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UNDERSTANDING THE MECHANISTIC ROLES OF *INTEGRIN ALPHA 6* IN TUMOR
DEVELOPMENT USING A HUMANIZED ZEBRAFISH (*DANIO RERIO*) MODEL SYSTEM

By
Ashley B. Williams

THESIS
Submitted in partial fulfillment of the requirements
for the degree of Bachelor of Science in Biology with Honors
in the College of Science and Mathematics of
Georgia Southern University

Research Mentor:
Dr. Vinoth Sittaramane
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ABSTRACT

Present day cancer incidence and mortality rates indicate the need for effective cancer diagnostic tools and targeted cancer therapeutic strategies. Recent studies have focused on the biological pathways of cells and tumor microenvironments to identify putative biomarkers and potential drug targets as diagnostic and therapeutic tools. Human integrins, adhesion receptors, have become the focal points in these studies, specifically Integrin Alpha 6 (ITGA6) which has been implicated in major tumor progression roles: metastasis and angiogenesis. These characteristics make ITGA6 an excellent candidate for potential drug or diagnostic target, however, the mechanism by which ITGA6 facilitates tumor progression remains unclear. Cell culture studies have indicated ITGA6 could be cleaved extracellularly to increase metastasis but, zebrafish with organismal structures and vascular network, present a complete *in vivo* model to track metastasis. Our previous studies indicate that truncated ITGA6 overexpression significantly upregulates tumor metastasis compared to full-length ITGA6 overexpression. Similarly, mutated ITGA6 significantly decreases tumor metastasis. These results suggest that cleaved ITGA6 increases tumor metastasis, potentially aiding in extracellular matrix remodeling. In this study, we aim to identify the cellular role of ITGA6 by transplanting ITGA6 siRNA and DNA transfected PC3 cells into zebrafish tumor xenografts. We anticipate these experiments will help establish the cell and non-cell autonomous roles of ITGA6 during tumor development. Further, we expect to use high-resolution imaging techniques to track the migration of single cancer cells in an *in vivo* system to understand the dynamics of metastasis.

Key Words: Cancer, Integrin Alpha 6, Metastasis, Humanized Zebrafish

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Dr. Vinoth Sittaramane

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CHAPTER 1 INTRODUCTION

1.1 Cancer

Cancer has been classified as a global epidemic, as present-day cancer incidence and mortality rates continue to rise (WHO, 2017). Cancer is a class of diseases characterized by the fundamental abnormality of uncontrollable cell division. Cancer cells are able to ignore the molecular signals which control normal cell growth and behavior, thus allowing them to proliferate in an unregulated fashion. In addition to sustained proliferative signaling, cancer cells acquire the ability to induce angiogenesis, resist apoptosis, metastasize and invade, as well as the ability to avoid immunological attacks (Hanahan & Weinberg, 2011; Figure 1.1). To date, over 100 different types of cancer have been identified, as abnormal proliferation can occur in all cell types. Due to the high level of variability amongst cell types, different types of cancers behave differently at the cellular and molecular levels.

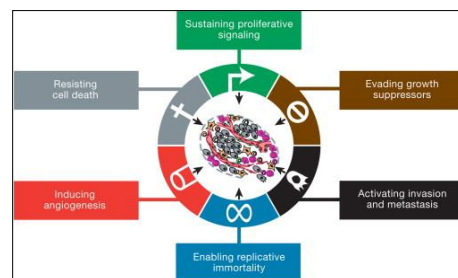


Figure 1.1: Cancer Hallmarks. Cancer cells acquire new properties, or hallmarks, which allow for tumor growth and development as well as metastatic invasion, (Hanahan and Weinberg, 2011).

Another source in cancer type variability is the etiology of cancer, which is vast and includes environmental factors, lifestyle choices, and genetic mutations. Environmental exposure to carcinogens is a driving force in cancer progression and tumor development (Parsa, 2012). Several external stimuli have been shown to be associated with cancer development, and have become classified as risk factors. More specifically, aging, family history, smoking, alcohol, radiation, chemical exposure, viral infections, hormone therapy, diet/obesity, and pollution have all been linked to cancer development (Parsa, 2012; Rossi, et al. 2008; Hassan, et al., 2008; Blackadar, 2016). Genetic causes of cancer have been well-studied and well described; numerous DNA alterations have been observed in almost every tumor type (Futreal, P.A., et al., 2004). Genetic mutations are considered the main mechanism by which cancer presents, as the diverse array of cancer causing factors ultimately act on cancer genes or proto-oncogenes (Griffiths, et al., 2000). These actions are typically in the form of cancer gene mutations which activate proto-oncogenes to their oncogenic state (Griffiths, et al., 2000). The conversion of proto-oncogenes to oncogenes

is facilitated by a variety of environmentally or spontaneously induced mutations (Chial, 2008; Griffiths, et al., 2000). Tumor suppressor genes are responsible for encoding proteins which inhibit cancer cell proliferation. Genetic mutations in tumor suppressor genes has also been identified as major mechanism in tumorigenesis, and several studies have described this class of tumor-associated genes (Rivlin, et al., 2011; Lodish, et al., 2000, Eshghifar, et al., 2017; Stewart, et al., 2014).

The term ‘tumor’ refers to an abnormal mass of tissue which may contain solid tissue or fluid. Tumors are often associated with cancer, however not all tumor growths are cancerous. Tumors may be classified as benign, non-cancerous, or malignant, cancerous and typically metastatic. Malignant tumors are monoclonal and develop over a significant of period time in a process called multistage carcinogenesis (Buffler, P., et al., 2004; Figure 1.2). The inception of tumor development can be initiated by genetic mutations caused by the previously described factors (i.e. chemicals, radiation, viruses, etc.). Tumorigenesis, or the process of tumor development, has been categorized into three stages: initiation,

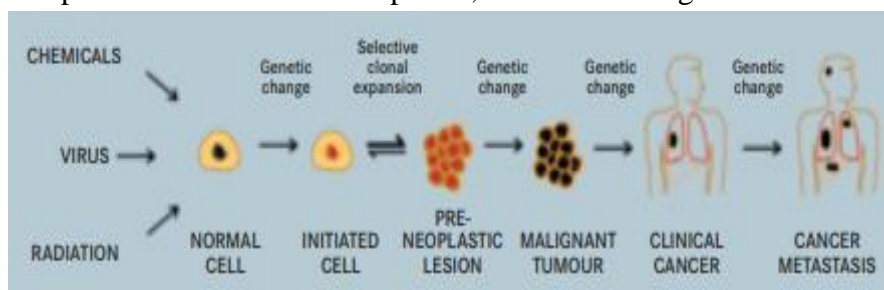


Figure 1.2: Carcinogenesis is a multistep process: Cancer causing agents transform normal cells to ‘initiated’ cancer cells via genetic mutations. Initiated cells continue to grow and develop as genetic changes occur, ultimately resulting in formation of a metastatic

progression, and metastasis (Buffler, P., et al., 2004 & Wang, M. et al., 2017).

Initiation refers the point at which normal cells are altered by carcinogens such that their genetic composition and cellular behaviors are changed. Progression encompasses the processes involved in tumor development including uncontrollable cell division, angiogenesis, and invasion. Lastly, metastasis describes the stage in which tumor cells spread to distant sites in the body. Briefly, these stages are a result of three main causes: the activation of proto-oncogenes, and the inactivation of both tumor suppressor and genomic stability genes (Buffler, et al., 2004).

1.1.1 Tumor Developmental Processes

As mentioned in the stages of tumorigenesis, tumor cells have the ability to penetrate and circulate through the vascular system ultimately establishing at another site (Buffler, et al., 2004 & Nishida, et al., 2006). The extension of the vascular network is a crucial component in the metastatic spread of tumor cells (Folkman, 2002 & Nishida, et al., 2006). The process of developing new blood vessels from preexisting blood vessels is referred to as angiogenesis. The vascular support provided by angiogenesis and lymphangiogenesis, the formation of lymphatic vessels, allows for metastasis and for an adequate supply of oxygen, nutrients, and waste removal (Nishida, et al., 2006 & Ziyad, et al., 2011). Angiogenic growth factors are a class of molecules which act as biological signals to mediate cross-talk between endothelial cells (EC) and tissue cells (Crivellato, 2011). A delicate balance of pro- and antiangiogenic factors is altered to favor angiogenesis in the tumor microenvironment (Merchan, et al., 2015 & Gordon, et al., 2009). Pro-angiogenic growth factors, like vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor-2 (FGF-2), or basic fibroblast growth factor (bFGF), and cytokines

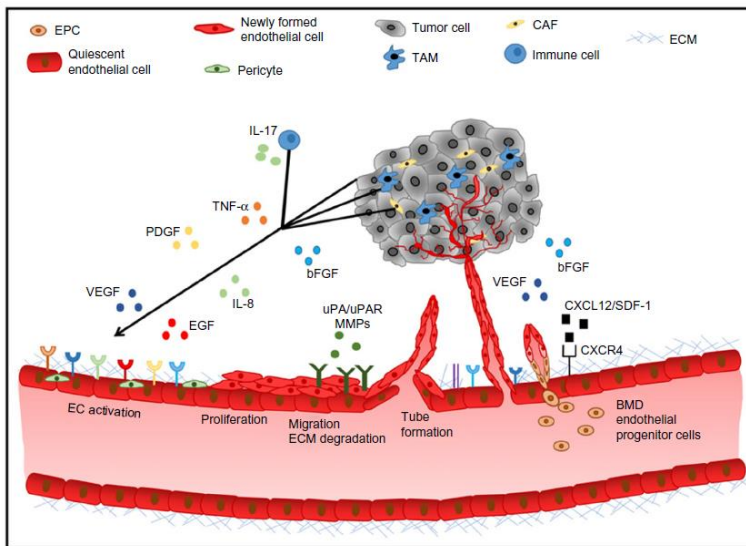


Figure 1.3: Tumor Angiogenesis cascade: Tumor cells secrete angiogenic factors and cytokines to stimulate endothelial cells (ECs; red). EC stimulation results in the production of proteases which degrade ECM proteins and allow for proliferation. Tube formation results from the binding of angiogenic factors to appropriate receptors and occurs due to a loss of pericytes (green) (Merchan, J., and Bejarano, T., 2015).

stimulate ECs to produce proteolytic enzymes (Campbell, et al., 2010; Figure 1.3). The production of proteases, such as metalloproteinases (MMPs), results in the degradation of the extracellular matrix (ECM) and basement membrane, proliferation of

ECs and in cell migration and invasion (Campbell, et al. 2010). Angiopoietin-2 (Ang-2), a proangiogenic factor,

also works to loosen the ECM and to detach pericytes. A pericyte deficiency paired with a degraded ECM gives rise to the formation of a new capillary tube (Gordon, et al., 2009). Blood vessel maturity is mediated by the binding of angiogenic growth factor, Ang-1, to

its receptor, Tie-2, stimulating pericyte recruitment to the newly formed tubes. Tumor angiogenesis however, tends to produce fairly immature blood vessels due to the oxygen-deprived tumor regions. These conditions require a continuous cycle of angiogenesis in the tumor microenvironment which further stimulates tumor growth and development (Gordon, et al., 2009).

