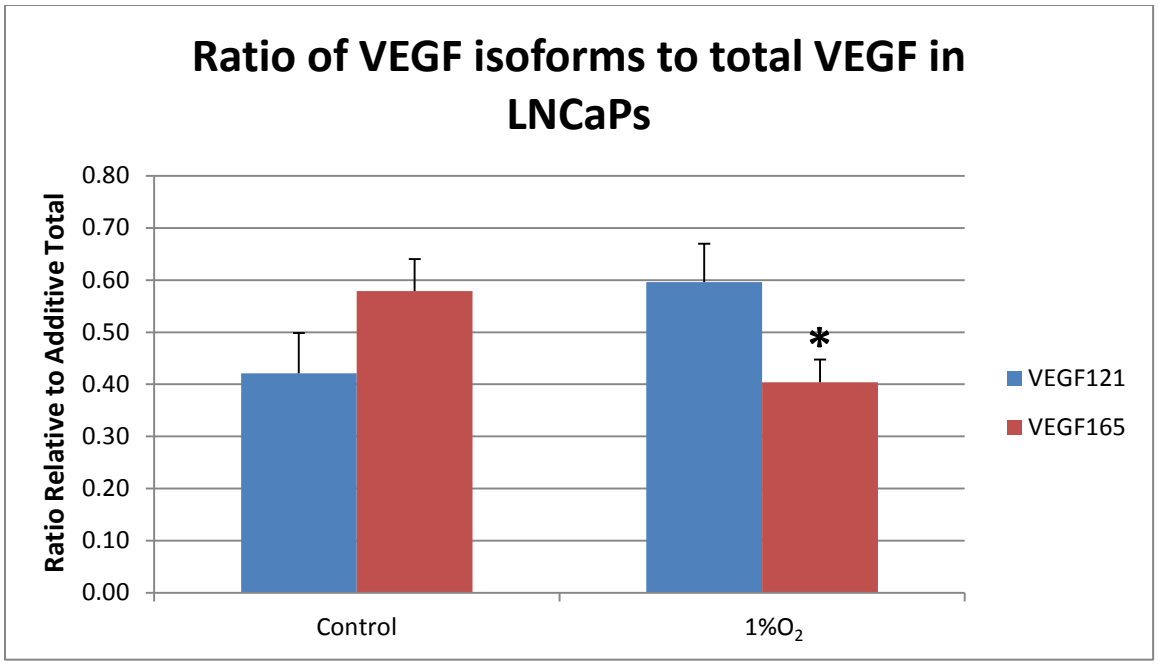

Figure 6: Hypoxia alters the ratio of the major VEGF splice forms in LNCaPs. This figure depicts a different analysis of the same raw data presented in Figure 5. The results above are shown as ratios of each VEGF splice form to the additive total VEGF mRNA in either normoxia (control) or hypoxia (1% O_2). In the control experiment, VEGF121 made up a smaller proportion of the total VEGF than VEGF165. However, in hypoxia, the VEGF121:total VEGF ratio increased while VEGF165:total VEGF ratio decreased. Although the change in the VEGF splice form proportion from normoxia to hypoxia was dramatic and reproducible, the results of the one-tailed t-test analysis of significance, as described in Figure 5, did not meet the standards for statistical significance, with the exception of the ratio change in VEGF165 (*$p<0.05$).

In LNCaPs exposed to normal oxygen conditions, VEGF121 made up a smaller proportion of the total VEGF than did VEGF165 (Figure 6). The ratio of VEGF121:total VEGF was approximately 0.42, whereas the ratio of VEGF165:total VEGF was around 0.58. However, the proportion of total VEGF that was made up of VEGF121 increased from normoxia (0.42) to hypoxia (0.60), while the proportion of VEGF165 actually decreased from 0.58 to 0.40. These experiments were done in triplicate and the general trends were reproduced twice, but the actual proportions varied between experiments. Although the differences did not reach the set level of statistical significance, the same trend was observed across the experiments in terms of the change in the proportion of the total VEGF that was made up of each isoform: the proportion of VEGF121 was always found to be increased in hypoxia while the VEGF165 splice form proportion was always found to be decreased in hypoxia.

