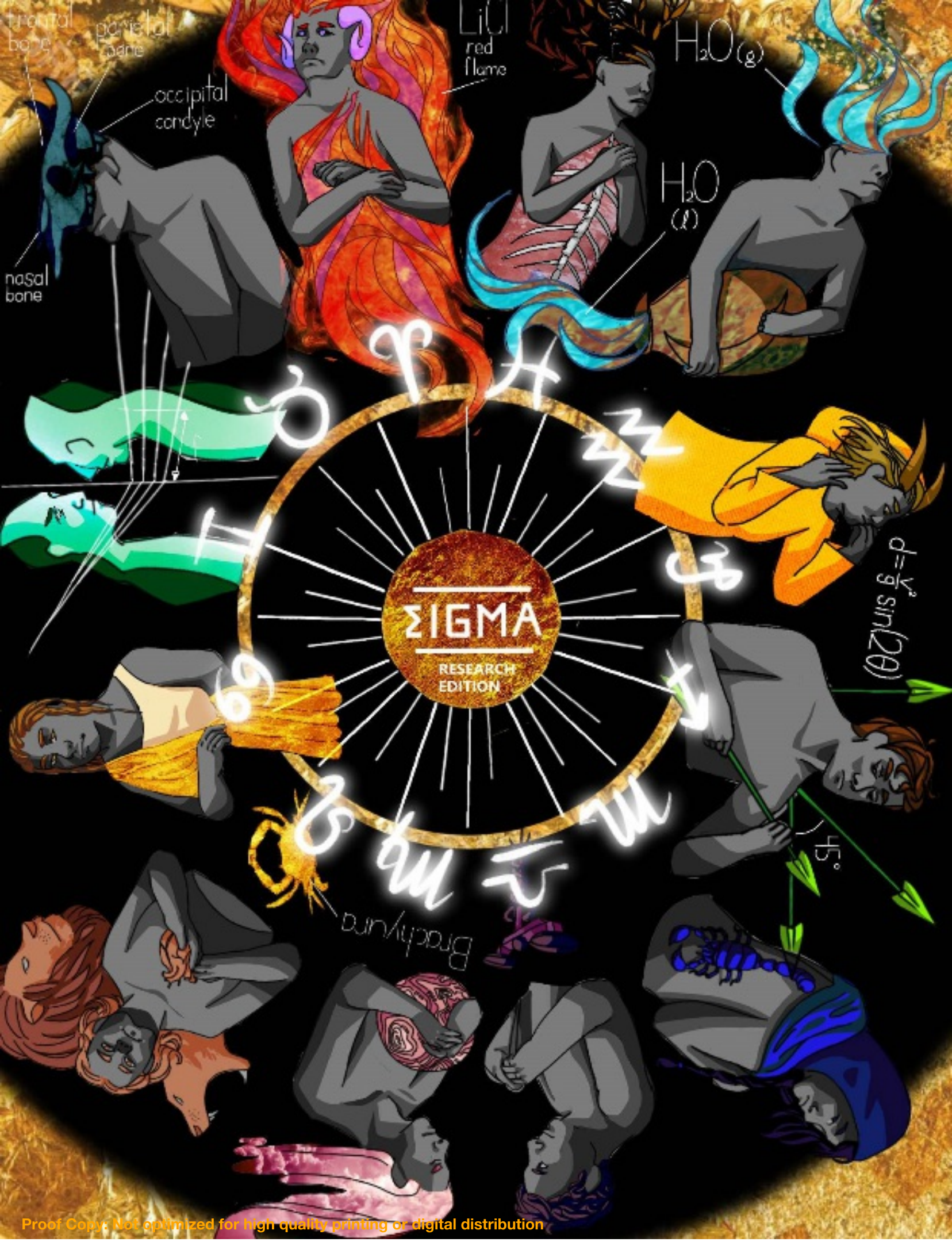

Stuyvesant STAR Mission

The Science Technology and Research Club is created to facilitate student research in a myriad of topics. One of its main goals is to revive Sigma, the science magazine, in order for more members of the Stuyvesant community to have access to scientific material written by their peers. By encouraging its members to write articles, the club aims to hone the writing skills of future scientists. Additionally, the club will expand and strive to compete in science competitions by forming groups of members into teams based on their interests and levels. The club hopes to motivate its members to actively engage in scientific discussions and earn a valuable experience by receiving feedback to improve their scientific writing and teamwork.The Science Technology and Research Club is created to facilitate student research in a myriad of topics. One of its main goals is to revive Sigma, the science magazine, in order for more members of the Stuyvesant community to have access to scientific material written by their peers. By encouraging its members to write articles, the club aims to hone the writing skills of future scientists. Additionally, the club will expand and strive to compete in science competitions by forming groups of members into teams based on their interests and levels. The club hopes to motivate its members to actively engage in scientific discussions and earn a valuable experience by receiving feedback to improve their scientific writing and teamwork.

The purpose of this publication is to disseminate Intel research acheived by Stuyvesant students. Intel research consists of a wide range of STEM topics (computer science, mathematical studies, physics, chemistry, biology, and engineering).

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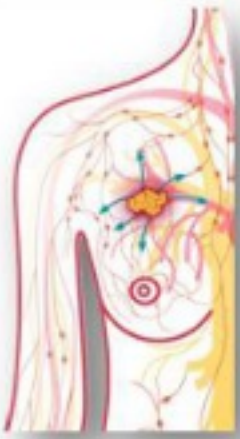
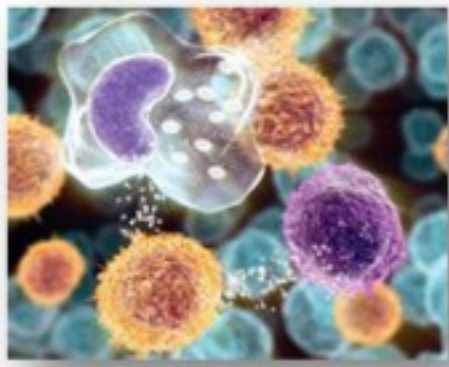
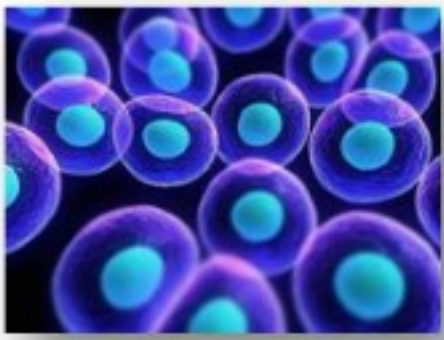
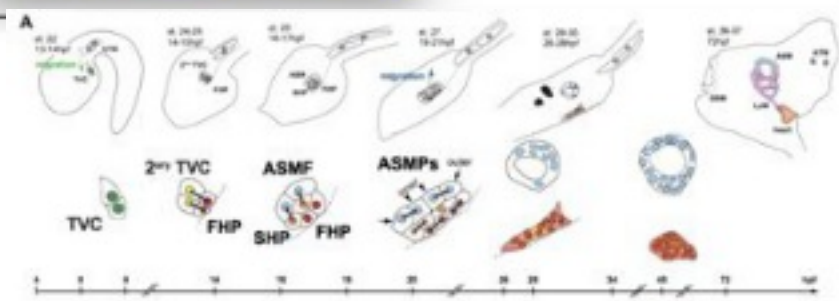
RESEARCH EDITION









Credits

Faculty Advisor
Mr. Thomas
Mr. Econome

Executive Department
Kimberly Ho
Benedict Ho
Jasmine Zhang

Technology Department
Annie Li
Oliver Chen

Editing Department
Janny Chen
Alvin Zhu
Marie Ivantechenko
Vijay Deo
Areesh Naz

Layout Department
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Julia Lu

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Jan Kowalski
Michael Zheng
Jessica Yuen
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Jacopo Siniscalco
Sehee Jun
Iris Tao

CHARACTERIZING THE ROLE OF RAB GTPASES AS MEDIATORS OF EXOSOME BIOGENESIS IN METASTATIC BREAST CANCER

Alexis Kushner

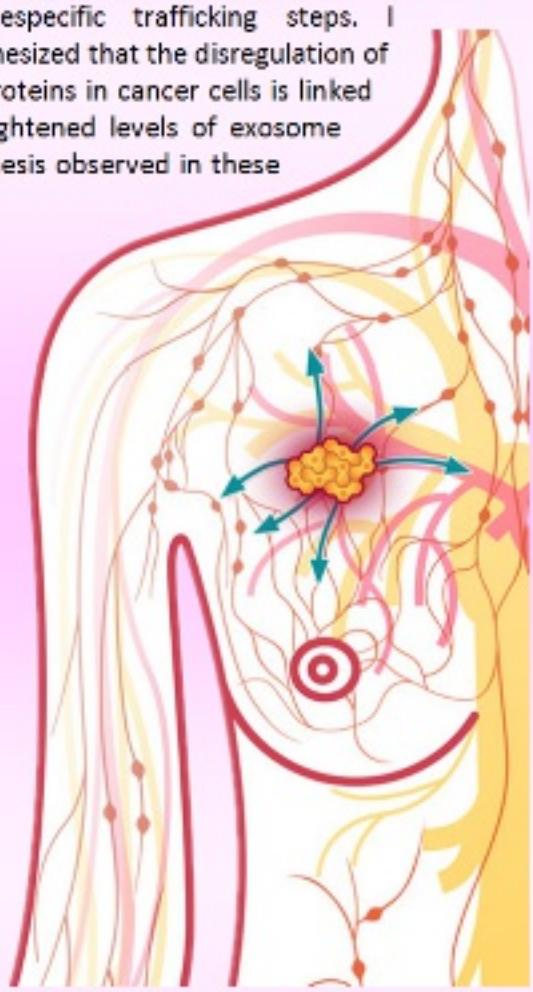
Abstract

Traditional definitions of cancer often portray it as a disease of aberrant proliferation. In recent decades, research at the molecular, cellular, and organismal levels has demonstrated the presence of much more complex and dynamic processes governing the initiation and progression of cancer.

An emerging field of cancer research is the study of the tumor microenvironment. Tumor microenvironment is a broad term describing the cellular and molecular components that contribute to the disease in a variety of mechanisms, including the stromal component of a tumor made up of immune cells, blood vessels, fibroblasts, and bone marrow derived endothelial cells. The tumor microenvironment also contains secreted extracellular vesicles, which have been implicated in cancer and in the formation of the future metastatic sites, referred to as the pre-metastatic niche.