Tumor metastasis is the process by which tumor cells invade the circulatory system and spread locoregionally or systematically to distinct organs where they establish secondary and tertiary tumor sites. Despite the fact that metastasis is responsible for 90% of all cancer related deaths, effective therapeutic strategies against metastatic spread remain unknown (Seyfried, 2014). Tumor metastasis can be categorized into four stages: invasion, intravasation, extravasation, and proliferation/colonization in distinct organs (Figure 1.4). Cell adhesion plays a major role in metastasis as the loss of cell-cell adhesion allows tumor cells to dissociate from the primary tumor site (Martin, et al., 2013). Adhesion molecules are not completely responsible for metastasis; however, they mediate important and required steps in the metastatic process. These molecules facilitate cell-cell interactions (homotypic adhesion) as well as cell-ECM interactions (heterotypic adhesion) (Harlozinska, 2005).

The detachment of a tumor cell from its primary site is an example of homotypic adhesion, and marks the first stage of metastasis, tumor invasion. Tumor cells acquire motile phenotypes and are able to migrate as single cells or as epithelial sheets. Single migrating cells may be characterized as amoeboid-like, round-bodied cells with diverse protrusion types, or as mesenchymal, elongated cells with long protrusions (Glark, et al., 2015). Collective migration is accomplished by cells that maintain cell-cell adhesion and may present in a linear lineup or as sheets. Collective groups of cells may also display epithelial or mesenchymal phenotypes (Glark, et al., 2015). In preparation for metastasis, tumor cells show changes in their plasticity as demonstrated by the classic epithelial to mesenchymal

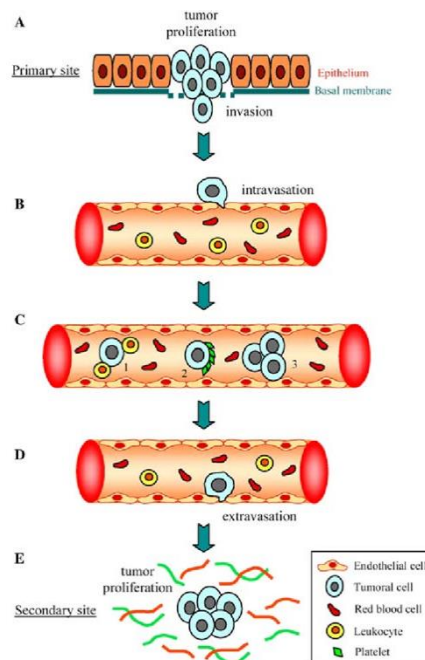


Figure 1.4: Stages of Metastasis: Tumor cells from the primary site invade and intravasate the blood vessels. Tumor cells then extravasate and establish at a secondary site (Gout, S., Tremblay, P., Huot, J., 2007).

transition (EMT), the collective amoeboid transition (CAT), and the mesenchymal to amoeboid transition (MAT) (Zijl, et al., 2011).

The epithelial-mesenchymal transition is the process by which polarized epithelial cells undergo a series of biochemical changes which allow for the acquisition of

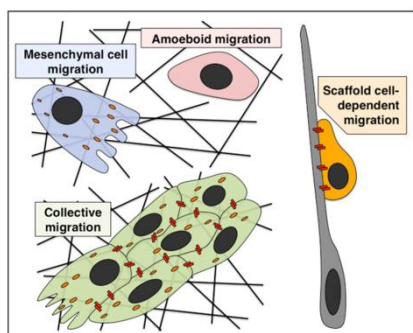


Figure 1.5: Cell migration types: Cells may undergo EMT maintaining cell-ECM adhesions (orange dots), MAT (loss of cell adhesion), or migrate collectively maintaining cell adhesions (red rectangles) and cell-ECM adhesions. ECMs are shown as black lines (Kawauchi, T., 2012).

mesenchymal phenotypes such as resistance to apoptosis, cell invasion, and enhanced cell migration (Kalluri, et al., 2009; Figure 1.5). A complete EMT is marked by basement membrane degradation and by the migration of newly formed mesenchymal cells (Kalluri, et al., 2009). The transition of cancer cells to the mesenchymal type has been termed the “path generating” mode (Hecht, et al., 2015). Cells that undergo the mesenchymal to amoeboid transition (MAT), thus adopting an amoeboid type enter a “path finding” mode (Hecht, I., et al., 2015). Unlike,

mesenchymal cells, amoeboid cells are unable to degrade the ECM but are still effective in cell migration due to acquired flexibility traits and loss of cell adhesion (Hecht, et al., 2015). The amoeboid state seems to be favored in times of metabolic stress suggesting that it may be more energetically conservative compared to the mesenchymal state (Hecht, et al., 2015). These changes in plasticity are extremely important for single-cell motility and have major implications for the metastatic ability of tumor cells. Moreover, these changes in plasticity allow cancer cells to navigate and migrate through different ECM environments. The process of cancer cell invasion is critical to the metastatic cascade and paves for the way for intravasation and extravasation.

The process of establishing a network of microvasculature via angiogenesis allows cancer cells to access vessels readily. Newly formed capillaries are comprised of disjoint basement membranes and are leaky, allowing tumor cells to infiltrate easily compared to mature blood vessels (Weidner, 2002). Intravasation is the entry of tumor cells into the circulatory system via blood or lymphatic vessels and can occur via active or passive mechanisms. Evidence for passive intravasation suggest that cells are propelled through the circulatory system by forces resulting from restricting blood vessel size and by cells

growing in a confined space (Bockhorn, et al., 2009). Active intravasation may also occur as the expression of matrix metalloproteinases (MMPs) has been shown to digest ECM proteins and to degrade vessel basement membranes (Bartolome, et al., 2004 & Bockhorn, et al., 2009). Once inside the host's circulatory system, cancer cells must survive the haemodynamic stressors and the response of the immune system. Extravasation occurs when tumor cells reach the desired point and initiate biochemical interactions with endothelial cells (Martin, et al., 2013). This resulting adhesion penetrates the endothelium allowing the cells to establish and proliferate at the secondary site (Martin, et al., 2013).

1.1.2 Breast and Prostate Cancer

Breast cancer is the leading cause of cancer death in United States women. Breast cancer refers to abnormal cell division in the breast tissue, typically originating in the milk ducts

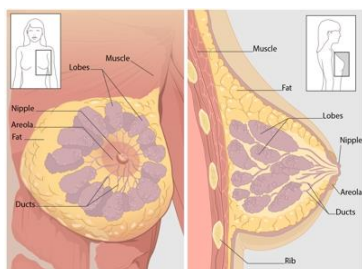


Figure 1.6: Female Breast Anatomy. The image depicts the anterior and cross-section view of the breast. Breast cancer is most commonly formed in the ducts and lobules (CDC, 2017).

or the lobules (Sharma, G.N., et al., 2010; Figure 1.5). The breast is composed of two tissue types, glandular tissue and stromal tissue. Stromal tissue refers to the supportive-connective tissue regions located inside the breasts.

Glandular tissue contains the lobules which are responsible for producing milk. There are several types of breast cancer, though invasive ductal carcinomas (IDC) and invasive lobular carcinomas (ILC) remain the most common. IDC accounts for 80% of all breast cancer diagnoses and ILC

accounts for approximately 10% (Sharma, et al., 2010). In addition to the standard causes of cancer, breast cancer may be caused by hormone replacement therapies (e.g. birth control pills), significant family history, and a previous history of breast cancer (i.e. a woman who has developed breast cancer previously). Breast cancer patients often undergo breast reduction surgery as a viable treatment option. Surgical options include breast conserving-surgery, Lumpectomy, Quadrantectomy, and Mastectomy. In breast-conserving surgery, only the tumor and a sample of normal tissue are removed, whereas a mastectomy removes all breast tissue (Sharma, et al., 2010).

Prostate cancer is a malignant cancer that forms in the prostate gland and mainly affects American men. Though several factors including age and ethnicity/race affect prostate cancer susceptibility, it can be estimated that 1 in 9 men will be diagnosed in his lifetime (American Cancer Society, 2018). The prostate is located between the bladder and penis and is responsible for producing the alkaline seminal fluid expelled during ejaculation (Figure 1.7). Androgens are a group of hormones required for male sexual development and have been implicated in prostate cancer progression. Specifically, mutations in androgen receptors have been shown to decrease ligand specificity (Heinlein, et al., 2004; Brooke, et al., 2009; Lallous, et al., 2016). The most common type of prostate cancer is adenocarcinoma, a malignant cancer that originates in the glandular tubes and duct lining. Other types of prostate cancers include sarcomas, small cell prostate cancer, transitional cell carcinomas, and neuroendocrine tumors. Similar to breast cancers, prostate cancers can be highly metastatic, and treatment options include surgery, hormone therapy, or chemotherapy.

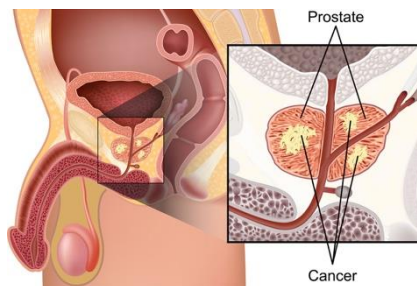


Figure 1.7: Anatomy of the Male Reproductive System. The prostate gland is responsible for producing seminal fluid and projecting the fluid through the urethra during ejaculation. Here, the prostate gland is shown with cancerous tissue (yellow) (Chundsell Medicals, 2017).

1.1.3 Cancer Diagnostics & Therapeutics

Cancer diagnostics are a critical component of cancer care as they allow physicians and scientists to not only detect, but monitor and track disease progression. Commonly used diagnostic methods include biopsy, imaging procedures (e.g., X-rays, PET/CT, MRI, etc.), laboratory tests (e.g., blood and urine), physical examinations, and genetic tests. Early detection of cancer significantly increases one's chances for successful treatment and survival, resulting in an emphasis on cancer screening and surveillance research. Biomarkers, or indicators of disease, are commonly used for screening, staging, diagnosing and monitoring cancer (Schiffman, et al., 2000). Despite these current diagnostic methods, the need for an early cancer detection test which exhibits a high efficacy and can be applied across multiple types of cancer, still exist.

