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The Role of Integrin Alpha 6 on Tumor Metastasis

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An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By
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ABSTRACT

Cancer research is a large topic in science, because over 14 million people are diagnosed worldwide, and of those, 8 million will die annually. Traditional therapies are the mainstays of treatment strategies, but a cure less invasive or with less side effects have not yet been identified. In order to develop a better cure, we need to understand the tumor and molecules present in its tissues. Integrins, cell surface proteins that aid in cell communication, have been found in the tumor tissues of several types of cancers, including prostate, lung and bone. Integrin alpha-6 (ITGA6), in particular, has been found to have a large effect on the progression of tumor metastasis. In tumor tissues, we have seen an overexpression of the ITGA6 that leads to tumor metastasis and overall tumor progression. We have also seen an in vitro cleaving mechanism, in which only a cleaved, or extracellular portion of the protein is expressed in the microenvironment. To develop a therapeutic strategy, it is important to understand the role of ITGA6 in tumor development. Using a humanized zebrafish model, we hypothesized that increasing ITGA6 and cleaving ITGA6 will increase tumor metastasis. To study this question, we overproduced ITGA6 by injecting human ITGA6 RNA and reduced the gene using the morpholinos. We also injected cleaved human ITGA6 RNA and non-cleaved, or mutated ITGA6 RNA that could be not removed from the surface of the cell. We then injected human prostate cancer cells and allowed a human tumor microenvironment to develop over two days. Using confocal imaging, we visualized non-metastatic versus metastatic embryos. We utilized a t-test and found p-values of <.01 when comparing the full length ITGA6 and cleaved ITGA6 to the control. We concluded that increased and cleaved integrin alpha 6 allow for the cell to develop cancerous abilities such as increased aggressiveness, motility through the ECM, invasion and metastasis in blood vessels to create secondary tumors. Studies are now showing that ITGA6 can be used a biomarker, a molecule that can predict the susceptibility of several cancers. In the future, we would like to use ITGA6 manipulations and drug screenings in a favorable zebrafish model to create a preventative strategy for cancer.

Keywords: cancer, metastasis, integrins, prostate, Zebrafish

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Introduction

1. Cancer

Cancer is the foremost generic word that creates severe panic within a person, family, or community. Cancer **[Figure 1]** is defined as a disease characterized by abnormal growth of cells due to uncontrollable cell division. It can be caused by external factors that include one's behavior such as smoking, drinking, diet, and exercise. It can also be caused by internal factors such as genetics, hormones, and immune conditions (1). This panic is supported by the gruesome reality of cancer with approximately 14 million people being diagnosed worldwide each year (2,3). Of those diagnosed, over 50% of these cases come from undeveloped countries. The most prominent types of cancer include lung, female breast, bowel and prostate cancer, with 4 in 10 cases stemming from one of these groups (3). Of those diagnoses, over half, 8 million, will die. Most deaths come from lung, liver, bowel, and stomach cancer, with lung cancer accounting for one-fifth of all deaths (3). In the female population, breast cancer is the most deadly. Approximately 240,000 new cases will be diagnosed in women, and 40,000 women will die (2). Prostate cancer, however, is very prevalent in males, being the second most

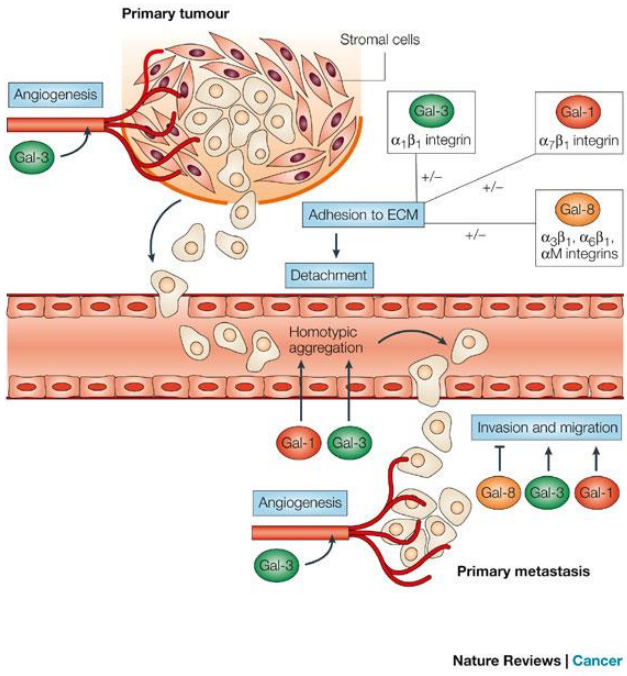


Figure 1. This schematic shows the overall progression of tumor development, where a cancer cell loses adhesion to the tissue. The cell invades and metastasizes into the blood vessels creating secondary tumors.

http://www.nature.com/nrc/journal/v5/n1/images/nrc1527-f4.jpg

common cancer in males after skin cancer. In the United States, there are approximately 230,000 new cases each year and over 3 million people living with the cancer today (2). The death rate of prostate cancer is one of the most with about 12% (1 in 38) of all diagnoses ending fatally (1).

1.1 Cancer Management

In order to have the best chances of managing a patient's cancer, early diagnosis and appropriate therapeutic strategies are the two major steps to effectively fight the disease (4). Early diagnosis of the cancer gives a better chance of it not already becoming too big or spreading to other vital organs (4). An important tool in early diagnosis is the use of biomarkers. Biomarkers are any substance or molecule that can indicate the presence of cancer in the body (4). In addition to early diagnosis, it is vital to use the most appropriately fitting therapeutic strategy to treat the three factors that cause cancer to be so difficult to control.

1.2 Three Factors of Cancer: Angiogenesis, Metastasis, and Cancer Stem Cells

First, angiogenesis occurs in a patient. Angiogenesis **[Figure 2]** is the creation of blood vessels that stem from the tumor into the blood stream (5).

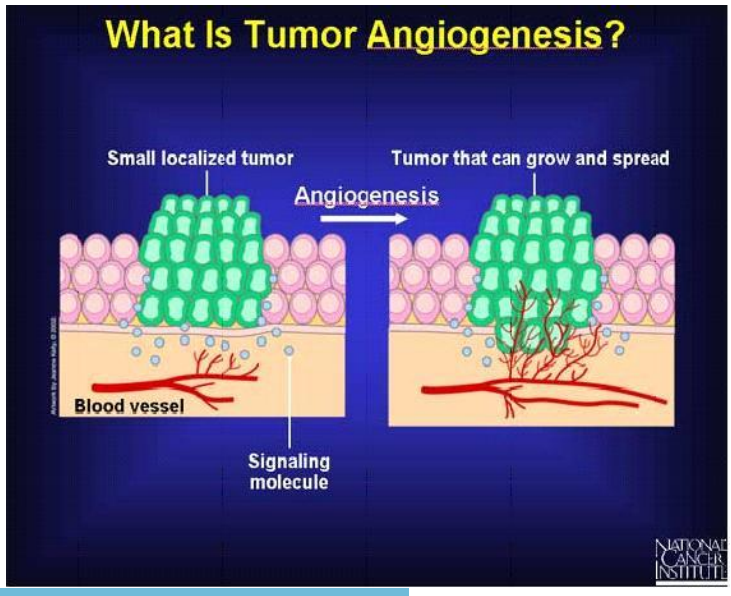


Figure 2. Newly formed blood vessels are stemming from the tumor into the microenvironment created via angiogenesis.

http://systemsbiology.case.edu/OLD%20WEBSITE%20STUFF/images/angiogenesis/angiogenesis_pic1.jpg

These blood vessels allow the tumor to receive nutrients that are pertinent to its growth and survival. They are also

a pathway for the cancer cells of the tumor to travel through the bloodstream to reach other organs (5).

The dissemination of cancer cells **[Figure 3]** throughout the body using these blood vessels is known as metastasis, the second factor of cancer (6).