Aim 3:

Hypoxia affects the production of the major VEGF splice forms in PC3 cells

As expected, hypoxia was shown to upregulate VEGF121, VEGF165, and total VEGF mRNA in PC3 cells when compared to the normoxic controls (Figure 7). In the hypoxia-treated cells, VEGF121 mRNA increased slightly more than 2.5 times its control amount, whereas VEGF165 was only increased around 1.8 times greater than its control; thus VEGF121 was upregulated to a greater extent than VEGF165. This trend in upregulation shown in Figure 7 is similar to the trend shown by the LNCaPs in Figure 5, however the LNCaPs produced VEGF and its splice forms to a greater extent in hypoxia

than the PC3 cells. For example, LNCaPs upregulated VEGF121 around eleven times greater than its normoxia control group, while PC3s only upregulated VEGF121 around 2.6 times greater than its controls. Total VEGF levels also did not increase as greatly in the PC3 cells as they did in LNCaPs. Overall, hypoxia is shown to upregulate the splice forms of VEGF and total VEGF mRNA (Figure 7). The fold change in VEGF121 mRNA was shown to be greater than the fold change in VEGF165 mRNA. Total VEGF mRNA was shown to be modestly upregulated in hypoxia, although the data was not statistically significant.

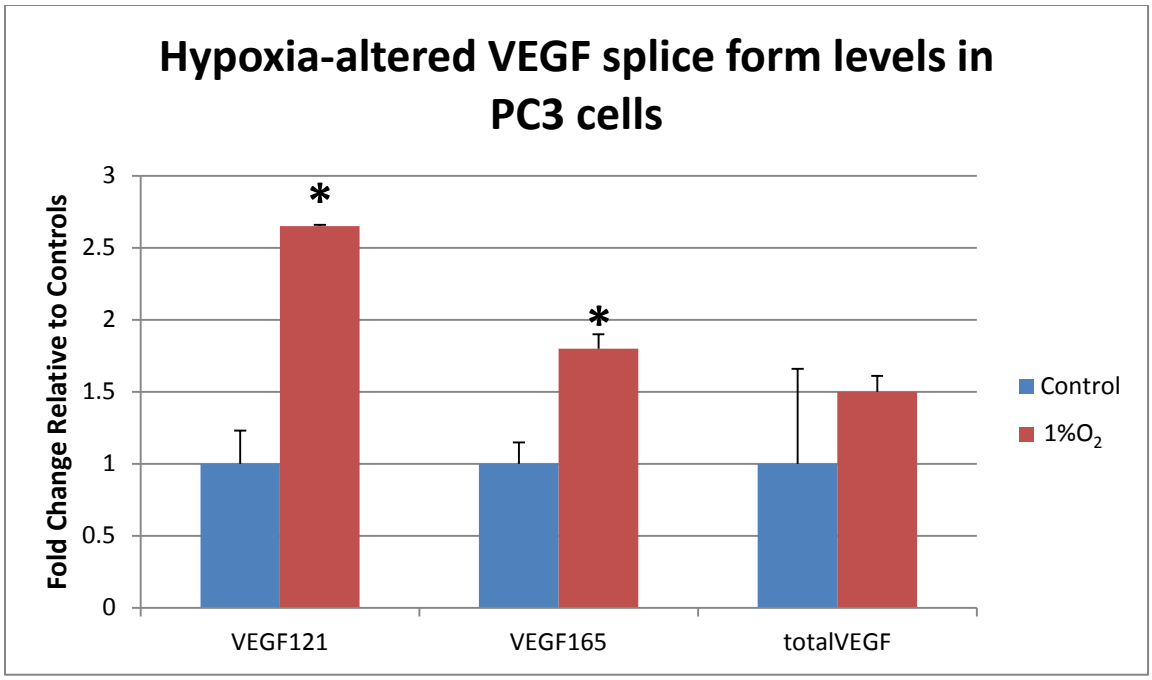

Figure 7: Exposure of PC3 cells to hypoxic conditions induces an increase in the VEGF splice forms 121 and 165 as well as an increase in the total VEGF mRNA. PC3 cells were cultured, seeded, and prepared for hypoxia as described in the methods. Cells were incubated in either normoxia or hypoxia for approximately 48 hours and RNA was isolated, reverse transcribed, and quantified using TaqMan primers. The figure shows the fold change in expression of VEGF121, VEGF165, and total VEGF normalized to the 18S transcript values; data is shown relative to the control (normoxia) levels. Experiments were done in triplicate and reproduced. The statistical significance of the fold change between control and hypoxia was determined by means of a one-tailed t-test (*$p<0.05$).