Present day cancer incidence and mortality rates are indicative of ineffective cancer therapeutics. Despite the increase in cancer mortality over the decades, several cancer

treatment options exist. Specifically, cancer patients are recommended to use a combination of current cancer treatments: Surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplants, and precision medicine. By far, radiation therapy, surgery, and chemotherapy are the most common cancer treatments. Radiation therapy is the application of ionizing, or high-energy, radiation to the DNA of cancer cells to inhibit cell proliferation, ultimately resulting in cell death (Baskar, et al., 2012). Chemotherapy refers to the use of cytotoxic agents which inhibit microtubule function, protein function, or DNA synthesis causing an inhibition of cellular growth or cell death (Vanderbilt-Ingram Cancer Center, 2016). Chemotherapeutics typically target the cell cycle heavily relying on the differences in proliferation speeds that exist between normal and cancerous cells. Obstacles to chemotherapy include the hypoxic tumor microenvironments, the heterogeneity of cancer, and drug resistance (Sak, 2012). In addition, surgical techniques are often used to remove malignant growths, prevent cancer (i.e. mastectomy), diagnose and grade cancer, as well as repair tissues damaged by other treatment types. Though each of these treatment options has demonstrated marginal success, they are each accompanied by host of side effects, and are unable to cure cancer.

1.2 Integrins

Integrins are a superfamily of transmembrane proteins and cell adhesion receptors that facilitate cell-cell and cell-ECM communication. Integrins are heterodimeric molecules consisting two non-covalently bound glycoproteins, the α and β subunit (Figure 1.8). At least 18 alpha and eight beta subunits have been identified in humans, each combining to form 24 distinct integrin heterodimers (Betsy, et al., 2010 & Takada, et al., 2007). Further, integrins are responsible for mediating adhesion to the basement membrane, cell migration through ECM, the formation of ECMs, bacterial and viral entry during infectious diseases, and the formation of platelet aggregates (Danen, 2013). The extracellular region of integrin proteins binds to ECM

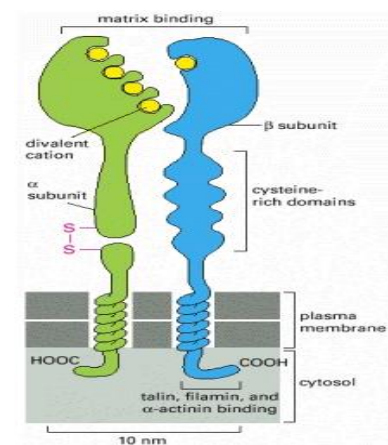


Figure 1.8. Integrin Protein Structure. Integrins are heterodimeric molecules composed of an alpha and beta subunit. These proteins are considered transmembrane linkers as they bind to the matrix (extracellularly) and the cytoskeleton (intracellularly). The integrin shown is a fibronectin receptor which displays an alpha chain that has been cleaved into two portions, and a beta chain with a cysteine-rich domain (Alberts B, Johnson A, Lewis J, et al., 2002).

glycoproteins like laminins, collagens, and fibronectins. Integrins also function as transmembrane linkers between the ECM and the actin cytoskeleton. In addition, these cell adhesion receptors are able to modulate signaling cascades which regulate cell motility, survival, and differentiation (Danen, 2013). The role of integrins in the regulation of cell motility through ECM interactions has been well studied and is the basis for implications of integrins in cancer progression.

Several studies have demonstrated the roles of integrins in tumor development, highlighting their roles in ECM remodeling, cell motility/invasion, tumor cell survival and modulation of metastatic signaling (Schaffner, et al., 2013; King, 2008; Ganguly, 2013; Aoudjit, F., et al., 2012; Chen, et al., 2014). Integrins receive environmental cues from interactions with the ECM and depending on the signal are able to promote cell survival or cell death. This is mainly regulated by integrin ligation; ligated integrins tend to relay survival messages while unligated integrins promote apoptosis (Degrosellier, et al., 2015). In addition, integrin adhesion to the ECM mediates tumor cell invasion by controlling ECM protein expression and various signaling pathways (Degrosellier, et al., 2015 & Koistinen, et al., 2013). Integrins are able to induce the expression of ECM proteases, namely MMPs, and are able to control MMP activity through direct binding (Koistinen, et al., 2013; Kessenbrock, et al., 2010; Yue, et al., 2012). Specifically, the production of MMPs is the result of the cytoplasmic binding of integrin subunits to intracellular proteins. The combination of ligated integrins and an intact cytoskeleton promotes the activation of focal adhesion kinase (FAK) which is able to bind to integrin subunits β_1 , β_2 , and β_3 (Koistinen, et al., 2013). Ligated integrins also enable FAK autophosphorylation triggering the MAPK/ERK/JNK pathway and MMP production (Koistinen, et al., 2013). The ability of integrins to modulate cell signaling and MMP expression serves as one example of integrin mediated cell invasion.

1.2.1 Integrin Signaling

As previously discussed integrins are essential for adhesion to the ECM and for signal transduction. These proteins are able to transmit information bidirectionally through two modes: “inside-out signaling” and “outside-in signaling” (Figure 1.9). Outside-in signaling refers to the binding of ECM ligands to the extracellular domains of integrins which

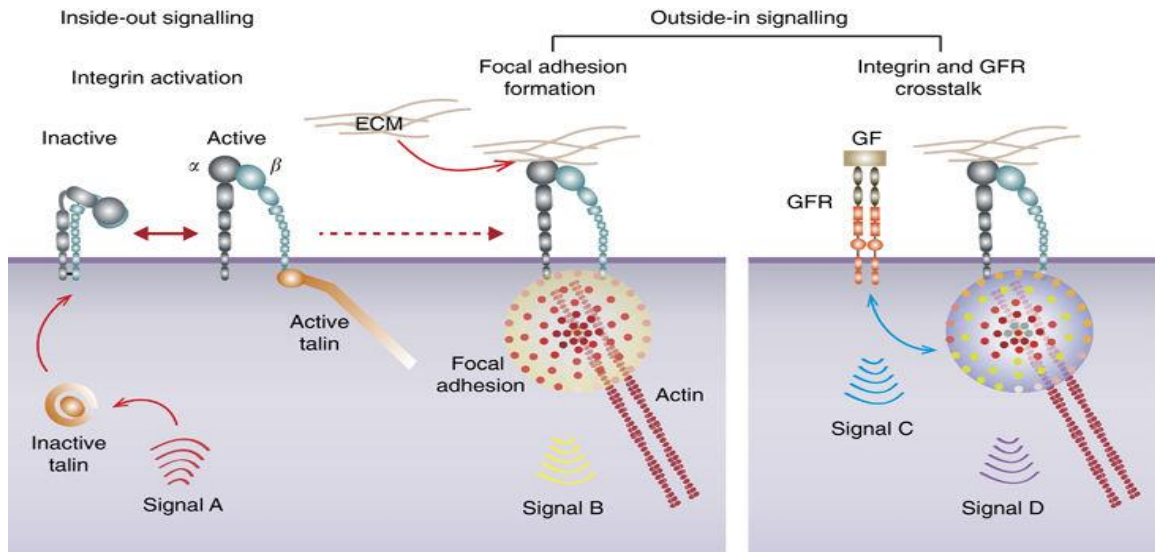


Figure 1.9: Integrin Signaling Modes. Inside-out signaling occurs via the activation of talin by an intracellular signal (signal A). Talin binds to the β subunit of the integrin and facilitates an unclasp event allowing for ECM ligand binding. The binding of an ECM ligand stimulates outside-in signaling by inducing the formation of focal adhesion and cytoskeletal reorganization (signal B). Growth Factor Receptors (GFR) can elicit signals (signal C) or participate in crosstalk with integrins to transmit information (signal D) (Hamidi, H., et al., 2016).

activates FAK. The autophosphorylation of FAK initiates a signaling cascade that ultimately transmits external signals to the inside of a cell (Menter, et al., 2012). Inside-out signaling is initiated intracellularly and depends on activator proteins, talin and kindlin, binding to the cytoplasmic tails of integrin β subunits (Menter, et al., 2012 & Yue, et al., 2012). When the cell is stimulated it induces a conformational change which exposes the head region of the talin protein. The head domain of talin is then free to bind to the β cytoplasmic tail. This binding displaces the α tail and results in an unclasp event between the non-covalently bound subunits (Qin, et al., 2004). These intracellular events ultimately lead to the activation of the adhesion receptor and an increase in affinity for ECM ligands. In Outside-in signaling, the binding of an ECM ligand triggers the recruitment of adaptor and signaling proteins inside the cell inducing focal adhesion formation and reorganization of the actin cytoskeleton (Hamidi, et al., 2016). Additionally, growth factor receptors can generate signals or participate in crosstalk with integrins to

transmit messages in response changes in the extracellular environment (Hamidi, et al., 2016).

1.2.2 Integrin Alpha 6 in Cancer Progression Roles

Integrin Alpha 6 (ITGA6) is the alpha subunit of two proteins, integrin $\alpha 6 \beta 1$ and integrin $\alpha 6 \beta 4$, which serve as laminin receptors. Integrin $\alpha 6$ is required for the normal embryonic development of vertebrates and invertebrates and plays an important role in epithelial regeneration and wound healing in adults (DiPersio et al. 1997; Cress et al., 2017; Zehr & Sittaramane, 2015; Larjava et al., 1993). In the absence of Integrin $\alpha 6$, mice exhibit vascular development defects, severe blistering of the skin, and die immediately after birth (Primo et al., 2010; Cress et al., 2001; Lee et al., 2006). Similarly, *itga6* deficient zebrafish embryos present with abnormal physical characteristics, involuntary shaking, a reduced formation of central arteries in the hindbrain, as well as an increase in blood vessel dilation (Zehr & Sittaramane, 2015). In addition to these developmental roles, Integrin $\alpha 6$ has been implicated in several pro-tumorigenic roles including the promotion of angiogenesis, metastasis and cancer stem cell propagation (Primo et al., 2010; Lee et al., 2006; Rabinovitz, et al., 1995; King, et al., 2008; Lathia et al., 2010; Cariati, et al., 2007).

As described previously, angiogenesis is a developmental process in which new blood vessels are formed from preexisting ones. This process is crucial to tumor development as this network of blood vessels work to supply the tumor with nutrients and serve as pathways for metastatic movements. Angiogenesis is heavily regulated by the production of proangiogenic factors such as VEGF-A and FGF-2 and by antiangiogenic factors. It has been shown that stimulation with these proangiogenic factors significantly upregulates ITGA6 expression in human EC *in vitro* (Primo, et al., 2010). The inducement of ITGA6 expression by angiogenic factors suggest that ITGA6 may play an important role in the beginning stages of tumor angiogenesis (Primo, et al., 2010). In addition, downregulation of ITGA6 on laminin-containing matrices has been linked to a reduction in migration and to a defect in the formation of tubular structures (Primo, et al., 2010). Several studies have presented similar findings implicating upregulated ITGA6 expression tumor angiogenesis (Cruz and O'Connor, 2008; Lee et. al., 2006; Bouvard, et. al., 2012).