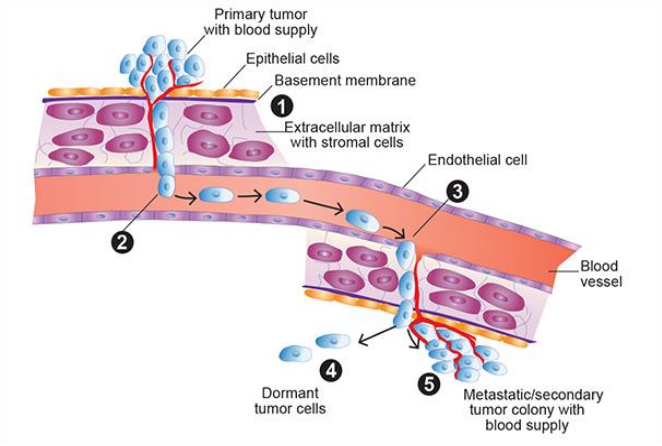


Figure 3. The cells moving from the primary tumor into the blood stream via newly formed blood vessel into a previous blood vessel is the process known as metastasis that creates secondary tumors in another tissues.

http://www.nature.com/nrc/journal/v5/n1/images/nrc1527-f4.jpg

Metastasis is the most deadly element of cancer as it is the cause of over 90% of the deaths related to cancer. Once cancer cells reach other organs, they have the potential to create secondary tumors, facilitated by cancer stem cells, the third and least understood mechanism of cancer (6).

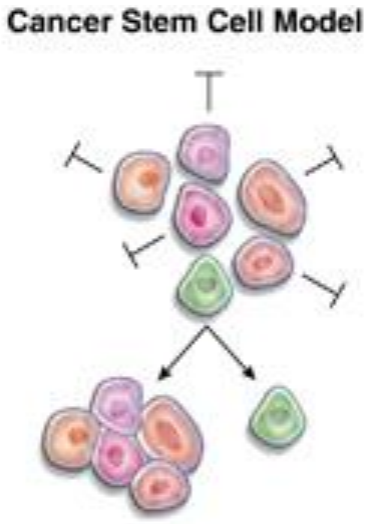


Figure 4. The purple, orange, and pink cells signify cancer cells with variations. The green cells represent cancer stem cells that have the ability to form new tumor masses in different tissues.

http://www.nature.com/labinvest/journal/v91/n5/images/labinvest201150f1.jpg

Cancer stem cells (CSC) **[Figure 4]** are similar to normal, human stem cells, because they are self-renewing (7).

These cells originate from stem cells or progenitors, targeted stem cells specific to cancer (7,10). In a tumor, there can exist multiple populations of CSCs or they can co-exist among normal cancerous cells (10). CSCs can influence the start of metastasis, as well as, relapse in a patient that is in remission (10).

1.3 Traditional Treatments

Modern, conventional therapeutic strategies that try to control the factors of cancer include chemotherapy, radiation, and surgery. Chemotherapy uses anti-cancer drugs to shrink and eliminate the tumor (8). Radiation uses ionizing radiation to kill cancer cells by damaging their DNA (9). Surgery for cancer patients has a number of different uses, ranging from diagnostic to curative surgeries (10). Both chemotherapy and radiation have severe adverse effects, because the strategies are non-specific. They both kill all types of cells, which leads to severe damage in other vital tissues and organs (9,10). Surgery can and very often leads to deadly infections that can kill the patient (10). What all three therapeutic strategies have in common is that they aim to treat cancer, but they have no mechanism to cure the cancer (9,10). In order to have the best chance of curing any type of cancer, a molecule needs to exist that can be a biomarker, as well as, be used appropriately as a therapeutic strategy (10). Integrinα6 (ITGA6) is a molecule that is a biomarker for several types of cancer and can potentially be used as a therapeutic strategy to treat cancer (10).

2. Integrinα6 in Cancer

2.1 ITGA6 Protein

ITGA6 is a part of a group of transmembrane proteins that aid in cell adhesion, cell-to-cell communication, intracellular communication, and act as ECM receptors that were

discovered approximately 25 years ago (11). These proteins are most notably known for binding to extracellular glycoproteins such as laminin, vascular cell adhesion molecule-1 and collagen, to enhance cell adhesion, proliferation, cell differentiation, and cell migration (11). Integrins are made of two subunits, alpha and beta, that are bound via noncovalent interactions to make heterodimeric proteins (6, 11). Integrins vary in size, depending on higher order function, and have been found in all mammals and most fish, such as sponges, nematodes, zebrafish, and humans (11). All integrins have a basic structure **[Figure 5]**, with an intracellular domain that interacts

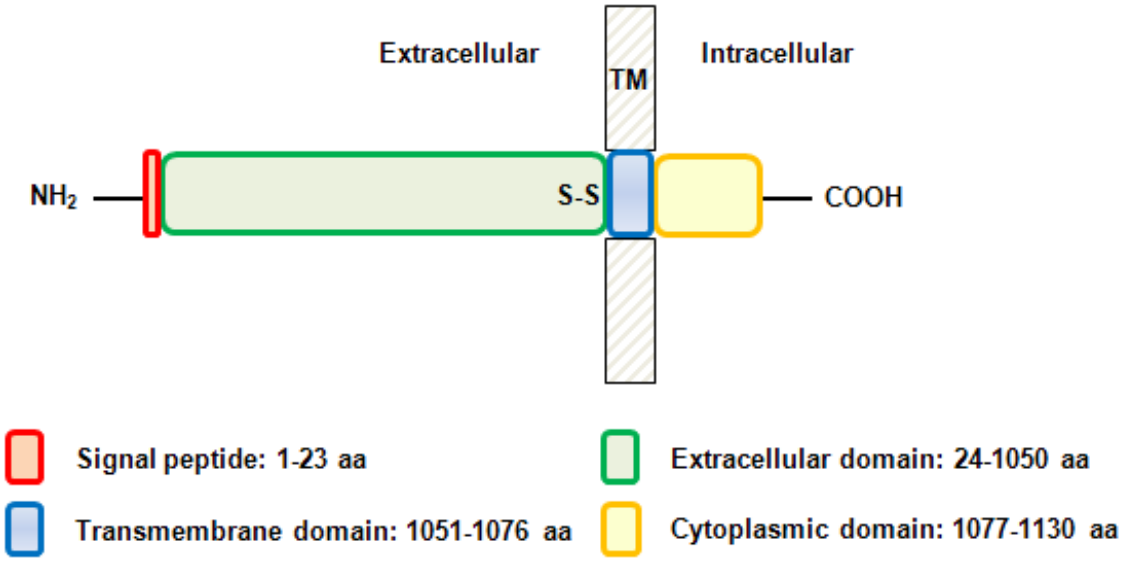


Figure 5. This schematic shows the structure of a basic integrin. It is transmembrane with the intracellular portion highlighted in yellow, and the extracellular portion highlighted in green.

http://atlasgeneticsoncology.org/Genes/Images/ITGA6Fig2.png

with signals in the cytoplasm and cytoskeletal elements, a very thin transmembrane domain, and an extracellular domain that acts as a receptor for binding to several different molecules, proteins and to relay signals between cells (11). Integrins are distinguished from other integral proteins and receptors on the cell surface in that they are bidirectional. This directionality occurs because integrins are

able to transmit signals from the extracellular to intracellular domain and vice versa, leading to their large roles in immune responses, hemostasis, and overall cell development (11). While there are several different types of integrins including 18α and 8β subunits, ITGA6, comprised of α6β1 and α6β4, have received special attention due to its role in tumor development (11).

