THE CREATION OF REAGENTS FIBULIN-1C/D AND TRUNCATES FOR PROTEIN PURIFICATION AND APOPTOTIC ABILITY ON PCA CELL LINES

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Fall 2018

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ACKNOWLEDGMENTS

Foremost, I would not be where I am today without the strength and resilience of my mother. On this long and wild academic journey, even when it looked bleak, she still had faith and hope in me to succeed. I cannot express enough gratitude to her in being the biggest supporter and advocate for me over the years. I would also like to thank my girlfriend for being so supportive outside the lab and understanding the long hours it takes to complete a masters.

Lessons in life sometimes come easy, but the ones that stick with you will be learned the hard way. I attribute Dr. Sikes hands-off technique in the laboratory to me developing many laboratory skills that I will keep for my entire career. The most important techniques that I have developed in Sikes lab are the ability to independently think as a scientist and perseverance, for which I am grateful.

Every Mckinly Lab resident has had a diverse outcome when it came to acquiring a new laboratory home. By myself I would not be given an entire lab to work out to which caused me to worry about if I would have a home. My one time neighbor in Wolf Hall, Dr. Neunuebel's Lab, offered placement in their lab. I appreciate their generosity assimilating me into their lab, giving me the full lab experience as well as taking time to teach me their techniques. Their encouragement and guidance gave me the tools to succeed.

Lastly, I would like to thank Dr. Selva for always being available to answer questions and providing insight to laboratory problems. Her critical analysis with me on my research has helped me succeed.

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LIST OF ABBREVIATIONS

ADAMTS	a disintegrin-like and metalloprotease with thrombospondin motifs
ACT	α_1-Antichymotrypsin
ADT	Androgen Deprivation Therapy
AT	Anaphylatoxin
BM	Basement Membrane
BMP	Bone Morphogenic Protein
BMPRI	Bone Morphogenic Protein Receptor I
BMS	Bone Marrow Stromal
BSA	Bovine Serum Albumin
CAF	Cancer Associated Fibroblast
CAM	Cell Adhesion Molecule
Cath	Cathepsins
cbEGF	Calcium binding epidermal growth factor
Coll VI	Collagen type IV
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRPC	Castrate Resistant Prostate Cancer
CM	Conditioned Media
DDK	Dickkopf-related protein
DMEM	Dulbecco's Modification of Eagle's Medium
DPCs	Disseminated Prostate cancer Cells
DRE	Digital Rectal Exam
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
HBME	Human Bone Marrow Endothelium
HRP	Horseradish Peroxidase
HS	Human Stromal
HSC	Hematopoietic Stem Cell
IGF	Insulin-like Growth Factor
IL	Interleukin
iMC	Immature Myeloid Cell
IP	Immunoprecipitation
MDSC	Myeloid-derived suppressor cell
MEM	Minimum Essential Medium Eagle
MMP	Matrix Metalloprotease
MS/MS	Tandem Mass Spectrometry
MCS	Multiple Cloning Site
MSF	Macrophage Stimulating Factor
NCC	Neural Crest Cells
NFDM	Non-Fat Dry Milk

OPG	Osteoprotegerin
PBS	Phosphate Buffer Saline
PCa	Prostate Cancer
PSA	Prostate Specific Antigen
PVDF	Polyvinylidene Difluoride
PTHrP	Parathyroid Hormone related Peptide
Px	Patient
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RANK	Receptor Activator of the Nuclear factor kappa-B
RANKL	Receptor Activator of the Nuclear factor kappa-B Ligand
Runx-2	Runt-related transcription factor 2
SF	Serum Free
SHBG	Sex Hormone Binding Globulin
shRNA	Short Hairpin RNA
SOC	Super Optimal broth with Catabolite repression
TAE	Tris-Acetate EDTA
TAK-1	TGF beta activated kinase 1
TAM	Tumor-Associated Macrophage
TAN	Tumor-Associated Neutrophil
TGF beta	Transforming Growth Factor beta
TGFBR	Transforming Growth Factor beta Receptor
TIMP-1	Tissue Inhibitor of Metalloprotease-1
TNF	Tumor Necrosis Factor
uPA	Urokinase-type Plasminogen Activator

ABSTRACT

Prostate Cancer (PCa) is the most diagnosed type of cancer in men in the United States. The 5 year survival rate in men for nonmetastatic cancer is around 100%, but when the cancer turns metastatic the 5 year survival rate drops to 30%. The issue for doctors is that it is difficult to tell whether a cancer will turn metastatic and if antibody therapy and excision of the tumor are not enough then patients do not have a successful options for therapy. Sikes' lab studied the bone microenvironment and how bone cells interact with PCa cells to aid in PCa invasion and proliferation. It was found that HS-5 cells secret a factor that induces apoptosis in PCa cells. That factor was found to be fibulin-1. At metastatic sites, cancers recruit other neighboring cells to aid in the PCa invasion and proliferation due to the factors the PCa secretes or upregulates in other cell types. One of those factors highly secreted in tumor supportive cells is TGF beta, once HS-5 cells are incubated in TGF beta they lose their ability to induce apoptosis to PCa, as well as the expression of fibulin-1. This has led to the supposition that the bone is originally a hostile place for PCa metastasis until HS-5 is remodeled. This being said, fibulin-1 has potential to be utilized as a marker in serum of PCa patients to determine the relative risk of the prostate cancer spreading and possibly a treatment.

Chapter 1

INTRODUCTION

The prostate is about the size and shape of a pre-cracked walnut. It is located in front of the rectum and below the bladder while surrounding the intersection where the urethra and ejaculatory duct intersect. Although it is not necessary for reproduction, the prostate operates as an accessory organ along with the seminal vesicles in the male reproductive system to vastly increase the sperm viability and motility as well as to protect the sperm. The prostate secretes a slightly alkaline fluid (7.2-7.8) that works to aid the sperm by combating the vaginal acidic environment. Another key secretion is citrate, which the sperm use as an energy source to power their flagella the prostate also secretes proteolytic enzymes to liquify the semen after ejaculation(1). Ironically although not necessary for life after puberty, it is the most common site for cancer development in men. (2) Although there are elevated risks with certain ethnicities, such as African Americans, on average any given boy will have an 11.6% chance of developing PCa over the course of his life(3).

Like all cancers early detection is key in PCa, the blood can be tested for Prostate Specific Antigen (PSA) as well as conduct a Digital Rectal Exam (DRE). A PSA test shows the serum levels of PSA which is a protein synthesized mainly by prostate cells. Normally there a PSA level of 4.0ng/ml or lower in serum, any level above that could indicate an above normal level of prostate cell growth. Since every male has varying biological levels, there can be many false positives and false negatives. Another test, DRE, physically detect lumps through the anterior wall of the

rectum which borders the prostate. All cancers are nonrandom in their choice of metastasis. The most common site for PCa metastasis is to the bone, where 90% of men with advanced PCa will acquire bone metastasis (4). The mechanisms by which PCa metastasizes to bone remains complex and elusive, when coupled with the struggle to determine which PCa will cause metastasize to bone has lead Sikes' Lab investigations in the direction of how PCa interacts with the bone microenvironment.

1.1 **Prostate Cancer Treatments and Statistics**

Nixon's war on cancer was the inertia breaking step to earlier detection, better treatments and finding cures for cancer patients. There have been many breakthroughs in the past half century such as increasing the 5 year survival rate from ~10% in 1972 to ~90% in childhood leukemia in 2012 (5). Although cancer has been at the research forefront for decades but alas, still takes the lives of millions of people every year, in the US alone 609,000 cancer deaths are projected. PCa is the most diagnosed cancer among men in the United States with 172,258 diagnoses and 28,343 deaths in 2014 and projected to have over 164,000 new cases in 2018 (2, 6, 7). In the course of a lifetime as a male there is a 1 in 9 chance he will develop prostate cancer. PCa tends to be more prevalent in economically developed countries along with French colonized countries (8). A study in 2012 showed that four of the top ten countries for the highest PCa incidence are France or French colonized countries; Martinique, France, New Caledonia, French Polynesia. PCa accounts for 19% of all cancer diagnosis in men(3). The risk of PCa increases as men get older with 97% of all PCa diagnosis being in men 65 years of age or older.

As a part of a normal prostate check-up a Digital Rectal Exam (DRE) or Prostate Specific Antigen (PSA) are used as diagnostic markers for PCa development. If markers determine there is potential for developed PCa then a biopsy is necessary to confirm the presence of PCa, which would be given a Gleason score. Partin tables are used to correlate PSA levels and Gleason scores to the probability of PCa organ confinement, extraprostatic extension as well as metastasis to lymph nodes or seminal vesicles. PSA and DRE tests alone produce many false positives and false negatives. The PSA test is done by drawing blood and testing the serum levels of a protein, PSA, that is mainly found in the prostate. When PSA levels are high it could indicate BM degradation in the prostate, growth of cells in the prostate or prostate cells are growing outside the prostate. The threshold value for this test is 4.0ng/ml. The test seems to be straightforward but there is a large variability in basal PSA values that result in 70% of all patients having values above 4.0ng/ml not having cancer basically these are false positives. On the contrary, 15% of all patients have PSA readings below 4.0ng/ml that actually have cancer; these are false negatives. It is more accurate to base the PSA test on previously recorded PSA levels from that Px. A DRE detects lumps in the prostate by physically examining the prostate through the wall of the rectum. Together, the PSA and DRE can determine if there if the patient has an statistical likelihood for PCa and a biopsy is performed.

A biopsy takes up to 15 tissue samples from the prostate to determine tissue normality. The histologist will examine the glandular architecture to determine the deviation of the tissue sample from the normal, whether it appear poorly or well-differentiated. The progressive loss of identifiable glandular features yields a score from 1 to 5, respectively, from each biopsy specimen. The two scores occurring most

often are added together to get a Gleason sum or score from 1-10. The Gleason score is one system for identifying histological grade(9). A score of 6 or lower has relatively low risk for development of distant metastasis. A score of higher then 8-10 is considered to be high risk for metastatic PCa. A score of 7 is problematic and difficult to categorize due to the nature of the primary number being either 3 or 4. Although PCa diagnosis is prevalent, not all PCa needs treatment. When the PCa is localized to the prostate gland, the patient has a 5-year survival rate of almost 100% but a patient with metastatic PCa has a survival rate of less than 30%.

Determining how far the cancer has spread is called staging. The stages of cancer denote the extent to which the cancer has detached from its localized environment and spread to different sites around the body, there are four broad stages. The first stage is having the cancer only at the primary tumor site where sub stages are given to the size of the tumor. Stage two means that the cancer has spread to away from the primary site but is still contained in the prostate. Stage three is where the tumor has spread to nearby organs such as the bladder neck or the seminal vesicles. Stage four, the final stage, is where the cancer has spread to distant organs and have created secondary tumors also called metastatic sites (9). At times it is difficult to accurately diagnosis of the stage of cancer because some metastatic tumors may not be apparent until the tumors are more well developed and start to present symptoms.

If risk is low shown by the biopsy, the standard care for the patient is surveillance, radiotherapy and/ or prostatectomy. For a patients with metastatic PCa, androgen deprivation therapy (ADT) is given by means of chemical or physical castration (10). Treatments work to deprive the body from production of testosterone from the testis as well as the adrenal glands. ADT tends to fend off the PCa for 1.5-3

years but after that the Px starts to develop a more aggressive, ADT unresponsive form of PCa termed, castrate resistant prostate cancer (CRPC), in which the PCa no longer needs testosterone to grow and thus will not respond to ADT (11). Once CRPC develops the average survival time for the patient drops to less than 2 years. Prior to 2004 only palliative care was given after diagnosis of CRPC, now docetaxel in combination with a glucocorticoid which has shown to increase survival rates slightly and is now the gold standard (12). Docetaxel binds to and impedes microtubule formation in order to cause apoptosis in rapidly growing cells. Glucocorticoids competitively bind to androgen receptors in PCa cells to which attempts to limit the further proliferation of PCa cells(13). This chemotherapy comes with complications and the average addition to the Px life is around 2 months, development of more effective and less harmful treatment is needed.

1.2 **Mechanism of Prostate Cancer Bone Metastasis**

Although other mechanisms for metastasis are possible, the most common type of hematogenic metastasis from prostate cancer is to the bone, with 85% of dying patients containing these lesions (14). PCa metastasis is correlated highly with the long bones and/or marrow producing bone where there are three types of bone lesions caused by PCa, osteoblastic, osteolytic and combination of the two, osteosclerotic (15). Osteolytic metastasis causes the breakdown the hydroxyapatite in bone, which causes the bone to become more fragile. Different from osteolytic metastasis, osteoblastic and osteosclerotic bone metastasis causes deposition of bone which creates an unusual pattern of compact bone. The osteoclast/blast start from the marrow spaces, endosteal, instead from the trabecular surface. This new bone is considered woven bone which is fibroblast-like instead of calcified. Not only does woven bone

compromise the structural integrity of the bone, making it more susceptible to break or fracture, but it also tends to come with considerable amount of bone pain. These together these symptoms greatly reduce the quality of life for PCa Px(16).

Bone metastasis being one of the major underlying causes for Px death, the mechanism behind PCa bone metastasis remains elusive. For over a century the presumed theory of PCa metastasis to bone was Dr. Stephen Paget's 'seed and soil' theory created in 1889. It was originally developed as an explanation why primary tumors have a preference to initiate metastasize in certain tissues rather than others. This suggests that the cancer acts as a seed and that every seed has a favorable soil to grow in and the soil being the microenvironment of any tissue (17) As for PCa, the thought was that the bone was a favorable environment for growth and metastasis, but recent studies have shown that the bone can actually be a hostile environment to PCa (18).

In general, cancer metastasis is a multistep process including invasion, local migration, intravasation, circulation and extravasation, colonization of the new metastatic niche followed by expansion (19). For PCa cells, in order to start this process the cancerous cell will detach from surrounding cells. This is done by epithelium to mesenchymal transition (EMT), which is a fine-tuned process in embryonic development to convert epithelial cells to mesenchymal cells. This process is signaled from an external growth factor or cytokine(s) that activates master regulators SNAI1 and Twist. These master regulators downregulate epithelial adherence factors (E-cadherin) and upregulate mesenchymal adherence factors (N-cadherins). PCa tumors with EMT have been shown to have a higher Gleason score as well as an increase in migration and invasion (20).

Once the PCa is freed from neighboring cells it is still barred from moving about the extracellular matrix (ECM) or going through the basement membrane (BM). In order to move about in the local environment the ECM must be degraded. The ECM and BM in the prostate are made from glandular epithelial cells. These cells lay down a meshwork of collagenous protein such as collagen type (coll) I and III along with non-collagenous proteins such as elastins and fibronectin(21, 22). In order to disassemble the ECM these proteins need to be degraded this is done by the secretion of proteolytic enzymes by PCa cells. Previous studies have shown that PCa cells secrete matrix metalloproteinases (MMP) to degrade the ECM more specifically MMP-2 and -9 for which coll IV is a target. Some studies suggest that decreasing the inhibitor of MMPs, tissue inhibitor of metalloproteinase-1 (TIMP-1), also can promote motility. Moreover the increase of MMP-2 and -9 to TIMP-1 ratio allows for the degradation of ECM proteins (21). Also cysteine proteases such as Cathepsins (Cath) degrade many ECM components including laminin, coll IV and fibronectin which can be inhibited by a protease inhibitor; stefin. As opposed to benign prostate hyperplasia (BPH) and normal tissue, PCa cells have been shown to have a higher ratio of Cath-B/-S to stefin, which aids in the metastasis in PCa cells (23). Moreover, another protease, PSA, can degrade fibronectin. Like MMP's, PSA has inhibitors such as α_1-antichymotrypsin (ACT). With a higher ratio between PSA and ACT there has been a correlation to PCa rather than BPH, due to the over activity of the protease PSA on fibronectin(24). Lastly urokinase plasminogen activator (uPA), a serine protease, has been shown to cleave plasminogen among other proteins. Plasminogen is then converted to another serine protease, plasmin, which further degrades parts of the

ECM and BM. It also activates GFs to produce MMP-2 and -9 to degrade the ECM further(25).