In addition to this proangiogenic role, Integrin Alpha 6 has been implicated in tumor metastasis, the spread of cancer cells to distant sites. It has been reported that ITGA6

interacts with Fn14, a receptor in the tumor necrosis super family, to promote the migration and invasion of lung carcinoma cells (Jandova, et. al., 2015). Fn14 induces ITGA6 expression and strengthens the formation of ITGA6-dependent anastomosing structures contributing to an increase in angiogenesis, tumor development, and metastases (Jandova, et. al., 2015). The upregulation of ITGA6 expression has also been shown to induce metastasis in esophageal squamous cell carcinoma (ESCC) cells (Kwon et al., 2013). Further, ITGA6 knockdown via siRNA transfection was shown to reduce ESCC cell proliferation and invasion (Kwon et al., 2013). Similar results have been replicated in breast cancer and prostate cancer cell lines (Yang, et. al., 2008 & Rabinovitz, et. al., 1995). Recent investigations of ITGA6 expression in tumor metastasis have revealed the presence of a novel structural variant referred to as A6p (King, et. al., 2008). This variant is produced by the truncation, or cleavage, of the ITGA6 protein. The ITGA6/B1 complex is post-translationally modified by urokinase plasminogen activator receptor (uPAR) to produce A6p which lacks the ligand binding extracellular domain (Kacsinta, et. al., 2014). The cleavage of ITGA6 has been linked to an increase in migration, invasion, and metastasis while the inhibition of ITGA6 cleavage has been shown to reduce or delay metastasis (Kacsinta, et. al., 2014 & Landowski, et. al., 2014).

The upregulation of ITGA6 has been well-studied and is linked to the aforementioned cancer progression roles. However, the downregulation of ITGA6 has also been linked to metastasis and angiogenesis. It has been shown that missense single nucleotide polymorphisms (SNPs) for the ITGA6 gene are associated with the development of papillary thyroid carcinomas (PTC) (Kim et al., 2011). In addition, ITGA6 mutations which result in low levels of ITGA6 expression have been associated with prostate cancer susceptibility and development. More specifically, the ITGA6 locus rs12621278 was found to significantly increase the risk of prostate cancer progression by 2.4-fold (Cheng et al., 2010). A deficiency in endothelial ITGA6 has been linked to an elevation of VEGF-mediated angiogenesis further suggesting that the downregulation of ITGA6 plays an important role in tumor development (Germain, et al., 2009). Based on these studies it appears that a reduction in ITGA6 expression may be responsible for the cancer progression roles associated with this protein. When compared with other studies of ITGA6, it seems that there is a discrepancy in the literature; both the upregulation and

downregulation of ITGA6 have severe consequences in terms of cancer progression. Based on these studies and the identified discrepancy, the significance of ITGA6 expression should be investigated.

1.3 Zebrafish (*Danio rerio*) Model System

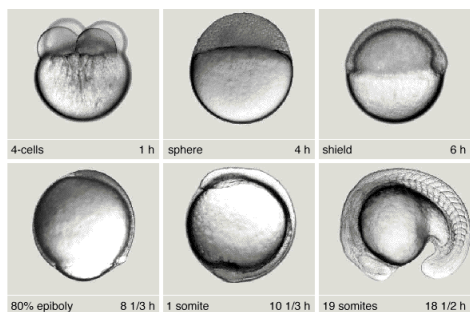


Figure 1.10: Zebrafish Development. Zebrafish develop quickly and in a transparent fashion. Development can be visualized from fertilization to the larval stage. Within 24 hours of fertilization, a swimming larva can be observed (Browder, L., Iten L., 1998)

The zebrafish (*Danio rerio*) is a tropical freshwater fish native to Indian rivers. These Southeast Asian fish emerged as a model organism in the 1960s. They are currently used as models for drug discovery, toxicology, cancer, development, and several other areas of study (Mione, et al., 2010; Dai et al., 2013; Shi, et al., 2015; Kithcart, 2017). Evidence supporting the many advantages of the zebrafish model chronicles its developmental properties as well as the many investigative tools

which have been developed. Adult females have a high fecundity and are able to produce up to 400 eggs under optimal breeding conditions. These eggs are fertilized externally and develop quickly, allowing for the study of early developmental stages (Figure 1.10). As larvae, these fish are transparent allowing for visualization of development and its mechanisms. In addition, zebrafish are able to recapitulate human diseases, making them an excellent model for cancer based studies. They are amenable to genetic manipulations and several methods can be used to alter their gene expression patterns. The complete zebrafish genome has been fully sequenced, and zebrafish share 70% of their genes with humans. Another major attribute of the zebrafish model is the application of transgenic technology. Zebrafish genetics allow for the expression of gene(s) of interest, or for the visualization of particular organs and tissues with fluorescent protein (Stoletov, 2008). The use of zebrafish in long-term studies is beneficial economically as they are small, easy to house and cheap to maintain compared to similar models.

1.3.1 Humanized Zebrafish Model System

Here, we employ a humanized zebrafish model system which will allow for the *in vivo* investigation of human ITGA6. As previously described, zebrafish present a stable

and genetically amenable model system. To humanize this system for the purpose of visualizing cell interactions and tumor metastasis we employ several well-developed genetic tools. By introducing human ITGA6 RNA to recently fertilized zebrafish embryos (1-2 cell stage), we can ensure the expression of human ITGA6 protein in the zebrafish. In addition, human cancer cells can be injected into this system, further humanizing the fish system. These manipulations result in a humanized animal model in which we are able to study the interactions between human cancer cells and human ITGA6 *in vivo*. This type of model will enable us to address the significance of ITGA6 expression by allowing for the generation of multiple human constructs. Humanized animal models have been well established and are a critical component to the study of human disease (Walsh et al., 2017; Morton, et al., 2016; Santoriello et al., 2012; Brehm, et al., 2011).

1.4 Present Study Aims & Significance

As initially discussed, cancer has become a global epidemic due to the rising accounts of cancer incidence and mortality rates worldwide. Each year 14 million individuals are diagnosed with cancer and despite current treatments, cancer incidence is projected to rise 70% over the next 20 years. This evidence highlights the need for effective diagnostic strategies and for targeted cancer therapeutics. Recent studies have focused on investigating the biological pathways of cancer cells, exploring interactions within the tumor microenvironment, and identifying mechanisms of tumorigenesis. The major implications of these research focal points are to identify putative biomarkers and potential drug targets which may combat current cancer incidence and mortality rates. The present study aims to explore the tumor microenvironment, specifically focusing on integrins, or cell adhesion proteins. The ultimate aim of the study is to identify the *in vivo* role of Integrin Alpha 6 in tumor development. Previous studies have implicated ITGA6 in tumor angiogenesis and metastasis; however, the mechanistic roles of this protein in tumor development remain unclear. This is largely due to competing hypotheses which implicate both the upregulation and downregulation of ITGA6 in pro-tumorigenic roles. In addition, the cleavage of ITGA6 may be important to this protein's cancer progression abilities.

Based on the discrepancies of ITGA6 expression in primary literature, we have designed a study which will interrogate ITGA6 expression on three levels: host level, cellular level, and the ECM level (Figure 1.13). Our previous studies focused on the host level by manipulating ITGA6 expression within the humanized zebrafish system. More specifically, human ITGA6 RNA constructs were used to increase expression levels while non-injected and morpholino (MO) groups were used to represent decreased ITGA6 expression. Zebrafish possess two copies of the integrin alpha 6 gene: *itga6a* and *itga6b*. For the purpose of these host level experiments, antisense nucleotides were used to target the *itga6a* version of the protein. Preliminary data from host level experiments reveal that an increase in ITGA6 expression results in an increase in tumor metastasis compared to ITGA6 null controls. Further, the role of ITGA6 cleavage was investigated by comparing the free-floating extracellular domain of ITGA6 to a mutated, non-cleavable version. We found that the extracellular domain of ITGA6 also increases tumor metastasis when compared to controls. Embryos injected with the non-cleavable version of ITGA6 or the knockout of *itga6a* exhibit tumor metastasis rates comparable to the non-injected controls.

The current study is a continuation of these experiments and focuses on ITGA6 manipulations on the cellular level. On the cellular level, we are aiming to understand how ITGA6 expression affects cell behavior and tumor metastasis independent of host ITGA6 manipulation. Based on preliminary host level data, **we hypothesize that decreasing ITGA6 expression will decrease tumor metastasis.** The prostate cancer cell line, PC3, is employed for all cell manipulation experiments. ITGA6 constructs will be introduced to the cell line via siRNA and DNA transfections. To decrease expression, PC3 cells are transfected with ITGA6 siRNA. Two versions of siRNA, s7492 and s7493, are employed each with different ITGA6 targets. siRNA s7492 targets exon 2 and s7493 targets exon 4 in the ITGA6 gene. Labeled and transfected PC3 cells are injected into zebrafish embryos (48 hpf) and analyzed for *in vivo* metastasis. Based on the assertions of previous studies and host level data, we know that the upregulation of ITGA6 plays an important role in tumor metastasis. **We hypothesize that increasing ITGA6 expression in cancer cells will lead to an increase in tumor metastasis.** To investigate this role on the cancer cell level, PC3

cells are transfected with ITGA6 DNA constructs. Transfected cells are introduced to the zebrafish (48 hpf) via microinjections. **Lastly, we hypothesize that the introduction of truncated ITGA6 will increase tumor metastasis in zebrafish embryos (48 hpf).** Once injected, zebrafish embryos (48hpf) are monitored for metastasis.