Exosomes, microvesicles 30100 nm in diameter, are a type of secreted vesicles that are created through the endosomal pathway. Exosomes carry proteins, lipids, and genetic material as cargo and have recently been demonstrated to play a central role in the establishment of the premetastatic niche. The effect of disregulation of the endosomal pathway on exosome biogenesis has yet to be completely identified.

Rab GTPases are a major family of proteins involved in regulation of the endosomal pathway. Rab GTPases are a subset of the Ras protein superfamily that regulate the majority of membrane trafficking processes in cells. These processes include internalization from the cell surface; recycling of receptors, cell adhesion molecules, and transport machinery; degradation and biogenesis of organelles; and celltypespecific trafficking steps. I hypothesized that the disregulation of Rab proteins in cancer cells is linked to heightened levels of exosome biogenesis observed in these cells.



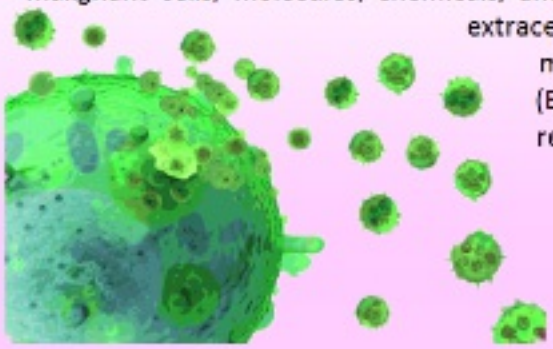
Introduction

Cancer is currently the second leading cause of death in America with an estimated number of 589,430 total deaths in 2015. As a result, there is an incredibly high demand for effective cancer treatments and possible cures. Traditionally, cancer is portrayed as a disease of aberrant proliferation of homogeneous cancer cells. In recent decades, research at the molecular, cellular, and organismal levels has demonstrated the presence of more complex and dynamic processes governing the initiation and progression of cancer. Douglas Hanahan and Richard Weinberg discussed the idea of certain hallmark capabilities of cancer: sustaining proliferative signaling; evading growth suppressors; resisting cell death; enabling replicative immortality; inducing

angiogenesis; activating invasion and metastasis; genome instability and mutation; tumorpromoting inflammation; reprogramming energy and metabolism; And evading tumor destruction. These hallmarks reveal that cancer progression and tumor maintenance are not solely driven by cancer cells themselves, but rather in conjunction with the tumor microenvironment.

The tumor microenvironment is a broad term that describes the cellular and molecular components that contribute to cancer in a variety of mechanisms. This includes the stromal component of a tumor, made up of immune cells, blood vessels, fibroblasts, and bone marrow derived endothelial cells. The actual tumor, though initially thought to be homogeneous, is comprised of several genetically different cells, resulting from mutations, a feature referred to as tumor heterogeneity. Cancer Stem Cells (CSCs) also contribute to the heterogeneity of a tumor and allow for metastasis to go unnoticed and for cancer to remain dormant within the body since CSCs lack HLA and MHC receptors. CSCs also have the potential to differentiate into endothelial cells and assist in the maintenance of tumors.

The tumor stroma, made up of non-malignant cells, molecules, chemicals, and the extracellular matrix (ECM), results from both



stromal and tumor cells, and functions to support the tumor and enable tumor progression. Endothelial cells comprise blood vessels, which serve to provide the tumor with blood and access to vessels for metastasis. Pericytes provide paracrine support for the tumor and structural support for endothelial cells and promote angiogenesis by secreting low amounts of vascular endothelial growth factor (VEGF). Certain immune inflammatory cells inhibit adaptive immunity while promoting angiogenesis and causing inflammation. Cancer associated fibroblasts appear as a structural foundation for epithelial cells and as myofibroblasts, which promote angiogenesis and inflammation.

The tumor microenvironment also contains secreted extracellular vesicles, which have been implicated in cancer and in the formation of the future metastatic sites. One type of secreted vesicle is exosomes. Exosomes are small membrane vesicles of 30100 nm created through the endosomal pathway. Early sorting endosomes formed through endocytosis mature into late endosomes, also known as multivesicular bodies (MVBs), through the accumulation of internal vesicles. These internal vesicles mature within the endosome and are released when the MVB merges with the plasma membrane, at which point they become exosomes.

Exosomes are shed by all cells during the physiological process, in mammals, bacteria, archaea, fungi and parasites alike; however, several other types of secreted vesicles, such as microvesicles and ectosomes, exist as well. Microvesicles and ectosomes are generally larger than exosomes with diameters of 1,00-1,000 nm and most likely differ in protein, lipid, and RNA composition. Very little is known about the exact differences between the various types of microvesicles, but efforts are being made to compare secreted vesicles.

Exosomes are comprised of proteins, lipids, and genetic material largely because of their endosomal origin. Proteins found in exosomes are often involved in membrane trafficking and MVB biogenesis, specifically Rab GTPases, integrins and tetraspanins (CD9, CD63, CD81, CD82), cytoskeletal proteins (actin, moesin), and chaperones such as Hsc73 and Hsc90 . Sphingomyelins are the main lipids shared across all exosomes. Both RNA and microRNA exist within exosomes; however, the 4 mechanisms of transfer of RNA from endosome to exosome remain unclear.

Over the past decade, much attention has been devoted to the role of exosomes in cancer metastasis. Specifically, tumor exosomes have been shown to influence growth of tumor cells and site of metastasis in in vivo experiments. It has been revealed that exosomes recruit proangiogenic factors and are able to transfer these molecules, as well as the genetic material within exosomes, to other cell types.

Tumor exosomes are capable of traveling through the bloodstream to distant organs and establishing new sites for cancer metastasis. While cancer cells cannot travel through the bloodstream undetected, tumor secreted exosomes have the capacity to do so. Tumor exosomes further embody the heterogeneity of tumors since they, too, differ. Prior to the formation of a tumor, various soluble angiogenic factors and inflammatory chemokines, including vascular endothelial growth factor A (VEGFA), tumor necrosis factor alpha (TNFα), and transforming growth factor beta (TGFβ), are recruited to form a favorable environment for tumor growth, referred to as the premetastatic niche. Bone marrow derived hematopoietic progenitor cells (HPCs) and endothelial progenitor cells (EPCs) also help establish the premetastatic niche. Tumor secreted exosomes influence the behavior of endothelial and bone marrow cells and upregulate the secretion of proangiogenic molecules.

Similarly, exosomes can recruit certain factors, such as VEGF, TNFα, and TGFβ, in order to maintain the primary tumor. In addition to the recruitment of molecules to the tumor microenvironment, tumor exosomes are also capable of reprogramming it. Exosomes also participate in crosstalk between tumor cells and may transfer proteins with oncogenic activity, such as epidermal growth factor receptor (EGFRvIII) or mutant KRAS alleles, from one cell to another. Exosomes can inhibit immune responses of T-cells, which otherwise could destroy the tumor, through the activation of myeloid-derived suppressor cells (MDSC). Not only can exosomes inhibit immune responses, but they may also promote immune inflammatory responses to enhance 5 tumor dissemination. MicroRNAs in exosomes can silence the transcripts of Toll-like receptors (TLR) in macrophages, stimulating the release of proinflammatory cytokines, which serve to maintain the primary tumor and facilitate its spread.

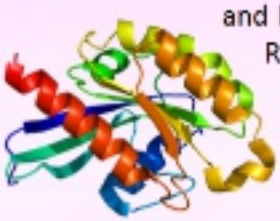
Rab GTPases are the largest subgroup of the Ras superfamily with approximately 70 known Rab proteins in humans. They are responsible for regulating the majority of membrane trafficking processes in cells, including internalization from the cell surface, recycling of receptors, cell adhesion molecules, transport machinery, and the degradation and biogenesis of organelles and celltypespecific trafficking steps. Notably, Rab proteins assist in the secretion and sorting of cargo (i.e. integrins, receptors) of exosomes.

Rab GTPases alternate between a GTP bound 'on' form and a GDP bound 'off' form. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, activating the protein, while GTPase activating proteins (GAFs) stimulate the hydrolysis of GTP to GDP, thus, inactivating the protein. Rab GDP dissociation inhibitors (GDIs) function to regulate the membrane cycle of the Rab, allowing it to remain inactive and soluble in the cytosol. GDI dissociation factors (GDFs) reverse the actions of GDIs and return the Rab to an active form in which they serve as specifically localized integral membrane proteins.

Rab proteins activate and recruit several specific effector molecules that play a crucial role in implementing downstream effects of the Rab GTPase. Effectors include sorting adaptors, tethering factors, kinases, phosphates, and motors. Each Rab GTPase can recruit various different effectors, each of which carries out a different action. Additionally, Rab GTPases may share effector proteins, functionally coupling one Rab to another in a network cascade.

Several Rab proteins have been shown to be associated with cancer. Notably, Rab25 (11C) has been shown to act as both an

oncogene and a tumor suppressor in ovarian and breast cancer respectively.



Rab27A/B play an integral role in exosome secretion, and a knockdown of either Rab reduces the number of exosomes secreted. Because exosomes play a crucial role in both the maintenance and progression of tumors, a decrease exosome biogenesis would have an adverse effect on cancer metastasis.

Results

I hypothesized that the disregulation of Rab proteins in cancer cells is linked to heightened levels of exosome biogenesis observed in these cells. For these studies, I employed a model system comprised of three MDAMB231 human breast cancer cell lines with distinct metastatic organ-specific potential: 4175 lung tropic, 1833 bone tropic, and Yoneda brain tropic cells. In order to determine patterns of Rab protein cargo in exosomes, I analyzed quantitative mass spectrometry data of exosomes each cancer cell line. Mass spectrometry identifies small molecules, such as Rab proteins, through four steps: ionization, acceleration, deflection and detection. Proteins are ionized through electrospray ionization, causing them to accelerate. The proteins are then deflected based on mass and charge so that those with the smallest mass and most positive charge are deflect the most. Electronic detection of proteins occurs in an ion chamber and is based off of a current of electrons moving to neutralize the positive ion.