Once free from the microenvironment and passed into the circulatory system, the PCa is up against its toughest battle through the whole process. Coupled with the added sheer stress from fluid flow sheer in the circulation cancer cells will have to fend off the immune system. Cancer cells sometimes will have the ability for more mutations to arise based off a mutation that damages the cells ability to repair its own genome. These will eventually cause some proteins to have incorrect amino acids, termed passenger mutations, which the adaptive immune system will recognize the cell and induce extrinsic apoptosis. A disseminated PCa cell that is destroyed by the immune system cannot be metastatic seed. Our immune system is coordinated to recognize disseminated cells, target and induce apoptosis (26). There are two main ways PCa cells evade this pathway: 1. Inactivate the apoptotic pathway and/or 2. Cloak themselves as a hematopoietic stem cell. As for the apoptotic pathway, the under expression or inactivation causing mutation of proapoptotic effectors such as Bax, Apaf1 and caspases as well as overexpressing the antiapoptotic proteins Bcl2, BclXL and Mcl1 can be useful mechanisms for PCa to evade apoptosis (27) (28). PCa can also trigger a factor on platelets, TCIPA, which activate platelets to attach to the PCa cells and shield them from the immune system.

Although there is such a high incidence of PCa metastasis to bone the mechanism is not entirely elucidated for the preference. More and more studies are backing chemotaxis as the possible cause for this homing of disseminated prostate cancer cells (DPCs) to bone. To get into the bone, discontinuous blood vessels called sinusoid are passed through to enter into the bone marrow. Commonly used by HSCs,

these sinusoids in the trabecular bone are more easily perforated by cells then normal circulatory endothelium because of the discontinuous basal lamina in the epithelial lining(29). To aid in the entrance into bone, some PCa cells have been shown to have homotypic aggregate properties in order to further slow the perfusion rate(30). Another driving force to enter the bone is the chemotaxis of PCa cells to bone. PCa cells produce CXCR4 and the marrow stromal cells produce its only known ligand CXCL12. When a neutralizing antibody is used to sequester free CXCL12, the homing ability of PCa cell lines to bone decreases(31). After CXCL12 binding, PCa cells will produce αvβ3 integrin, which works with other cell adhesion molecules to bind to bone marrow (BM) stromal cells. Endothelial cells in the BM express constitutively high levels of adhesive proteins such as P/E selectins, intercellular adhesion molecule (ICAM) and vesicular cell adhesion molecule (VCAM1) for which endothelial cells in other tissues need an inflammatory stimulus to express these proteins. These interactions aid in the localization and docking of PCa to BM endothelium (32).

1.3 **Bone Microenvironment and Prostate Cancer Adherence**

1.3.1 **Interaction of Osteoblast/Osteoclast/Osteocyte in Bone**

Once localized to the bone the disseminated PCa cells (DPC) must interact with the bone microenvironment to grow and colonize. To understand how the disseminated PCa cell hijacks the bone microenvironment, the bone microenvironment needs to be well understood without the presence of the PCa. Then the remodeling process of the bone by PCa can be fully understood.

The bone microenvironment consists of a biologically active heterogenous mixture of various cell types such as osteocytes, osteoblasts, osteoclasts,

osteoprogenitor cells, fibroblasts, immune cells and endothelial cells along with various growth factors as well as various ECM proteins. The bone also is mineral based in the cortical and spongy bone consisting of hydroxyapatite but within the minerals are imbedded growth factors, which are deposited upon formation of the bone by osteoblasts. PCa bone metastasis occurs primarily bones that contain hematopoietic stem cells or currently undergo hematopoiesis, such as the pelvis, vertebra, sternum, ribs and femur. Most of these bones are long bones which contain a periosteum, which is a layer that is contiguous with ligaments and fibers in order to connect the bone to other bone and muscles. The inside of the periosteum also contains osteogenic stem cells that will mature into osteoblasts for bone remodeling(33). Under the periosteum the cortical bone is found which constitutes a majority of stability for the bone. Cortical bone consists of elongated, overlapping cylinders of bone called osteons, which descend down the bone alongside one another. In each osteon there is a Haversian canal containing blood and lymphatic vessels to nourish the bone. The PCa will utilize this microenvironment in order to invade and colonize the bone.

One of the most important signaling pathways in bone is the Nuclear Factor-κB (NF-κB)/ receptor activator Nuclear Factor-κB Ligand (RANKL) pathway. In the bone marrow, osteocytes and preosteoblasts causes macrophage precursors to differentiate into osteoclasts by releasing NF-κB(34). Preosteoclasts contain the receptor activator nuclear factor-κB (RANK), which, when activated by RANKL causes the formation of NF-κB, which is a regulator of osteoclast genes.

In quiescent bone the osteocyte releases Dickkopf-related protein -1/-2 (DDK-1/-2) and sclerostin, which inhibit Wnt signaling to impede osteoblast formation. An

increase in mechanical load on an osteocyte suppresses sclerostin whereas a decrease in mechanical load will induce sclerostin expression to inhibit and induce bone formation respectively (35). The remodeling process begins by osteocytes sensing mechanical load in the form of fluid flow sheer stress the full mechanism is still up to debate but ultimately results in osteocyte apoptosis. At first sense of increased mechanical load DDK-1/-2 and sclerostatin are downregulated, which activates the Wnt pathway and in concert depletes osteoprotegerin (OPG) (36, 37) This simultaneously inhibits osteoblastogenesis by depleting β-catenin and also causes osteoclastogenesis by increasing the RANKL/OPG ratio. Unloaded bone conditions cause osteocytes to release active TGF beta, which inhibits osteoclastogenesis but once load is sensed that signal is removed and allows for osteoclastogenesis while also expressing other osteoclastogenic factors such as RANKL and macrophage stimulating factor 1 (MSF-1) to induce bone remodeling (34). Once the multinuclear osteoclast is formed it secretes HCl into a hemivacuole in order to burrow a cone into the bone also causing the ionization of hydroxyapatite as well as apoptosis of osteocytes in the way of the path of the osteoclast allowing for further degradation (38, 39). After degradation of the bone by activating osteoclasts, the growth factors that previous osteoblasts laid down in the bone promote new osteoblast differentiation and subsequent bone formation in the osteon. Osteoblasts lay down a meshwork of type I collagen as well as embedding other growth factors such as insulin-like growth factors (IGFs), bone morphogenic proteins-2/-4 (BMP), thrombospondin, Wnt, TGF beta and protease inhibitors such as TIMP-1 (40, 41). These proteins embedded in the bone matrix will be released by osteoclasts to promote new osteoblasts. The activation of Wnt pathway leads to osteoblastogenesis.

Osteoblastogenesis is regulated by runt-related transcription factor 2 (Runx2). This gene is transcribed by the activation of, Smad-dependent, canonical TGF beta and BMP signaling, as well as their non-Smad-dependent non-canonical pathways. Once TGF beta receptor I (TGFβRI) and BMP receptor I (BMPRI) are activated by their external ligands, which cause the phosphorylation of Smad2/3 and Smad1/5/8, respectively (42).

1.3.2 **PCa and Bone Microenvironment**

In the bone microenvironment, metastatic PCa cells compete for the same niche as hematopoietic stem cells (43). In vitro, PCa cells have been shown to metastasize preferentially to human bone marrow endothelium (HBME) rather than other endothelium (44). When a co-culture of HBME and PC-3 cells were grown on ECM proteins from the bone, kidney and placenta there was a significant increase amount of adhesion in the PC-3 to the bone ECM rather than the kidney. This study went on to show how ECM proteins found in the bone like collagen I and laminin play a large role in movement and adherence, of the PCa, from the endothelial cell to the ECM. To impede attachment and slow PCa progression, targeted therapies have been implemented to target the cascade of intracellular events focusing on the inhibition of formation of the intermembrane proteins like αvβ1-4 which binds to proteins like fibrinogen, laminin and collagen (45).

PCa bone metastases have been shown to activate or mimic osteoclasts and osteoblasts in order to create mixed lesions known as osteosclerotic lesions, leading to the creation of woven bone. PCa cells release RANKL, which interacts with RANK on preosteoclasts to induce osteoclastogenesis leading to bone resorption, osteolysis(46). Upon bone resorption growth factors that were imbedded in the matrix by prior rounds

of osteoblast activity are released, such as, TGF beta1, IGF-1 and IL-6, which promote the proliferation of both osteoblasts and PCa cells (47). In recent years RANK/RANKL targeted therapies have arose such as Denosumab, a monoclonal antibody toward RANKL, in order to inhibit osteoclastogenesis; however, the treatment was not approved due to the resulting osteonecrosis of the jaw (48). Like osteoblasts, PCa cells can activate latent TGF beta1 to its active form but use proteins like PSA and uPA instead of osteoclast derived protease (41). In early stages of PCa TGF beta is tumor suppressive, but in the later stages becomes tumor supportive by activating supporting cells in the tumor.

Parathyroid hormone related peptide (PTHrP) induces osteoblast maturation by activating RANKL and downregulating OPG in osteoblasts. In PCa cells, PTHrP is overexpressed, which inhibits osteoblast apoptosis and stimulates osteoclast maturation (41). This allows osteoblasts to release more inactive TGF beta that is activated by uPA cleavage or by osteoclast derived protease for increased osteoclast activity to aid in osteosclerotic lesions resulting in the "vicious cycle" (49). Furthermore, PTHrP expression causes a decreased expression of DDK1, which allows for the activation of the Wnt pathway (50). This leads to added pressure for osteoblast maturation and proliferation.

1.3.3 **Bone Marrow and the hematopoietic niche**

The bone marrow makes a good host for PCa metastasis is due to its ability to aid in PCa tumor growth and production of more metastasis by utilizing the "vicious cycle". The "vicious cycle" is a complex of interactions between PCa cells, osteoclasts, osteoblasts, the mineralization of the bone matrix and a cast of tumor of

other tumor supporting cells. The various growth factors released by one of the cell types causes the activation, release or disbarment of growth factors from another.

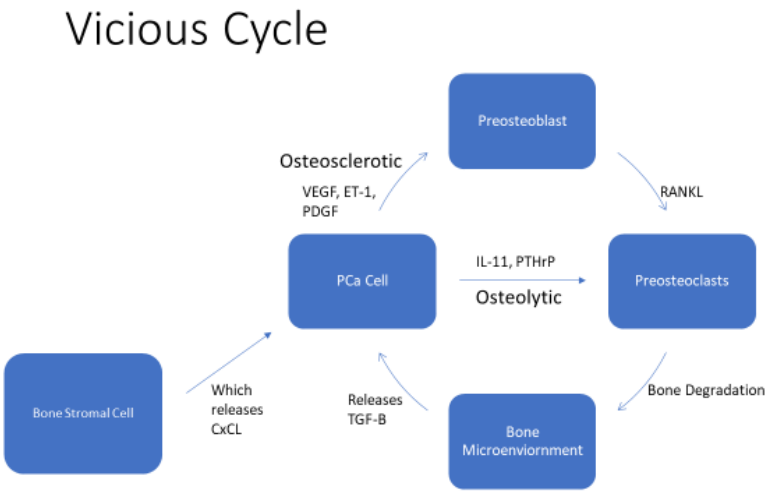


Figure 1: Illustration of two of the types of bone lesions caused by PCa, the bone breaking down then reforming osteosclerotic and the bone breaking down osteolytic.

The colonization of PCa in the hemopoietic niche can cause further complications as well as promoting tumor growth and proliferation. In normal prostate tissue there is cell mediated immune responses (51). So it is no surprise that PCa metastatic sites utilize immune cells in order to proliferate. The bone marrow is the location where native immature myeloid cells (iMCs) differentiate into various white blood cells; neutrophils, granulocytes and macrophages. When iMCs interact with the bone microenvironment and PCa tumor, the normal differentiation is blocked and differentiation is funneled into producing myeloid-derived suppressor cells (MDSCs). MDSCs support tumor growth by secreting angiogenic factors, quelling immune cell activation as well as the ability to respond to RANKL to differentiate into osteoclasts

(52, 53). This osteoclast activity causes further bone resorption causing the release of more growth factors. When coupled with MDSCs production of TGF beta1, which causes further osteoblastogenesis and PCa proliferation occur that accelerate the vicious cycle.

PCa tumors usually are accompanied by innate immune cells that can aid the mass in a wide variety of ways. MDSC's can further differentiate into tumor associated macrophages (TAMs) as well as tumor associated neutrophils (TANs). Macrophages differentiate into either a pro-inflammatory phenotype by lipopolysaccharides or anti-inflammatory phenotype by IL4/10/13, M1 and M2 respectively(54). TAMs have been shown to have more of a M2 phenotype, which could be due to PCa overexpression of IL-6 but more research would need to done for validation. TAM's have been correlated with poor prognosis in PCa by secreting proteolytic enzymes, chemotactic ligands, angiogenic factors as well as contributing to immune suppression. This aids in the PCa cells mobility as well as evasiveness (55, 56). TANs develop and differentiate from MDSCs largely because of active TGF beta and as we learned in the last section about bone remodeling process releasing pro-TGF beta, which the PCa can activate, allows the TANs to associate with the PCa forming a relationship in which both cells aid in each other's proliferation (41, 47, 56). The blocking of TFG beta in mice changes the characteristic of these cells to more of an anti-tumorigenic phenotype(57). In TFG-β-rich tumors neutrophils have been shown to produce angiogenic factors and MMPs (58).

Lastly cancer associated fibroblasts (CAFs) have been shown to play a role in promoting tumor growth and invasion. CAF's are the most abundant cells in tumor stroma mainly because they are specifically recruited by the tumor by cell-secreted

platelet-derived growth factor. (54) CAF's have been shown to secrete enhanced levels of TGF-β and migration stimulating factor (MSF). TGF beta acts by enhancing the "vicious cycle" by promoting osteoblast differentiation. MSF works by activates mTORC1 signaling through the Atk pathway in order in increase protein synthesis and inhibit autophagy.

Like cancer cells, T cells utilize sugar as an energy source. The activation of mTOR in PCa enhances the rate of glycolysis in and around the tumor. This depletion of glucose in turn leads to enhanced immunosuppression (59). Lowering the serum glucose lowers the functionality of T cells (60). These cell types together leave a repository of growth factors and cytokines for the growth and proliferation of PCa cells (61).

1.3.4 **Bone Marrow Stromal Cells and Prostate Cancer Colonization**

In order to obtain a cell line that accurately represents bone marrow stromal cell secretions to support hematopoiesis in vetro, multiple BMS cell lines were created by Roecklein and Torok-Storb(62). Cells were harvested from the bone marrow of a healthy, 30 year old male donor and seeded on a culture dish under long-term culture. The adherent cells were immortalized using a retroviral vector LXSN-16, containing the human papilloma immortalizing genes E6 and E7. Clones were selected under G418 pressure. This produced many cell lines that were termed Human Stromal (HS) 1-27 (62). HS-5 and HS-27α are used frequently in studies due to their wide range of abilities that mimic the hematopoietic niche. HS-5 to secrete a variety of cytokines that support the growth of bone marrow progenitor cells. HS-27α does not secrete any known cytokines to enhance bone marrow progenitor cells but does express hJagged1, which is a Notch1 ligand, that is proposed to influence bone marrow progenitor cell

fate(63). With these cell lines being so integral to the hematopoietic niche it is postulated that these cell lines could be one of the first cells that the PCa cell would interact with once extravasated.

Previous research in the Sikes Lab investigated the effects of these cell lines on PCa. Since both HS-5 and HS-27α play a large role in matrix composition by secreting proteins into the matrix, the conditioned media (CM) from these cell lines were taken and treated on LNCaP progression cell lines. The HS-5 CM induced a high level of apoptosis in the LNCaP cells and the surviving cells acquired a neuroendocrine phenotype whereas the HS-27α had no such effect. Since PCa Px with metastasis tend to have higher serum levels of TGF beta, HS-5 and HS-27α were treated with 10ng/ml TGF beta(64). Afterward the CM of HS-5 was treated onto LNCaP cell lines and it was found that the HS-5 cells lost their ability to induce apoptosis to the PCa cells. This lead to the speculation regarding Dr. Stephen Pagett's original "seed and soil' theory for cancer where specific tissue environments are inherently favorable to cancers then others. To be refined to follow our observations suggesting that the bone microenvironment is inherently hostile toward the cancer but must be remodeled in order to be a fertile soil for the invading PCa. In order to determine the biomolecule responsible for the apoptotic action, CM was treated with trypsin digestions, heat treatment, high and low pH, disulfide bond reduction and size exclusion to determine what type of molecule is causing this effect. It was found to be a protein between 30-100kD. Analysis was then done on the excreted proteins of HS-5, by MS/MS, to see the changes in protein levels before and after TGF beta treatment. The most prominent candidates for selection were 3 MMPs and a protein named Fibulin-1. A CRISPR/Cas9 knockdown of Fibulin-1 was done in HS-5 cells and the apoptotic

ability was knocked down and thereby providing a strong association between Fibulin-1 and PCa cell apoptosis(18).