2.2 ITGA6 in Angiogenesis

Angiogenesis is the formation of blood vessels that grow from the tumor into the blood stream. These blood vessels allow cancer cells to migrate to other organs. In order for angiogenesis to occur, proangiogenic factors are essential (5). In a study using hindlimb ischemia as a model for peripheral arterial disease (PAD), researchers found ITGA6 levels of expression were increased by vascular endothelial growth factor and fibroblast growth factor, two major proangiogenic factors (5). Once there were high levels of gene expression, *in vitro* vascular tube formation was enhanced significantly (5). This study concluded that high levels of ITGA6 are needed in order to make the blood vessels that stem from the tumor (5). Another study researching how angiogenesis occurs found that upregulation of ITGA6 induces the endothelial cells to begin vasculogenesis (12). A study using human endothelium cells (EA cells) that closely resemble vascular endothelium cells, saw an *in vitro* formation of a network tube like structures, favoring blood vessels and the cellular process, angiogenesis (13). The experiment concluded that a G-protein mediated signal was needed in order to form the tube structures. With this information, they used pertussin toxin (PT), as means to block the signal, and ultimately block angiogenesis (13). However, the researchers saw that PT was able to modestly block angiogenesis, but when monoclonal antibodies against ITGA6 were used in the medium, the results saw that tube like formation was completely block. Scientists concluding that ITGA6 was the protein of most influence in angiogenesis (13). Lastly, an experiment was conducted based

off the previously supported hypothesis that tumors have cells with increased basic fibroblast growth factor (bFGF), one molecule that initiates that increased formation of blood vessels (14). It was found that in cells with bFGF, there was severe upregulation of α6β1, α6β4 and other integrins. These cells also had much better adherence to laminin, fribonection, and collagen 1, prime factors thought to have a role in tumor progression (14). The experiment concluded that integrins, ITGA6 specifically, led to increase angiogenesis in the endothelial cells (14). In conclusion, several research groups have concluded that ITGA6 upregulation plays a significant role in angiogenesis; however, scientists have not yet concluded what the increased amounts of ITGA6 exactly do in angiogenesis and why normal levels of the same protein do not seem to have an impact (14).

2.3 ITGA6 in Metastasis

Metastasis is the dissemination of cancer cells throughout the body via blood vessels created during angiogenesis, accounting for approximately 90% of all deaths. In metastasis, several researchers have looked in-depth into how ITGA6 is accommodating this largely important factor of cancer (15). In a study using human prostate cancer cells and mice as a model, researchers knocked down the levels of ITGA6 using a blocking-function antibody (15). Their results showed that there was only 40% of the metastatic tumor progression in comparison to the control (did not receive the antibody) which saw 80% progression. The study concluded that ITGA6 played a role in inducing metastasis (15). A different experiment wanted to discover if there was a biomarker for the highly metastatic cancer of the female breast. Their research saw that ITGA6 had high levels of expression in brain metastasis (16). They concluded that gene profiling to look for the levels of expression for ITGA6 would be an important biomarker for early diagnosis for cancer, and hopefully being able to treat the cancer before it begins (16).

Another study investigating the effects of metastasis and ITGA6 used esophageal squamous cancer cells as a model (17). The results of the experiment concluded there were significant levels of ITGA6 and CTHRc1, a protein found at high levels in esophageal cancers, in the cells at a rate of 71% (17). These cells and the expression of the two proteins were compared to the levels in normal esophageal cells using immunohistochemistry, and they were able to confirm lower levels of expression in the normal cells (17). They also concluded that metastatic cells in squamous population also had higher levels, 50% higher, of ITGA6 (17). They concluded that ITGA6 has a large role in metastasis of squamous cancer cells, and may be a potential marker of esophageal cancer (17). Lastly, an experiment was conducted to study the role of *SNAI1* protein and its role in epithelial-mesenchymal transition (EMT), an important factor in metastasis (18). Using prostate cancer cell lines, LNCaP and PC3, they saw that knockdown of the *SNAI1* protein also led to downregulation of ITGA6 which in turn, led to decreased metastasis, and overall cancer cell death in both lines (18). Researchers saw that ITGA6 played a large role metastasis (18). In conclusion, we have been able to determine that ITGA6 levels can be indicative of metastasis, but more research needs to be directed into the correlation of these levels and increased progression and susceptibility (18).

2.4 ITGA6 in Cancer Stem Cells

Cancer stem cells are self-renewing cells that co-exist within normal cancer cell populations and give rise to secondary tumors and relapses in the disease. In the last factor of cancer, CSCs, ITGA6 has also been seen to play a profound role in the stem cell's ability to control angiogenesis and metastasis (19). In an experiment studying the CSCs responsible breast cancer's high metastasis rate, they used an *in vitro* knockdown approach to reduce gene expression of ITGA6. They saw a large decrease in secondary tumors, as well as, the loss of

renewal ability in the CSCs (19). Another study in prostate cancer focused on what gave rise to the cancer (19). The scientists used gene profiling of progressed human cancer cells to see if there were biomarkers of the cancer (19). The profile showed ITGA6 as an important biomarker due to its relatively high amounts of gene expression in the entire prostate cancer population and in the sub-population of prostate CSCs (20). In CSCs, high levels of ITGA6 have been seen in several cancers to play a role in CSC population, but they have not yet seen determined what these high levels contribute to (20). In conclusion, research has been able to discovery that ITGA6 upregulation, high levels of gene expression plays a role in proangiogenesis, metastasis, and cancer stem cell markers (20).

2.5 Down Regulation of ITGA6

In another direction, some researchers have even looked into what effects low levels of ITGA6 can lead to in cancer progression. While high levels of ITGA6 has been found to promote tumor progression, mutations in ITGA6 also can lead to tumor susceptibility and progression (21,22). A study in papillary thyroid cancer found that a single missense mutation, a single change in a codon that leads to a different mutation, may be responsible for the cancer (21). This hypothesis was also supported by a study in prostate cancer in which five different intron single nucleotide polymorphisms in the ITGA6 gene led to increased progression in the cancer (22). These studies support the notion that even low levels of ITGA6 can lead to cancer progression and susceptibility, a contradicting conclusion to high levels also increasing progression (21,22).

2.6 ITGA6's Potential Diagnostic/Therapeutic Strategy

In all, we understand that high levels of ITGA6 can promote any factor of cancer. In another aspect of ITGA6, research has suggested that mutated ITGA6 can lead to lower levels of

progression in comparison to truncated or cleaved ITGA6. Cancer researchers know that low levels of ITGA6 can also lead to progression and susceptibility, two very contradicting statements. However, what we do not yet understand is how exactly both high and low levels ITGA6 lead to cancer susceptibility and how a mutated gene can decrease cancer progression.

In conclusion, ITGA6's ability to decrease tumor progression can be examined and isolated to potentially provide a therapeutic strategy of minimalizing the cancer or serving as a biomarker for the susceptibly to different cancers. With this information, the race to find a cure for cancer will be greatly accelerated.

3. Hypothesis/Predictions

In my study, I plan to study the two questions**: 1) will high or low levels of ITGA6 protein induce tumor metastasis.** We predict that increased levels of ITGA6 will induce tumor metastasis. **2) will cleaved, extracellular ITGA6 induce tumor metastasis.** We hypothesize that cleaved ITGA6 will induce tumor metastasis, also. The second question stems from *in vitro* cell culture, where they have seen a cleaving mechanism of the protein. With this mechanism, an enzyme cleaves the extracellular portion of the protein, and this product is expressed in the microenvironment. A cell culture assay does not allow for the cell-to-cell interactions that are present in the tumor microenvironment, and therefore, this mechanism needs to be explored in an *in vivo* model to confirm the mechanism and to determine its effects in tumor progression. We pTo explore both questions, we will use a Zebrafish tumor xenograft model. In this model, we will transplant human prostate cancer cells into zebrafish embryos, as well as use ITGA6 RNA to manipulate the levels of human ITGA6 in the zebrafish.