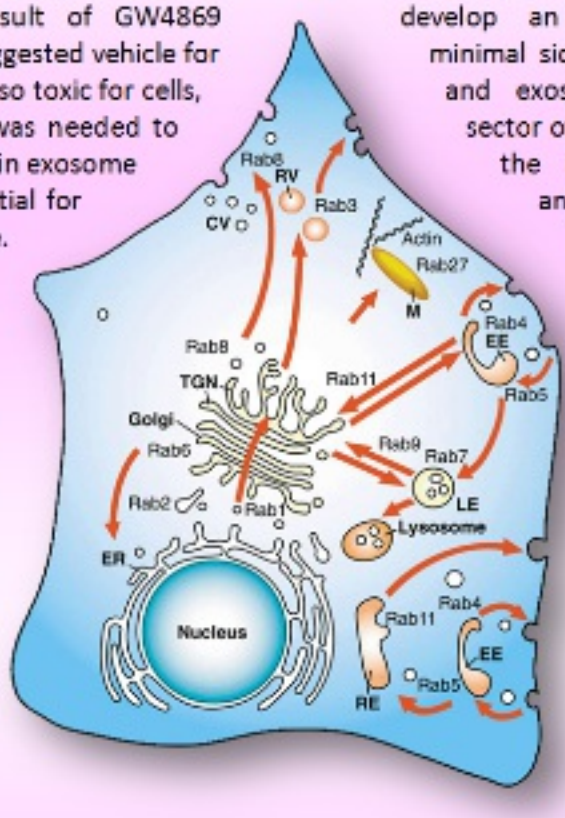
From the raw data collected, I normalized the protein level to βactin, a protein often used as a housekeeping gene because it maintains constant levels across cell lines, and compiled a chart outlining the relationships between the Rab proteins in the lung, bone, and brain and tropic cancer exosomes (Figure 1). Upon this analysis, in conjunction with available literature, I chose to focus on Rabs 5, 7, and 11. Rab5 and Rab7 have been implicated in the sorting of cargo and formation of early endosomes while Rab11 is involved in the targeted delivery of materials and Rab11C has been identified as an oncogene. All three Rab proteins have at least one isoform present in exosomes from each cell line. To validate the mass spectrometry data, I isolated exosomes from conditioned media and performed immunoblots to confirm the presence of all three rab proteins in each cell line. I also performed immunoblots on cell lysates to determine if Rab content of cells would differ from that of exosomes. Again, I used βactin for normalization.

The lung tropic cells had the highest exosome yield and cell lysate concentration, which I determined through protein quantification. To create the master mix to load into the gels, I calculated the value in µl of 15 µg of lysates and exosomes and then adjusted the volume accordingly and ultimately loaded 33 µl per lane (Figure 1). Both charts contain values normalized to βactin. Red indicates the highest value, green indicates the lowest. a) This chart compares the presence of Rab proteins between cell lines. The bone tropic exosomes appear to have the most abundant Rab content, while the brain tropic cells contain lower levels of Rab proteins than the other two cell lines, except in regards to Rab 5C. b) This chart compares the level of Rab proteins relative to one another within one cell line. Figure 2 shows the results of Western Blots for cell lysates and exosomes in lung tropic, bone tropic, and brain tropic cell lines. All cells and exosomes are found to contain Rab proteins. However, the bands vary in intensity, not only between cell lines, but also between cells and exosomes of the same line. Interestingly, proportionate to actin, there is an enrichment in Rab7 in exosomes which matches the mass spectrometry data. In addition, Rab11 cells show two bands, while exosomes only show one, meaning that there is some specification in the packaging of exosomes. The second band is most likely an isoform of Rab11. Though the antibody used is intended to pick up Rab11A, the sequence is very similar to that of Rab11B, thus it is not unlikely that the antibody also picked out Rab11B. 8 Figure 2. I also treated the cell lines with GW4869, a drug targeting the

enzyme neutral sphingomyelinase, to investigate the cell lines's different sensitivity to the inhibition of lipid molecules essential for exosome biogenesis. I cultured cells in regular media, serum free media with a vehicle (DMSO), and serum free media with GW4869.

Prior to this step, I cultured cells in serum free media and regular media, to ensure that cells would grow adequately in serum free media and that any reduction in cell count would be primarily the result of GW4869 treatment. DMSO was the suggested vehicle for GW4869, however DMSO is also toxic for cells, thus another control group was needed to further ensure that reduction in exosome biogenesis was more substantial for GW4869 than for DMSO alone.

I prepared 1:3 dilutions of exosomes from the three different plates for each cell line with PBS, using Nanoparticle tracking analysis (NTA) software to determine the amount of exosomes present (Figure 3). Using the raw data, which I obtained from NTA, I compiled a chart comparing the average concentration of exosomes isolated from varying conditioned media between cell lines (Figure 4).

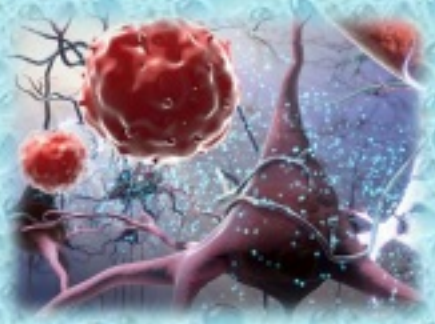


Discussion

I set out to try to understand how Rab proteins and endosomal system affect exosome biogenesis and cancer progression. Through my experiments, I have shown that Rab proteins are present in both cells and exosomes, though levels of Rab protein differ from cell line to cell line. Differences between Rab expression in cell lines and exosomes displayed in my Western Blots indicate that there are certain mechanisms in the endosomal system that package exosome cargo in a specific and purposeful manner. The decrease in exosome production in cells treated with GW4869 prove that disruptions in the endosomal system in any way affects exosome production. Thus, a disregulation of Rab proteins, which serve to control the endosomal system in cells, could lead to heightened levels of exosomes and cancer. Most notably, my experiments uphold the notion that that cancer is an incredibly complex disease. Levels of three different Rab proteins differed drastically between closely related cell lines as did exosome biogenesis. This reaffirms that treatment for cancers must target cancer specific vulnerability in order to develop an effective therapy with minimal side effects. 11 Rab proteins and exosomes represent a small sector of cancer research, however, the implications of both are anything but. Exosomes are responsible for tumor maintenance and metastasis. Thus, a reduction in exosome biogenesis would have adverse effects on tumors and cancer itself. Rab proteins present themselves as an ideal target for future therapies to limit exosome production and prevent tumor metastasis. Much more research is necessary in order to understand what causes the disruption of Rab proteins and the endosomal system. If the process is at all reversible, it will provide a clear path for development of specific treatments. It may also provide knowledge for earlier detection, diagnosis, and reveal individuals that may be susceptible to cancer. Even with this knowledge, cancer still presents itself as a daunting challenge. There are so many other factors that still need to be explained, however, a deep understanding of Rab GTPases and their role in exosome biogenesis will help establish new therapies and solve part of the puzzle that is tumor metastasis.

APOL1 Risk Variants Enhance HIV Replication in Peripheral Bone Mononuclear Cells through Increasing Pro-inflammatory Cytokines Production

Carol Wang



Abstract

Clinical studies show that African Americans (AAs) have higher rates of HIV infection and mortality compared to other races. However, the mechanism behind this discrepancy (association) remains unclear. This study investigated the effect of *Apolipoprotein L1* (APOL1) gene variants on HIV replication because its variants are predominant among these individuals. It was observed that constitutively expression of *APOL1* in peripheral blood mononuclear cells (PBMCs) and T-cells, as well as lentiviral over-expression showed a significant increase in the gene expression of the HIV genome in these cells. Further, among individuals with the APOL1 variants, we found that there was an increase in the secretion of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α along with the accelerated HIV replication rate.

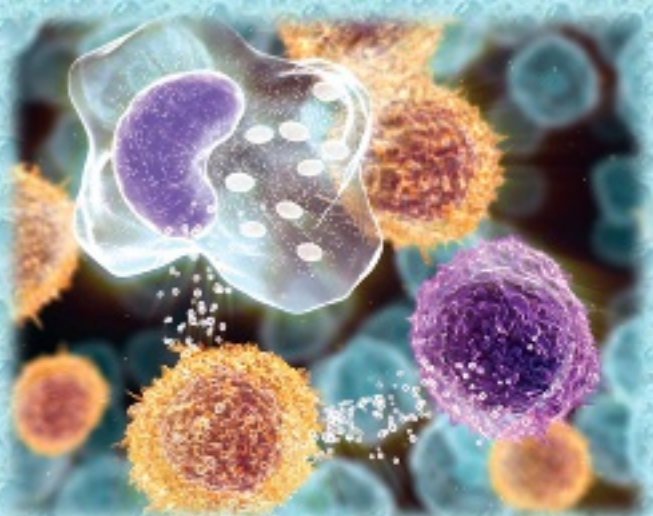
Our studies showed that HIV replication was attenuated when treating PBMCs with IL-6 and IL-1β neutralizing antibodies. This suggests that *APOL1* risk variants induce the expression of pro-inflammatory cytokines which then induce the expression of the HIV genome and lead to increased HIV replication. Increased HIV replication leads to a higher viral load and faster disease progression. This study gained insights into the molecular mechanisms involved in the higher rate of HIV incidence among AAs and will highlight some novel strategies for induced HIV prevention and therapeutic practice for this population.

Introduction

Clinical studies have shown that African Americans (AAs) are a high risk group for Human Immunodeficiency Virus (HIV), as they make up approximately twelve percent of the United States population but represent nearly half (44%) of newly reported HIV cases and one third of all people living with HIV in 2011]. Data collected from 2009-2010 also showed that the rate of HIV incidence among AAs is nearly eight fold that of European Americans (EAs). Another study of patients diagnosed with HIV infection in 2008-2012 found that AAs have an estimated rate of death that is fourfold higher than those of other races. Therefore, AAs have an increased rate of HIV infection as well as higher mortality rates. Although various socioeconomic factors have been suggested as the cause of this disparity, other studies have demonstrated that African Americans are at a higher risk for HIV infection even when their behavior is "normative". Another study found that young female AAs were the least likely to show high-risk behaviors but more likely to report sexually transmitted diseases (STDs), suggesting that risk behavior patterns do not necessarily correlate with STD prevalence. These findings suggest that the disparity in

infection rates is not due to the risk behaviors associated with certain socioeconomic statuses but rather a biological or genetic factor whose mechanism is currently unknown.

Besides higher HIV incidence, AAs have also been shown to have higher rates of other conditions such as leukemia, high blood pressure, and heart disease. Similar to the mechanisms of HIV, the underlying mechanisms for these other conditions have not been confirmed. However, a genetic mechanism behind higher rates of chronic kidney disease in AAs has been partially elucidated. Clinical studies have illustrated that AAs have four to fivefold higher rates of chronic kidney diseases, such as focal segmental glomerulosclerosis (FSGS), end stage kidney disease (ESKD), and HIV-associated nephropathy (HIVAN), compared to EAs. It is well established that this discrepancy is due to the variants of the gene coding for *Apolipoprotein L1 (APOL1)*, a component of circulating high density lipoprotein (HDL) found only in primates. These protein variants, coined G1 and G2, have been predominantly selected over the wild-type (G0) in people of African ancestry (34% of AAs carry both risk alleles). Meanwhile, the variants remain virtually nonexistent from European and Asian population (0.3% of EAs), indicating its relatively recent origins from after the first modern human beings migrated from Africa.