1.4 **Characterization of Fibulin Proteins**

The Fibulin protein family consists of seven genes encoding glycosylated secretory proteins that reside in the basement membrane of the ECM. Fibulins contain three core domains; I, II and III. Domain I consists of a signal peptide and a variant region which either contain an anaphylatoxin (AT) domain as in Fibulin-1 and -2, an immunoglobulin domain as in Fibulin-6 or no variant region at all, as seen in Fibulin-3,-4 and -5. Anaphylatoxin aids in the immune response and host defense by binding to complement peptides to produce a local inflammatory response (65). Working toward the C-terminus, in domain II there are a series of calcium binding epidermal growth factor (cbEGF) domains, normally found in membrane bound proteins, are used as spacers for protein-protein interactions or structural stabilization of the protein (66). Lastly, at the C-terminus in domain III a fibulin domain resides which contains a globular like structure that is unique to each fibulin.

The fibulins are divided into 2 sub-groups, seen in figure 1, dependent on the presence or absence of the anaphylatoxin domain. Fibulin-1/-2 are in the first sub-group, whereas fibulins -3 to -6 are all in the second sub-group. The fibulins in the first sub-group containing the anaphylatoxin domain are larger than the other fibulins, except fibulin-6, being between 100-200kDa. The anaphylatoxin group also could give fibulin-1/-2 a specialized immune function because of a role in the host immune response. This immune response can be activated by proteolytic cleavage. Once activated these anaphylatoxins trigger secretions or degranulation from endothelial cells, mast cells or phagocytes, which can cause the release of histamine, vasodilation

and smooth muscle contraction to name a few (67). More research needs to be done on the specific function of AT in fibulin proteins to see if cleavage occurs to create an immune response. Fibulin-6, the largest of the fibulins at 615kDa, contains a 44 tandem immunoglobulin repeat and 6 thrombospondins type-I modules bulking up the protein (68). More recently discovered there is a fibulin-8, which closely resembles the fibulin-6 structure (69).

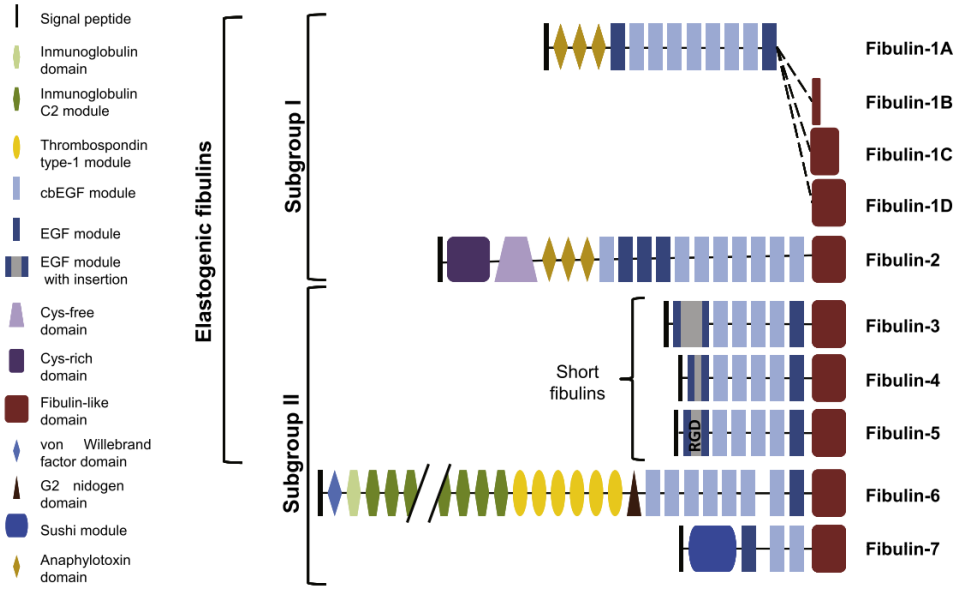


Figure 2: Structure of all the known proteins in the Fibulin family and all known splice variants. Different subgroups of fibulins are shown.(68).

1.5 **The Fibulin-1 Subfamily**

Fibulin-1 was the first to be discovered in 1989 by Argraves et. Al. and has been studied wildly over the 30 years since (70). As of now there are four known splice variants of fibulin-1, denoted A,B,C and D. These proteins all contain the same amino acids in domain I and II; however, the proteins differ in domain III. Domain I contains the signal peptide and 3 AT domains. Domain II contain seven cbEGF

domains with an EGF domain flanking each side. Domain III is splice variant specific where all the fibulin-1 proteins contain a different module; variant A creates a stop codon to have no extra amino acids, B has 35 extra amino acids, C has 117 and D has 137. Also, a version of Fibulin-1D has been found that is not secreted and contains a similar C-terminus but lacks the signal peptide and AT domains (71). This protein most likely has a different start site on the gene.

Basement membranes are thin layers of ECM that separate two morphologically distinct tissues from another, which play a major role in the development, maintenance, adherence, migration and proliferation of tissues. The basement membrane is comprised of many proteins that make up a supramolecular structure. Some of the main components are collagen IV, perlecan, laminin and nidogen. In many tissues Fibulin-1 is deposited into the ECM of basement membranes by fibroblasts. Fibulin-1 was also shown to co-localize with Fibulin-2 in elastic fibers located in the aorta and neuronal structures(72). Due to their similar structure, the fibulins may be co-localizing because of having the same binding partners rather than binding to one another(72). Fibulin-1 and -2 both bind to the α2 subunits in laminins as well as the γ2 domain in laminins to aid in epithelial adhesion. The G domain in the laminins play roles in cell adhesion, migration and integrin binding. In addition, Fibulin-1 also binds to α1 subunit of the G domain. The γ2 subunit is found in laminin-322 is an element of the anchoring subunit to link cells to the basement membrane (73). Fibulin-1 and -2 also bind the globular domains of nidogen-1 through their Fibulin and EGF domains. Fibulin variant 1D domain region does not bind as strongly to nidogen-1 compared to 1C. Nidogen-1 has been identified as a binding partner of perlecan and laminin-322. The interaction of these Fibulins with the

basement membrane may provide the foundation to support tissue adherence (74, 75). Fibulin-1 was identified by being a binding partner of the β subunit of fibronectin receptor(70). Another binding partner in the ECM is endostatin (76).

In the ECM, the chondroitin sulfate proteoglycans versican and aggrecan bind to Fibulin-1. Versican is found in articular cartilage of the knees and femoral head. Aggrecan contains large chondroitin sulfate chains, which form large aggregates with other protein complexes to provide mechanical strength and resist compression in joints. Fibulin-1 seems to play a role in stabilization of cartilage. ADAMST (A disintegrin-like and metalloprotease with thrombospondin motifs) is a metalloprotease, which cleaves aggrecan among others. Fibulin-1 binds to ADAMTS-1 along with aggrecan to form a tertiary complex to enhance aggrecan turnover as well as tissue remodeling(77).

Circulating Fibulin-1 was found to be at a relatively high concentration in human and mouse serum samples (10-50µg/ml) (78). Fibulin-1 also binds to fibrinogen and is incorporated into fibrin clots while also mediating platelet adhesion. Fibulin forms a bridge between fibrinogen, collagen I/IV and fibronectin (75). Around 1-2% of all testosterone is freely circulating in the blood and thus biologically active. Sex-hormone binding globulin (SHBG) binds to free hormones in the blood rendering them temporarily inactive. Fibulin-1 has been shown to bind to SHBG, which then inhibits SHBGs ability to bind to testosterone. This shows that Fibulin-1 may play a role in hormonal regulation (79).

Fibulin-1 is imperative to proper vertebrate development, homozygous knockout mice for Fibulin-1 have perinatal lethality(80). Lack of Fibulin-1 inhibited the development of many cardiac tissues or blood vessel stages including the

myocardial wall, aortic arch and ventricular septum defects to name a few. Defects in cranial nerves also were seen in the Fibulin-1 null mice. What connects these defects, from a developmental standpoint, is where these tissues are derived from; neural crest cells (NCCs). NCCs must delaminate from the neural tube/epidermal boarder after the neural tube is formed and migrate ventral and caudally by expressing MMPs specifically ADAMTS. NCCs must undergo a full or partial EMT, results from the null fibulin-1 mice are similar to the results seen when NCCs are completely ablated from the embryo. Fibulin-1 could interrupt this process by either or the combination of the halting the EMT or the absence of fibulin-1 binding to ADAMTS, decreasing protease activity. ADAMTS has shown to be involved in the myocardial compaction and closure of the secondary plate (81).

1.6 **Fibulin-1 C/D and Cancer**

The Fibulin-1C/D isoforms have shown to bind to many of the same proteins; however, they have various effects on tumor attachment and growth. To date, studies have shed light on their effects on bladder, prostate, breast and ovarian cancers. Fibulin-1 expression has been shown to be altered in various cancers with some difference in expressed isoforms. In some cases the ratio of 1C to 1D in tumor tissue is higher then in normal tissue (ovarian, breast, prostate); however, in other cases, Fibulin-1 is absent by means of promoter methylation (bladder, gastric, hepatocellular carcinoma, renal cell carcinoma).

In non-muscle invasive bladder cancer, researchers showed that hypermethylation of the promoter region of fibulin-1 downregulated its gene expression. When non-muscle invasive bladder cancer cells were subjected to Fibulin-1 in conditioned media it significantly inhibited cell growth and as increased apoptosis

levels (82). This study shows that Fibulin-1 can be a novel marker for identification of non-muscle invasive bladder cancer. The Fibulin-1 isoform that was used in this study was not divulged.

Breast and ovarian cancers contain many similarities when it comes to Fibulin-1 and its regulation. Estradiol induction of the Fibulin-1 promoter causes a 3 fold increase in Fibulin-1C expression in breast cancer cell lines. Mutagenesis of the Sp1 binding site -57bp from the transcriptional start site completely abolished the ability of estradiol to induce Fibulin expression. The study goes on to show that estradiol induction lowers the half-life of the Fibulin-1D mRNA but more research would need to be done to elucidate a mechanism (83). Interestingly, TGF beta causes a 2 fold increase in Fibulin-1 expression in breast cancer cell lines, but in HS-5 cells TGF beta causes the abolishment of Fibulin-1 expression suggesting crosstalk of repressive and activation pathways (18, 84). Studies have been done with the breast cancer cell line, MDA-MB-361, on Matrigel®, which mimics a specific ECM environment to which doxorubicin was added. The Matrigel®, which contains Fibulin-1, have shown to decrease the effect of the doxorubicin. When antibodies were used to extract Fibulin-1 from the Matrigel® the function of doxorubicin was restored concluding that Fibulin has a protective effect on breast cancers. The results showed that with the Fibulin not present in the Matrigel® the MDA-MB-361 cells had a higher apoptotic level. This study also could not elucidate between the Fibulin-1 variant that was being studied because there is no isotype specific Fibulin-1 antibody (85).

In metastatic PCa it has been shown that inflammation around the PCa induces the release of CXCL1/GROα. These factors have been shown to correlate with increased ATK activation and IκB kinase α phosphorylation leading to the activation

of NF-κB. NF-κB leads to the activation of HDAC1, which in turn leads to deacetylation of H3and H4 on the histones that attach to the Fibulin-1 gene leading to the downregulation of Fibulin-1D mRNA. This mechanism somehow does not affect the transcript level of Fibulin-1C(86). Fibronectin being one of the major ECM proteins to coordinate cell adhesion and motility when Fibulin-1D is bound it blocks the ability of fibronectin to bind to tumor cells, an effect not seen with Fibulin-1C(76). This inhibits the cancer cells from binding to the matrix. Splice-variant expression could be an underlying cause of tumor cell adherence and proliferation(18). Previously, the Sikes' lab has shown that HS-5 cells induce apoptosis in LNCaP cell lines by Fibulin-1; the PCa cells that lived differentiated into a neuroendocrine phonotype. After qRT-PCR of the Fibulin-1 isoform secreted by HS5 it was determined that HS-5 expresses 5 fold higher levels of variant 1D then variant 1C(18). Leading to the hypothesis that Fibulin-1D is the variant from HS-5 cells inducing apoptosis in LNCaP progression model cell lines whereas Fibulin-1C induces the neuroendocrine phenotype.

Chapter 2

MATERIALS AND METHODS

2.1 **Miniprep**

While fusing multiple procedures, this miniprep I have found to give the greatest yield as well as the most pure DNA while also attempting to cut down on the time required to do this miniprep.

2.1.1 Reagents

STE =Tris-HCl 1mM, EDTA = 0.1mM, NaCl= 0.1M

Alkaline Lysis Solution I = 50mM Glucose, 25mM Tris-HCl (pH=8.0), 10mM EDTA, (pH = 8.0)

Alkaline Lysis Solution II (< 2 weeks old) = 1% SDS, 0.2N NaOH

Alkaline Lysis Solution III = 3M KOAc, 0.115M Glacial Acetic Acid dissolved in water

Ethanol 100%

KOAc 3M pH=5.2

2.1.2 **Procedure**

All centrifugation at max speed at 14,000xg, unless otherwise stated. Centrifuge 1.5mL of overnight culture for 1 minute then aspirate supernatant. Proceed to resuspend pellet in 0.25 v/v of original culture, in this case ~0.4ml. Then centrifuge for 1 minute and aspirate off supernatant. Resuspend completely in 100µl Alkaline Lysis Solution I and incubate for 2 minutes. Add 200µl of Alkaline Lysis Solution II and incubate for 3 mins on ice. Next, add 150µl of Alkaline Lysis Solution III incubate for 5 minutes on ice. Pellet in 4°C centrifuge for 10 minutes at ≥14,000xG,

afterward pour supernatant into new 1.5ml Eppendorf tube (Eppendorf, Hamburg, Germany) containing 1mL of -20C° 100% EtOH. Incubate on dry ice for 10mins then centrifuge at 4°C for 10 mins at ≥14,000xG. To remove RNA, add 300µl of TE buffer containing 20µg/ml of RNase A (Fisher Scientific, Pittsburgh, PA) incubate for 10 mins. After, add 300µl of Phenol/Cholroform/Isoamyl Alcohol 25/24/1 (P/C/I) (Fisher Scientific, Pittsburgh, PA) and vortex for 5 seconds then centrifuge for 5 mins. Carefully remove 200µl from the top layer being cautious not to perturb the interface between the 2 phases (contains proteins). Add 20µl of 3M KOAc for a final concentration of 0.3M and vortex for 5 seconds. Add 1ml of -20C° 100% EtOH and mix fully by tapping the bottom of the tube while upside down. (If it is not fully mixed after the dry ice step there will be frozen liquid where a pellet would form) Incubate on dry ice for 10 mins then centrifuge at 4°C for 10 mins.

2.2 **Preparation of Chemically Competent Cells**

Chemically competent cell protocol was received from Dr. William Cain (UD Department of Biological Sciences). Previous attempts at the process yielded transformation frequencies of 10^8Transformants/ µg of plasmid DNA. I was able to achieve $5x10^6$ transformants/ µg of DNA which was sufficient for my cloning.

2.2.1 **Reagents**

Transformation Buffer

Add 3.35g PIPES (10mM), 2.2g $CaCl_2 \cdot 2H_2O$ (15mM), 18.64g KCl (250mM) to 950mL of ddH_2O. The pH was adjusted dropwise to 6.7 with 1M KOH. To make this solution add 10.9g of $MnCl_2 \cdot 4H_2O$ (55mM) then the water level was brought to

1L with the addition of ddH_2O. The liquid was then sterile filtered (Millipore, Burlington, MA) and stored at 4°C.

All media solution reagents were bought from Thermo Scientific, Waltham, MA) or otherwise stated, those solutions were:

Luria-Bertani Medium (LB)

10 g/L Tryptone, 5g/L Yeast Extract and 10g/L NaCl then autoclave

Super Optimal Broth (SOB)

(20g/L) Tryptone 5g/L Yeast Extract and 0.5g/L NaCl and stir till dissolved. Add KCl (Fisher Scientific, Pittsburgh, PA) in H_2O to achieve a final concentration of 2.5mM, then autoclave on liquid cycle. Once finished add a preautoclaved solution of $MgCl_2$ (Fisher Scientific, Pittsburgh, PA) to achieve a final concentration of 10mM.

Super Optimal broth with Catabolite repression (SOC)

Use SOC and add glucose (Fisher Scientific, Pittsburgh, PA) to a final concentration of 20mM

LB agar plates

Take LB medium and add 15g/L of agar then autoclave. After cooling if antibiotics are needed add accordingly after temperature has reached below 50°C then mix. Pour LB agar onto 10cm plates and wait to solidify.