ITGA6 will be used will be regulated in three constructs, full length RNA, truncated RNA, and mutated RNA. To study the first question, full length mRNA will be compared to embryos with the unchanged or regular levels of ITGA6. Full length mRNA **[Figure 6]**

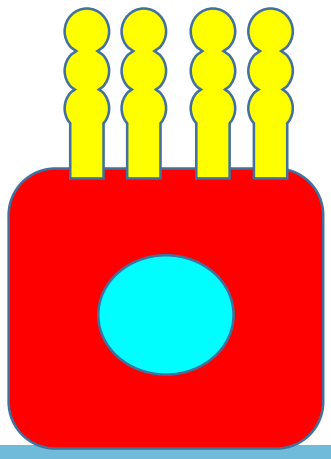
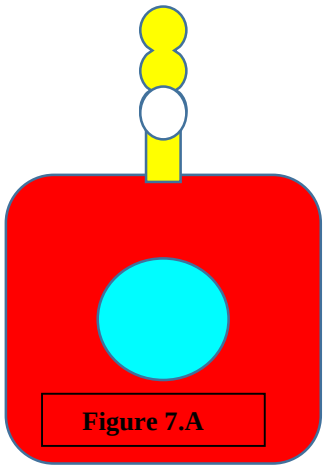


Figure 6. In the FL-ITGA6 construct, there is an increase of the protein on the cell surface.

was created by overproducing the extracellular domain of the protein on the surface of the cell. Instead of having only one extracellular domain portion, like that of the control embryos, full length embryos will have several extracellular domain protein, in order to study these effects on tumor progression. We hypothesize increase levels of ITGA6 will induce tumor metastasis and overall tumor development.

To answer the second study, mutated mRNA and truncated mRNA will be used to model the *in vitro* mechanism. Mutated mRNA **[Figure 7.A]** was translated with a mutation in the cleaving site of the enzyme that is believed to cut the extracellular domain of ITGA6. With this mutation, the cleaving cannot occur, and the extracellular domain stays attached to the cell surface. In truncated mRNA **[Figure 7.B]** only the extracellular domain of the protein is produced, so that there is no ITGA6 on the surface of the cell, and the protein is able to be dispersed within the ECM and microenvironment of the tumor. We believe truncated protein will increase tumor metastasis and mutated ITGA6 will lead to decreased levels of tumor metastasis and overall tumor development.



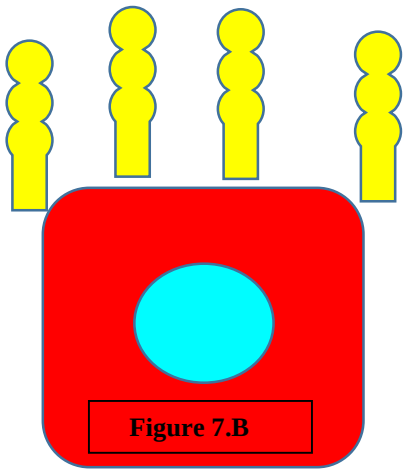


Figure 7.A shows the mutated ITGA6 construct, in which the cleaving site was mutated, so the integrin was fixed to the cell surface.

Figure 7.B The α6p construct only expresses the extracellular component of the integrin, in which it has been cleaved.

Materials

4. Cell Culture

4.1 Inoculation

Prostate cancer cells, line PC-3, from GeneScript® were obtained from another lab on campus. These cells are labeled immortalized cell lines, as they are more homogenous and stable than primary cancer cells in *in vitro* assays. Cell culture must be conducted in a biosafety level two lab, with a biosafety cabinet with laminar flow, due to their potential pathogenic properties. To begin growing the cells, one bottle of Opti-Mem 1X w/ glutamine w/ HEPES w/o| phenol red media, five milliliters of penicillin streptomyocin (P/S), and 50mL of Fetal Bovine Serum (FBS) are placed into a 37^0C water bath for 30 minutes. While the contents are heating, aseptic techniques were conducted. The hood was opened half way and sprayed with 70% ethanol. The 70% ethanol was dried using paper towels, and the appropriate amount of 10 mL and 25 mL pipettes for the experiment were obtained and sprayed with 70% ethanol before being placed inside of the hood. When the media had 10 minutes left, one vial, containing one milliliter of cells, was removed from liquid nitrogen and placed into the 37^0C water bath. All contents were taken out of the water bath after ten minutes, and sprayed with 70% ethanol before being placed inside the hood. The growth media was prepared by adding 50mL of FBS and five milliliters of P/S to one bottle of growth media. The solution was pipetted up and down, and placed aside. Two T-75 cell culture flasks from Corning® were obtained, sprayed with 70% ethanol and placed inside the hood. 20mL of media were pipetted into each cell culture flask, and 500uL of cells were pipetted into each flask. The flasks were labeled with the initials, date, and passage one, signifying its first splitting from the original line. The flasks were placed into the 37^0C

incubator. To clean the hood, all contents and pipettes were placed into the biohazard waste bag and all glassware was placed into the sharps container. The media was placed in the four degree Celsius refrigerator, and the hood was again sprayed with 70% ethanol. The ethanol was dried, then hydrogen peroxide was sprayed in the hood. The hood was dried again, closed, and the UV light was turned on for one hour. The flasks were checked every 24 hours until confluency reached 60-75%, usually spanning five to six days.

4.2 Harvesting

When cells have reached confluency, they are ready to be split and used for experiments. To begin splitting, growing media and 1X phosphate saline buffer (PBS) were placed into the 37^0F water bath for 30 minutes. While the media and PBS heated, aseptic techniques were again performed. 70% ethanol was sprayed in the hood and the pipettes were sprayed before being placed into the hood. The cell flasks were retrieved from the 37^0C incubator and sprayed before entering the hood. After 20 minutes, 1X trypsin-EDTA was placed into the warm water bath. After 10 minutes, all contents were taken out of the water bath, sprayed and put inside the hood. A PBS-1X trypsin solution was made by adding 3mL of 1X PBS to 7mL of 1X-trypsin EDTA (1.5mL PBS and 3.5mL trypsin for one flask), and they were mixed by pipetting up and down. The solution was placed to the side, and the vacuum was used to discard of the previous media from each flask. Next, 10mL of PBS was added, and the flasks were rocked back and forth to wash all walls. The PBS was discarded, and 5mL of the trypsin-PBS solution was added to each flask. The flasks were then placed back into the incubator for 15 minutes. The cells were taken out every five minutes and tapped gently on the palm to detach the cells from the bottom. After 15 minutes, the cells were sprayed and placed back into the hood. Eight milliliters of fresh media was added to each flask, then the 13mL solution was transferred to a 15 mL Eppendorf tube. 20

mL of fresh media were added to the flasks. The flasks were labeled with initials, date, and passage 2 (or corresponding passage) and placed back into the incubator.

4.3 Counting and Labeling of Cells for Experimentation

To use the cells for implantation, the 15mL solutions were counted using a hemocytometer. 10uL of the solution was pipetted on to the hemocytometer, and each of the four quadrants of the hemocytometer were counted, divided by four and multiplied by a factor of 10^4. Next, this concentration was used to determine final volume needed to achieve a desired concentration of one million cells per milliliter. This final volume was pipetted from the solution into a new falcon tube, and centrifuged at 970 RPM for five minutes. The supernatant was discarded and the pellet of cells was broken up. Next, the final volume of R-1 media, media without FBS and P/S, was added to the cells. Next, DiI, red in color, was added to the solution (5uL per final volume), and pipetted up and down. The solution was put in the incubator for 15 minutes, and then centrifuged at 500 RCF for two minutes. The supernatant was discarded, pellet broken up, and the final volume, calculated using C1V1=C2V2, of fresh media was added to the cells. The cells are viable for up to four hours in the incubator.