Slight differences between the VEGF isoform ratio changes were also found in PC3 cells compared to LNCaPs. Figure 8 shows that, in PC3 cells under either control or hypoxic conditions, VEGF121 made up a smaller proportion of the total VEGF than VEGF165. These proportions are very similar to the splice form proportions in the LNCaP control group shown in Figure 6. VEGF121 increased in proportion of total VEGF from 0.40 in normoxia to 0.47 in hypoxia. VEGF165, however, decreased from 0.60 in normoxia to 0.53 in hypoxia. Although the proportion of VEGF165 actually decreased in hypoxia, it still made up a greater proportion of the total VEGF than did VEGF121. In hypoxia, however, VEGF121 was upregulated in LNCaP cells so that it was more prevalent than the VEGF165 isoform, whereas in PC3s VEGF121 was still found to be less prevalent than VEGF165 (Figures 6 and 8).

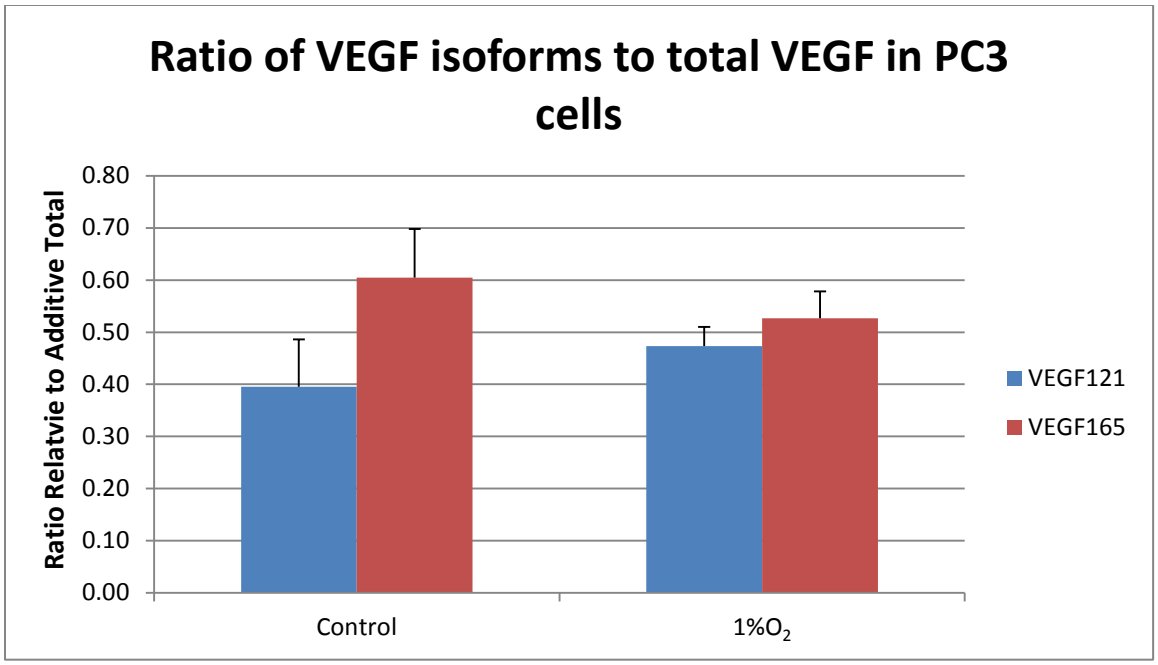

Figure 8: Hypoxia alters the ratios of VEGF121 and VEGF165 to total VEGF in PC3 cells. This figure presents a different analysis of the same raw data shown in Figure 7. The results above are shown as ratios of each VEGF splice form to the additive total VEGF in normoxia (control) or hypoxia (1% O_2). In both the control and hypoxia samples, VEGF121 made up a smaller proportion of the total VEGF than VEGF165. However, the proportion of VEGF121 did increase slightly whereas the proportion of VEGF165 decreased from normoxia to hypoxia. In hypoxia, the two major splice forms made up very similar proportions of total VEGF. A one-tailed t-test was performed in order to assess the statistical significance of the splice form:total VEGF ratio; however, the ratios were not found to be statistically significant (*$p<0.05$).