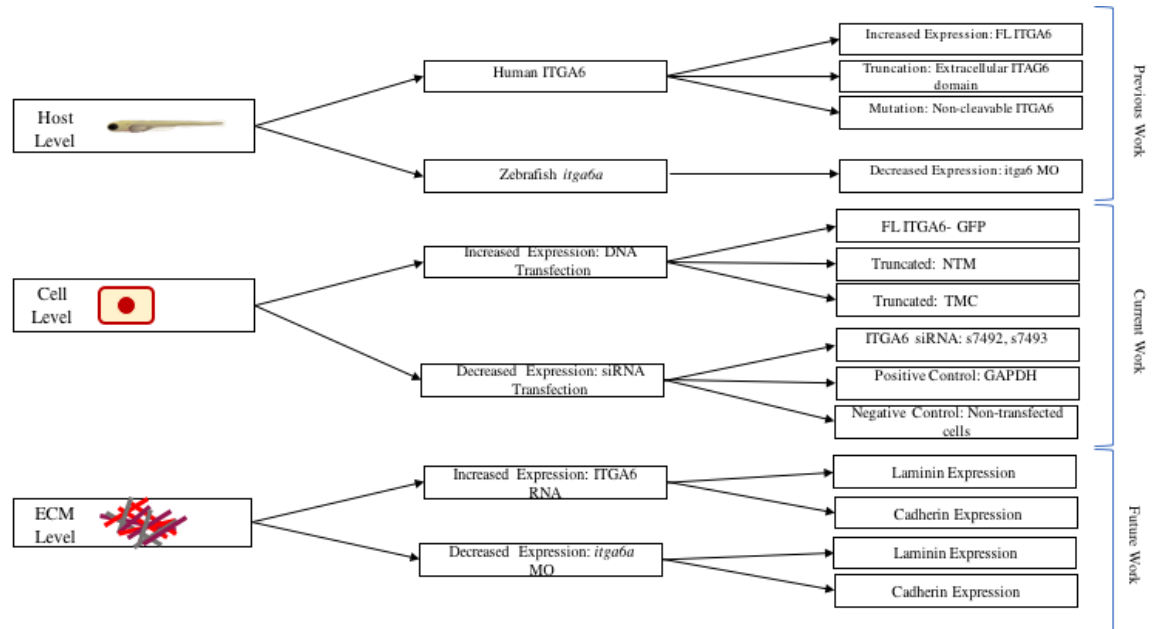
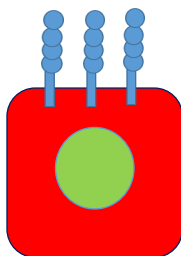


Figure 1.11: Schematic of Experimental Design: ITGA6 expression is manipulated on three levels, host, cell, and ECM to provide a complete understanding of mechanistic roles. Expression is manipulated using RNA injections as well as siRNA and DNA transfections.

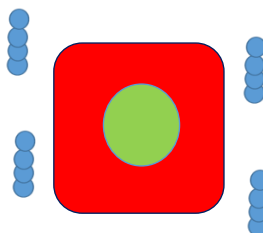
CHAPTER 2 CONSTRUCTS

Host Level Constructs



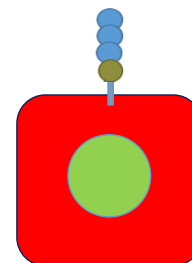
Human Full Length ITGA6 RNA

- Human ITGA6 protein overexpressed in zebrafish.
- Increased ITGA6 in tumor microenvironment.



Human Truncated ITGA6 RNA

- Cleaved version of human ITGA6 expressed in zebrafish.
- Tumor microenvironment flooded with ITGA6 free-floating domain.



Human Mutated ITGA6 RNA

- Non-cleavable version of human ITGA6 expressed in zebrafish.
- Absence of cleave product in tumor microenvironment.

Cell Level Constructs



Human Full Length ITGA6: GFP DNA

- Human ITGA6 protein overexpressed in PC3 cells.
- GFP tag to allow for visualization of protein



Human NTM ITGA6 DNA

- Cleaved version of human ITGA6 expressed in PC3 cells
- NTM: amino terminal of ITGA6: free-floating domain equipped with signal peptides.



Human TMC ITGA6 DNA

- Cleaved version of human ITGA6 expressed in PC3 cells
- TMC: carboxy terminal of ITGA6, also referred to as A6p.



Human ITGA6: siRNA

- Human ITGA6 expression reduced in PC3 cells.
- Two siRNAs, s7492 and s7493 are used.



Human ITGA6: GAPDH siRNA

- Human GAPDH expression reduced in PC3 cells.
- Serves as positive control



Human ITGA6: Negative siRNA

- Negative Silencer siRNA
- Serves as Negative control

CHAPTER 3

Figure 2.1: ITGA6 Constructs: Schematic of host level constructs, full length ITGA6, Truncated ITGA6 RNA, and Mutated ITGA6 RNA. Cellular constructs are also shown and include Full-length ITGA6-GFP DNA, NTM ITGA6 DNA, TMC ITGA6 DNA, ITGA6 siRNA (s7492 and s7493), GAPDH siRNA (positive control), and Negative Silencer siRNA (negative control). In addition, not injected and non-transfected controls were used but are not shown above.

MATERIALS AND METHODS

3.1 Zebrafish Husbandry

Adult Zebrafish were housed in the AQUAneering system located in the Georgia Southern animal facility. All experiments were performed in accordance with GSU Institutional Animal Care and Use Committee guidelines. To collect fertilized eggs, adult breeding tanks containing males and females (1:1) were set up. Crosses were setup at night to allow fish a suitable acclimation period. Fish water changes occurred early each morning, as daylight triggers zebrafish spawning. To obtain eggs, the breeding chamber with the adult fish was removed, and the egg-containing fish water was filtered to separate embryos from fish waste. Harvested embryos were resuspended in Embryo Medium (E3), a water based solution containing methylene blue. For all collected embryos, E3 was changed daily or as needed. For all experiments, zebrafish used were < 7 days post fertilization (dpf). Experiments described employed Wild-Type and Tg (*fli1a: GFP*) embryos.

3.2 Preparation of ITGA6 DNA Constructs

The isolation of three plasmids, FL-GFP, NTM, and TMC, was used to begin the transformation process and generation of ITGA6 DNA constructs. These plasmids are all in a mammalian vector and can be identified through Myc-tag immunostaining. Lyophilized plasmids were dissolved in 20 uL of nuclease free water and centrifuged. Next, 100 ng of each DNA construct was added to a 1.5 mL Eppendorf tube containing 50 uL of competent cells at optimal cell density. The tubes were placed on ice for 30 minutes before being exposed to 42°C for exactly 30 seconds. After the heat shock, the tubes were placed back on ice for two minutes. 950 uL of SO enriched medium was added to each tube. All tubes were then incubated and at 37°C for one hour in gently shook. A bacterial lawn was created on LB agar plates using a steel rod. Bacteria were allowed to grow for 12 hours on ampicillin containing LB broth. A plasmid extraction kit was used to isolate plasmid DNA. Glycerol stocks of pcDNA for each constructed were created and stored at -80°C. The pcDNA3.1(+) plasmid was used for experiments. This mammalian plasmid is resistant to ampicillin and has a CMV promoter. Stock pcDNA for each construct was used in all cell transfections of ITGA6.

3.3 Cell Culture

3.3.1 Thawing

Breast cancer cell line, MDA-MB-231, and prostate cancer cell line, PC-3, (GeneScript®) were used in all experiments. MDA-MB-231 and PC-3 cells are human epithelial lines isolated from patients with an adenocarcinoma. MDA-MB-231 cells were isolated from a female patient, while PC-3 cells were isolated from a male patient. Vials containing 1×10^6 cells were stored in a liquid nitrogen Dewar until thawed for use. In preparation for thawing, frozen cells were placed in the vapor phase, or in the -80°C freezer for 24 hours. RPMI 1640 media and frozen cells were warmed in a 37°C -water bath. The media was warmed for approximately ten minutes, while frozen cells were warmed until the ice was dissolved. After spraying the biological safety cabinet (BSC) with 70% isopropyl alcohol (IPA), the media, cells, and T-75 flasks were placed in the hood. Next, 20 milliliters (mL) of warm media were pipetted into each flask. Quickly, 500 microliters (uL) of cell solution were pipetted into each flask, and the flasks were mixed by gently pipetting up and down. Flasks containing thawed cells were placed in a 37°C incubator with 5% CO_2 .

3.3.2 Harvesting

All reagents used for cell harvesting are warmed in a 37°C -water bath and sprayed with 70% IPA before being placed in the BSC. To prepare culture media, 50 mL of fetal bovine serum, five mL of penicillin and streptomycin solution are added to 500 mL RPMI 1640. Culture medium is used to sustain and grow cells between experiments and is changed 24-48 hours before each passage. Phosphate buffered saline (10 X PBS) was diluted to 1 X PBS using DNase-free distilled water. Serine protease, trypsin (1X) is used to dissociate adherent cells from tissue-culture treated surfaces. To harvest the cells, the old media was removed and replaced with ten mL of 1X PBS. The flask was gently rocked to wash the PBS across the bottom and sides of the flasks. After removing the PBS, five mL of trypsin-PBS solution are added to the flask. The flask is then placed in incubator and the cells are trypsinized for three to five minutes. Following trypsinization, the flask is gently tapped to release adherent cells. Once the cells are in solution, 8 mL of culture media are added to the flask in order to terminate trypsinization. Next, the culture solution was placed in 15 mL falcon tube and centrifuged at 970 revolutions per minute (RPM) for five

minutes. After centrifugation, the cells are resuspended in fresh media and counted using a hemocytometer. The desired cell concentrations are calculated and placed in a new flask or plate depending on the experimental set-up.

3.3.3 Cell Labeling

During harvesting, cells intended for labeling are aliquoted into 1×10^6 cells per microcentrifuge tube. Each aliquot was centrifuged at 500 relative centrifugal force (RCF) for two minutes. The supernatant was removed and replaced with one mL of R-1 media, or serum-free media. After resuspending the cells, five uL of CellTracker Green CMFDA Dye (Thermo Fisher C2925) were added to the microcentrifuge tube. The tube was then rocked back and forth before a 15-minute incubation period at 37°C. The cells were again centrifuged at 500 RCF for two minutes after incubation. At the completion of cell labeling, the cells were resuspended in 200 uL of fresh culture media in preparation for microinjection. Labeled cells were incubated for up to four hours after final centrifugation.

3.3.4 Immunostaining of Cells

After harvesting, cells intended for immunohistochemical analysis were plated in 4 well chamber slides (Sigma-Aldrich C6807). Each chamber contained 2×10^4 cells suspended in 500 uL of RPMI 1640 media. A solution of PBS++ which contained 0.9 mM Calcium Chloride (CaCl_2), 0.52 mM Magnesium Chloride (MgCl_2), and 0.16mM Magnesium Sulfate (MgSO_4) was used as a washing buffer. Cells were washed once with serum-free media, followed by two washes with PBS++. Cells were then blocked for 15 minutes with PBS-BSA, a PBS++ solution containing 1% Bovine Serum Albumin (BSA). Next, cells were washed two times with PBS++, fixed with 4% paraformaldehyde (PFA) for 20 minutes. Excess fix was removed with three PBS++ washes, and cells were permeabilized for 10 minutes with 0.2% Triton-X-100. Following three additional washes with PBS++, cells were again blocked with PBS-BSA for 15 minutes. Cells were incubated with 100 uL of Integrin Alpha 6 (primary antibody) dilution after two washes with PBS++. Three PBS++ washes then preceded a final 15-minute blocking period with PBS-BSA. After being washed twice with PBS++, cells were incubated in a dark environment with 100 uL Alexa Fluor 555 dilution. Finally, cells were washed three times with PBS++ and prepared for imaging analysis.