4.4 Zebrafish Husbandry

Zebrafish are a great non-mammalian cancer model for several reasons. Zebrafish are widely used as a cancer model, because they can spontaneously develop almost any type of cancer that are present in humans (23). They also can mimic the severity of a cancer, such as a cancer that shows little metastasis versus a cancer that is highly metastatic. With this, the development **[Figure 8]** of the embryo is *ex-utero* and transparent which allows for observation of the cancer progression in a live model without harming or killing the embryo. Zebrafish are

also not adversely affected by genetic knockouts and expression of transgenes, which are necessary techniques in regulating levels of human ITGA6 expression (23). Zebrafish are also a better tool than other models such as mice, because they are cheaper and can be studied within a five day post injection period, while a mouse would have to take one to two months to fully develop the cancer. They can also be injected with fluorescent cells **[Figure 9]** to see the progression of cancer and ITGA6 via imaging software *in vivo*. In all, a zebrafish tumor xenograft model would be the most efficient model to study the progression and effects of prostate cancer and different levels of ITGA6 of a human.

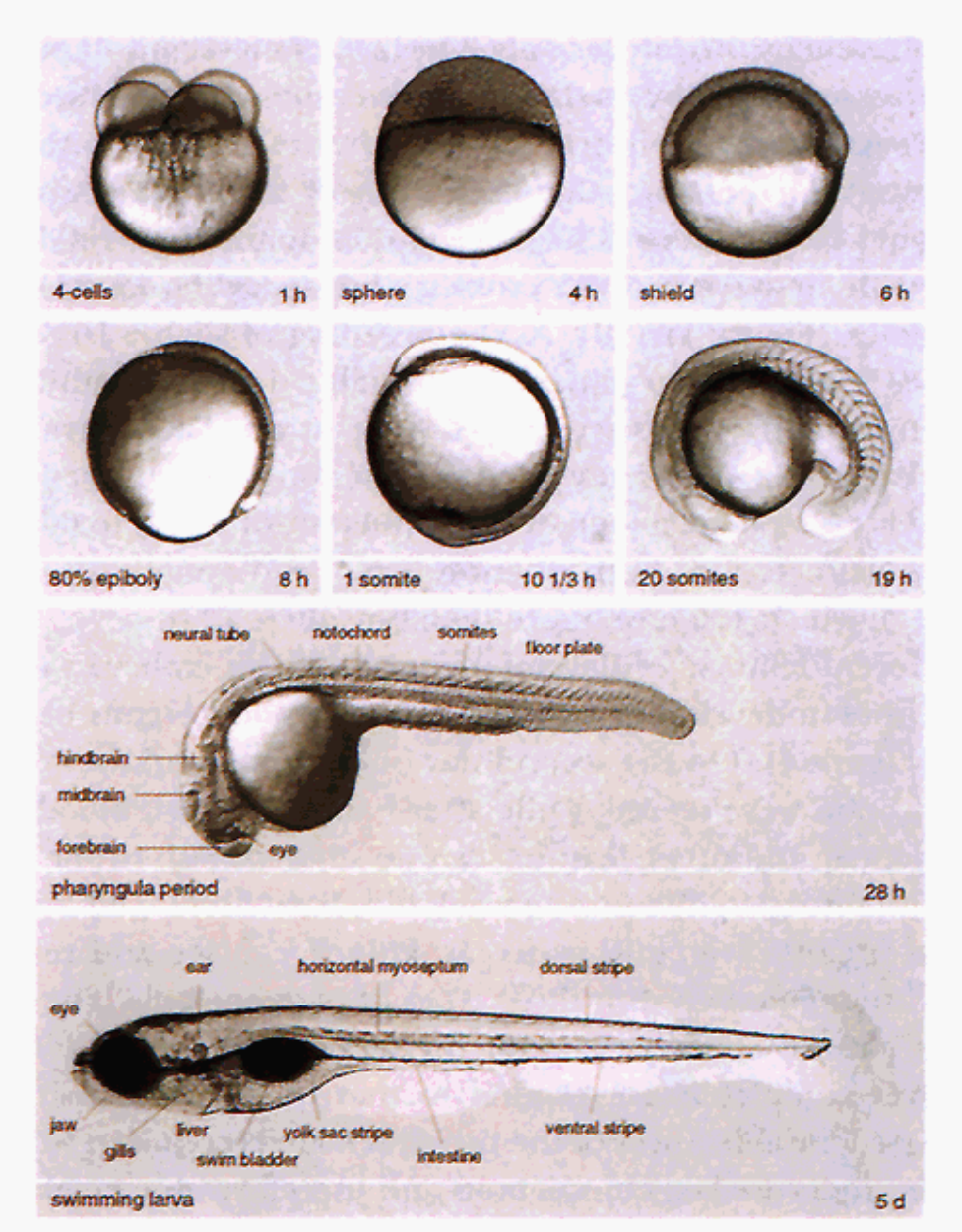


Figure 8. The figure shows the development of the zebrafish from 0hpf to five days old.

http://gos.sbc.edu/n/nv/graphics/nv6.gif

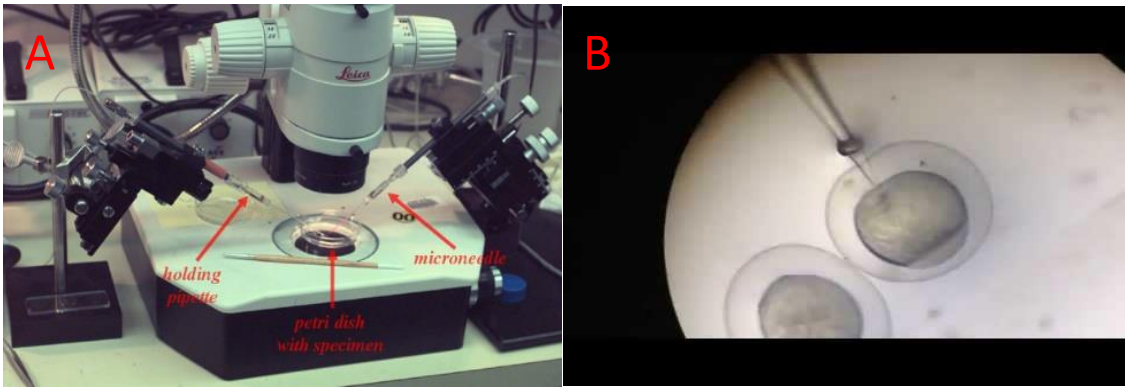


Figure 9. Panel A shows the injection microscope used in injecting ITGA6 RNA and human prostate cancer cells. Panel B shows where the ITGA6 RNA is injected into an embryo at 0hpf.

A. http://zfish.nichd.nih.gov/Intro%20Page/intro_pix/angio2.jpg

B. https://i.ytimg.com/vi/7N83MzkzIns/hqdefault.jpg

The zebrafish provided in the experiment were obtained from a self-maintained laboratory on campus. The fish were kept up to standard using approved protocols, and permission was obtained following an IUPAC proposal. To breed the fish to yield fertilized eggs, two separate tanks are set up, and eggs are collected the following morning. Transgenic fish of the strain, *Tg (fli l a: GFP)* were used in the making of the xenograft model, as the *fli la* expresses a green fluorescent protein in the blood vessels.