Chapter 4: Discussion

Hypoxic conditions exist within the inner tumor environment (Weinberg, 2013; Dai et al, 2011; Alqawi et al, 2007). The lack of oxygen leads to an upregulation of different factors, one of the more important being hypoxia-inducible factor 1α (HIF1α) (Weinberg, 2013). HIF1α is involved in the transcription of a variety of genes which promote cell survival and angiogenic growth in hypoxic conditions (Tsai and Wu, 2012; Alqawi et al, 2007). VEGF is one such factor that is upregulated in hypoxia by HIF1α (Weinberg, 2013; Robinson and Stringer, 2001); however, to our knowledge there have not been any studies which have looked at the regulation of the various VEGF splice forms in prostate cancer cell lines under hypoxic conditions.

Our results showed that the while both VEGF121 and VEGF165 were upregulated significantly in both PC3s and LNCaPs, VEGF121 seems to have been preferentially increased over VEGF165 when compared to the control levels in each cell line. Although no previous studies were found which investigated VEGF splice form regulation in hypoxic conditions in prostate cancer cell lines, our results are supported by those of other studies that show a trend in upregulation of the smallest diffusible isoform of VEGF, VEGF121, in cancerous tissue relative to normal tissue (Zhang et al, 2000; Fenton et al, 2004; Catena et al, 2007). Since normal tissue is unlikely to be hypoxic, but tumors can contain hypoxic areas, this seems consistent with our results. In a comparison of VEGF isoform production between normal human prostate and prostate cancer tissues

and cell lines in normoxia, VEGF121 was found in lower levels than VEGF165 in normal prostate tissue; however, in prostate cancer tissue and cell lines, VEGF121 became the more abundant isoform and the percentage of VEGF165 decreased (Catena et al, 2007). It could be argued that hypoxia exaggerates these differences between VEGF121 and VEGF165 production in some prostate cancer cells, which is how our results parallel those of this tumor tissue study.

One of the supporting arguments for the upregulation of VEGF121 over VEGF165 is that because VEGF121 lacks the exons which bind heparin, it is not bound on cellular surfaces or in the extracellular matrix, so it is able to diffuse more distantly and elicit a stronger, more rapid angiogenic response from major blood vessels in order to ameliorate the low oxygen levels within the tumor (Zhang et al, 2000). In MCF-7 breast cancer cell tumors, VEGF121 was found to induce angiogenesis in a quicker fashion than the VEGF189 and VEGF165 isoforms (Zhang et al, 2000). Similarly, overexpression of VEGF121 in the same line of cells increased the oxygenation throughout the tumor by increasing the density of the blood vessels and decreasing spacing between the vessels; in contrast, overexpression of VEGF165 in this same study also decreased vessel spacing but did not oxygenate the tumor to the same extent as with VEGF121 (Fenton et al, 2004). However, others have found VEGF165 to be the more predominant isoform in other tumor types such as colorectal cancer and non-small cell lung carcinoma (Cressey et al, 2005). These differing preferences in isoform upregulation across various tissues may be caused by unknown differences in isoform function between tissues (Cheung et al, 1998; Guo et al, 2001).

Aside from VEGF's hypoxia-mediated transcriptional upregulation, it is possible that the upregulation of VEGF165 might be related to the vessel patterning that seems to be induced by this isoform. In mice, the VEGF isoforms are each smaller in length than their corresponding human isoform by one amino acid (VEGF120, 164, and 188) (Grunstein et al, 2000). Studies of the functions of these murine isoforms may expand on what is already known about the functions of their human counterparts. One group investigated the effect that individual murine isoforms had on the blood vessel formation in tumors grown in immunocompromised mice through the creation of retrovirally infected cancer cells which only expressed single isoforms (Grunstein et al, 2000). The isoform-specific transduced cells were injected into the mice, and the resulting tumors showed distinct patterns of blood vessel formation. Interestingly, the wild type tumors and the VEGF164-specific tumors had the most similar blood vessel patterns: vascular densities were similar between the two, VEGF was present in a soluble form within the vessels as well as bound to the ECM, and both recruited the larger systemic blood vessels and induced significant internal vascularization of the tumor as a whole (Grunstein et al, 2000). Since VEGF165 is the predominant isoform in normal human prostate, this finding was not surprising. VEGF120 was shown to only induce systemic vessel recruitment around the outer edge of the tumor, while the VEGF188-expressing tumor showed excessive internal smaller vessels. Based upon these findings, we could conclude that, because the inner tumor environment is hypoxic, the tumor cells would prefer to induce the formation of smaller internal capillaries (microvessels) to increase the oxygen diffusion in the center of the tumor and recruit the larger systemic vessels (macrovessels)