The selection for *APOL1* variants in people of African ancestry resulted from the lytic properties of *APOL1* towards the trypanosomes associated with the African sleeping sickness. While *APOL1G0* can readily lyse *Trypanosoma brucei brucei* through the formation of anionic pores in its endolysosomal membranes, *Trypanosoma brucei rhodesiense* has developed a resistance by generating Serum Resistance-Associated (SRA) proteins that interact with the C-terminal helix of *APOL1* to neutralize and degrade the proteins through cysteine proteases. The novelty of the variants is that they both formed due to mutations in the C-terminus, the SRA-interacting domain. The two missense mutations in *G1* (Ser342Gly and Ile384Met) and the two amino acid deletions in *G2* (N388 and N389) prevent the SRA protein from neutralizing them. *APOL1* variants are thus able to maintain their trypanolytic properties while the wild-type is not, and this adaptive advantage has caused these variants to prevail in sub-Saharan Africa.

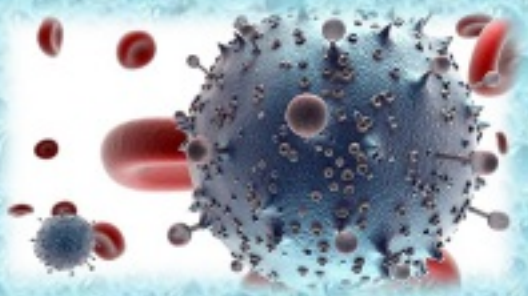
Although a single parental *APOL1* variant is sufficient to protect against trypanosomes, two copies of the risk alleles increase rates of chronic kidney disease in AAs. Podocytes are highly differentiated cells located inside the kidney that maintain the filtration barrier in the glomerulus. The loss of this filtration barrier due to podocyte injury can lead to serious conditions such as proteinuria—abnormal amounts of protein found in urine—and glomerulosclerosis—scarring or hardening of glomeruli tissue. *APOL1* variants confer toxicity through the enhancement of lysosomal permeability, causing the lysosome's hydrolytic enzymes to damage the podocytes. It has been

suggested that the enhanced lysosomal membrane permeability compromises the podocytes' actin cytoskeleton and induces necrosis in the cells.

It is currently unknown whether *APOL1* risk variants also contribute to the higher rates of HIV infection among AA's as they do to the rates of chronic kidney disease. Although HIV can infect multiple types of cells in the body, its main target is the CD4 lymphocyte, also called helper T-cells. For this reason, I conducted my study on Jurkat cells—immortalized T lymphocytes—and Peripheral Bone Mononuclear Cells (PBMCs): lymphocytes, monocytes, and macrophages.

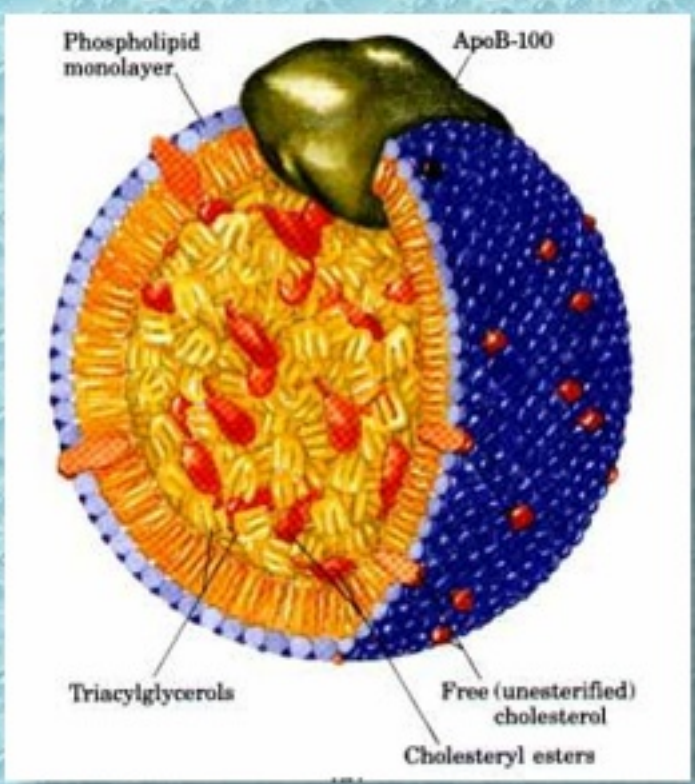
Conclusion

My research discovered that *APOL1* variants increase HIV replication by inducing the expression of HIV genes in infected cells. The variants do so by inducing the expression of pro-inflammatory cytokines that have been shown to augment HIV replication. Although there have not been published papers reporting similar conclusions, other studies have found that IL-1β, IL-6, and TNF-α—alone and in conjunction with each other—increase HIV replication through multiple mechanisms. Furthermore, it is believed that these cytokines seem to interact with HIV in a



positive feedback manner, meaning that the level of cytokines increase as the disease progresses until cells are so defective that they can no longer produce cytokines. This suggests that the induced expression of pro-inflammatory cytokines is capable of creating an effect pronounced enough to cause higher rates of HIV infection and mortality in AAs.

The induction of HIV replication by *APOL1* variants through pro-inflammatory cytokines is a novel idea discovered from my project. A significant aspect of my project was that APOL1 variants-induced HIV replication was attenuated by blocking IL-1β and IL-6 cytokines. Since several anti-IL-6 and anti-IL-1β are available and have been used in clinical trials for other diseases, a logical next step would be testing the efficacy of these cytokine antagonists to attenuate APOL1-induced HIV replication in an animal model. FDA-approved antagonists that could be considered for testing based on clinical trials include siltuximab, an IL-6 monoclonal antibody that has shown success in treating patients with multicentric Castleman's Disease, and anakinra, an IL-1β receptor antagonist that is safe and effective in treating rheumatoid arthritis.

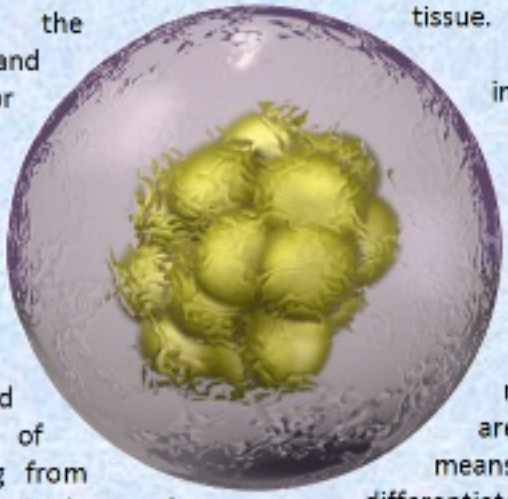


MESENCHYMAL STEM CELLS AND TISSUE REGENERATION

Vanathi Ganesan

Abstract

Recent years have brought to light the potential of human umbilical cord tissue for tissue regeneration, particularly because it contains mesenchymal stem cells (MSCs) in Wharton's Jelly. This study aims to determine the optimal conditions needed to maintain cord cell survival and growth. We tested the dependency of oxygen and serum nutrients for human fetal umbilical cord tissues (obtained, after baby deliveries, as discarded tissues). Thin tissue slices were cultured in various serum concentrations (1%, 5%, and 10%) and under various levels of carbon dioxide, ranging from 2%-14% CO_2 levels. After 24 hours of tissue culture, the tissue slices were cryofrozen and cryosections were prepared for H&E staining. The numbers of intact cells were quantified under microscope and the total area of each section was measured by imaging tool. As a control, a tissue section before culture was included in analyses. Our results showed that under 14% CO_2 and 10% serum, the cord cells were the highest (637 cells/mm^2). Thus, our study suggests that human fetal umbilical cord tissues need a hypoxic environment and depend on serum nutrients.



Introduction

In recent years, the potential of using Mesenchymal Stem Cells (MSCs) in regenerative techniques has been brought to question. Initially, the bone marrow was a source of abundance for MSCs, as discovered by A.J. Friedenstein in the 1960s. These cells are multipotent stem cells and can differentiate into a variety of different cells. Specifically, there are three criteria that must be met to be characterized as a MSC: (1) they must be plastic-adherent (2) they must express C105, CD73, and CD90 and not CD45, CD34, CD14, CD11b, CD79 or CD19 and HLA-DR surface molecules by flow cytometry and (3) they must be capable of differentiating into osteocytes, adipocytes, and chondrocytes. The MSCs are found also in the cord blood, adipose tissue, and, recently discovered, the umbilical cord tissue.

These cells are currently in high demand because of their potential to self-renew with high proliferation, their undifferentiated states, and their immunomodulatory properties. Not only are they undifferentiated, but also, unlike normal multipotent stem cells, these are much more primitive; this means that they will not differentiate with traits specific to the types of cells in the region they are located in, but rather into any type of tissue. This opens up avenues to use the cord tissue stem cells in a variety of ways unlike other stem cells.

Since the discussion of the potential of these cells has been recent, information regarding the profile of these stem cells are limited. The tissue's stem cells are the most favorable since they are present in abundance in the Wharton's Jelly and obtaining these cells can be done simply without invasive procedures on a patient since it is being extracted from discarded tissue and thus not crossing any ethical lines.

Before attempting to use these cells for tissue regeneration, however, it is crucial to complete an accurate profile of these cells, and thus a necessity to answer a number of questions. First, how many times greater is the number of cells in the

Wharton's Jelly compared to the bone marrow? What are the most optimal conditions necessary to ensure maximum potential of cell development and growth? Additionally, since these cells are labeled as "primitive" since it is connected to fetal tissue, how do these cells express traits and functions differently compared to other MSCs found in other regions of the body? How do the organelles in these cells function and are they all as equally viable as those in a normal somatic cell?

This paper attempts to add to this limited profile of MSCs with several aims. First, it is crucial to quantify the umbilical cord tissue as a whole, and specifically if it is predominant in particular parts of the tissue (beginning, middle, end). It is then important to determine the most optimal conditions needed for culturing these stem cells.