2.2.2 **Procedure**

To precede the preparation of competent cells, DH5α were streaked on a LB plate then grown at 37°C overnight. The next day a single, isolated colony was picked and placed in 5mL of LB in a 10mL polystyrene culture tubes (Fisher Scientific, Pittsburgh, PA) at 37°C and shaken at 225rpm for 7 hours. 100mL of SOB was then inoculated with 1mL of the starter culture and grown at 20°C and 200rpm overnight.

Early in the morning the OD600nm was examined and monitored until it reached 0.5-0.75 absorbance units. Once reached the culture was transferred to ice and gently swirled periodically to allow for even cooling over 10 minutes. The culture was then transferred to 2, 50mL polypropylene Falcon tubes (Fisher Scientific, Pittsburgh, PA). Cultures were centrifuged at 2500xg for 10 minutes at 4°C using a Thermo Scientific Sorvall Legend XTR centrifuge. Supernatant was discarded and the pellet was resuspended gently in 17mL of Transformation Buffer and put on ice for the next 10minutes. Th cells were pelleted again at 2500g for 10 minutes at 4°C and the supernatant discarded. The pellet was resuspend gently in 4mL of Transformation Buffer and 140µL of DMSO was added. Cells were then put on ice for 5 minutes. Next an additional 140µL of DMSO was added and the cells were put on ice for 10 minutes. The cells were flash frozen in 500µL aliquots of competent cells in 1.5mL Eppendorf tubes in dry ice and EtOH, store in -80°C until needed.

2.3 **Ligation Protocol**

For ligations 800ng of cut and purified vector DNA (7.0pM) was pipetted into a PCR strips (Invitrogen, Waltham, MA) along with a 1:3 molar ratio and 1:6 molar ratio of vector to insert. 2µL of 10x T4 ligase buffer (Thermo Scientific, Waltham, MA) then was added and the volume of the reaction was brought to 19µL with ddH_2O. The stock T4 ligase (Thermo Scientific, Waltham, MA) was in concentration of 5U/µL and was diluted to have 1U/µL. 1U was added to the reaction to bring the final volume to 20µL. The reaction was placed in a thermocycler overnight at 16°C with a hot lid to prevent evaporation onto the lid. Transformation followed ligation.

2.4 **Transformation Protocol and Colony Growth**

To transform, 5µl of the ligation mixture was added to 100µL of competent E.coli, strain DH5α. All transformations were kept on ice for 30 mins then heat shocked for 45 seconds at 42°C then put back on ice for 2 minutes. 300µl of Super Optimal Broth (SOB) was added to the transformation and the cells were grown at 37°C for 1 hour with shaking. 250µL of transformation mixture was plated on LB plates with 100µg/mL of ampicillin. The next day, colonies were picked and grown in LB with 100µg/mL of ampicillin at 37°C in a 10mL polystyrene culture tubes at 225rpm and grown overnight.

2.5 **Creation of V5 and myc/His epitope in pcDNA3.1A**

In order to be able to add different ratios of Fibulin 1C/D and be able to quantify after purification, different epitope tags were needed for Fibulin 1 C/D, I chose to use myc/His and V5, respectively. The eukaryotic expression vector pcDNA3.1 myc/His A (Thermo, V80020) was used for transient expression of Fibulin 1C/D (NM_006486). In order to amplify the V5 epitope a V5-Par4 vector was graciously supplied by Dr. Donna Woulfe (UD Department of Biological Sciences) pGFP^2-N2 (6310014). After amplification, I directly dropped the V5 epitope into the region where the myc/His had previously resided. Overhangs were designed on the primers for V5 so that there were 5 random bases before the XbaI (Thermo, FD0684) and after the PmeI (Thermo, ER1342) cut sites for 5' and 3' ends respectively. Shown below are the primers used to amplify the V5 fragment.

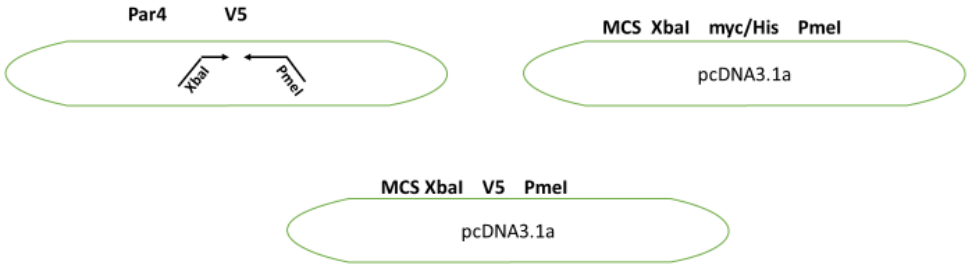


Figure 3: Host vector on the top left was used to amplify its V5 epitope tag with overhanging XbaI and PmeI cut sites. Restriction digest were done to pcDNA3.1A and amplified V5 fragment. Digests were purified and ligated together. The end product was the vector on the bottom. MCS = Multiple cloning site

5′ V5 = 5′ GCATA TCTAGA ATGGGAAAGCCGATTCCTAAT 3′

Random Bases — XbaI Cut Site — 5′ V5

3′ V5 = 5′ GCATA GTTTAAAC TTACCCGCTCTCGTCGTAGACGCT 3′

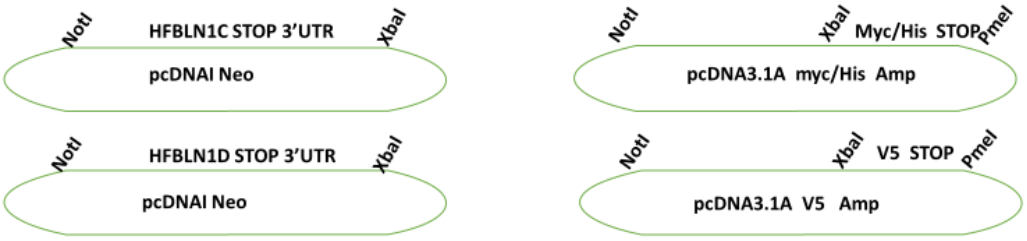
Random Bases — PmeI Cut Site — 3′ V5

Figure 4: Primers used to amplify the V5 epitope for ligation into pcDNA3.1 A

Amplification of V5 epitope was done with DreamTaq and DreamTaq green buffer for 35 cycles with an initial denaturation temperature = 95°C for 30 seconds, Tm=52°C for 30 seconds and ramped at 2°C/second to reach an extension at 72°C held for 30 seconds. PCR product was confirmed on a 1.5% gel. Subsequent cut with 0.5U XbaI (Thermo, FD0684) and 1.5U PmeI (Thermo, ER1342) in 1x Tango buffer in 30µl. The vector, pcDNA3.1, was cut also in a similar fashion, afterward the vector was EtOH precipitated and subsequently incubated with CIAP (Thermo, Waltham, MA).

2.6 Creation of recombinant Fibulin 1C/D Proteins with V5 & myc/His epitopes

The human Fibulin 1C and 1D cDNAs were given to the Sikes lab by Dr. Marion Cooley from The Medical University of South Carolina in pcDNA1 (kanamycin resistant) and pcDNA3.1 (ampicillin resistant), respectively. The cut sites NotI (Thermo, FD0595) , 5', and XbaI (Thermo, FD0684), 3', were used to excise the inserts of Fibulin 1C/D and were ligated into pcDNA3.1 A V5 and pcDNA3.1 A myc/His respectively. These clones were called NF1C V5 and NF1D myc/His (NF=Coding Sequence Not Fused to Epitope Tag).



- **Human Fibulin 1 = HFBLN1**

Figure 5: Vectors on the left were used to excise Human Fibulin-1C/D with NotI and XbaI and ligated into the vectors on the right pcDNA3.1A with the same cut sites. Completed clones are NF1C myc/His and NF1D V5 (NF= signal peptide Not Fused to coding sequence)

2.7 Removal of the 3' UTR

Due to the presence of the 3' UTR in both the 1C and 1D, the epitope tag was not continuous with the coding sequence, basically, a STOP codon would have prevented the translation of the epitope tag. To remove this 3'UTR reverse PCR using DreamTaq polymerase from the start of the V5 and finishing at the end of the coding

sequence of Fibulin-1C. Also DreamTaq polymerase was used to amplify the start of the myc/His epitope to the end of the coding sequence of Fibulin-1D along with XbaI overhangs of 1C and 1D was amplified. The sequences were amplified using 35 cycles of 30 seconds at 95°C followed by 30 seconds at 55°C and lastly hold at 72°C for 6 minutes.

5' V5 = 5' GCATA (Random Bases) TCTAGA (XbaI Cut Site) ATGGGAAAGCCGATTCCTAA (5' V5 homology) 3'

5' myc/His = 5' ACTGA (Random Bases) TCTAGA (XbaI Cut Site) GGGCCCTTCGAACAAAAACTC (5' myc/His homology) 3'

3' 1C = 5' GCATA (Random Bases) TCTAGA (XbaI Cut Site) GAGCTCTGCAGACACAAAGAT (3' Fibulin-1C homology) 3'

3' 1D = 5' GCATA (Random Bases) TCTAGA (XbaI Cut Site) GAACCAGTACTCATAGACGAA (3' Fibulin-1D homology) 3'

Figure 6: Primers used for the reverse PCR of Fibulin-1C V5 and Fibulin-1D myc/His in order to fuse the coding sequence of the Fibulins with their epitopes.

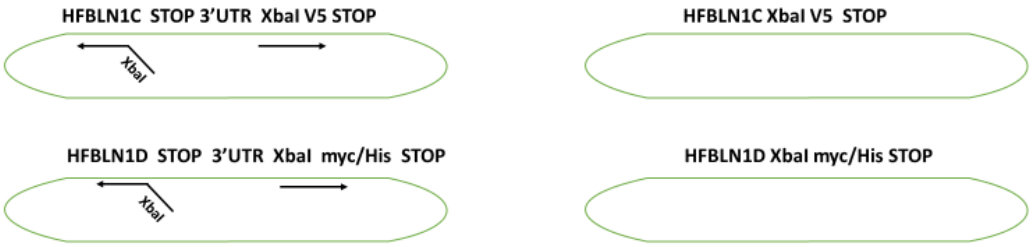


Figure 7: Primers were designed to amplify the entire plasmid on pcDNA3.1 A Fib1C myc/His and pcDNA3.1A Fib1D V5 in order to eliminate the STOP codon and 3'UTR in both plasmids. The coding sequence was fused to the 3' epitope with an XbaI cut site.

The amplicon was purified with P/C/I and EtOH precipitates. The purified amplicons were cut with XbaI (Thermo, FD0684) and DpnI (Thermo, ER1701) using

1x Tango buffer. Amplicons were EtOH precipitated and ligated with T4 ligase (Thermo, EL0014) overnight at 16°C with a 50°C lid. 5µL of ligation was transformed and incubated on ampicillin an plate overnight. Colony growth was examined the next day. Minipreps were done on 3 to 6 colonies all clones were sent for sequencing.

2.8 **Removal of errors from Fibulin 1D**

To remove these errors the Gibson cloning method would be used so that primers homologous were made that contained the base correction for both mutations. The bolded bases are the mutation correction.

F1825 = 5’ TGA GAA C**T**A CCG CCG CTC 3’
R1825 = 5’ GGA GCG GCG GT**A** GTT CTC A 3’
F2243 = 5’ ATC TTC GTC T**C**T GAG TAC TG 3’
R2243 = 5’ CAG TAC TCA **G**AG ACG AAG AT 3’

Figure 8: Primers for Gibson Cloning error removal in Fibulin-1D myc/His. Bolded letters were the corrected bases.

The R1825 and F2243 were used to amplify the portion in between the mutations to get a band size of 428bp as well as F1825 and R2243 that created a band of ~7.7kbp that have the base corrections at 1825 and 2243. The insert was amplified with Radiant HiFi polymerase under standard reaction protocol with a Tm= 56°C. After amplification DpnI (Thermo, ER1701) digestion in 1x Tango buffer was done for 1 hour to cleave template DNA. To further purify, P/C/I phase extraction and subsequent EtOH precipitation was done to the PCR products. The Gibson Cloning was performed by NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, E2621S) 0.1pmol of the large segment was used and 0.01pmol of the small

segment was used. The reaction was set at 50°C with the lid at 95°C for 15mins then immediately transformed into DH5α. The cells were then plated on LB plates with 100µg/ml of ampicillin and incubated overnight at 37°C. The next day colonies were picked and grown in LB with 100µg/mL of ampicillin at 37°C at 225 rpm using I2500 Series (New Brunswick Scientific, Edison, NJ). The next day minipreps were done on all cultures and purified plasmids were sent to Genewiz for sequencing.

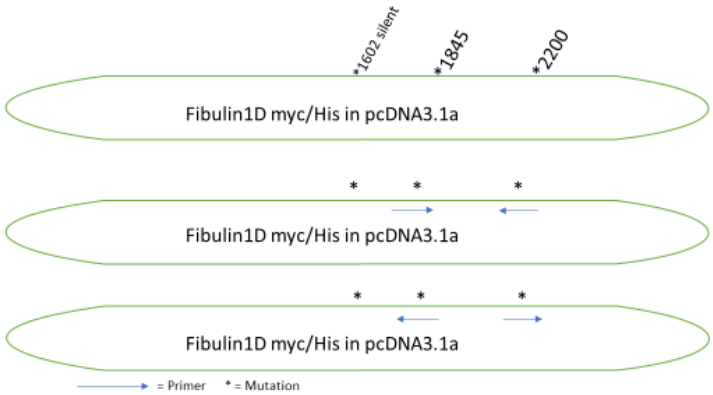


Figure 9: Mutations and direction of error correcting primers are shown. Primers were homologues of each other with a single base change included corresponding to the mutations found in the coding sequence.

2.9 **Removal of 13 extra amino acids on F1C V5**

After sequencing F1C there was 39 nucleotides at the end of the V5 tag before a STOP codon. This was done on purpose when I chose my primers, when I made the primers I did not think would be that detrimental to the protein in my first semester at UD. The area at the end of the tag was very GC rich and I figured it would create problems priming. I later found out I was wrong in both instances.

Since I had so much experience with reverse PCR I once again chose this method to sneak in a STOP codon immediately after the V5 tag in the pcDNA3.1 A

V5 F1C vector. The V5 tag was amplified with an overhanging STOP codon and an overhanging ApaI cut site.

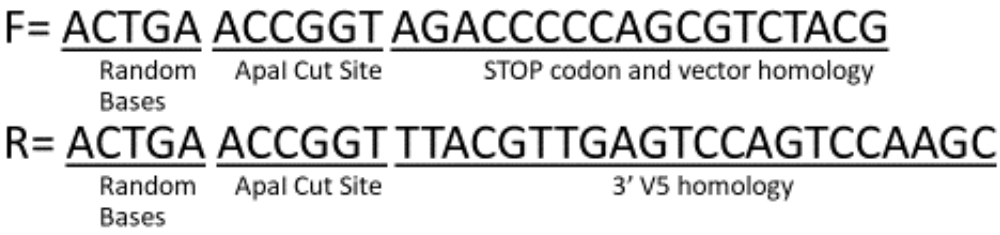


Figure 10: Primers shown that were used to eliminate 29bp between the end of the V5 epitope and the stop codon.

2.10 **Creation of recombinant Fibulin 1C/D truncates**

Creation of truncated proteins was rather simple, reverse PCR was once again implemented where the forward primer started at the beginning of the variant C-terminal region. The reverse primer began at the end of the signal peptide. Both primers contained overhanging BamHI cut sites so that after amplification restriction digestion and ligation could be done to complete the vector.

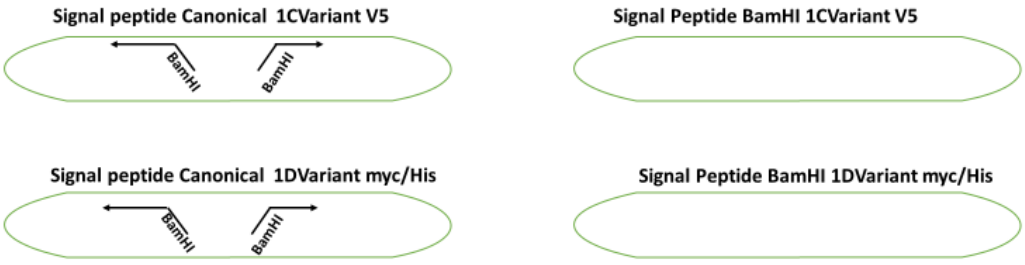


Figure 11: Depiction of F1C V5 and F1D V5 (left) Reverse PCR to eliminate canonical region is shown with BamHI cut sites to create F1Ct V5 and F1Dt myc/His (right).