4.5 Manipulation of ITGA6

4.5.1 RNA Microinjections

Synthetic RNA constructs were made creating full length, mutated, and truncated forms of ITGA6. The sequences for each construct and pCS2+ vector **[Figure 10]** were determined *in vitro* and sent to Genscript® for

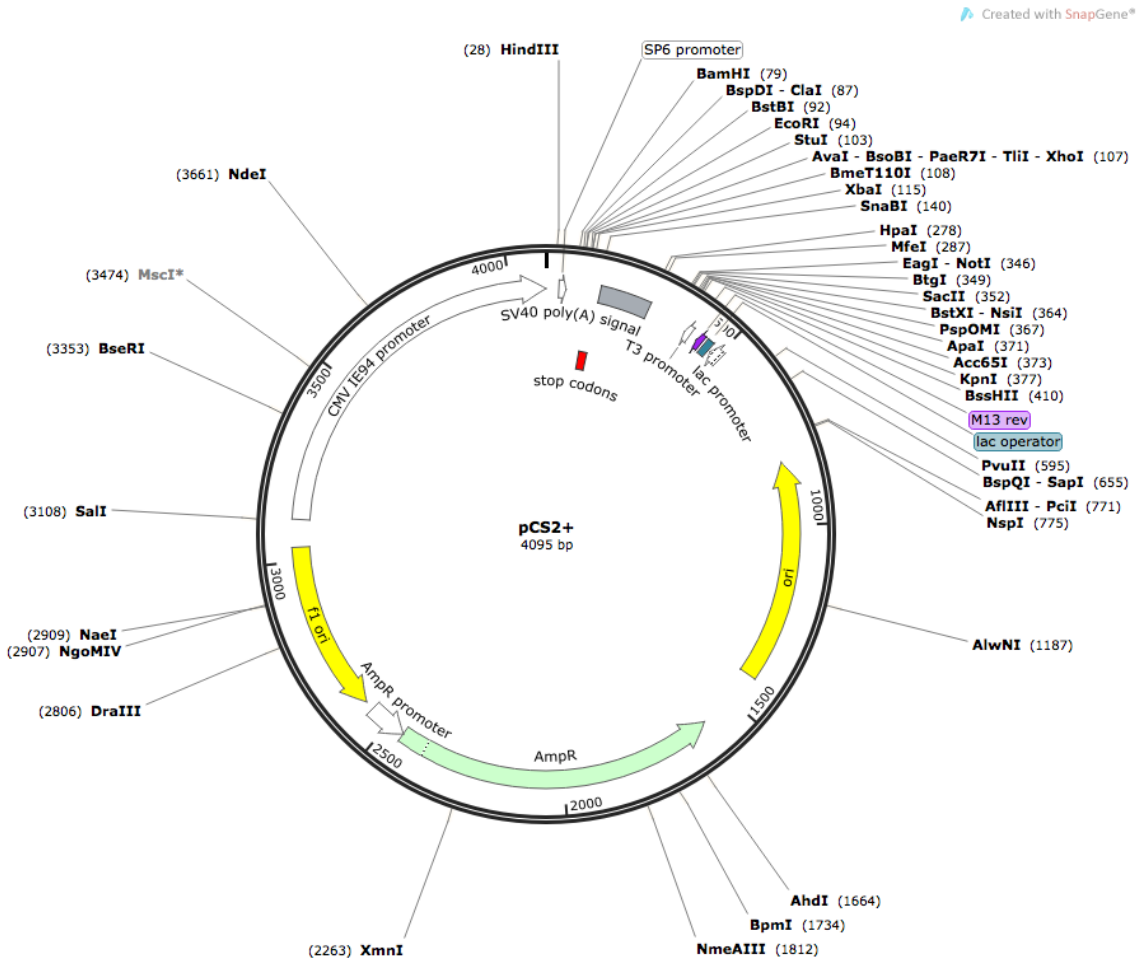


Figure 10. pCS2+ vector used to make ITGA6 constructs (full length, mutated, and truncated) using restriction enzymes ClaI, XbaI, BamHI, and EcoRI.

http://www.snapgene.com/resources/plasmid_files/image_consortium_plasmids/pCS2+/pCS2+_1x.png

synthesizing. In order to confirm the presence of each RNA using immunohistochemistry, a MYC tag was added to the C-terminus of the protein.

The three RNA injections were made to change the protein's normal structure and function. Full length RNA is altered to produce more of the protein on the surface of the cell. Truncated RNA mimics the *in vitro* cleaved protein. With this construct, the protein's transmembrane structure is found in the microenvironment of the tumor, instead of the plasma membrane of the cell. The last construct, mutated ITGA6 RNA, mimics the *in vitro* non-cleaved product. With this, the protein's site of enzymatic cleavage is mutated, allowing the enzyme to not be able to cut the protein. In this construct ITGA6 cannot be removed from the cell surface.

4.5.2 ITGA6 Knockdown

The "knockdown" approach used in the experiment was the morpholino technique. This approach leads to no gene expression of ITGA6 in the zebrafish cell. Morpholino powder was ordered from Gene Tools LLC, and resupsended in nuclease free water. The solution was then diluted in nuclease free water and dextran tetramethyl-rhodamine. The morpholinos were titrated and were then ready to be used in the experiment.

4.6 Microinjections

At zero hours post fertilization (hpf), the embryos are injected with their respective RNA or antisense RNA. Needles for injection are made using three and one half inch Drummond replacement tubes (#3-000-203-g/x) in a Model P-1000 Fleming/Brown Micropipette Puller. The solution for each construct is in 0.2% phenol red is pipetted into the needle, and oil is added to the needle to remove the remaining air. The tip of the needle was broken using micro tweezers and then injected, approximately three nanograms per nanoliter, into the embryo using a Nanoject II™ Auto-Nanoliter Injector. The embryos are then placed into the incubator, and will be ready in 24 hours for the second round of injections.

Next, the cultured prostate cancer cells will be used to inject into the embryo. The cells, at a concentration of 1,000,000 cells/mL, were loaded into the microinjection needle. Oil was then injected into the needle to remove air, and the tip of the needle was broken using micro tweezers. The zebrafish are then taken out of the incubator, and placed into tricane, a light anesthetic. The cancer cells are injected into the embryos, and then are washed with embryo medium to take away the effects of tricane. The embryos are then placed back into the incubator.

4.7 Observations and Confocal Imaging

4.7.1 Observations

After injections, the embryos are assayed for metastasis and angiogenesis for two days (observations beginning when embryos are 48hpf). To conduct the assays, the embryos are taken out of the incubator and placed in tricane, a light anesthetic. The embryos are observed for angiogenesis looking at the embryos under the GFP 470 filter. This filter is used, because the blue fluorescence allows the blood vessels to illuminate green, allowing for easy visualization of the red-labeled cancer cells. The Rhodamine filter is used to observe metastasis, as this filter fluoresces green and illuminates the cancer cells in red. The embryos are then taken out of tricane, washed with embryo medium, and placed back into the incubator. Assays are then performed again, approximately 24 hours later, under the same conditions.

4.7.2 Confocal Imaging

After observations on the second day, confocal imaging begins. Confocal imaging allows for both blue and green fluorescence enabling the visualization of the red cancer cells in the green blood vessels. Two embryos showing metastasis and angiogenesis and one without is imaged per treatment. To begin confocal imaging, each embryo is live mounted onto a slide.

Live mounting is conducted by placing the embryo in tricane, a light anesthetic, for approximately one minute, or until it is not moving. It is then pipetted into a drop of methyl cellulose that is in the depression of a depression slide. The embryo is manipulated so that the body is on its side, with the head pointing to the right. It is then placed in the laser confocal microscope. The microscope is set to the 488 wavelength, and a time lapse video of approximately one minute and a half is created. The embryo is washed with embryo medium, placed back into the incubator, and the next fish was imaged. After all imaging is conducted, the embryos are fixed with 4% Paraformaldehyde for preservation.