Literature has noted that hypoxic conditions lead to better stem cell growth; however, there is not much described about the effects of different levels of carbon dioxide on the growth rate and viability of the cells. Instead, many studies use the standard culture conditions of 5% CO_2 and 37°C when culturing the tissue. This study will take on a different approach of culturing the cells by modifying the medium used and not using fetal bovine serum (FBS) and instead will use the cord blood serum from humans. This study will present a novel approach to effectively culture the tissue and identify the potential for MSCs.

Results and Discussion

The highest concentration of cells per square millimeter was noted to be at conditions 14% CO_2 and 10% serum, where it was close to 637 nuclei/mm^2. In this study, the staining showed the nuclei present in the tissue, but given that the MSCs are in a primitive form in the umbilical cord tissue, it is highly unlikely that there are two nuclei merged into one cell, so each count would be representative of one cell.

To note the changes in the 14% CO_2 incubation, the highest value resulted from 10% serum, followed by 1% serum, and then 5% serum. This narrows discussion as to whether 1% or 10% would produce the most optimal results since both yielded a similar concentration of cells. A previous study noted that bone marrow MSCs were confluent in both serum-free and serum-containing medium, but the way the cells were organized and the appearance varied.

Despite the variations between the bone marrow MSCs and those of the cord tissue, it can be deduced that a similar phenomenon will occur in the latter type where one can assume that 1% serum is representative of "serum-free" conditions and 10% serum is "serum-containing." In this scenario, it would be assumed that the 10% serum is denser and has larger cells; in comparison, qualitatively, the 10% serum did not seem to have these characteristics, which instead held true for the 1% serum group.

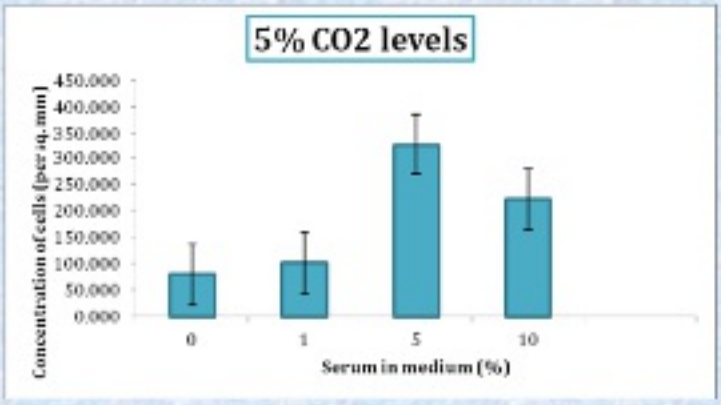
Additionally, it is interesting to note the second largest cell concentration; at 2% CO_2 and 1% serum, there are about 569 nuclei/mm^2 of tissue, which surpasses the 14% CO_2 concentration by about thirty nuclei. The general trend of 2% CO_2 at different concentrations of serum is that the concentration of cells decreases with 10% serum yielding the least number of intact cells. This indicates, disregarding the 14% trial, that 1% serum would produce the most optimal results to ensure the most number of viable cells.

Comparing these results with the control group of the incubated samples, 5% CO_2 incubation does not actually lead to the most viable cells overall and is ranked as one of the lowest for both 1% and 10% serum concentrations. Generally, cell culture held at 5% CO_2 allows the cells to adapt and tolerate the changes in PO_2 and pH in the environment. In the case of stem

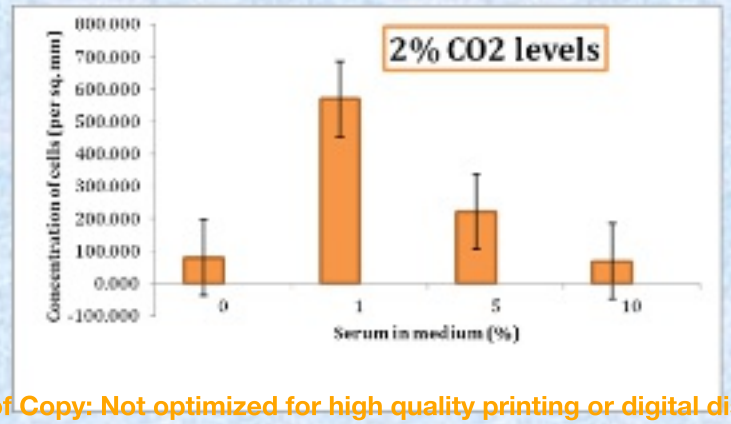
cells, the decreased viability of cells proves that a specific range of CO_2 favors increased cellular count. This selects the top carbon dioxide levels, 2% and 14%, and suggests looking into ranges around these points to determine the most suitable range for the cells.

Qualitatively, there are also certain key observations that come to conflict with the quantitative results. Generally, higher the CO_2 concentration results in greater disintegration of the tissue. At 2% CO_2, the tissues were the most intact and at 14% CO_2 they were the least intact. Specifically at 14%, the tissue was more faded and in some parts barely visible; yet the nuclei could still be seen. Some of these nuclei were more rounded and regular compared to the nuclei in the 2% group; this shows that the increased CO_2 concentration contributes to not only the disintegration of the tissue, but also the configuration of the nuclei.

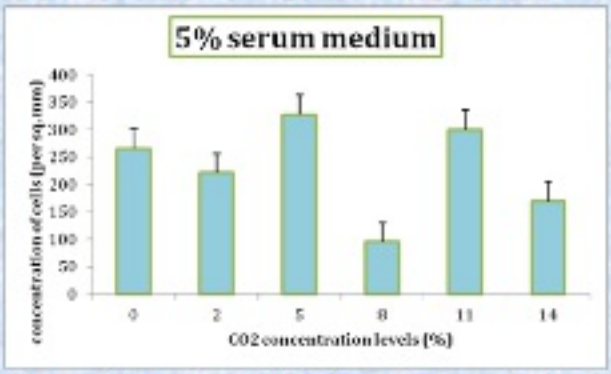
The overall structure of the tissue caused the tissue to disintegrate unevenly. The muscular parts of the cord tissue, which are closer to the vein and arteries,



remained relatively intact the entirety of the trials, beginning to break apart

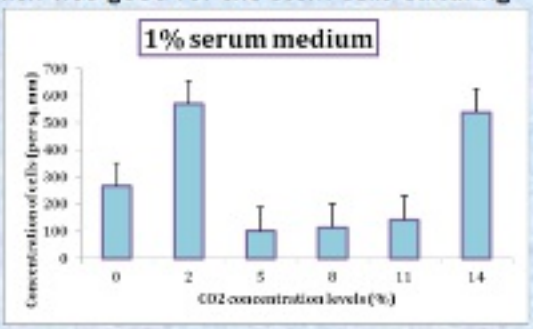


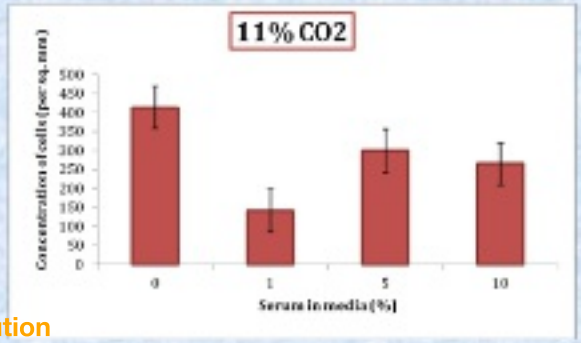
noticeably at 14% CO_2. The outer tissue became weaker and thus was quick to disintegrate. With serum concentrations, this also may have caused varied patterns of loss in tissue: typically at 1% serum, the tissue remained more intact comparatively than at 5% or 10%. These added



observations then shifts the advocacy that 14% CO_2 and 10% serum are the most viable since there was also a large amount of disintegrated tissue to 2% CO_2 and 1% serum may be the optimal set of conditions.

Lastly, these trials, while performed at desired CO_2 levels and at a constant temperature of 37°C, the O_2 levels were not at the standard 21%, but at more hypoxic conditions. That is each set of trials had more hypoxic conditions than the last so that 2% CO_2 had 13.9% O_2 while 14% CO_2 had tissues subjected to 8.2% O_2. The best O_2 conditions for stem cells in general range from 0.7%-7%. (Van Der Sander 2012) The last trial's conditions were the most hypoxic, which was good for the stem cells culturing

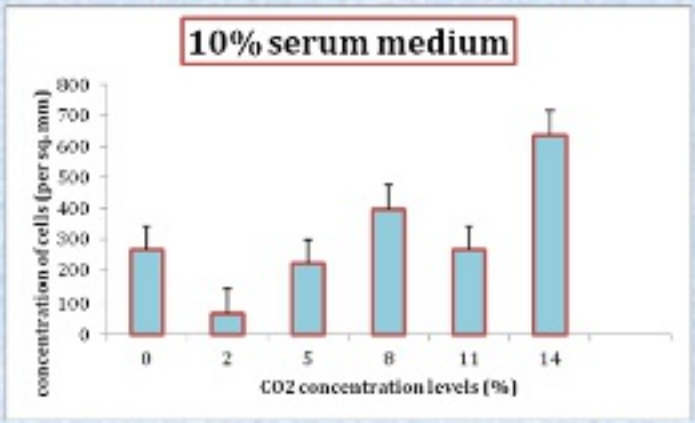




and thus promoted a large amount of viable nuclei. However, it is contradicting when at 11% CO_2 the O_2 levels were at 8.4%, and yet it was still not able to preserve as many viable cells.