2.11 **Cell Lines and Cultures**

MDA-MB-231, a human breast adenocarcinoma cell line, and HS5, a human bone marrow stromal cell line, were both cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning CellGro, Tewksbury, MA) supplemented with 10% Fetal Bovine Serum (FBS) (Atlas Biologicals, Fort Collins, CO) (v/v) and 100X Antibiotic-Antimicotic (Gibco, Franklin, TN). All cells were incubated in a sterile incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Media was changed every 3 days or less depending on confluency. To passage, DPBS, trypsin (Corning CellGro, Tewsbury, MA), DMEM (Corning CellGro, Tewsbury, MA) were warmed to 37°C in a water bath. Media on 10cm dishes (Eppendorpf, Hamburg, Germany) were aspirated and rinsed with 5mL of DPBS 2 times. 1.5mL of 0.25% trypsin (Corning CellGro, Tewsbury, MA) was added to the dishes and placed in the 37°C incubator for 2 minutes. Cells were analyzed under a microscope to detect detachment from the plate. 3.5mL of DMEM was added to plates and 5mL of DMEM/trypsin mixture was added to a 15mL Eppendorf tube. Tubes were centrifuged for 3 minutes at 300xg at room temperature. Supernatant was aspirated and cells were resuspended in 5mL of DMEM and added to new 10cm plates to a total volume of 10mL. Passage numbers were between 20 and 30.

2.12 **Fibulin-1 Collection from Conditioned Media (CM)**

CM was collected from 100% confluent plates. When plates were at 70-90% confluency new 10% FBS containing media for 24 hours. At 24 hours cells were rinsed with 37°C DPBS (Corning cellgro, Tewksbury, MA) and near or at 100% confluency they were then rinsed with DPBS two times and were replaced with serum free media (SF) SF-DMEM (Corning cellgro, Tewksbury, MA) supplemented with

100X Antibiotic- Antimicotic (Gibco, Franklin, TN). Serum was collected at 48 hours and cells were replenished in DMEM with 10% FBS and Antibiotic-Antimicotic. CM's were sterile filtered through 0.22µm hydrophilic polyethersulfonate filter (EMD Millipore, Burlington, MA).

2.13 **Transformation and selection**

MDA-MB-213 cells were plated the day before transfection so that cells would be 70-90% confluency the next day. Lipofectamine 3000 (Invitrogen, Waltham, MA) was used for transfection the following day. About 1 hour before transfection, cells were washed with 5ml DPBS and washed with 5ml of Opti-MEM (Gibco, Franklin, TN) then given Opti-MEM. The manufacturers protocol for lipofectamine was followed for $10cm^2$ cell culture dish scaled up and pEGFP-C1 (ClonTech, Palo Alto, CA) vector was used as a transfection control and subsequent control for Geneticin selection. For each 10cm dish transfection reaction, media on plates were aspirated washed with DPBS and given 10mL of Opti-MEM. In a separate reaction 500µL of Opti-MEM was mixed with 30µL of Lipofectamine 3000 reagent. In a separate tube 500µL of Opti-MEM was mixed with 35µL of P3000 reagent and 20µg of DNA. The two reaction tubes were mixed and let sit for 10 minutes. Afterward 1mL of the reaction was pipetted onto the 10cm dishes.

Selection on MDA-MB-231 cells was done by resuspending 1g Geneticin (G418) (Thermo Scientific, Waltham, MA) and creating a 350µg/µl solution after compensating for the potency of 714mcg/mg. This gives a 500x solution for an active concentration of 700µg/ml, which was used for MDA-MB-231 cells. Prior to selection cells were given fresh complete DMEM 24 hours before selection. Cells were

incubated with complete DMEM with 700µg/ml of Geneticin and given fresh media with Geneticin every 3 days for 2 weeks. Transformation and selection efficiency was determined by totaling the number of cells with GFP compared to all cells. Cells were analyzed every 3 days along with fresh media delivery to detect cell viability.

2.14 **Size Selection**

HS-5, MDA-MB-231 and MDA-MB-231 with Fused Fibulin-1 C V5 (F1C V5) and Fused Fibulin-1D myc/His (F1D myc/His) CM were filtered through Amicon Ultra-15 centrifugal filters for 30 kDa cutoff (Millipore, Burlington, MA) MDA-MB-231 with Fused Fibulin 1C truncated V5 (F1Ct V5) and Fused Fibulin-1D myc/His (F1Dt myc/His) were filtered through Amicon Ultra-15 centrifuge filters for 10 kDa cutoff (Millipore, Burlington, MA). After adding 12mL of CM to filter devices the tubes were centrifuged at 4000 x g for 35 minutes and 20 minutes for 30 kDa and 10 kDa cutoff respectively.

2.15 **Western Blotting**

After CM was collected protein concentration was determined by a Bradford Protein Assay Dye (Bio-Rad, Hercules, CA) using BSA to standardize the curve. Twenty micrograms were subjected to a 6x solution of Laemmli SDS sample buffer, non-reducing (Fisher Scientific, Pittsburgh, PA) (85%) mixed with 2-Mercaptoethanol (Fisher Scientific, Pittsburgh, PA) (15%) (v/v) and incubated for 5 mins at 99°C in order to denature proteins. Samples were loaded into a Bio-Rad TGX Stain Free FastCast 10% polyacrylamide gel (Bio-Rad, Hercules, CA). Gels were run at 250V for

30 minutes in Tris-Glycine SDS 1x buffer (KD Medical, RGH-0390). Before transfer, blotting papers (Bio-Rad, Hercules, CA) were soaked in Trans-Blot Turbo buffer (Bio-Rad, Hercules, CA) and Polyvinylidene Difluoride (PVDF) Transfer Membrane (Thermo Scientific, Waltham, MA) was soaked in Methanol (Fisher Chemical, Pittsburgh, PA). In a semi-dry transfer apparatus Trans-Blot Turbo (Bio-Rad, Hercules, CA) was used and the stacking in the apparatus started with one soaked blotting paper was used as the base followed by the soaked PVDF membrane followed by the gel and lastly a final soaked blotting membrane. Gels were transferred to the transfer membrane at 25V for 7 minutes. Membranes were stained with 0.1% Ponceau in 5.0% glacial acetic acid in dH_2O for 5 minutes then washed with water until protein bands become apparent. ddH_2O was exchanged every 5 minutes until red bands disappeared. Membranes were blocked with 5% non-fat dry milk (NFDM) (Boston Bio Products, Ashland, MA) in PBST (0.05% Tween 20®) by w/v for 1 hour. All primary antibodies were diluted in 5% NFDM in PBST and incubated at 4°C overnight. The next day the membranes were washed 3 x 5 minutes with 5% NFDM in PBST and diluted secondary antibody in 5% NFDM in PBST was added for 1 hour. After an hour they were washed 3 x 5 minutes with 5% NFDM in PBST. Primary antibodies used were rabbit anti Fibulin-1 monoclonal antibody (Invitrogen, Waltham, MA) (PA5-51612) at 1:500 dilution, rabbit Anti-V5 Epitope Tag polyclonal antibody (Millipore, Burlington, MA) (AB3792) at 1:5000 dilution and mouse IgG1 anti-myc antibody (Thermo Scientific, Waltham, MA) (46-0603) at 1:5000 dilution . Secondary antibodies used are goat anti rabbit IgG conjugated with horseradish peroxidase (Pierce, Waltham, MA) (31460) at 1:40,000 dilution and rabbit anti mouse IgG

conjugated with horseradish peroxidase (Abcam, Cambridge, England) (ab97046) at a 1:20,000 dilution.

Chapter 3

RESULTS

3.1 Vectors

Amplification of V5-Par4 vector by DreamTaq was successful and ligation into pcDNA3.1 A using XbaI and PmeI cut sites were used to replace the myc/His epitope with V5. After transformation, colony selection and miniprep I tested the ligation of V5 with PCR seen in Figure 11.

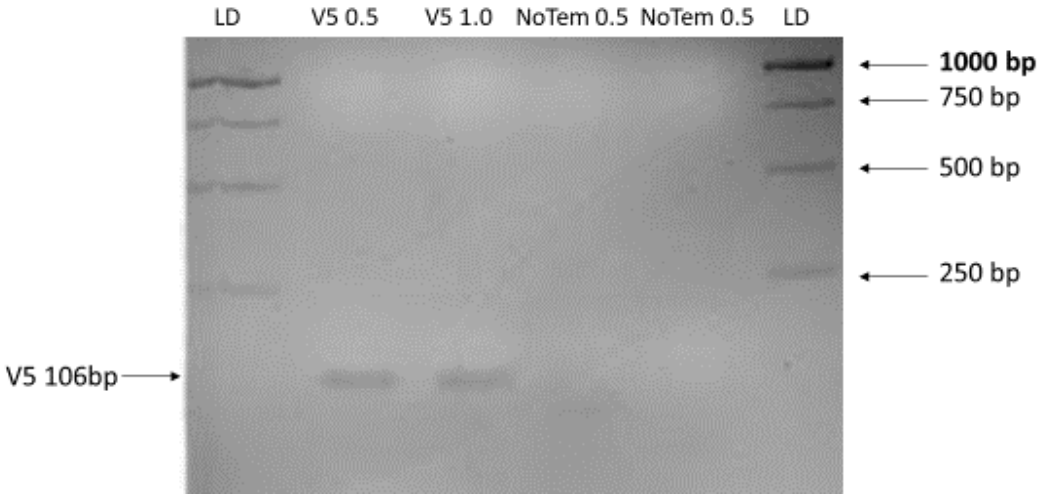


Figure 12: After ligation of V5 into pcDNA3.1a the vector was used to amplify V5 of see if the V5 was inserted. Band indicated is around 106bp and the expected size of amplified V5. PD = Primer Dimer

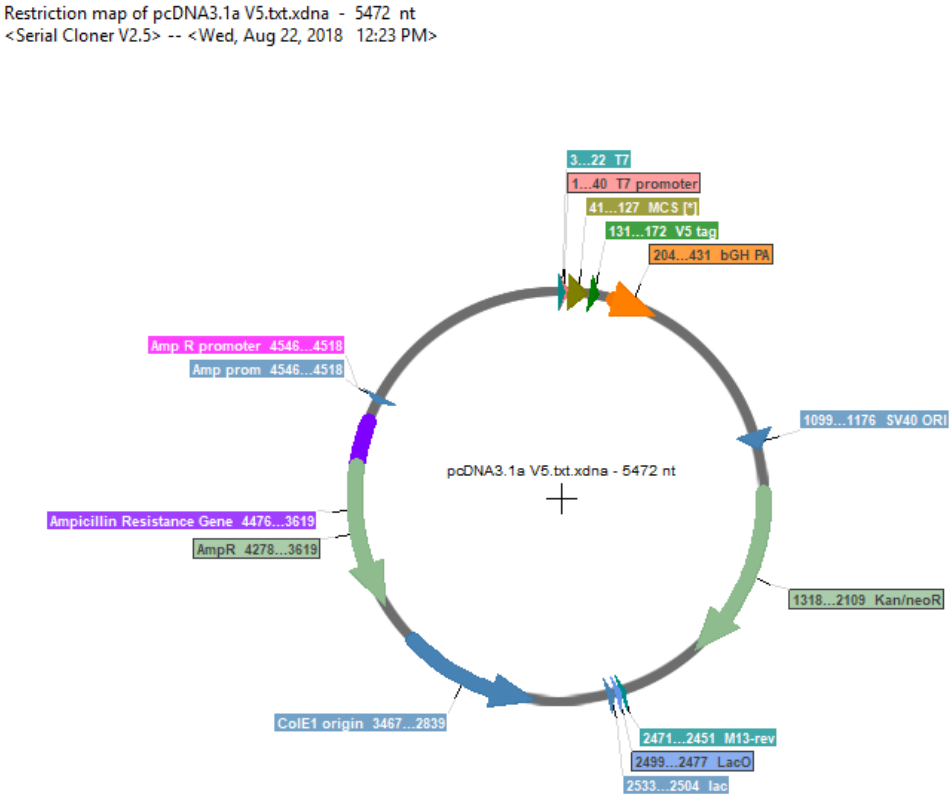


Figure 13: Vector map of pcDNA3.1A V5

The next step in cloning was to cut out the full length Fibulin-1C and Fibulin-1D out of their host vectors, pcDNA1, using restriction digests and into pcDNA3.1 A V5 and pcDNA3.1 A myc/His, respectively. Fibulin-1 C/D coding sequences were excised from host vectors using NotI and XbaI for the 5' and 3' ends, respectively. This was the full length mRNA and included a 5'UTR, Signal Peptide, START and STOP codons and the 3'UTR to go along with the coding sequence.

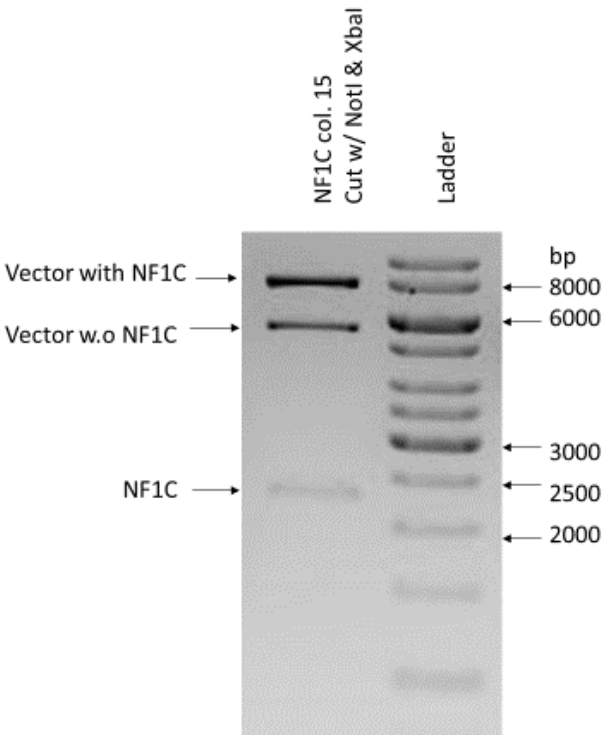


Figure 14: After ligation of coding sequence of Fibulin-1 C into pcDNA3.1 V5 plasmids were cut using NotI and XbaI. Bands for NF1C9,10,11 and 15 dropped out our insert. Black arrow shows insert, ~2.2kb, dropping out which is the size of Fibulin-1C with UTRs. Yellow arrow indicates incomplete digestion of insert and plasmid. (NF-Coding sequence is Not Fused to epitope tag) (1C- Fibulin-1C)

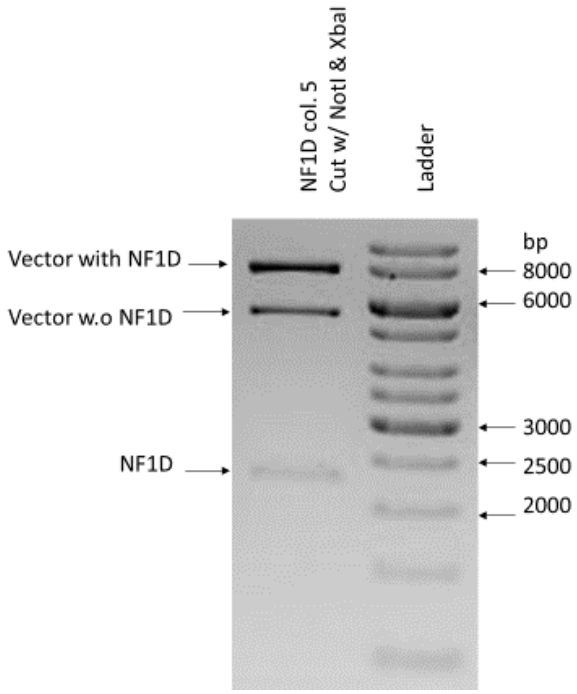


Figure 15: After ligation of coding sequence of Fibulin-1 D into pcDNA3.1 myc/His plasmids were cut using NotI and XbaI. Bands for NF1D1 & 5 dropped out Fibulin 1D insert. Black arrow shows insert, ~2.2kb, dropping out which is the size of Fibulin-1D with UTRs. Yellow arrow indicates incomplete digestion of insert and plasmid. (NF-Coding sequence Not Fused to epitope tag) (1D- Fibulin-1D)

Removal of 3'UTR in the Fibulin-1 clones was completed by utilizing primers to amplify around that region and conjoin the sides with XbaI cut site. Cut with XbaI then ligating the cut sites together. Figures 10 and 11 show the amplified reverse PCR products before clean up and cutting.