3.3.5 ITGA6 Small Interfering RNA (siRNA) Transfections

PC3 cells were plated in a 24 well tissue culture treated plate; each well containing 5×10^4 cells. The plate was incubated for approximately 48 hours before initializing the transfection protocol. Lipofectamine RNAiMAX reagent (Thermo Fisher 13778-075) was diluted by adding 150 μ L of Opti-MEM medium to 9 μ L of Lipofectamine in a microcentrifuge tube. Stock solutions of the siRNA constructs were diluted to 10 μ M solutions: GAPDH, Negative Control, siRNA s7492, siRNA s7493, siRNA s7494. The siRNA constructs were further diluted by adding 50 μ L of media to three μ L of 10 μ M siRNA. Next, the diluted siRNA constructs were mixed with the diluted Lipofectamine RNAiMAX in a 1:1 solution. The siRNA-RNAiMAX solution was then incubated for five minutes at room temperature. Cells were transfected by adding 250 μ L of the incubated solution to the appropriate wells. The cells were then incubated at 37°C for 48 hours. Control cells were transfected with a solution containing only Lipofectamine RNAiMAX and Opti-MEM medium.

3.3.6 ITGA6 DNA Transfections

PC3 cells were seeded at 80% confluency in a 24 well tissue culture treated plate and incubated for two days. For each well, 3.75 μ L of Lipofectamine 3000 reagent (Thermo Fisher L3000008) was diluted with 125 μ L of Opti-MEM cell culture medium. Next, each of the DNA constructs were diluted by adding 5 μ g of DNA to 250 μ L of Opti-MEM medium and 10 μ L of P300 Reagent. Next, 125 μ L of diluted DNA construct were added to 125 μ L of diluted Lipofectamine 3000 reagent. The resulting solution was incubated for 15 minutes at room temperature. Lastly, 250 μ L of the 1:1 DNA/Lipofectamine 3000 dilution were added to the appropriate wells. DNA constructs included Full-length GFP, NTM, and TMC. A 1:1 Lipofectamine 3000/Opti-MEM solution was used on control cells. Cells were incubated at 37°C with transfection reagents for 48-72 hours.

3.4 qPCR

3.4.1 RNA Extraction

RNA extraction was performed on previously transfected PC3 cells. Media containing transfection reagents was aspirated and transfected cells were washed once with ice cold 1 X PBS. Ice cold PBS was replaced with 1 mL of TRIzol reagent, and cell scrapers (location) were used to gently detach adherent cells. TRIzol/cell lysate was transferred into

a 1.5 mL Eppendorf tube and incubated at room temperature for five minutes. After incubation, 250 uL of Phenol chloroform were added to each tube. The tubes were shook vigorously using a vortex for approximately 20 seconds. Following a five-minute incubation at room temperature, samples were centrifuged at 4°C for five minutes at 10,000 RPM. The aqueous phase resulting from centrifugation was collected and transferred to a sterile 1.5 mL Eppendorf tube. Next, 550 uL of isopropanol were added to each tube, and the tubes were again incubated at room temperature five minutes. Due to the expectation of a low yield, tubes were centrifuged at maximal speed (13,500 RPM) for 30 minutes. Centrifuged samples were then placed on ice and washed with 1 mL of 75% ethanol in nuclease-free water. The tubes were mixed gently and recentrifuged at 9,500 RPM for ten minutes. Ethanol washes were repeated once to enhance purity of sample RNA. Following centrifugation, ethanol was poured off and pellets were air-dried. The Nano-drop was used to determine purity and concentration of extracted RNA. Samples with 260/280 ratios of less than 2.0 were precipitated to improve RNA purity. To precipitate RNA, 50 uL of filtered 3M sodium acetate (pH = 5.5; location) were added to each tube. Additionally, 150 uL of ice cold 100% ethanol were added the tubes. Samples were stored at -80°C overnight to allow for precipitation. The following day, the samples were centrifuged at maximal speed for 30 minutes at 4°C. The supernatant was removed, and the pellet was washed with 500 uL of ice cold 70% ethanol. The tubes were then recentrifuged for 10 minutes, the ethanol was poured off. Pellets were allowed to air-dry and were resuspended in nuclease-free water. All RNA samples were stored at -80°C until needed for further analysis.

3.4.2 cDNA Synthesis

The Invitrogen cDNA synthesis kit (Life Technologies 11752-050) was used to generate first-strand cDNA. The standard protocol provided by Life Technologies was used for all samples. Purity and concentrations of cDNA were determined used the Nano-drop. All cDNA samples were stored at -20°C until needed for further use (Life Technologies, 2018). Samples with 260/280 ratios of less than 1.8 were not used for further analysis.

3.4.3 qPCR

To begin quantitative gene expression analysis, cDNA samples, TaqMan® Gene Expression Assays 20X (catalog numbers (GAPDH, & ITGA6), TaqMan® Universal Master Mix II, no UNG (Thermo Fisher, PN 4440047) were thawed on ice. Each sample was prepared in triplicate on the semi-skirted qPCR plates. Each well contained 1 uL of 20X TaqMan® Gene Expression Assay, 10 uL of master mix, 100 ng of cDNA template, and RNase—free water. The total volume for each well was 20 uL, and the plate remained on ice during preparation steps. Two TaqMan® Gene Expression Assays were used, GAPDH and ITGA6. GAPDH was used a housekeeping gene, while ITGA6 assay was used to detect expression changes in the gene of interest. Each sample was analyzed with GAPDH gene assay and with ITGA6 gene assay, each performed in triplicate. Optimization studies also included sample wells with no gene assay, as well as sample wells with no cDNA template. The QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher 4485694) and QuantStudio Real-Time PCR software were used to analyze gene expression. To begin the experimental set up on QuantStudio software, a fast comparative CT for 96 well block (0.2 mL) was selected. Next, targets, samples, and endogenous controls were defined and assigned to the appropriate well locations. It should be noted that FAM and NFQ-MGB were used as reporter and quencher respectively, while ROX was used a passive reference. The run method applied consisted of two stages, a hold stage and PCR stage. The hold stage was one step at 95°C for five minutes. The PCR stage contained three steps: Step 1 (95°C for 15 seconds), Step 2 (60°C for one minute) and Step 3 (72°C for 30 seconds). The PCR stage was run for 40 cycles, and the run method was optimized for a 20 uL reaction volume. The comparative CT mean and gene expression plots were used for data analysis.

3.5 Microinjections

Cultured and labeled Human PC3 cells were injected into 2 dpf dechorionated zebrafish embryos. Embryos were transferred into a gridded agar plate and treated with 0.04% Tricaine, an anesthetic. Each embryo was injected with PC3 cells into either the perivitelline space or brain ventricles using the Nanoject II™ Auto-Nanoliter Injector. Injection needles were prepared using the Flaming/Brown Micropipette Puller (Sutter

Instrument, P-97) and loaded with labeled PC3 cells (or MDA-MB-231 cells), followed by mineral oil, which acts as a pressure agent. Embryos were washed three times to remove the effects of the anesthetic and placed in an incubator for later observation and imaging.

3.6 Imaging Analysis

3.6.1 Immunohistochemical Analysis of Zebrafish

Zebrafish embryos were fixed in 4% paraformaldehyde solution overnight. The embryos were washed four times in incubation buffer (IB), each wash lasting 30 minutes. The embryos were washed once in a solution of IB and 1% horse serum for 30 minutes. The embryos were washed in a solution of IB + 1% horse serum + mouse primary antibody in a 1:250 dilution, overnight. The embryos were then washed for four 30 minute washes in IB, followed by a wash in IB + 1% horse serum. The embryos were washed overnight in a solution of IB + 1% horse serum + Goat anti mouse (secondary antibody) in a 1:500 dilution. Embryos then received 3, ten minute washes with 1X PBS before being fixed in 4% paraformaldehyde overnight. The following day embryos were washed 3 times in ten minute intervals with a solution of 1X PBS. Embryos were incubated in 1 mL of 25% glycerol solution for ten minutes, then incubated with 1 mL of 50% glycerol for 10 minutes and finally placed in 70% glycerol and stored in the 4°C refrigerator.

3.6.2 Mounting

Zebrafish embryos were fixed in 1.5 mL Eppendorf tubes with 1 mL 4% PFA. Fixed embryos were stored at 4°C in 24 well plates or 1.5 mL tubes. Fixed embryos were removed from the 4°C refrigerator and pipetted onto a slide (one embryo per slide) with minimal 70% glycerol. The embryo was oriented under a microscope in symmetrical position, with the yolk facing down and the head aligned with the tail. A coverslip was gently placed on top of the embryo and held in place with Vaseline. The coverslip and embryo were sealed with the 70 % glycerol solution, and stored in a dark environment until used for imaging. Live Zebrafish embryos were anesthetized with Tricaine solution before being mounted in chamber slides. A 3% agarose solution was microwaved briefly and approximately 250 μ L of agarose were pipetted into the chamber. The anesthetized zebrafish embryo was then

pipetted into the agarose and oriented with a small pin. After the agarose solidified, the chamber slide was filled with a Tricaine/embryo medium solution. The mounted embryo can then be imaged over a varying period of time.

3.6.3 Confocal Microscopy

Embryos mounted in chamber slides were placed on the laser confocal for visualization of injected cells and blood vessels. The laser scanning confocal microscope uses ZEN software to collect and analyze images or time lapses. Using the ZEN software, preferred settings were applied to enhance fluorescent visualization of the embryo and cancer cells. Once settings were selected, mounted embryos underwent Z-stack imaging or time lapse experiments.

3.6.4 Analysis of Cell Morphology

PC3 cells were harvested, plated in a 4-well chamber slide and transfected as previously described. Transfected and control cells were labeled with Green Cell Tracker and imaged at 10X using the laser scanning confocal and ZEN software. Tilescan images were analyzed using FIJI. Images were made binary and corrected to exclude edges, fill holes, and remove debris. Binary images were analyzed using the analyze particle functions on FIJI. Cell area, circularity and aspect ratio were analyzed. Statistics were calculated using Graph Pad Prism Software.

CHAPTER 4 RESULTS & DISCUSSION

4.1 Expression of ITGA6 in Prostate Cancer (PC3) Cells

Integrin Alpha 6 is expressed in several types of human tumors including gastric, breast, prostate, leukemic, astrocytoma, and ovarian cancers (Lathia, et al., 2010 & ITGA6-Gene Cards. To confirm the presence of ITGA6 expression in PC3 cells, immunostaining of non-manipulated, control, cells was performed. PC3 cells were fixed and stained using an ITGA6 primary antibody (Life Technologies). The immunostaining revealed that ITGA6 is expressed in the PC3 cell line employed for all other experiments (Figure 4.1).