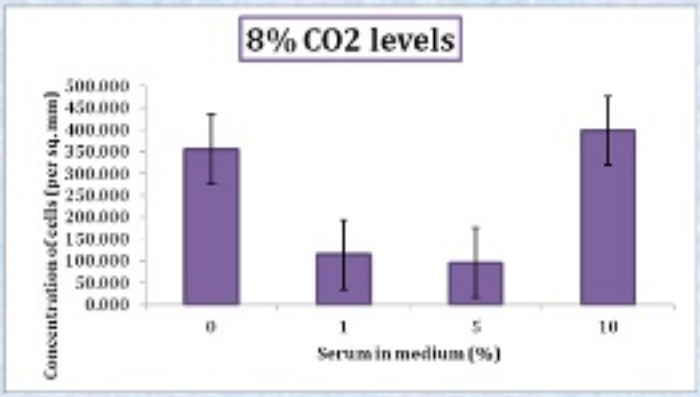
Conclusion

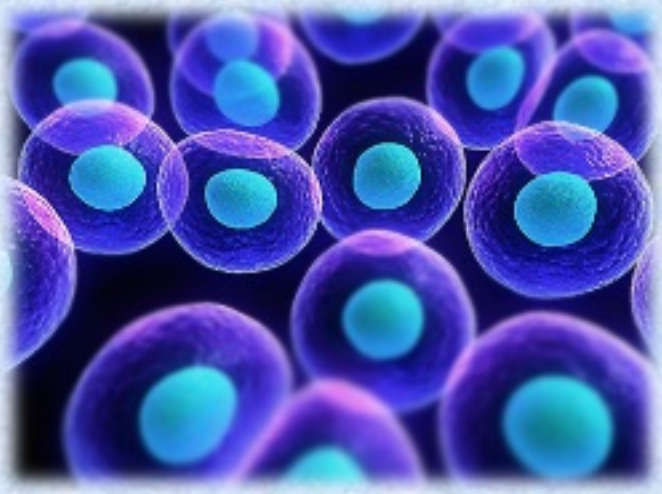
From conducting these sets of trials, it can be narrowed down to two critical points that need to be further tested to solidify the most optimal CO_2 concentration and the best hypoxic value to culture the cells in. This study's results show that it is crucial to have a serum concentration added to the medium along with these low oxygen levels to provide compatible conditions and preserve a large number of viable cells. For future trials, it would be best to focus on 2% and 14% CO_2 to confirm these findings; also, this would need to be followed up by trials conducted with smaller ranges of carbon dioxide values to best determine the specific criteria of survival for the cells. 5% CO_2 levels would also need to be kept to continue to serve as the control group for the testing values.



In addition to simply counting the viable nuclei, the study would need to expand to include the actual count of MSCs in the cord tissue. This way, the conditions can be continued to be refined according to the surviving number of stem cells in each group. The tissues would need to be stained with double markers to ensure that the cells located are not simply responding to the marker, but are truly stem cells.

Stem cells are very delicate cells that require keen fine-tuning of conditions to preserve them to maximize the viability of the cells. The relative versatility of such cells throughout the body is key to manipulating what is already present to design treatment methods specific to the person. Moreover, the primitive nature of these mesenchymal stem cells allows these cells to be regenerated into a variety of tissues. Further profiling of these stem cells is necessary to clarify the ability and nature of the cord tissue stem cells and put them to use.





Tissue-Specific CRISPR/Cas9-Mediated Editing of the *Tbx1/10* Cardiopharyngeal Regulatory Element In A Simple Chordate Model

Tina Jiang

Abstract

97% of our genome consists of non-coding DNA, typically involved in governing spatial-temporal activities like expression or transcription of specific genes. With CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRIPSR- associated with protein-9 nuclease) mediated gene editing, it is possible to probe the function of non-coding regulatory regions in its native context, as opposed to studying it in isolation with reporter assays. A putative *cis*-regulatory element, or "enhancer," controlling the expression of the transcription factor-coding gene *Tbx1/10* in the cardiopharyngeal mesoderm of *Ciona* embryos was previously identified by data generated from deletion analysis and an assay for transposase-accessible chromatin using sequencing (ATAC-seq). The *Tbx1/10* gene is associated with cardiopharyngeal cell fate, and loss of function in this gene can result in congenital diseases like DiGeorge Syndrome. Here I report the use of CRISPR/Cas9 to produce small and large deletions and insertions both in and flanking this enhancer. Mutations in the enhancer were first verified through DNA sequencing, then tested *in vivo* using a reporter assay, and finally through Whole Mount *In Situ* Hybridization (WMISH). Deletions in the putative enhancer were found to severely diminish the expression of both *Tbx1/10* reporter constructs and endogenous expression, suggesting this enhancer is indeed necessary for *Tbx1/10* activation in the cardiopharyngeal mesoderm. This is a rare demonstration that the minimal enhancer is necessary for gene expression. Showing that CRISPR/Cas9 can be used to create targeted genetic modifications in non-coding regions of DNA provides a new perspective for studying the role of *cis*-regulatory elements involved in genetic disease and development, not only in *Ciona*, but also in humans.

Introduction

The human genome contains over three billion base pairs, most of which scientists have yet to elucidate the function of. In 2000, data from the Human Genome Project seemingly confirmed original suspicions that most of the genome, more specifically 97%, contained "junk DNA." In 2012, however, data published from the Encyclopedia of DNA Elements (ENCODE) project revealed that 80% of the genome has a biochemical function, particularly so called "junk DNA" outside of the protein-coding regions, otherwise known as non-coding regions. Many studies point to the presence of regulatory elements, typically involved in governing spatiotemporally regulated expression or transcription of specific genes, in non-coding regions of DNA.

Until now, studies about *cis*-regulatory elements like enhancers have almost exclusively relied on the use of reporter transgenes, where the regulatory element is studied outside of its genomic context. Now, emerging breakthrough genome engineering technologies like CRISPR/Cas9 allow for the study of endogenous functions of non-coding regulatory DNAs and their impact on gene expression in their native environment. This is rather unprecedented and profoundly transformative. These technologies constitute a formidable

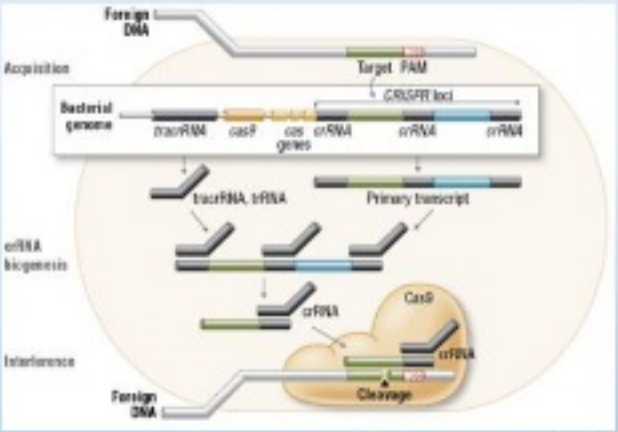


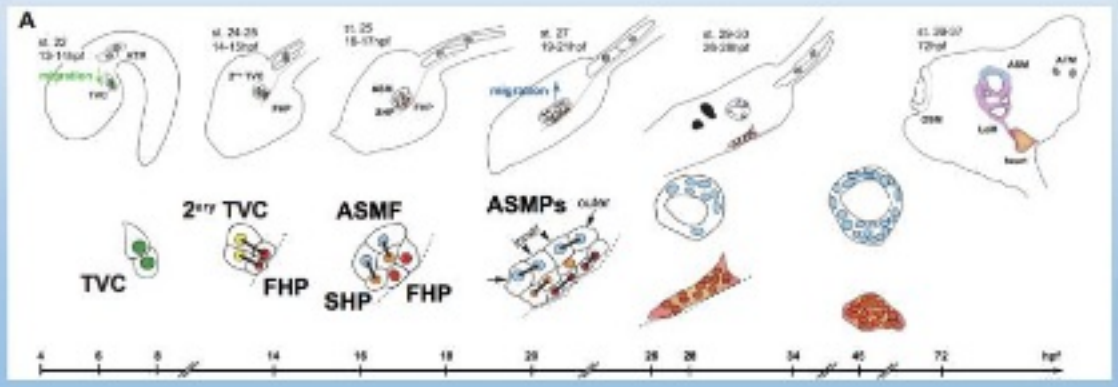
Figure 1
The CRISPR/Cas9 immune defense system in bacteria. The crRNA, tracRNA, and Cas9 form a complex that can create DNA double stranded breaks in the foreign DNA and thus mutate it.

stepping-stone to understanding the genomic code for gene expression, and eventually cell activity, development, and disease. CRISPR/Cas9 is an immune defense system from bacteria, which involves the insertion of foreign DNA, or protospacers, into its own DNA, forming a CRISPR. Transcription occurs at the CRISPR loci, producing site-specific crRNA and universal trans-activating crRNA (tracrRNA). The crRNA hybridizes to the protospacer in the original foreign DNA sequence, which is always situated next to a three base pair (bp)-long protospacer adjacent motif (PAM), which Cas9, the cleaving protein, will recognize and bind to[7] (Figure 1). In an artificially recreated CRISPR/Cas9 system for gene editing, the crRNA and tracRNA have been fused to create what is known as a single-chain guide RNA (sgRNA).

Unlike prior gene editing methods such as Transcription activator-like effector nucleases (TALENs) and Zinc Finger Nucleases (ZFNs), CRISPR/Cas9 offers several advantages including simplicity and cost[10]. Unlike ZFNs and TALENs, designing CRISPR/Cas9 sgRNAs does not require forming complex protein-coding sequences, but rather a short 20 nucleotide sequence. Because of the short set of nucleotides, the target sequence can be easily cloned using conventional techniques as opposed to the complicated ligation techniques necessary for TALENs and ZFNs. In addition to being less labor-intensive, CRISPR/Cas9 is also relatively cheap in comparison to the high costs of ZFNs and TALENs. Custom ZFNs can cost as much as $25,000 and custom TALENs can cost as much as $5,000. CRISPR/Cas9 sgRNAs, on the other hand, can be easily made in the lab for about $30 or less.

Ever since scientists discovered how to adapt the CRISPR/Cas9 system to genetic engineering, the use of this technology has exploded. It has largely been used to alter coding sequences of DNA, but there has been minimal research on using CRISPR/Cas9 to target *cis*-regulatory elements. Because the area of gene editing in non-coding regions is currently underdeveloped, the following descriptions of experiments are the only ones to have been published of late. The earliest use of CRISPR in this context was to target *cis*-regulatory sequences that drive *lin-3* expression in *C. elegans*. Recently, nuclease-null dCas9 protein fused to acetyltransferase p300 has been used to activate and manipulate gene regulation from promoters and enhancers. CRISPR/Cas9 has also been used to mutate CTCF binding sites (CBS) within the Pcdh enhancer to test the effect of CBS on chromatin looping. CRISPR has been used to mutate CTCF binding sites that localize to Hox cluster chromatin boundaries in an effort to elucidate the insulating functions of CTCF binding sites. Additionally, in mice, CRISPR/Cas9 has been used to mutate topologically associated domains (TADs) to demonstrate their function in human limb malformations. These papers are the only ones published that deal with CRISPR/Cas9 gene editing in non-coding regions of DNA.