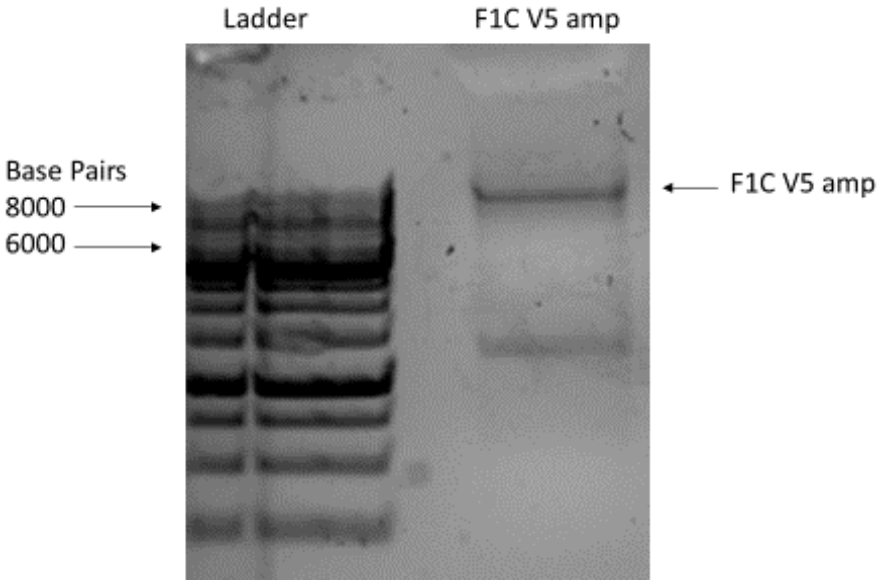


Figure 16: Gel shows reverse PCR product of Fibulin-1C V5. Product was around 8kb.shown in amplification F1C V5 1, 3 and 4.

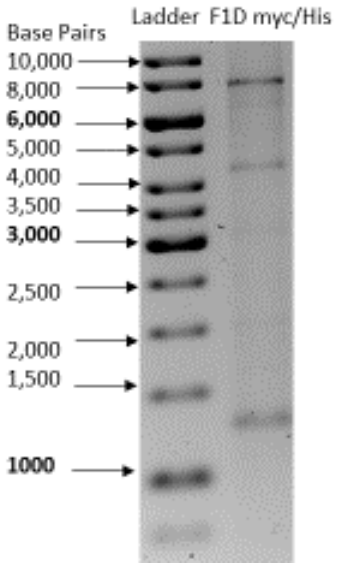


Figure 17: Gel shows reverse PCR product Fibulin-1D myc/His. Product was around 8kb shown in F1D 1 and 2.

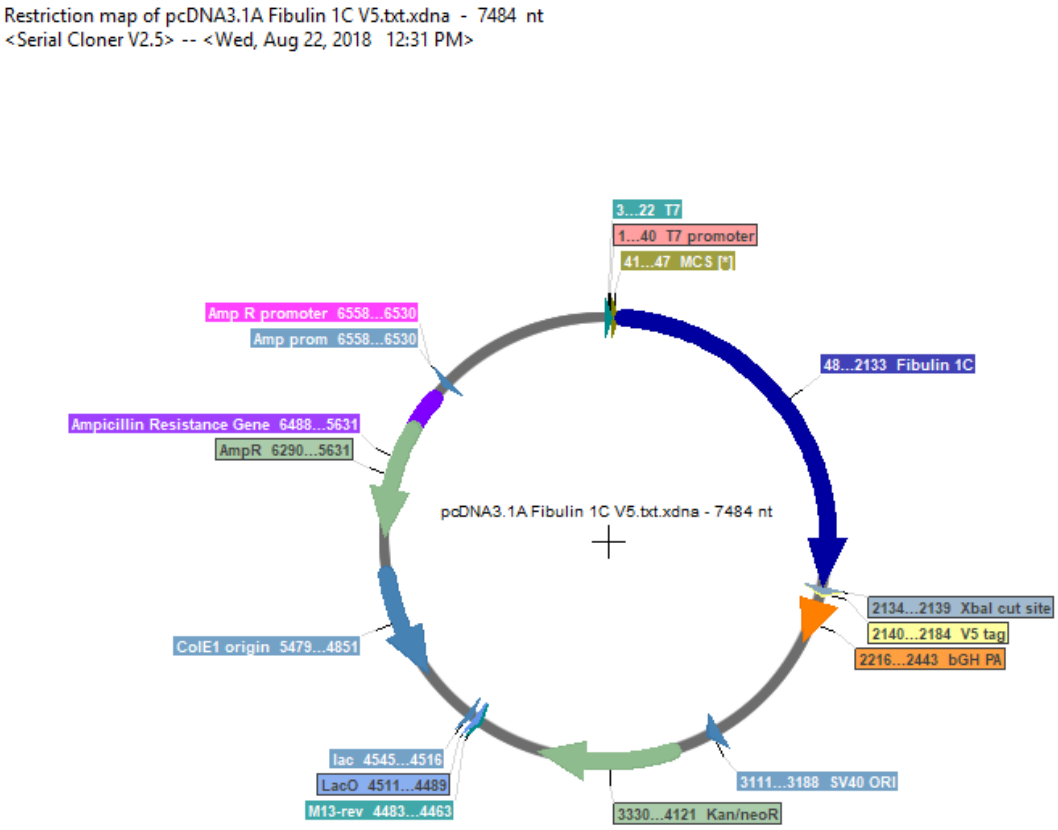


Figure 18: Vector map of pcDNA3.1A Fibulin 1C V5

3.2 **Vector Corrections**

After sequencing full length 1C/D fused coding sequence to the C-terminal epitope it was apparent that there were three mutations in F1D coding sequence. One of the three was a silent mutation at 1600 but the other two mutations at 1825 and 2243 caused amino acid changes. Errors are shown in Figures 12 and 14 from the clone pcDNA3F1D11. Errors were removed simultaneously with, PCR, reverse PCR and subsequent Gibson cloning techniques. When amplifying F1D vector the test

reaction of DreamTaq (Thermo, EP0701) worked with under standard conditions with a Tm= 51.4-56°C but when the reaction was repeated with the high fidelity polymerase, Radiant HiFi (Alkali Scientific, HF1020), there was no amplification. Thus the amplicon from DreamTaq was used for the large segment. Clones with errors removed are shown in Figures 13 and 15 show as F1D_12. After mutations were corrected truncates were created. Vectors were sequenced by Genewiz and compiled onto CLC Workbench.

Figure 19: Shows sequence analysis of the Fibulin-1D mutation at 1825. Mutation was changed from a thymine to an adenine. Sequencing provided by Genewiz, analysis of reads were compiled on CLC Workbench.

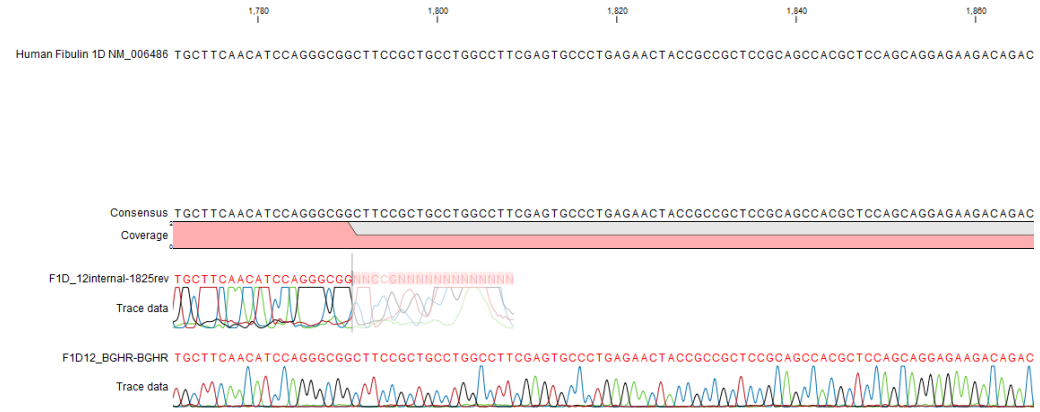


Figure 20: Sequence analysis of the error removal at 1825 in Fibulin-1D by means of Gibson cloning. Corrected Gibson primers changed the adenine to a thymine. Sequencing provided by Genewiz, analysis of reads were compiled on CLC Workbench.

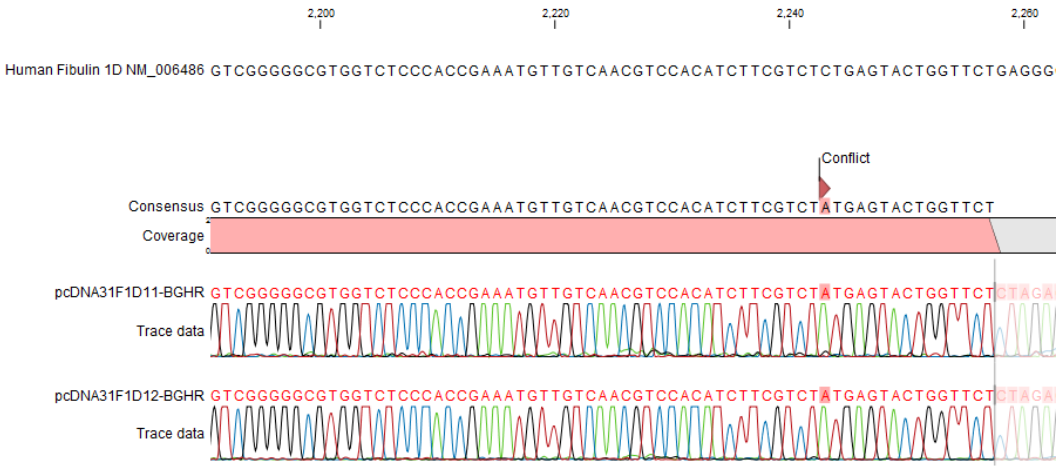


Figure 21: Shows sequence analysis of the Fibulin-1D mutation at 2243. Mutation was changed from a cytosine to a adenine. Sequencing provided by Genewiz, analysis of reads were compiled on CLC Workbench.

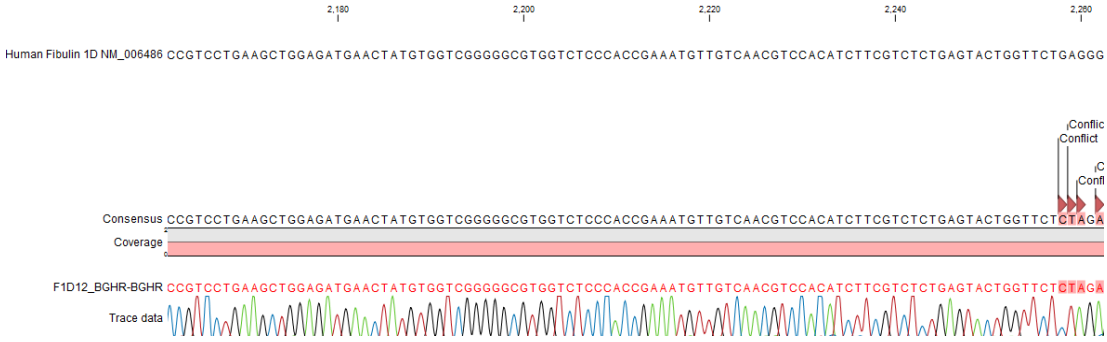


Figure 22: Sequence analysis of the error removal at 1825 in Fibulin-1D by means of Gibson cloning. Corrected Gibson primers changed the adenine to a cytosine. Sequencing provided by Genewiz, analysis of reads were compiled on CLC Workbench.

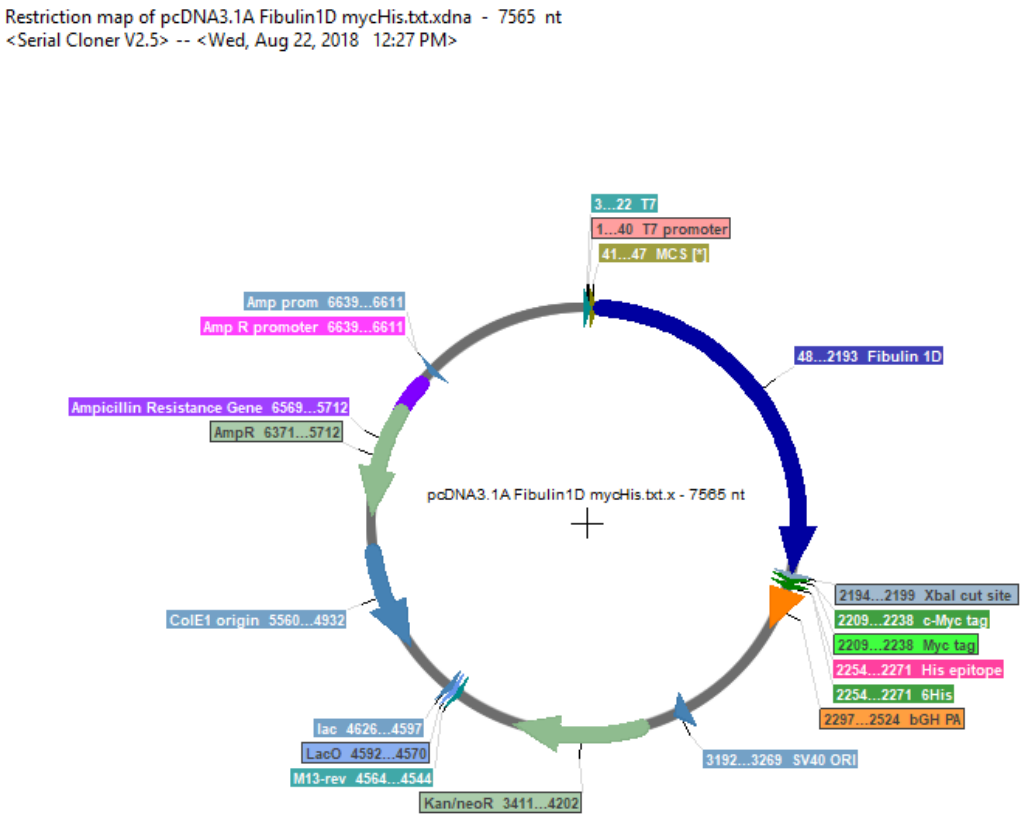


Figure 23: Vector map of pcDNA3.1A Fibulin 1D myc/His

3.3 **Creation of Truncated Variant Regions**

After creation of full length corrected of F1C V5 and F1D mvc/His clones, the next step was to eliminate the canonical region of the coding sequence to create F1Ct V5 and F1Dt myc/His. This was done by joining the Signal Peptide with the C-terminal variant region while also preserving the C-Terminal epitope tag. Figure 16 shows the amplified reverse PCR product of the variant C-terminal region to the end of the signal peptide as show in Figure 6. The PCR product was EtOH purified, cut with BamHI, ligated with T4 ligase, colony selected and sequence with Genewiz. The BamHI also created 2 new amino acids in the coding sequence, Gly and Ser, between the Signal Peptide and the C-terminal variant region.

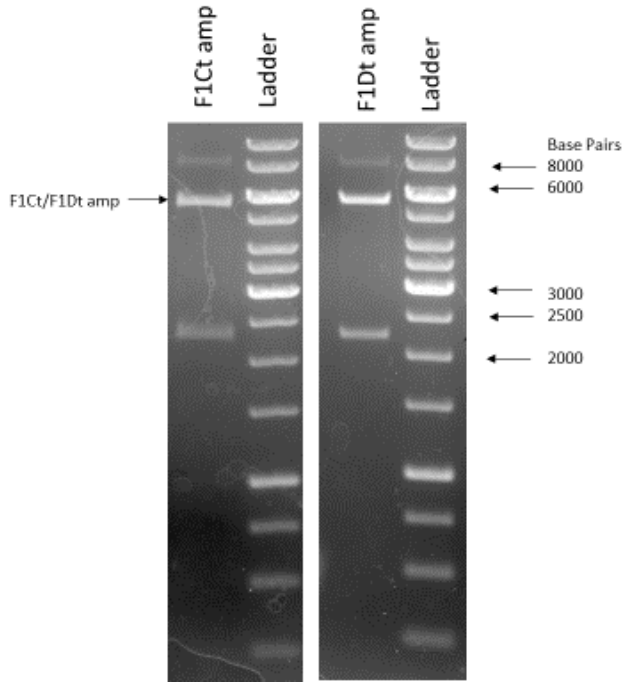


Figure 24: Figure shows the Reverse PCR of F1Ct in the first lane. In the third and fourth show amplification of F1Dt myc/His. Signal peptide and variant regions were conjoined by BamHI.