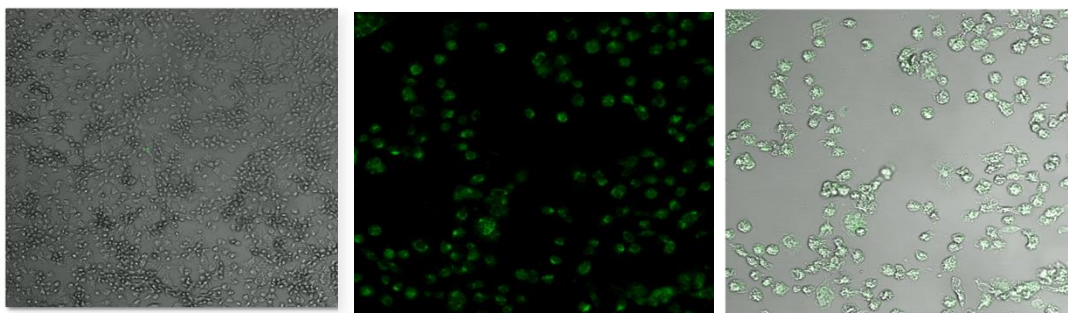


Figure 4.1: ITGA6 Expression in PC3 Cells: ITGA6 expression was visualized in fixed PC3 cells by immunohistochemical analysis. A) Control PC3 cells were not stained and therefore do not exhibit GFP expression. B) PC3 cells were fixed and stained using an ITGA6 primary antibody followed by an Alexa Fluor 488 secondary antibody (ITGA6 labeled in green).

4.2 siRNA Transfection of PC3 Cells Reduces ITGA6 Expression

We have previously shown that ITGA6 is expressed in PC3 Cells (Figure 4.1). Based on previous studies and preliminary host level data, we hypothesized that low levels of ITGA6 expression would decrease tumor metastasis. To effectively reduce ITGA6 expression in PC3 cells, ITGA6 siRNAs were transfected into the cell line. Two siRNAs, with different ITGA6 targets were employed: s7492 and s7493. To verify successful transfection of the ITGA6 siRNAs, a comparative CT analysis was performed (Figure 4.2). We show that ITGA6 siRNAs are able to reduce ITGA6 expression in PC3 cells. GAPDH siRNA was used as a positive control, while all samples were normalized to a control group. Control transfection cells were treated with Lipofectamine 3000, but were not transfected with siRNA.

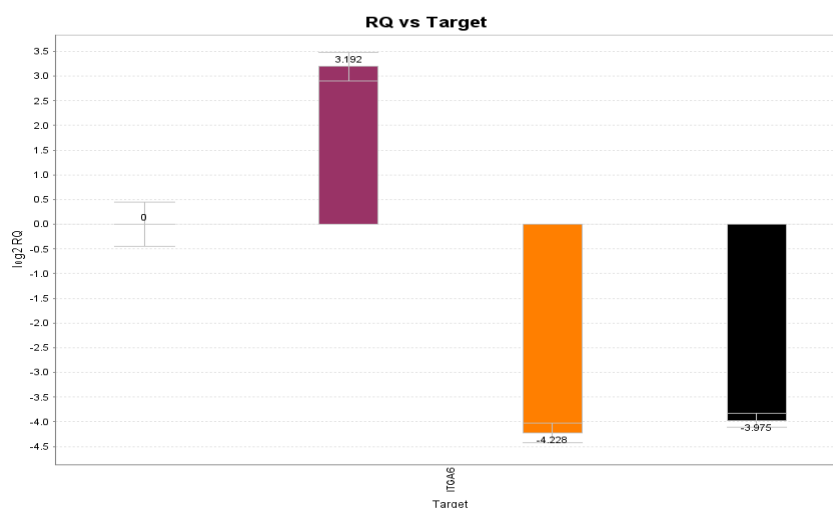


Figure 4.2: ITGA6 siRNA Transfection of PC3 Cells: The figure above demonstrates that both ITGA6 siRNAs, s7492 (orange) and s7493 (black) are able to reduce expression of the ITGA6 gene. The positive control, transfected with GAPDH siRNA, does not exhibit reduced ITGA6 expression. All samples were normalized to a non-transfected control.

4.3 Analysis of Cell

Morphology in ITGA6 siRNA Transfected PC3 Cells

Changes in cell morphology serve as important markers for metastatic potential. Often times when cancer cells undergo morphological changes they become more metastatic or lose their metastatic ability altogether. Due to the changes in the expression of adhesion receptor, ITGA6, changes in morphological characteristics should be assessed in a metastatic context. Here, we examine the differences of three morphological characters between transfected and non-transfected cells. An analysis of cell size in terms of area (μm^2) reveals no significant differences between controls and siRNA transfected cells (Figure 4.3). Circularity is measure of how close an object is to a perfect circle and ranges from 0 (a straight line) to 1 (perfect circle). Cells transfected with ITGA6 siRNA were found to be closer to a perfect circle than non-transfected cells. Additionally, cells transfected with s7492 were found to be significantly more circular than s7493 transfected cells. Lastly, aspect ratio, a measure used to describe the proportion between width and height, was analyzed. We find that siRNA transfected cells have a significantly lower aspect ratio when compared to non-transfected controls (Figure 4.4). These data suggest that when ITGA6 expression is reduced, cells become more circular and less elliptical in cell shape. It follows then that siRNA transfected cells would be less metastatic than control cells. Future experiments will be expanded to include DNA constructs and additional morphological parameters.

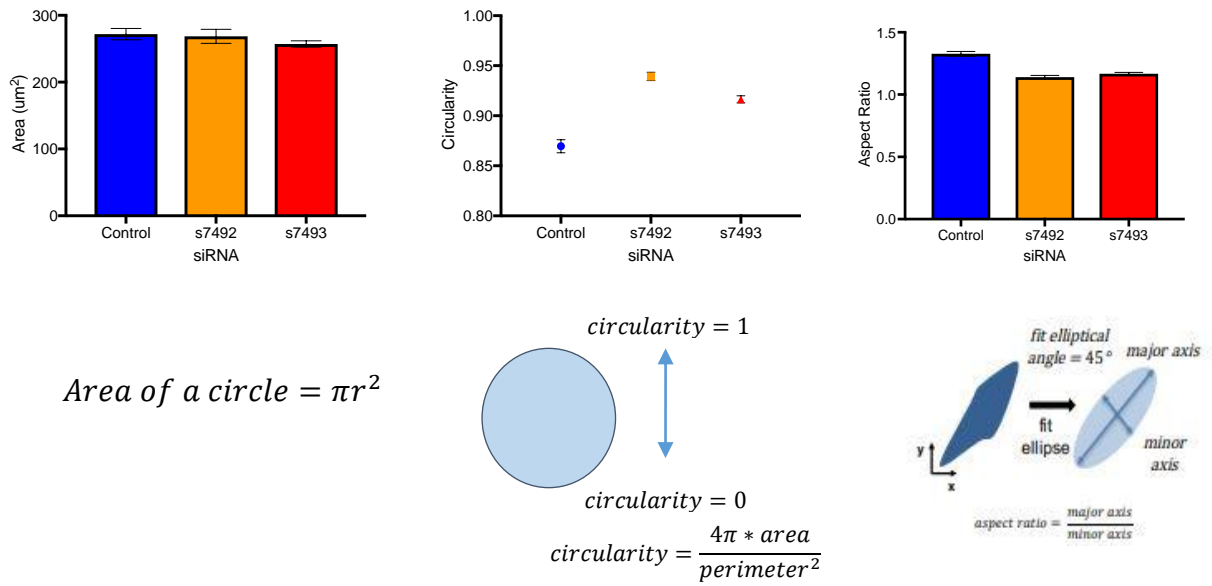


Figure 4.3: Effects of ITGA6 Knockdown on Cancer Cell Morphology: Three morphological characteristics of PC3 cells transfected with ITGA6 siRNA were analyzed: Area (μm^2), circularity, and aspect ratio. siRNA transfected cell shapes were compared to a non-transfected control. There were no significant differences in the cell size between s7492 (n=112), s7493 (n=277) transfected cells and non-transfected controls (n=212). Further there was no significant differences in cell size between the s7492 and s7493 transfected cells. Analysis of circularity reveals a significant difference between s7492 transfected cells and control cells, where transfected cells appear to be more circular ($p < 0.0001$). Similarly, s7493 cells also found to be significantly more circular than control cells ($p < 0.0001$). A comparison of the circularity between s7492 cells and s7493 cells shows that s7492 cells are significantly more circular than s7493 cells ($p < 0.0001$). Lastly, aspect ratio was used to describe the elliptical character of transfected and non-transfected cells. There is a significant difference in the aspect ratio between control (n=212) and transfected cells s7492 (n=112) and s7493 (n=277) ($p < 0.0001$). However, no significant differences were found s7492 and s7493 transfected cells. Unpaired, parametric t-tests using Welch's ($p < 0.01$) were used to analyze these data.

4.4 Preliminary Analysis of Tumor Metastasis with Transfected PC3 Cells

Upon demonstrating the successful transfection and action of ITGA6 siRNAs, we move to the introduction of this cellular construct to our *in vivo* model system to analyze the effects of ITGA6 knockdown on tumor metastasis. PC3 cells transfected with ITGA6 siRNAs were labeled and injected into the brain ventricle of Tg (*Flila*: gfp) zebrafish embryos (48 hpf). Zebrafish embryos were observed for tumor metastasis, where metastasis was defined as the spread of at least ten cancer cells from the primary injection site. Long-term time-lapse experiments of injected zebrafish embryos were performed to visualize metastasis *in vivo* (Analysis of time-lapse data is not shown). In addition, to live imaging, preliminary population based studies were performed to identify the number of embryos (%) exhibiting tumor metastasis within the different cellular constructs (Figure

4.3). Approximately 15% of zebrafish embryos injected with non-transfected controls exhibit tumor metastasis (Figure 4.3). Embryos injected with s7492 transfected cells did not exhibit tumor metastasis as defined, while only 5.4% of embryos injected with s7493 ITGA6 siRNA present with tumor metastasis (Figure 4.3). In addition, the effects of increasing ITGA6 expression in PC3 cells were examined. In the presence of FL-GFP transfected PC3 cells, 44.4% of embryos exhibit tumor metastasis; double the number of embryos observed in the control group (Figure 4.3). 10% of embryos injected with the ITGA6 TMC construct show tumor metastasis (Figure 4.3). The expression of ITGA6 was verified in control and siRNA transfected cells concurrently with tumor metastasis observations (Figure 4.3.1). Based on these preliminary data, we can form an initial conclusion implicating the increased expression of ITGA6 in PC3 cells as an enhancer of tumor metastasis. These data also show that a reduction of ITGA6 expression in the PC3 cells may contribute to a decrease in tumor metastasis.