in order to supply oxygen to the smaller blood vessel within the tumor. However, Catena et al (2007) postulate that there might be differences in isoform functions between the human and mouse models. In the immunocompromised mouse model using human tumor xenografts, VEGF165 was predominant in both normal prostate and tumor tissues, but in cultured human prostate cancer cell lines, they observed an increase in VEGF121 and a decrease in VEGF165 when compared to normal human prostate epithelial cells.

Our study also found differences in isoform expression between types of prostate cancer cells. In LNCaPs, VEGF121 was upregulated in hypoxia to a greater extent than in PC3s relative to their respective VEGF121 normoxic control levels. Catena et al (2007) found that LNCaPs had higher levels of the secreted isoforms of VEGF than PC3 cells, while PC3 cells had higher overall VEGF levels. Our study found that LNCaPs, under similar normoxic (control) conditions, expressed lower levels of VEGF121 than VEGF165; however, under hypoxic conditions, VEGF121 was present in a greater amount than VEGF165. We also saw that in the PC3 cell line, there was a greater upregulation of VEGF121 than VEGF165 in hypoxia versus the controls, yet the proportion of the total that was made up of VEGF121 was still less than VEGF165 in hypoxia. Catena et al (2007) also made the observation that total VEGF levels remained the same in both normal and cancerous prostate cell cultures. Our data shows that total VEGF levels increased in hypoxia, presumably as a consequence of increased HIF1α activity in the hypoxic setting. The difference in the relationship of VEGF121 and VEGF165 between these two cell lines may be related to the differences in metastatic

potential of the cells, since PC3 cells are known to have a greater metastatic potential than LNCaPs cells (Aalinkeel et al, 2004; Dozmorov et al, 2009).

Although each isoform seems to have slightly different functions within the tumor, studies have shown that the most effective blood vessel formation and patterning to enhance tumor growth is caused by a combination of all three of the major isoforms. Grunstein et al (2000) demonstrated that a combination of VEGF120, VEGF164, and VEGF188 induced the most potent tumor growth in mice, which was followed by the combination of VEGF120 and VEGF164, and then by VEGF164 and VEGF188. A second study found that these three isoforms work in a synergistic fashion in order to induce optimal blood vessel formation within the tumor (Whitlock et al, 2003).

Total VEGF production and the abundance of specific isoforms have been found to have the potential to act as prognostic indicators in cancer (Shen et al, 2000; Cressey et al, 2005). However, the isoforms upregulated in cancers may differ according to cell type or anatomical site (Carmeliet, 2003; Cheung et al, 1998). The different VEGF splice forms have been shown in previous studies to contribute different effects on the formation of tumor vasculature. VEGF189 has been shown to be sequestered in the ECM due to its heparin-binding domain, while VEGF121 is the most prevalent of the diffusible isoforms – since it lacks ability to bind heparin – and VEGF165 remains an intermediate form which has the potential to diffuse and bind (Robinson and Stringer, 2001).

Future Plans

Based on the data we have acquired, it seems that further analysis of the protein production of the isoforms in both human and mouse models in hypoxic conditions is

necessary. It may also be beneficial to quantify protein levels in conditioned media as well as those associated directly with the cell in order to get a more accurate view of the amount of each isoform being produced and secreted by the cells. To determine mechanisms controlling isoform-specific expression, it would also be critical to identify the splicing factors that function in the production of these isoforms both in normoxia and hypoxia, and to determine whether their function is affected by low oxygen conditions.

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