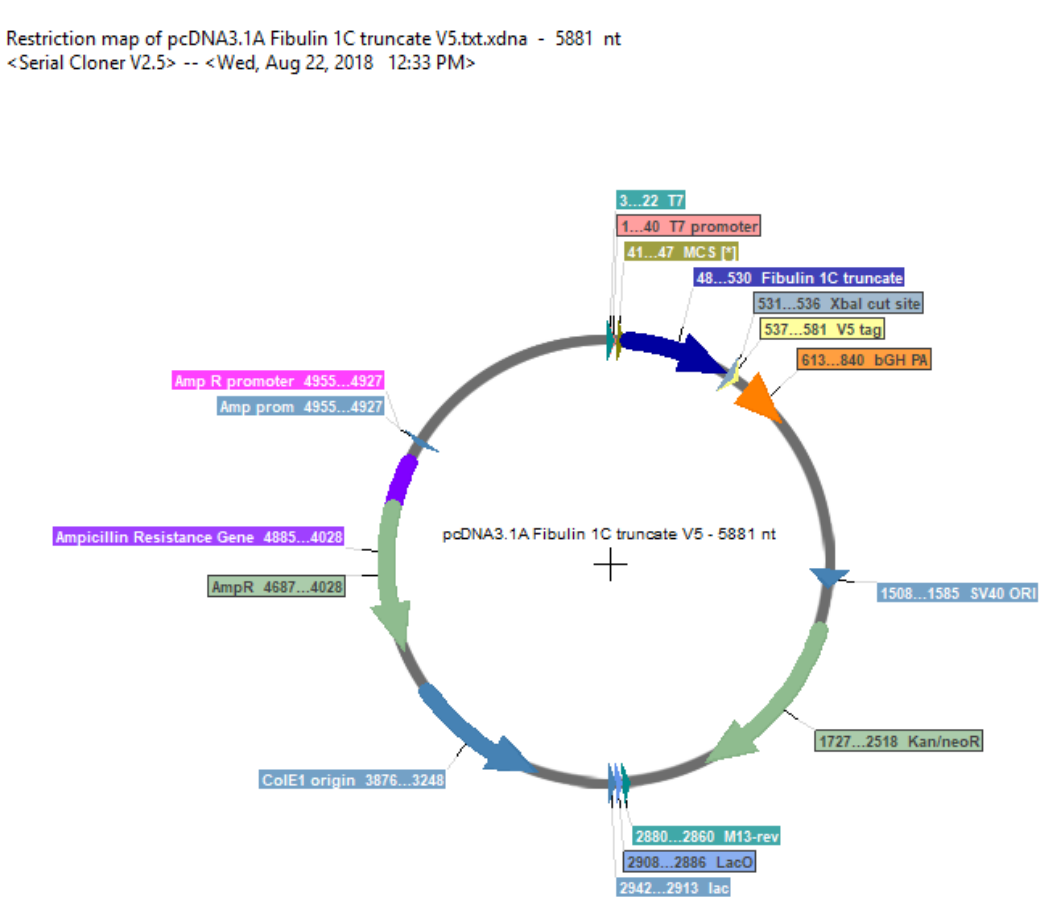


Figure 25: Vector map of pcDNA3.1A Fibulin 1C truncate V5

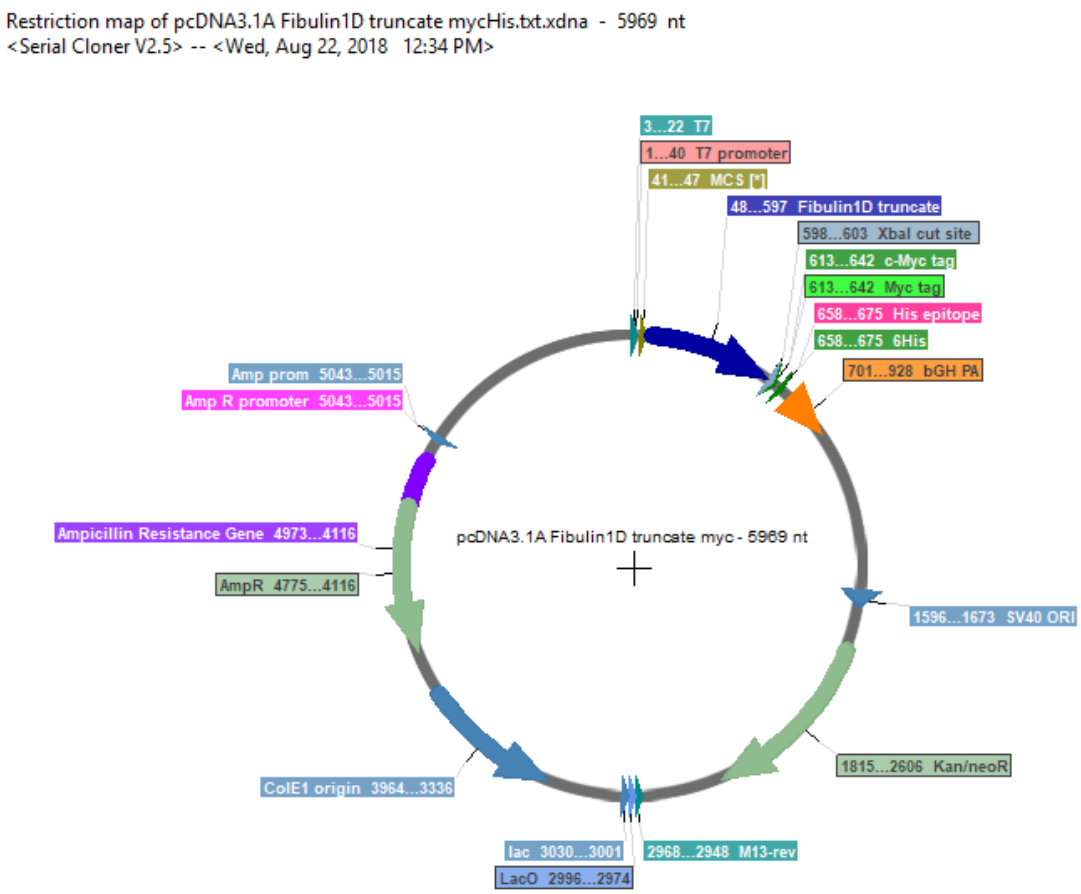


Figure 26: Vector map of pcDNA3.1A Fibulin 1D myc/His

3.4 **Protein analysis**

Prior to transfection, HS-5 cells which endogenously produce Fibulin-1 and MDA-MB-231 which we were testing for production of Fibulin-1, were seeded on 10cm cell culture dishes and conditioned media was taken and concentrated with 30kd MWCO spin filter (Millipore, Burlington, MA), after a day in serum free media. A western blot was done to confirm that HS-5 produced Fibulin-1 and that MDA-MB-231 did not express Fibulin-1 (Figure 21). MDA-MB-231 was transfected with all four clones, F1C V5, F1D myc/His, F1Ct V5 and F1Dt myc/His, into MDA-MB-231 along with a GFP control to show transformation efficiency. After a day transformation

efficiency was around 60% for the GFP control. Cells were recovered for a day in DMEM and 10% FBS and then serum starved for a day. Conditioned media was taken and used to confirm the presence of Fibulin-1 from the full length clones. Cell lysate also was collected. Conditioned media and lysate western blot analysis are shown on Figure 21. A 10 hour transfection of full length clones and GFP was also done and cell lysate was taken from them to test for the presence of Fibulin-1 shown in Figure 21.

Figure 27: Lanes from 1-10 (MDA MB 231=231) (1-Ladder, 2-CM HS-5, 3-CM in 231, 4-CM F1C V5 in 231, 5-CM F1D myc/His in 231, 6-cell lysate F1C V5 in 231, 7-cell lysate F1D myc/His in 231, 8- cell lysate GFP in 231, 9-cell lysate 10 hour transfection of F1C V5 in 231 and 10-cell lysate of 10 hour transfection of F1D myc/His in 231.)

Chapter 4

DISCUSSION

4.1 Vector Design

Fibulin-1C V5, fibulin-1C variant V5, fibulin-1D myc/His and fibulin-1D variant myc/His were sequenced and all coding sequence was in alignment after fusion. There are many issues that arise when creating novel proteins including folding and toxicity issues to cells. In hindsight it would have been more accurate and efficient to use the Gibson cloning method throughout my cloning escapade. The engineered internal cut site from BamHI in the truncated proteins denoted for extra 2 amino acids that are not native to the protein. The amino acids added by BamHI were Glycine and Serine, which glycine is harmless but the hydroxyl group could cause the protein to fold incorrectly. Less of a problem are the XbaI cut sites between the protein coding sequence and the epitope tag, due to the protein being entirely before the cut site. With Gibson cloning there would be no extra amino acids due to residual cut sites.

4.2 Toxicity of Fibulin to MDA MB 231

After analyzing my western blot of CM from transfections with F1C, F1D and GFP vectors from with anti-His and V5 there was no fluorescence. Due to not possessing proper controls to the V5 and myc antibodies I believed that there could be one of two issues; the first being either there was no Fibulin 1 present or I had problems with my antibodies binding to my epitope tags on Fibulin 1. So anti-Fibulin-1 was used as a control to see if there was full length Fibulin 1 present in the CM of transfected cells. As my fluorescence control I had Fibulin-1 in the HS-5 CM shown

as lanes 4 and 5 for 1C and 1D in Figure 17, respectively. Maybe being an issue of concentration or trouble being excreted out of the cell a cell lysate was done on the transfected MDA MB 231 cells to see if that could be the issue. Also shown in Figure 17 in lanes 6, 7 and 8 are 1C, 1D and GFP, respectively. Knowing that it is not being expressed in these cells after knowledge of successful transfection by showing GFP, successful lysate protein collection shown on a ponceau stain and successful antibody binding I believed the protein could be toxic to MDA MB 231.

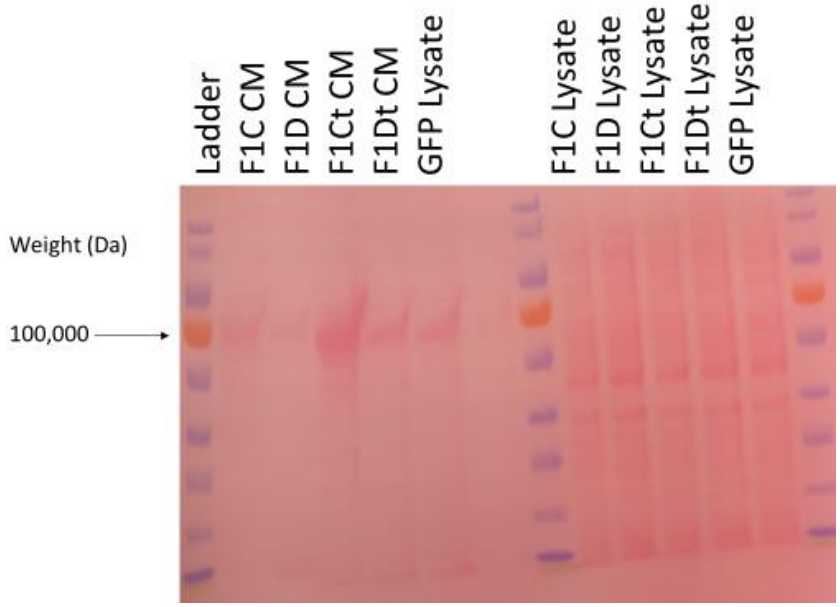


Figure 28: Ponceau Stain showing in lanes 2-6 CM collection and 30KDa MWCO of F1C, F1D, F1Ct, F1Dt and GFP after one day transfection one day recovery and one day of serum starvation. Lanes 9-13 show their cell lysates.

So I performed a 10 hour transfection. The transfection cell lysates of 1C and 1D are shown in Figure 17 as lanes 9 and 10, respectively.

This leaves the problem residing with the vector itself. Since I used DreamTaq to reverse PCR to fuse the coding sequence to the epitope tag, the DreamTaq could have easily caused a mutation in the promoter or elsewhere in the vector preventing the sequence from being transcribed.

4.3 **Cloning Issues**

In my first year on this project I was attempting to use pBABEpuro as a viral vector to add fibulin-1C/D and truncates into the genome of a mammalian cell in order to produce as much protein as possible in the easiest way. After attempting to cut this vector with restriction enzymes for a few months and aimlessly fiddling around with a very complicated miniprep to which I had no success. At first my PI and I believed that I was doing something wrong with my set up of the reaction but after a while I sent the vector out for sequencing and a majority of the MCS was missing. As I explained before, I also received a coding sequence that was mutated in multiple locations which I had to fix with Gibson Cloning, to give an accurate representation of the protein. If I learned anything from this experience is to sequence every piece of DNA prior to attempting to clone. Also it can be applied to premade clones for protein production. You cannot trust items that are given to you from other people, as a scientist it is up to you to determine if all of your starting materials are what you believe they are. In my research if we would have known of these problems prior to starting cloning it would have saved me 9 months of time trying to clone into sites that didn’t exist.

Another thing I have learned was the importance of positive and negative controls. In all aspects of molecular biology research it is crucial to have at lease one

positive control to troubleshoot your problems. Without a control, a failed experiment is exactly what it is called but a failed experiment with controls gives you direction and can point you in the correct direction of where your errors are in your experiment. In terms of western blots, when you receive no signal in a blot with no controls there is no direction to where your problem could be. With a control if your control shows no band in a western there is an issue with your procedure of blotting or antibody binding. Also, if the control illuminates you can be sure there is none of your protein of interest in the sample you tested.

Chapter 5

FUTURE DIRECTION AND SIGNIFICANCE

5.1 Future Directions

Previous lab members in Sikes lab have shown that HS5 cells secreted a soluble protein that induced apoptosis in PCa cells. This protein was found to be Fibulin-1. The removal of Fibulin-1 from HS-5 conditioned media decreased apoptotic ability to PCa cells. In order to test this hypothesis of the apoptotic ability of Fibulin-1C/D and truncates on PCa, the proteins can be added to media with PCa cells to see if proteins alone without any other factors from HS-5. Also if truncates alone would be able to induce apoptosis or if the canonical region is imperative for induction of apoptosis. Further studies to confirm this hypothesis would be to use shRNA to specifically knockdown each variant individually in HS-5. After transfection, qRT-PCR could be used to determine mRNA levels and western blot using fibulin-1C/D specific antibodies for protein levels of each variant to see if knockdown was successful. Apoptotic ability could be analyzed by a live/dead assay.

In order to see specific variant expression antibodies for variant specific fibulins need to be developed in order to test for Fibulin-1C/D levels and ratios in vivo. First removing each variant separately from HS-5 CM to see if there is a loss or gain of apoptotic ability or if a neuroendocrine phenotype is shown. Based on previous data in the report there is a very good chance that the apoptotic inducing protein is Fibuin-1D rather than Fibulin-1C. We also created truncated versions of this protein in order to see if there is specificity for PCa cell death in only the Fibulin-1D variant region. If this proves true truncate binding partners need to be analyzed and determine how Fibulin-1D truncate can lead to apoptosis in PCa cells.

Serum levels of fibulin-1 and ratio between C/D could be measured in PCa patients to determine if there is a correlation to bone metastasis overtime. These results could result in Fibulin-1 being a biomarker for patients who are at a higher risk and further could lead to a treatment prior to obtaining any metastasis.

Immunoprecipitation and subsequent purification of full and truncated fibulin-1 proteins could be useful. Purified proteins can be added to PCa media to see if they alone can induce apoptosis or other factors are needed from HS-5. The mechanism by which Fibulin-1 induces apoptosis is not fully understood to which many proteins could be needed for Fibulin-1 to induce apoptosis. Fibulin-1 has been show to attach to many different proteins and could need the coordination of many proteins in order to induce apoptosis.

Co-immunoprecipitations (IP) could be done to fibulin-1C/D and truncates to determine possible differences in binding ability in CM to induce intracellular signaling pathway in PCa. Also proteins in the basement membrane could be tested to determine the ability to anchor cells. Knockout or knockdown of proteins that co-IP with Fibulin-1 could lead us to their importance to PCa cell death as well as fibulins role.

Furthermore, immunodeficient mice could be given a highly aggressive and tumorigenic PCa cell line like, C4-2B, in order to grow tumors in vivo. Varying levels of the recombinant Fibulin-1C/D or truncated domains could be given intravenously to see if tumor growth or metastasis decreases with each different variant, dose levels or ratios of 1C to 1D.