Results and Discussion

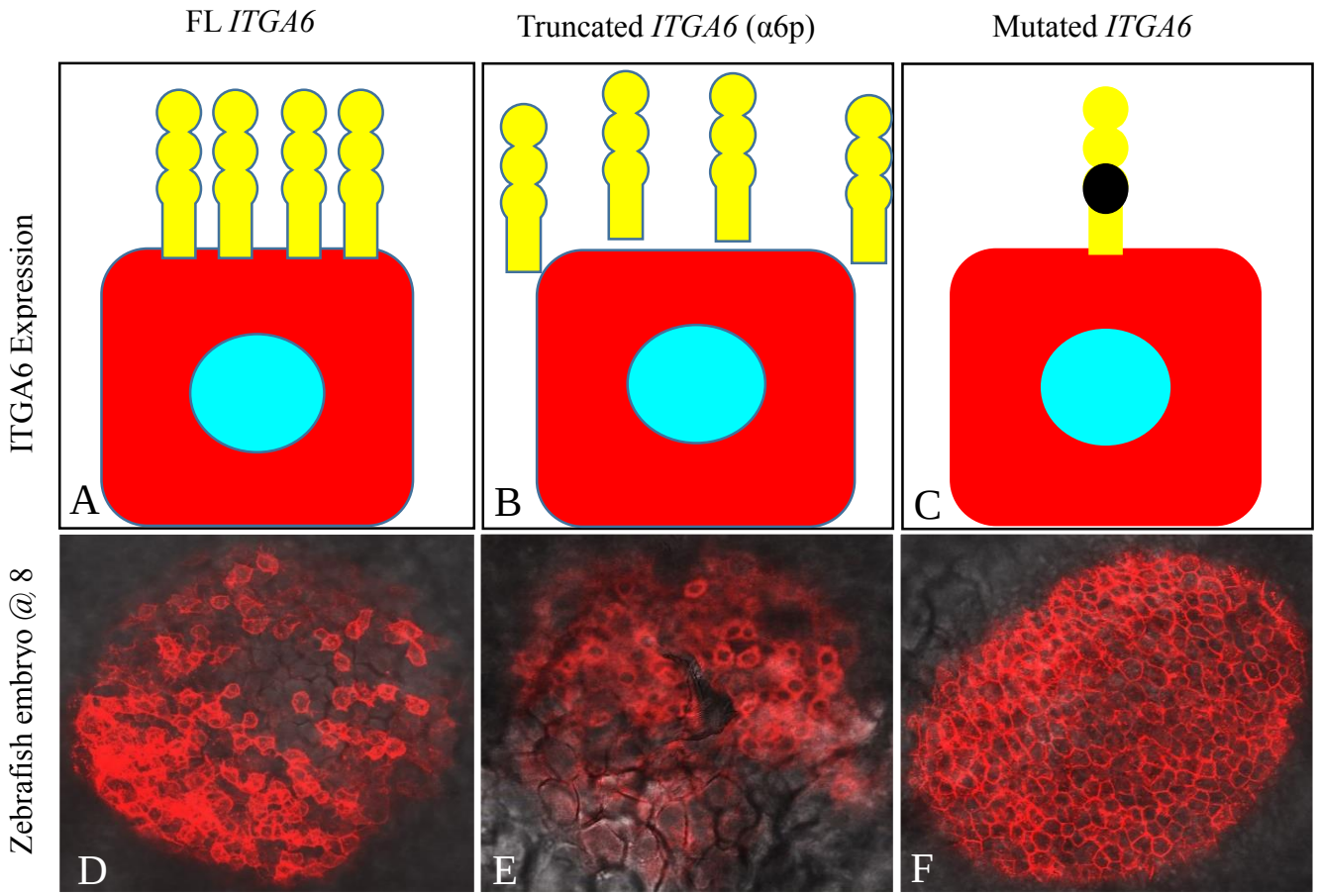


Figure 11. Panel A shows full length ITGA6 with increased protein on the cell surface. Panel B shows only the extracellular portion of ITGA6 was produced, and Panel C shows a mutation in the cleavage siting, allowing the protein to be non-cleavable. Panel D shows full length ITGA6 expression in an embryo at 8hpf. Panel E expresses the cleaved protein in the microenvironment of the 8hpf embryo, and Panel F shows the mutated ITGA6 only on the surface of the cells.

Using confocal imaging, we were able to visualize the expression of our ITGA6 constructs in the embryo. With the FL-ITGA6 embryos, we injected the full length ITGA6 RNA into the embryo at 0hpf. After eight hours, we could see the expression of the protein the embryo. We expected to see an increased amount of the protein in the embryo. We can see a lot of red, signifying the protein, indicating the RNA expressed the correct phenotype. In the α6p embryos, we expected to see less of the protein on the cell surface, and more in the

microenvironment, as we only expressed the extracellular portion of the protein. We saw this phenotype in the embryo. Lastly, we injected mutated ITGA6 RNA into the embryo, and we expected to see integrin on the surface of the cell only, as we mutated the cleaving site for the enzyme. We also saw this expression in the embryo. With these images, we were able to conclude that our RNA constructs are expressing the correct phenotype.

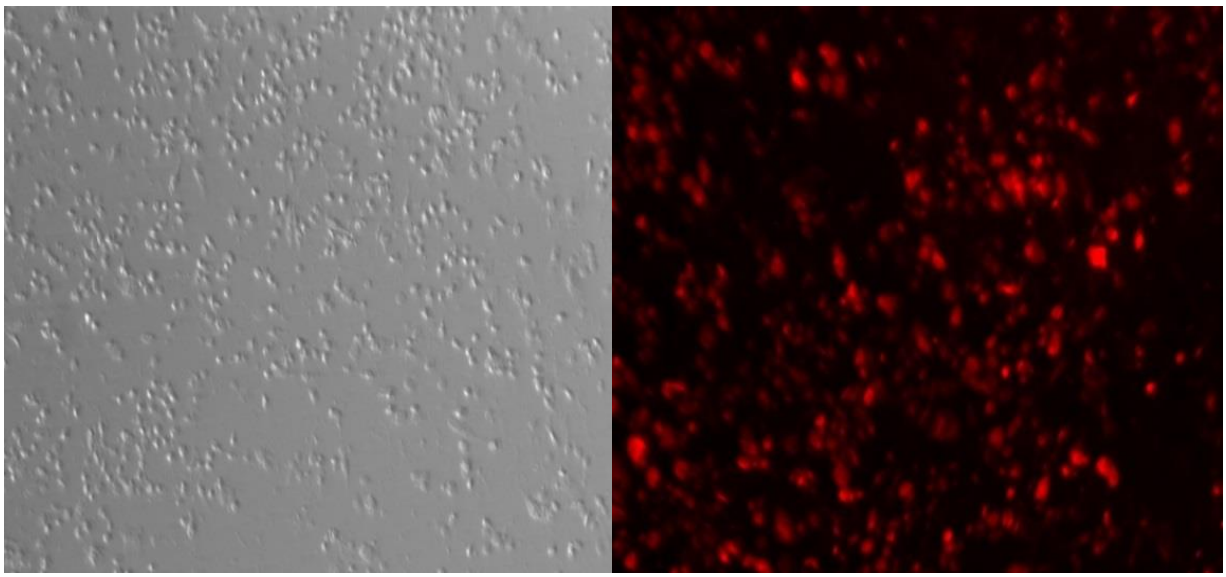


Figure 11. In this panel, image A shows labeled with DiI-red prostate cancer cells used to inject into the zebrafish to make the zebrafish tumor xenograft model. Image B shows the same cells under a fluorescence light.

In order to create our humanized zebrafish tumor xenograft model, it was imperative to use human prostate cancer cells to study the interactions between human integrin alpha 6 and human cancer cells. The red flourescences of the cells allowed for clear visiblity of the tumor, another feature important in study *in vivo* tumor progression.