There are several notable limitations in the present study:

- 1) ITGA6 siRNA and DNA Transfections: All cellular constructs were generated through the transfection of ITGA6 siRNA and ITGA6 DNA. The transfection protocol was optimized using ITGA6 siRNA constructs: s7492 and s7493. Knockdown of ITGA6 expression was verified using a comparative analysis (CT) analysis. Initial results from optimization studies revealed that ITGA6 siRNA transfections were capable of reducing ITGA6 expression in PC3 cells. Likewise, ITGA6 DNA was transfected to generate additional cellular constructs: FL-GFP, NTM, and TMC. Due to the low concentrations obtained during RNA extraction, comparative analysis (CT) analysis could not be used to verify the expression of DNA constructs. As such, we cannot assume that injected PC3 cells transfected with ITGA6 DNA were expressing the appropriate construct. The transfection set-up, reagents, and incubation time used for siRNA and DNA transfections were similar suggesting that overall transfection protocol was successful; however, ITGA6 gene expression should have been quantified in order to form concrete conclusions.
- 2) Sample Size: Due to the small sample sizes employed for population-based studies, these results can only be interpreted as preliminary. A series of replications is

required to solidify conclusions and to allow for statistical analysis. Sample sizes for each cellular construct are as follows: s7492 (n=41), s7493 (n=37), FL-GFP (n=9), TMC (n=10), Control (n=48).

- 3) Preliminary basis of studies: The ITGA6-tumor metastasis results discussed were obtained from only two experiments. Therefore, the conclusions drawn may only be considered preliminary. In addition, these studies and parallel studies are currently ongoing, and an analysis of complete data may alter initial conclusions.

Further, a concurrent consideration of both host-level data and cell-level data provides a better understanding of ITGA6 expression in tumor metastasis. Based on our host-level studies, we concluded that increasing ITGA6 expression in the tumor microenvironment results in a significance increase in tumor metastasis. In addition, we found that reducing ITGA6 expression or *itga6* expression results in a decrease in tumor metastasis. Host-level studies also implicate ITGA6 truncation as an enhancer of tumor metastasis. This finding is further supported by the decrease in tumor metastasis observed when a mutated, non-cleavable version of ITGA6 is introduced. Similarly, cellular studies reveal that decreasing expression of ITGA6 in PC3 cells results in a decrease in tumor metastasis, while simultaneously an increase in ITGA6 expression seems to promote metastatic spread (Figure 4.3). Taken together these studies suggest that the upregulation of ITGA6 expression acts as an enhancer of tumor metastasis independent of its location; in the tumor microenvironment or within cancer cells. More specifically, these data reveal that ITGA6 can regulate metastasis both inside and outside of the cell. If this is the case, then ITGA6 expression should be studied in the context of ECM and ECM remodeling. ITGA6 may work by altering the ECM through interactions with ECM proteins like cadherin, laminin, or fibronectin. As a laminin receptor, it follows that ITGA6 may be modulating ECM protein behavior by controlling ligand binding and by mediating signaling cues. In addition, we have shown that truncation of the ITGA6 extracellular domain seems to play an essential role in increasing tumor metastasis. Perhaps the truncated portion of ITGA6 serves as signal which induces ECM remodeling to produce a more navigable environment for cancer cells. Based on our studies, the mechanisms by which ITGA6 induces metastasis remain unclear, however these studies have contributed to the understanding of ITGA6 expression significance. Current and future studies are required to strengthen these results

and to expand our understanding of ITGA6 mechanics at the level of, host and cell, and potentially ECM.

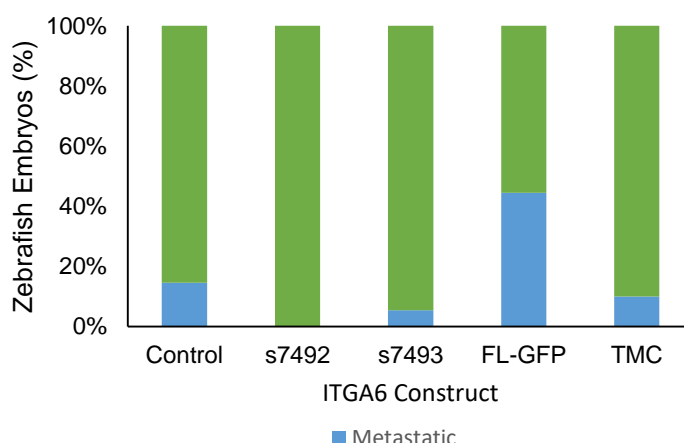


Figure 4.4: Tumor Metastasis in ITGA6 Cellular Constructs: The figure above demonstrates the effects of ITGA6 cellular constructs on tumor metastasis. Approximately 14.58% of zebrafish injected with control cells (n=48) exhibit tumor metastasis. Embryos injected with s7492 cells (n=41) and s7393 cells (n=37) show decreased levels of tumor metastasis. The Full-length GFP construct (n=10) appears to increase tumor metastasis, while the ITGA6 TMC (n=9) construct remains comparable to the control group.

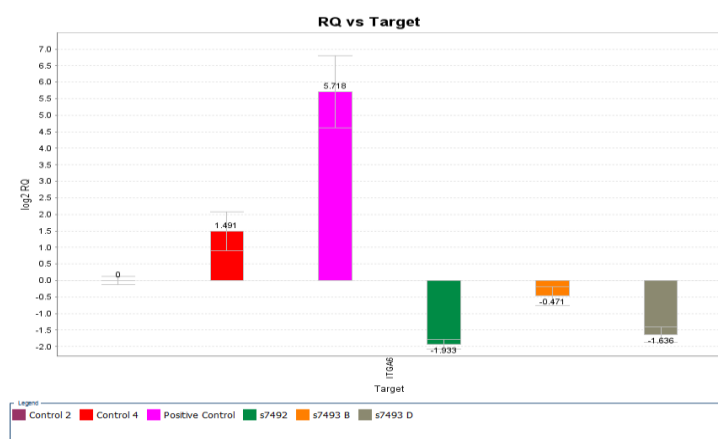


Figure 4.4.1: Gene Expression of ITGA6 siRNA Constructs: ITGA6 expression is reduced in PC3 cells transfected with ITGA6 siRNA (s7492, s7493). Samples were normalized to a non-transfected control (control 2). GAPDH siRNA was used a positive control.

4.5: An overview of current and future ITGA6 expression studies

To address the limitations, we have previously identified and to expand our knowledge on ITGA6 mechanics in tumor development, we will describe on-going and future investigations:

- *Use of time-lapses to visualize in vivo ITGA6 mechanics and cell behavior:*
The aim of this experiment is to visualize the effects of ITGA6 manipulation on cancer cell behavior and tumor metastasis. We hypothesize that ITGA6 DNA transfected cells will have many protrusions and will successfully metastasize out of the brain tissues into other embryonic tissues. Cell behavior can be observed and analyzed *in vivo* by employing confocal

microscopy, cell labeling techniques, and Fiji software. Trackmate can be used to follow individual cells as they metastasize.

- *Establish Significance of ITGA6 Expression on the Cellular Level:* We are currently repeating ITGA6 siRNA and ITGA6 DNA transfections and subsequent injection experiments. The aim of these experiments is to shed light on the roles of ITGA6 inside the cell. ITGA6 DNA constructs are used to model upregulation and truncation within PC3 cells. ITGA6 siRNA constructs are used to demonstrate the effects of decreased ITGA6 expression on metastasis. These experiments are ongoing, and several replications will be required to expand preliminary analyses. Failure to verify successful transfection of ITGA6 DNA constructs has presented an obstacle in these studies. In addition, the ITGA6 NTM construct was excluded from preliminary studies due to complications during transfection. We are currently working to repeat both siRNA and DNA transfections in order to visualize the impact of these constructs on tumor metastasis. Comparative CT analysis will continue to be used as a measure of gene expression. Population based metastasis studies are also ongoing and these additional data will be used to describe the effects of upregulation, downregulation and truncation on tumor metastasis.
- *Effects of ITGA6 on Cell Morphology:* Here, we described briefly how a decrease in ITGA6 affects the morphological characteristics of PC3 cells. We found that cells transfected with ITGA6 siRNA appear to be comparable in size to non-transfected controls; however, siRNA transfected cells (s7492 and s7493) are more circular and less elliptical compared to control cells. Future studies will expand on these analyses of cell morphology, as morphological characteristics may contribute to metastatic ability. A comparison of the cell morphologies observed in siRNA transfected and DNA transfected cells may reveal some clues as to why these cells behave differently. These *in vitro* analyses of cell morphology complement the *in vivo* studies. Further, a 3D cell culture model system should be used to explore ECM-ITGA6 interaction *in vitro*.

- *ITGA6 Expression and ECM Remodeling:* Based on our preliminary studies, the interactions between ITGA6 and ECM proteins may explain the role of ITGA6 in tumor metastasis. ECM remodeling refers to changes in the tumor microenvironment induced by cellular signals or molecular cues. In future studies, we hope to probe these interactions by using immunohistochemistry to visualize ECM protein expression in tissue cross sections. Using the previously discussed ITGA6 constructs, we can visualize how differential expression of this protein impacts ECM structure and composition. These data will also us to determine if ITGA6 works through ECM signaling to control tumor metastasis.

In conclusion, we have shown that increased levels of ITGA6 expression in PC3 cells leads to an increase in tumor metastasis. These data align with previous studies which have implicated upregulation of ITGA6 in cancer progression roles. More specifically, Jandova et al., showed that the upregulation of ITGA6 induces metastasis and angiogenesis through signaling mechanisms (Jandova et al., 2015). Similarly, Kwon et al., was able to demonstrate that increased ITGA6 expression contributes to metastasis while ITGA6 siRNA reduces metastasis. These findings were confirmed by our studies which have shown that siRNA transfected PC3 cells reduce metastasis compared to controls. Due to the limitations of our study we were unable to identify the rile of truncation in ITGA6 driven metastasis, however current and future studies have been designed to address these roles. Our findings of reduced ITGA6 expression resulting in decreased tumor metastasis conflict with other works which show that mutations for the ITGA6 gene amplify cancer progression roles and increase susceptibility (Cheng et al., 2010; Germain et al., 2009; Kim et al., 2011). Based on these results, we should investigate the interactions between the ECM and ITGA6 to provide a complete understanding of ITGA6 expression levels. Overall, ITGA6 remains a strong candidate for potential cancer therapeutics targets.

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