Due to HDAC1 coordinating the 1C and 1D variants in PCa cell lines, it would be advantageous to see FAIRE-seq data from this to see how the splice variants are

coordinated. To see how tightly packed the DNA is and further what histones the HDAC1 is actually deacetylating in that area to cause this splice difference.

5.2 **Significance**

PCa is the most diagnosed cancer in men and the second leading cause of cancer related deaths in men. 90% of men who die from PCa have bone metastasis upon death. Bone metastasis causes a decrease in mobility, life expectancy and increased bone pain. Today, prostate tissue Gleason scores combined with PSA tests are the standard for determining if a PCa has the potential to be metastatic and thus require treatment or excision of the growth(15). The difficult part of treatment is whether or not to excise the entire tumor while a majority of the time tumors are left alone. This research into Fibulin-1 could lead to another marker to show the potential risk for PCa cells to adhere and metastasize the bone microenvironment and possibly a treatment. This will allow doctors to more accurately diagnose PCa patients and excise tumors with more metastatic ability then others. More research into Fibulin-1 and PCa is needed to prove any of these assumptions.

REFERENCES

1. Du Plessis SS, Gokul S, & Agarwal A (2013) Semen hyperviscosity: causes, consequences, and cures. *Front Biosci (Elite Ed)* 5:224-231.
2. Anonymous (2017) *United States Cancer Statistics: 1999-2014 Incidence and Mortality Web-based Report . .* (Centers for Disease Control and Prevention, https://nccd.cdc.gov/uscs/).
3. DeSantis CE, *et al.* (2014) Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* 64(4):252-271.
4. Gandaglia G, *et al.* (2015) Impact of the Site of Metastases on Survival in Patients with Metastatic Prostate Cancer. *Eur Urol* 68(2):325-334.
5. Anonymous (2012) 5-Year Survival Rate | CureSearch. (CureSearch for Children's Cancer, https://curesearch.org/5-Year-Survival-Rate).
6. Siegel RL, Miller KD, & Jemal A (2018) Cancer statistics, 2018. *CA Cancer J Clin* 68(1):7-30.
7. Anonymous (*United States Cancer Statistics: 1999-2014 Incidence and Mortality Web-based Report . .* (Centers for Disease Control and Prevention, https://nccd.cdc.gov/uscs/).
8. Ferlay J, *et al.* (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136(5):E359-386.
9. Yang ZJ, *et al.* (2018) The combined pN stage and breast cancer subtypes in breast cancer: a better discriminator of outcome can be used to refine the 8th AJCC staging manual. *Breast Cancer* 25(3):315-324.
10. Raghavan D (2018) First-line use of novel hormonal agents in prostate cancer: a critical appraisal. *Clin Adv Hematol Oncol* 16(4):289-295.
11. Yeku O & Slovin SF (2015) Metabolism and pharmacokinetics of radium-223 in prostate cancer. *Expert Opin Drug Metab Toxicol* 11(5):843-849.
12. Nevedomskaya E, Baumgart SJ, & Haendler B (2018) Recent Advances in Prostate Cancer Treatment and Drug Discovery. *Int J Mol Sci* 19(5).
13. Culig Z (2014) Targeting the androgen receptor in prostate cancer. *Expert Opin Pharmacother* 15(10):1427-1437.
14. Soloway MS, *et al.* (1988) Stratification of patients with metastatic prostate cancer based on extent of disease on initial bone scan. *Cancer* 61(1):195-202.
15. Cheville JC, *et al.* (2002) Metastatic prostate carcinoma to bone: clinical and pathologic features associated with cancer-specific survival. *Cancer* 95(5):1028-1036.
16. Roudier MP, *et al.* (2008) Histopathological assessment of prostate cancer bone osteoblastic metastases. *J Urol* 180(3):1154-1160.
17. Paget S (1989) The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev* 8(2):98-101.

18. Lertsuwan K, Choe LH, Marwa IR, Lee K, & Sikes RA (2017) Identification of Fibulin-1 as a Human Bone Marrow Stromal (HS-5) Cell-Derived Factor That Induces Human Prostate Cancer Cell Death. *Prostate* 77(7):729-742.
19. Aranzubía OM, *et al.* (2009) Subcutaneous metastasis of prostate cancer. *Arch Esp Urol* 62(7):583-585.
20. Montanari M, *et al.* (2017) Epithelial-mesenchymal transition in prostate cancer: an overview. *Oncotarget* 8(21):35376-35389.
21. Stewart DA, Cooper CR, & Sikes RA (2004) Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer. *Reprod Biol Endocrinol* 2:2.
22. Frantz C, Stewart KM, & Weaver VM (2010) The extracellular matrix at a glance. *J Cell Sci* 123(Pt 24):4195-4200.
23. Sinha AA, *et al.* (2001) Ratio of cathepsin B to stefin A identifies heterogeneity within Gleason histologic scores for human prostate cancer. *Prostate* 48(4):274-284.
24. Zhang WM, *et al.* (1999) Measurement of the complex between prostate-specific antigen and alpha1-protease inhibitor in serum. *Clin Chem* 45(6 Pt 1):814-821.
25. Iiizumi M, Liu W, Pai SK, Furuta E, & Watabe K (2008) Drug development against metastasis-related genes and their pathways: a rationale for cancer therapy. *Biochim Biophys Acta* 1786(2):87-104.
26. Corthay A (2014) Does the immune system naturally protect against cancer? *Front Immunol* 5:197.
27. Igney FH & Krammer PH (2002) Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2(4):277-288.
28. Patel LR, Camacho DF, Shiozawa Y, Pienta KJ, & Taichman RS (2011) Mechanisms of cancer cell metastasis to the bone: a multistep process. *Future Oncol* 7(11):1285-1297.
29. Edlund M, Sung SY, & Chung LW (2004) Modulation of prostate cancer growth in bone microenvironments. *J Cell Biochem* 91(4):686-705.
30. Cho EH, *et al.* (2012) Characterization of circulating tumor cell aggregates identified in patients with epithelial tumors. *Phys Biol* 9(1):016001.
31. Sun YX, *et al.* (2003) Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo. *J Cell Biochem* 89(3):462-473.
32. Hensel J & Thalmann GN (2016) Biology of Bone Metastases in Prostate Cancer. *Urology* 92:6-13.
33. Chaudhry GR, Yao D, Smith A, & Hussain A (2004) Osteogenic Cells Derived From Embryonic Stem Cells Produced Bone Nodules in Three-Dimensional Scaffolds. *J Biomed Biotechnol* 2004(4):203-210.
34. Xiao W, Wang Y, Pacios S, Li S, & Graves DT (2016) Cellular and Molecular Aspects of Bone Remodeling. *Front Oral Biol* 18:9-16.
35. Divieti Pajevic P & Krause DS (2018) Osteocyte regulation of bone and blood. *Bone*.

36. Dallas SL, Prideaux M, & Bonewald LF (2013) The osteocyte: an endocrine cell ... and more. *Endocr Rev* 34(5):658-690.
37. Kenkre JS & Bassett J (2018) The bone remodelling cycle. *Ann Clin Biochem* 55(3):308-327.
38. Raggatt LJ & Partridge NC (2010) Cellular and molecular mechanisms of bone remodeling. *J Biol Chem* 285(33):25103-25108.
39. Berger CE, Rathod H, Gillespie JI, Horrocks BR, & Datta HK (2001) Scanning electrochemical microscopy at the surface of bone-resorbing osteoclasts: evidence for steady-state disposal and intracellular functional compartmentalization of calcium. *J Bone Miner Res* 16(11):2092-2102.
40. Franz-Odendaal TA, Hall BK, & Witten PE (2006) Buried alive: how osteoblasts become osteocytes. *Dev Dyn* 235(1):176-190.
41. Josson S, Matsuoka Y, Chung LW, Zhau HE, & Wang R (2010) Tumor-stroma co-evolution in prostate cancer progression and metastasis. *Semin Cell Dev Biol* 21(1):26-32.
42. Chen G, Deng C, & Li YP (2012) TGF-β and BMP signaling in osteoblast differentiation and bone formation. *Int J Biol Sci* 8(2):272-288.
43. Ye XC, Choueiri M, Tu SM, & Lin SH (2007) Biology and clinical management of prostate cancer bone metastasis. *Front Biosci* 12:3273-3286.
44. Cooper CR, *et al.* (2000) Preferential adhesion of prostate cancer cells to bone is mediated by binding to bone marrow endothelial cells as compared to extracellular matrix components in vitro. *Clin Cancer Res* 6(12):4839-4847.
45. Juan-Rivera MC & Martínez-Ferrer M (2018) Integrin Inhibitors in Prostate Cancer. *Cancers (Basel)* 10(2).
46. Park JH, Lee NK, & Lee SY (2017) Current Understanding of RANK Signaling in Osteoclast Differentiation and Maturation. *Mol Cells* 40(10):706-713.
47. Karlsson T, Sundar R, Widmark A, Landström M, & Persson E (2018) Osteoblast-derived factors promote metastatic potential in human prostate cancer cells, in part via non-canonical transforming growth factor β (TGFβ) signaling. *Prostate* 78(6):446-456.
48. Deng X, *et al.* (2014) Recent advances in bone-targeted therapies of metastatic prostate cancer. *Cancer Treat Rev* 40(6):730-738.
49. Cook LM, Shay G, Araujo A, Aruajo A, & Lynch CC (2014) Integrating new discoveries into the "vicious cycle" paradigm of prostate to bone metastases. *Cancer Metastasis Rev* 33(2-3):511-525.
50. Zhang H, *et al.* (2014) Parathyroid hormone-related protein inhibits DKK1 expression through c-Jun-mediated inhibition of β-catenin activation of the DKK1 promoter in prostate cancer. *Oncogene* 33(19):2464-2477.
51. Bostwick DG, de la Roza G, Dundore P, Corica FA, & Iczkowski KA (2003) Intraepithelial and stromal lymphocytes in the normal human prostate. *Prostate* 55(3):187-193.

52. Sawant A, *et al.* (2013) Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer. *Cancer Res* 73(2):672-682.
53. Olechnowicz SW & Edwards CM (2014) Contributions of the host microenvironment to cancer-induced bone disease. *Cancer Res* 74(6):1625-1631.
54. Ocaña MC, Martínez-Poveda B, Quesada AR, & Medina M (2018) Metabolism within the tumor microenvironment and its implication on cancer progression: An ongoing therapeutic target. *Med Res Rev*.
55. Shiao SL, Chu GC, & Chung LW (2016) Regulation of prostate cancer progression by the tumor microenvironment. *Cancer Lett* 380(1):340-348.
56. Fridlender ZG, *et al.* (2012) Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One* 7(2):e31524.
57. Fridlender ZG, *et al.* (2009) Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16(3):183-194.
58. Shojaei F, Singh M, Thompson JD, & Ferrara N (2008) Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci U S A* 105(7):2640-2645.
59. Ho PC & Liu PS (2016) Metabolic communication in tumors: a new layer of immunoregulation for immune evasion. *J Immunother Cancer* 4:4.
60. Sukumar M, Roychoudhuri R, & Restifo NP (2015) Nutrient Competition: A New Axis of Tumor Immunosuppression. *Cell* 162(6):1206-1208.
61. Miles FL & Sikes RA (2014) Insidious changes in stromal matrix fuel cancer progression. *Mol Cancer Res* 12(3):297-312.
62. Roecklein BA & Torok-Storb B (1995) Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood* 85(4):997-1005.
63. Torok-Storb B, *et al.* (1999) Dissecting the marrow microenvironment. *Ann N Y Acad Sci* 872:164-170.
64. Wu CT, Chang YH, Lin WY, Chen WC, & Chen MF (2015) TGF Beta1 Expression Correlates with Survival and Tumor Aggressiveness of Prostate Cancer. *Ann Surg Oncol* 22 Suppl 3:S1587-1593.
65. Fritzinger DC, Petrella EC, Connelly MB, Bredehorst R, & Vogel CW (1992) Primary structure of cobra complement component C3. *J Immunol* 149(11):3554-3562.
66. Stenberg Y, Julenius K, Dahlqvist I, Drakenberg T, & Stenflo J (1997) Calcium-binding properties of the third and fourth epidermal-growth-factor-like modules in vitamin-K-dependent protein S. *Eur J Biochem* 248(1):163-170.
67. Ogata RT, Rosa PA, & Zepf NE (1989) Sequence of the gene for murine complement component C4. *J Biol Chem* 264(28):16565-16572.

68. Obaya AJ, Rua S, Moncada-Pazos A, & Cal S (2012) The dual role of fibulins in tumorigenesis. *Cancer Lett* 325(2):132-138.
69. Cangemi C, Hansen ML, Argraves WS, & Rasmussen LM (2014) Fibulins and their role in cardiovascular biology and disease. *Adv Clin Chem* 67:245-265.
70. Argraves WS, Dickerson K, Burgess WH, & Ruoslahti E (1989) Fibulin, a novel protein that interacts with the fibronectin receptor beta subunit cytoplasmic domain. *Cell* 58(4):623-629.
71. Twal WO, Hammad SM, Guffy SL, & Argraves WS (2015) A novel intracellular fibulin-1D variant binds to the cytoplasmic domain of integrin beta 1 subunit. *Matrix Biol* 43:97-108.
72. de Vega S, Iwamoto T, & Yamada Y (2009) Fibulins: multiple roles in matrix structures and tissue functions. *Cell Mol Life Sci* 66(11-12):1890-1902.
73. Talts JF, Andac Z, Göhring W, Brancaccio A, & Timpl R (1999) Binding of the G domains of laminin alpha1 and alpha2 chains and perlecan to heparin, sulfatides, alpha-dystroglycan and several extracellular matrix proteins. *EMBO J* 18(4):863-870.
74. Sasaki T, Göhring W, Pan TC, Chu ML, & Timpl R (1995) Binding of mouse and human fibulin-2 to extracellular matrix ligands. *J Mol Biol* 254(5):892-899.
75. Sasaki T, *et al.* (1995) Structural characterization of two variants of fibulin-1 that differ in nidogen affinity. *J Mol Biol* 245(3):241-250.
76. Twal WO, *et al.* (2001) Fibulin-1 suppression of fibronectin-regulated cell adhesion and motility. *J Cell Sci* 114(Pt 24):4587-4598.
77. Lee NV, *et al.* (2005) Fibulin-1 acts as a cofactor for the matrix metalloprotease ADAMTS-1. *J Biol Chem* 280(41):34796-34804.
78. Argraves WS, Tran H, Burgess WH, & Dickerson K (1990) Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structure. *J Cell Biol* 111(6 Pt 2):3155-3164.
79. Ng KM, *et al.* (2006) Evidence that fibulin family members contribute to the steroid-dependent extravascular sequestration of sex hormone-binding globulin. *J Biol Chem* 281(23):15853-15861.
80. Cooley MA, *et al.* (2008) Fibulin-1 is required for morphogenesis of neural crest-derived structures. *Dev Biol* 319(2):336-345.
81. Nandadasa S, Foulcer S, & Apte SS (2014) The multiple, complex roles of versican and its proteolytic turnover by ADAMTS proteases during embryogenesis. *Matrix Biol* 35:34-41.
82. Xiao W, *et al.* (2014) Fibulin-1 is epigenetically down-regulated and related with bladder cancer recurrence. *BMC Cancer* 14:677.
83. Bardin A, *et al.* (2005) Transcriptional and posttranscriptional regulation of fibulin-1 by estrogens leads to differential induction of messenger ribonucleic acid variants in ovarian and breast cancer cells. *Endocrinology* 146(2):760-768.

84. Figueiredo NB, *et al.* (2014) Estrogen-responsive genes overlap with triiodothyronine-responsive genes in a breast carcinoma cell line. *ScientificWorldJournal* 2014:969404.
85. Pupa SM, *et al.* (2007) Regulation of breast cancer response to chemotherapy by fibulin-1. *Cancer Res* 67(9):4271-4277.
86. Kuo PL, Shen KH, Hung SH, & Hsu YL (2012) CXCL1/GROα increases cell migration and invasion of prostate cancer by decreasing fibulin-1 expression through NF-κB/HDAC1 epigenetic regulation. *Carcinogenesis* 33(12):2477-2487.

Appendix A

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Title: The dual role of fibulins in tumorigenesis
Author: Alvaro J. Obaya,Susana Rua,Angela Moncada-Pazos,Santiago Cal
Publication: Cancer Letters
Publisher: Elsevier
Date: 28 December 2012

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