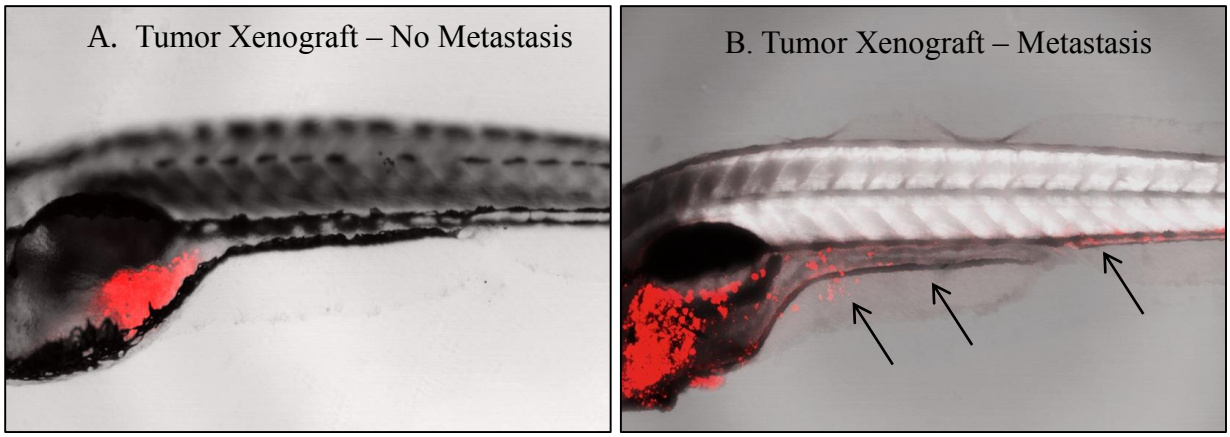


Figure 12. Image A portrays a fish with no metastasis. Panel B shows a fish with metastasis in the trunk.

The above embryos were imaged using confocal imaging. This was an important factor in being able to clearly distinguish between metastatic and non-metastatic characteristics of each embryo.

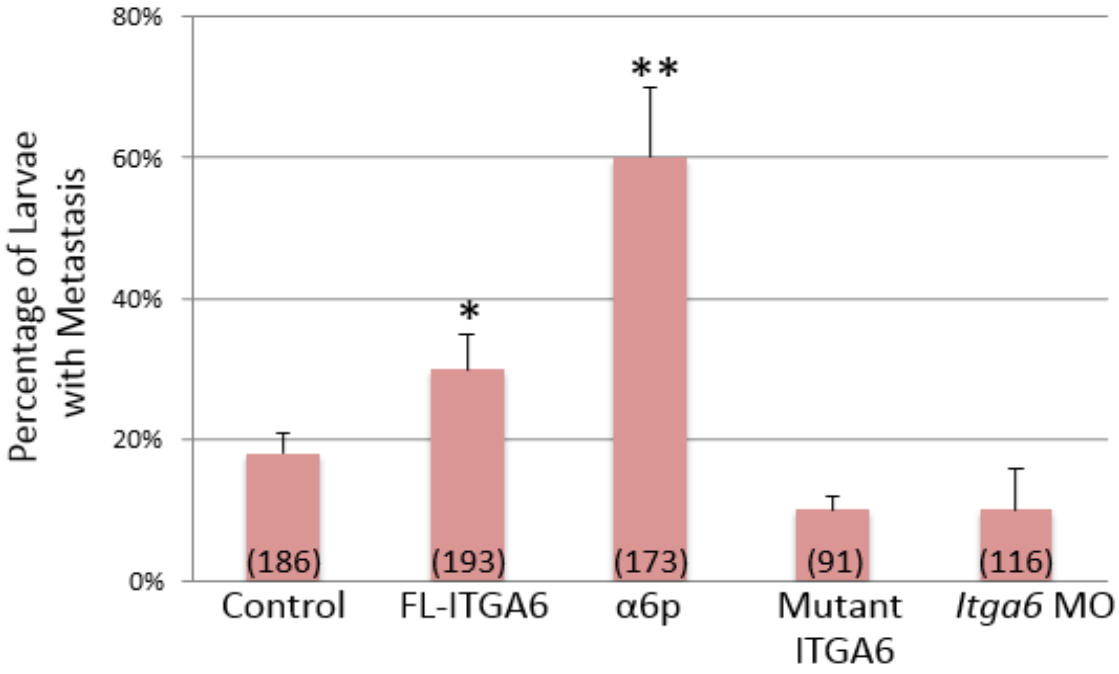


Figure 13. The graph shows the average amount (%) of metastasis of the embryos from each group. The number in bracket signifies how many total embryos were a part of the group. Each construct was compared to the control, using a t-test. Significant p-values of <0.01 were found in the FL-ITGA6 and α6p (truncated ITGA6) constructs.

The graph above measures the average percentage of tumor metastasis in five groups, with the numbers in parenthesis representing how many where in each group. The control group only received human prostate cancer cells as a measure to determine the metastasis of the cancer cells without manipulation of ITGA6. The FL-ITGA6 embryos expressed an increased amount of the protein on the cell surface. α6p fish expressed only the extracellular portion of the protein, and the mutant ITGA6 embryos expressed a mutation that did not allow for the enzyme to cleave, and therefore, was rooted to the cell surface. Lastly, we used a morpholino knockdown approach in the *Itga6* MO embryos to ablate the expression of integrin alpha 6. Using a t-test, we compared each group to the control. We found a significant statistical p-value of <.01 when comparing FL-ITGA6 to the control, suggesting that increasing ITGA6 induces tumor metastasis. However, when comparing α6p to the control, we found a smaller, more statistically significant p-value suggesting that truncated ITGA6 also induces tumor metastasis.

These results add clarity to what may be occurring in the microenvironment of the tumor. When a normal cell has undergone transformation into a cancerous cell, a change in the microenvironment allows the cell to lose its adhesion to the epithelial basement membrane. This change also enables the cell to invade a blood vessel and metastasis to a new tissue, creating secondary tumors. With these results, we suggest an increased amount or cleaved integrin alpha

6 protein in the microenvironment are one of the changes allowing these cells to lose adhesion, invade, and metastasize (16, 17, 18). With this, ITGA6 has been identified to be a biomarker predicting several different cancers such as stomach, oral, prostate, and several more cancers (24,25,26).

Looking further into the microenvironment of tumors, we speculated that signaling is the major factor that allows for the changes to be able to lose adhesion and metastasizes. When there is an increased amount of cleaved ITGA6 on the cell, we believe this allows for first, outside-in then inside-out signaling, that allows for these tumorigenic properties. To begin, we believe that outside-in signaling begins the changes. This type of signaling allows the cell to sense and respond to the changes in their environment (27). The signaling begins with a specific ligand-binding interaction, resulting in changes in the shape of the integrin (28). This changes allows for modified protein interaction sites and increased ligand affinity, transmitting the signal into the cell (27,29). We believe the signal the cell receives contains information that changes the programming of the cell, leading to changes in the morphology and behavior of the cell. A change in the morphology allows for an increased amount of membrane protrusions, making the cell spiked and able to penetration surfaces (29). The change in behavior allows the cell to be more aggressive and to gain increased motility, important factors in invading and moving through a blood vessel.

Next, inside-out signaling occurs. This signaling pathway accounts for the ability for the cell to regulate the activity of its integrin (27,28). Inside-out signaling occurs when other

receptors send signals, activating intracellular signaling pathways. After this, the cytoplasmic domain of the beta subunit binds specific cofactors, talin and kindlin, in the cell which activates ligand-induced bind sites on the extracellular portion of the protein (28,29). We believe that a specific substrate or interaction occurs which releases the enzymes into the extracellular matrix. We focused on a particular group of enzymes, metalloproteinases (MMPs), which allows the cell to degrade the extracellular matrix as it migrates and metastasizes (27,28,29)

To conclude, we believe that when a cell undergoes transformation into the cancer cell, changes in integrin alpha 6, that either upregulates it or cleaves it, allows the cell to lose cell adhesion to its tissue. Next, both outside-in and inside-out signaling pathways allow for the cell to become more aggressive, and move through the extracellular matrix to invade blood vessels and metastasize. With this, ITGA6 can be used a biomarker to detect the probability of these changes occurring. We believe that combining ITGA6 genetic testing with early diagnosis of cancer gives the best chances of completely eradicating or killing the cancer in its early stages. In the future, we would like to conduct drug assays with ITGA6 to devise a preventative strategy to cancer.

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