While scientists have just recently started to use CRISPR/Cas9 to study the

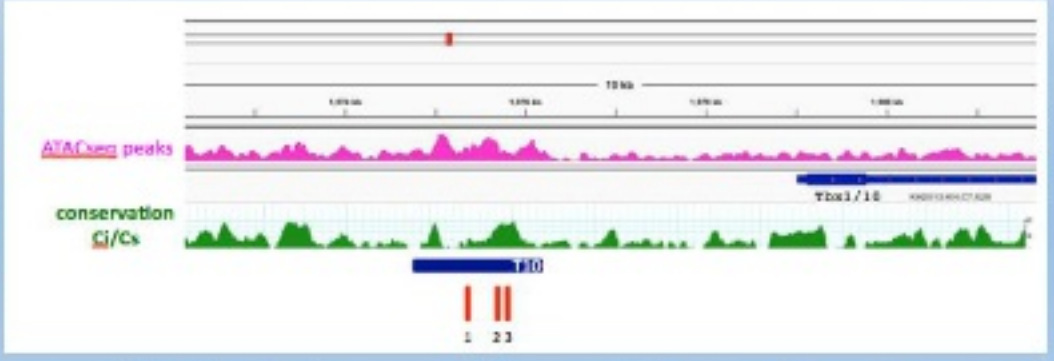


function of *cis*-regulatory elements, this editing system has yet to be used in *Ciona* for this aim. Showing that this is possible in *Ciona* provides several distinct advantages. *Ciona* are tunicates, which are the invertebrates most closely related to vertebrates, making it an invaluable model organism. *Ciona*'s unique phylogenetic position makes it likely that the study of the traits and regulatory networks in the organism will also be conserved in humans.

The relative simplicity of cell lineage patterns in *Ciona* allows for in depth studies on the regulatory networks controlling development. One cell lineage pattern of interest is that of the B7.5 blastomeres (Figure 2). In each of the bilateral B7.5 blastomeres, *Mesp*, a conserved cardiac determinant is expressed, leading to the birth of two Trunk Ventral Cells (TVCs). In late gastrula embryos, the TVCs migrate to the trunk where they divide asymmetrically and mediolaterally to form the First Heart Precursors (FHPs) and secondary TVCs (STVCs). The STVCs also divide asymmetrically and mediolaterally to form the Secondary Heart Precursors (SHPs) and Atrial Siphon Muscle Founder cells (ASMFs). Thus, the primordia of the adult heart and siphon (pharyngeal) muscles are formed.

In the TVC progeny, *Tbx1/10* is first expressed in the STVCs and continues to be expressed in the ASM precursor cells (ASMPs), but is down-regulated in the SHPs. At this point, Tbx1/10 expression in the ASMs, coupled with Fibroblast Growth Factor (FGF) signaling (Razy-Krajka et al., unpublished data), results in *Ebf* expression, and ultimately, the progression to pharyngeal muscle fate. It has been shown that without *Tbx1/10*, *Ebf/COE* expression will not occur, resulting in the specification of excessive heart precursors at the expense of pharyngeal/siphon muscles.

Figure 2



Tbx1/10 is responsible for many of the phenotypic effects of Velo-cardio-facial syndrome/DiGeorge Syndrome (VCFS/DGS). DGS is a developmental disease, characterized by defective cardiopharyngeal development, in which *Tbx1/10* is removed by the deletion of 22q11.2, a part of chromosome 22. Cardiopharyngeal development refers to the development of the early mesoderm, which will produce the right ventricle, outflow tract, and branchiomeric head muscles. Thus, working under the hypothesis that DGS/VCFS is a disease of multipotent cardiopharyngeal progenitors, this study seeks to better understand the cardiopharyngeal regulation and function of *Tbx1/10*, which appears to be conserved between *Ciona* and vertebrates.

Because of the unique role of *Tbx1/10* in pharyngeal muscle fate and thus in DGS, *Tbx1/10* became the focus of where CRISPR/Cas9 would be used. Prior research used an assay for transposase-accessible chromatin using sequencing (ATAC-seq), which generates data by creating peaks where the Tn5 enzyme cuts accessible chromatin regions of genomic DNA, to identify the potential fragment containing the minimal enhancer element of *Tbx1/10* (Figure 3). The rationale is that enhancers are more accessible, or less frequently occupied by nucleosomes, than other non-

coding regions. The fragment containing the minimal enhancer (T10) is a region of 1035 bp located from -3930 to -2896 from the *Tbx1/10* start codon. When the T10 region was cloned in a vector containing the *Tbx1/10* basal promoter upstream the coding sequence for Green Fluorescent Protein (GFP), fluorescence was observed in STVCs progeny, but not in FHPs, demonstrating that the T10 reporter gene is able to recapitulate *Tbx1/10* expression.

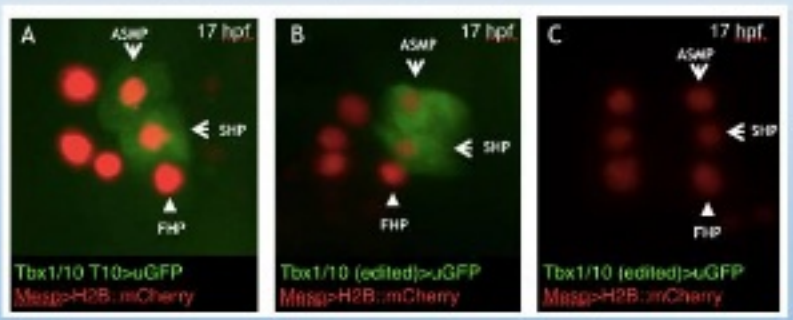


Figure 3

These reporter assays, however, are not proof that this enhancer is necessary for endogenous expression of *Tbx1/10*, even though it is consistent with the idea. It shows sufficiency in a transient assay that could also generate artifacts. CRISPR/Cas9 allows for a more thorough testing of the hypothesis that this T10 element is necessary for *Tbx1/10* expression specifically in the STVCs and their progeny. Thus, sgRNAs that would work with the CRISPR/Cas9 system were developed to target the minimal enhancer in the T10 region. The effect of these deletions were tested *in vivo* via electroporation to see the capability to direct cell lineage-specific expression of the GFP reporter, and through WMISH to evaluate if the genome editing in the *Tbx1/10* regulatory region influences *Tbx1/10* mRNA expression levels.

Results and Discussion

sgRNAs were designed to target the T10 fragment, a region containing the minimal enhancer that controls *Tbx1/10* expression specifically in the STVC progeny (the "STVC enhancer") (Figure 3). Deletions and their functional consequences on STVC enhancer activity and *Tbx1/10* expression were verified through DNA sequencing, a reporter gene assay, and WMISH, respectively.

Sequencing Verification

T10 sequences with deletions caused by individual or combined sgRNA expression cassettes that potentially removed parts of the STVC enhancer were obtained and sequenced. The CRISPR/Cas9 system results in a variety of mutations (deletions or insertions) of variable length, although short (6-16 bp) deletions were most common. sgRNA 1 was the most efficient, resulting in deletions of approximately 90 bp, along with an 18 bp insertion, within the STVC enhancer (Figure 7). sgRNA 2 and 3 were located outside of the STVC enhancer (Figure 3) and were also less efficient compared to sgRNA 1. sgRNA 2 resulted in a 16 bp deletion and 11 bp insertion. sgRNA 3 resulted in a 6 bp deletion (Figure 7). Because sgRNA 2 and 3 were less efficient and also located outside of the STVC enhancer, they were later used in combination with sgRNA 1 to produce larger deletions in the STVC enhancer. sgRNA 1 and 2 combined produced deletions of ~405 bp and sgRNA 1 and 3 combined produced deletions of ~662 bp (Figure 7).

***In Vivo* analysis of CRISPR/Cas9 edited *Tbx1/10* enhancer elements**

Sequences that showed deletions or insertions were tested *in vivo* using a reporter gene assay. Once clones were checked for mutations through DNA sequencing, the fragment was cloned in a vector containing the *Tbx1/10* basal promoter upstream the coding sequence for the GFP reporter gene. The constructs were tested by transfection into *Ciona* embryos *via* electroporation to test their ability to direct STVC-specific expression of the GFP reporter. In order to mark the B7.5 cell

lineage, constructs were co-electroporated with *Mesp*>H2B::mCherry, to trace the B7.5 progeny. Embryos were scored for GFP and mCherry (GFP^+ / $mChe^+$) or just mCherry (GFP^- / $mChe^+$) (Figure 6).

Figure 6

Because the T10 fragment is able to activate GFP expression in the ASMPs and SHPs, CRISPR/Cas9 mutations in the region should result in embryos showing no GFP expression in transfected STVC progeny, but still mCherry in B7.5 progeny (GFP^- / $mChe^+$), which includes the STVCs (Figure 2).

The wild-type T10 fragment was cloned in the same vector and used as a positive control (Fig. 6). 34.5% of the embryos electroporated with *Tbx1/10* T10+basal promoter>uGFP showed GFP expression in the STVC progeny. In embryos electroporated with plasmid DNA containing deletions made by sgRNA 1, only 0-3% of embryos were GFP^+ / $mChe^+$ (Figure 6-7). This data confirms that the deletion created by CRISPR/Cas9 in the T10 fragment, -3689 from the start codon of *Tbx1/10,* abolishes enhancer activity of this minimal element, considering that it was enough to completely stop GFP expression in STVCs progeny. In embryos electroporated with plasmid DNA containing instead an insertion created by sgRNA 1 in the T10 region, 21.5% embryos showed GFP expression in the ASMPs and SHPs, at, still lower than the 34.5% expression of $GFP^+/mChe^+$ in embryos electroporated with the control (Figure 7). Thus, the artificial insertion in the T10 region has a weaker effect on the activity of *Tbx1/10* enhancer in comparison to the drastic effects observed with the deletions.

In embryos electroporated with plasmid DNA containing deletions made by sgRNA 2, the activity of *Tbx1/10* enhancer was not affected at all; GFP expression was detected in STVCs in approximately 34% of embryos (Figure 7). When embryos were electroporated with the plasmid containing the deletion created by sgRNA 3, GFP expression was detected in 21% of larvae (Figure 6-7). Because expression patterns for deletions made by sgRNA 2 were very close to the control, the region that sgRNA 2 created mutations in was not essential to sgRNA activity. On the other hand, mutations created by sgRNA 3 resulted in embryos that had slightly lower expression of GFP^+ / $mChe^+$, suggesting the region sgRNA 3 is located in contains a regulatory fragment important to *Tbx1/10* activity.

To target a larger part of the T10 minimal enhancer, combinations of sgRNAs were used with CRISPR/Cas9. Combinations of sgRNAs targeting neighboring sequences can cause larger deletions in the region located between sgRNA target sequences. In embryos co-electroporated with plasmid containing the deletions created by sgRNA 1 and sgRNA 2 combined and sgRNA 1 and sgRNA 3 combined, GFP expression in the STVCs was completely absent (Figure 6-7), suggesting that the region downstream of sgRNA 2 contains essential components to STVC enhancer activity in the reporter assay.

Evaluation of the impact of T10 mutation on endogenous *Tbx1/10* expression by WMISH

The GFP reporter assay on the CRISPR/Cas9 edited *Tbx1/10* regulatory region suggested that mutations in the T10 element, induced by specific sgRNA combinations, would alter endogenous *Tbx1/10* expression, if STVC enhancer activity is indeed necessary *in vivo*. To test this hypothesis, WMISH was performed on embryos electroporated with sgRNA cassettes, *Mesp*>nls::Cas9::nls and *Mesp*>H2B::mCherry. Control animals were electroporated with just *Mesp*>Cas9 and *Mesp*>H2B::mCherry. The variable effect of CRISPR/Cas9 mutations should be kept in mind when interpreting data from the WMISH, because although certain mutations are highly expected, it is impossible to know exactly what deletions and insertions CRISPR/Cas9 are created in each embryo and whether or not one or both alleles of a gene were mutated.

50% of embryos electroporated with sgRNA 1 showed decreased expression levels of *Tbx1/10* (Figure 8A-B), 25% showed complete absence of *Tbx1/10* expression (Figure 8C), and the remaining 25% of embryos showed normal *Tbx1/10* expression as compared with control samples (Figure D).

30% of embryos electroporated with sgRNA 1+2 showed decreased expression levels of *Tbx1/10* (Figure A-B), 45% showed complete absence of *Tbx1/10* expression (Figure 8C), while the remaining 25% of embryos showed normal *Tbx1/10* expression as compared with control samples (Figure 8D). 25% of embryos electroporated with sgRNA 1+3 showed decreased expression levels of *Tbx1/10* (Figure 8A-B), 50% showed complete absence of *Tbx1/10* expression (Figure C), while the remaining 25% of embryos showed normal *Tbx1/10* expression as compared with control samples (Figure D). Because sgRNA 1+3 created a larger deletion than sgRNA 1+2, more embryos showed complete absence of *Tbx1/10* expression. sgRNA 1, 1+2, and 1+3 all have 75% of embryos with altered Tbx1/10 expression. However, the percentage of embryos containing no *Tbx1/10* increased, as the deletions generated by the CRISPR/Cas9 system get larger.

In the 75% of embryos electroporated with sgRNA 1, sgRNA 1+2, and sgRNA 1+3 that showed altered *Tbx1/10* expression, changes occurred specifically in STVCs and not in other *Tbx1/10* expression territories, such as the endoderm surrounding the sensory vesicle in the larvae head. This is because Cas9 was specifically expressed in the B7.5 lineage, thus introducing mutations only in the STVC progenitor cells. Taken together, these data confirm what was observed in the GFP reporter assay and reinforce the hypothesis that the designed sgRNAs target a region of the T10 fragment that contains the enhancer element necessary for *Tbx1/10* expression in cardiopharyngeal precursors.

Conclusion

Although CRISPR/Cas9 has been widely used to edit coding regions of the genome, as of 2015, only five papers have described the use of CRISPR/Cas9 to elucidate the function of non-coding regions. In fact, in *Ciona*, CRISPR/Cas9 has never been used to mutate an enhancer. Combined with the ease of reporter gene assay by electroporation in *Ciona*, CRISPR/Cas9-mediated mutagenesis of *cis*-regulatory elements will provide invaluable insights into enhancer functions during chordate development.

Importantly, these data demonstrate that the T10 element is necessary for the endogenous expression of *Tbx1/10* in STVCs. This was predicted by GFP reporter assays, but could only be ascertained by mutating the endogenous genomic DNA. While these data are clear evidence that the sgRNA cassettes designed for CRISPR/Cas9 create mutations and block *Tbx1/10* expression, it is impossible to formally know whether both alleles of the gene were altered and which mutations were present in which cells in the WMISH experiment, although certain mutations are highly expected. In the future, an experiment could be done using stable engineered lines with known mutations. The exact effect of mutations in the T10 element could then be quantified using more quantitative methods such as Single Molecule Fluorescent *In Situ* Hybridization, quantitative real-time PCR, and RNA sequencing. The latter would also explore the impact on candidate *Tbx1/10* downstream targets. This is important because Tbx1/10 is a DNA-binding transcription factor and should be compared to loss-of-function mutations targeting the coding region.

Using CRISPR/Cas9 to generate small and large deletions and insertions in the T10 region of *Tbx1/10* has widespread

implications in the study of developmental biology, as well as disease. Showing deleterious mutations can be made in *cis*-regulatory regions provides a new tool for scientists to answer questions about the role of these non-coding regions in gene regulatory networks. Because *Ciona* are tunicates and thus closely related to vertebrates, the function of certain regulatory regions in the *Ciona* genome, including the *Tbx1/10* enhancer, may be conserved in vertebrates, including humans. Since loss of *Tbx1/10* is heavily associated with the DGS, CRISPR/Cas9 can also further be used to elucidate the function of this gene in the disease.

demonstration of CRISPR/Cas9's ability to disrupt the function of non-coding regions of DNA could have a strong impact on the way scientists investigate how specific gene regulatory networks function in *Ciona* and humans.

Figure7

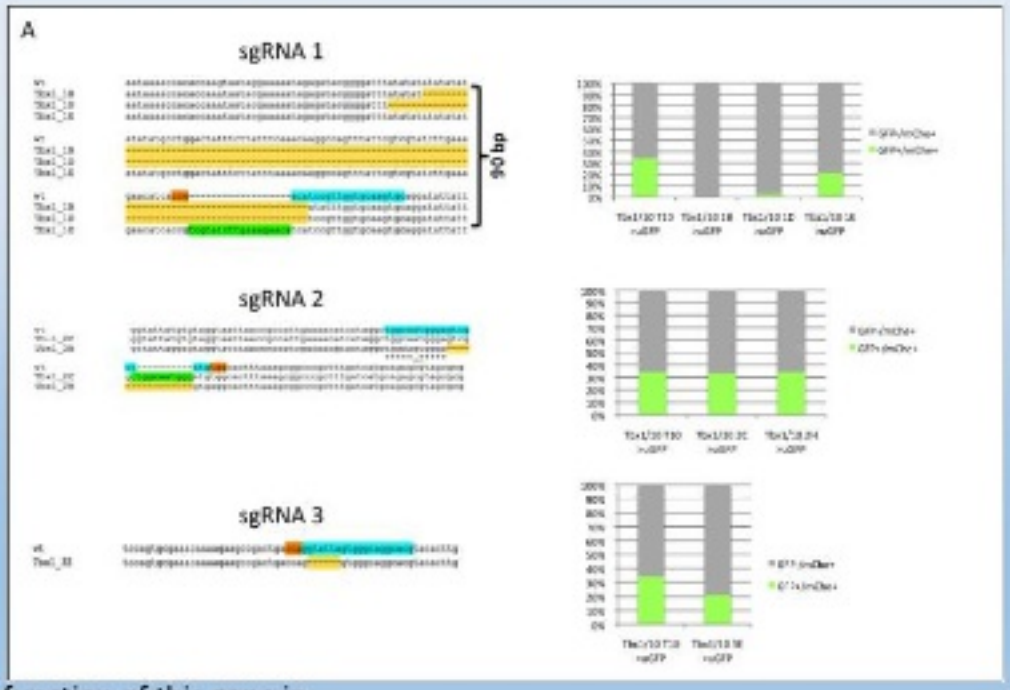
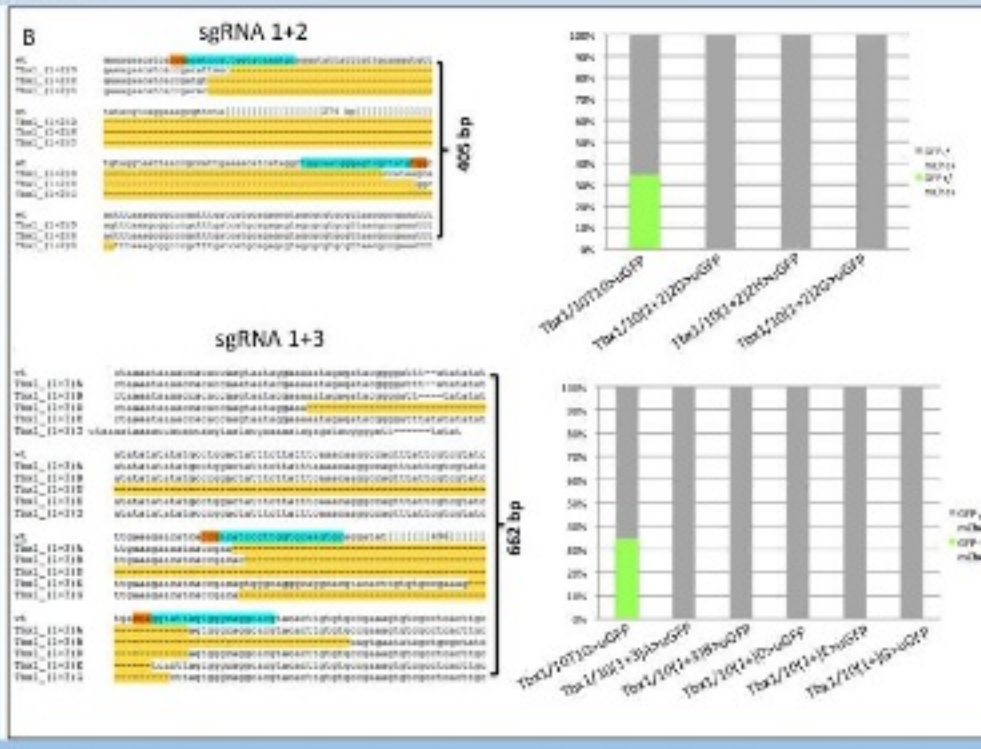


Figure 8



So powerful is CRISPR/Cas9 technology that in May of 2015, it was reported that CRISPR/Cas9 was successfully used to edit human tripronuclear zygotes. This advancement, while thoroughly exciting, also brings the question of ethics to the surface. CRISPR/Cas9 can be used to elucidate functions of genes, but when and where it is appropriate to do so is a point of contention. For now, CRISPR/Cas9 continues to be used in model organisms and human cell cultures, but its use in individual human beings will remain up to debate.

All in all, the CRISPR/Cas9 gene editing system has the potential to, and in fact has already started to, revolutionize the world. This study's

Contact Information

E-mail:

stuyvesantSTAR@gmail.com

Website:

stuySTAR.weebly.com

Facebook Page:

Science Technology And Research Club