Vasolin-Containing Protein (VCP) in Cardiomyocyte Survival and Growth via MTOR Complex Mediated Signaling

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doi: https://doi.org/10.57709/6SSD-6389

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Vasolin-Containing Protein (VCP) in Cardiomyocyte Survival and Growth via MTOR Complex Mediated

by

Sharadhi Siri

Under the Direction of Hongyu Qiu, M.D., Ph.D.

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Interdisciplinary Studies

in the Institute for Biomedical Sciences

Georgia State University

2022
ABSTRACT

Recent studies have demonstrated that the Valosin-containing protein (VCP), an ATPase–associated protein, plays a protective role in the heart against cardiomyopathies and injuries caused by various cardiac stresses, including ischemia and pressure overload. However, the underlying molecular mechanisms remain largely unknown. It has been shown that VCP presents in stressed hearts. Our hypothesis is that VCP plays an essential role in cardiomyocyte growth and survival through a dual regulatory effect on the signaling of the mechanistic target of rapamycin (mTOR). Thus, we used a functional gain and loss strategy to determine the critical role of VCP in regulating mTOR signaling in cardiomyocytes in vitro. By using recombinant adeno-virus system (Ad-CMV-GFP-VCP for overexpression and Ad-U6- siRNA-VCP for knockdown), we successfully overexpressed VCP and knocked down VCP in H9C2 cells respectively. This study aimed to better understand how VCP overexpression and knockdown affected certain proteins involved in the mTOR pathway – in particular phosphorylated Akt (pAkt T308 and pAkt S473), Raptor, Rictor and mTOR. Western blots were also done to confirm VCP knockdown and overexpression with GaPDH as the loading control. This preliminary study, although did not yield substantial results, can be designed as an in vitro system to better understand VCP and mTOR interactions.

INDEX WORDS: Sample keyword, Sample keyword, Sample keyword
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July 2022
ACKNOWLEDGEMENTS

I extend my heartfelt gratitude to my Committee chair and principal investigator Dr. Hongyu Qiu for her immense wisdom and support throughout the course of my project. I want to thank Dr. Chunying Li for agreeing to be a part of my Thesis Defense Committee. This project would not have been possible without the continued guidance of my mentor Dr. Xiaonan Sun, and for her infinite patience in teaching me. A special thank you to all the other members of the lab Amirah, Shi, Jing, and Xiaoxu for their help and support. I also want to extend my gratitude to the NIH grant that supported the project. Finally, a huge thank you to my family and friends for pushing me to perform my best.
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1. INTRODUCTION

1.1 Cardiomyocyte Growth and Survival, and Heart Diseases

1.1.1 The Character of Cardiomyocytes

The heart is a vital organ that functions as a pump maintaining the blood circulation around the whole body, which comprises various cellular subtypes, including cardiomyocytes, fibroblasts, vascular smooth muscle cells (SMCs), endothelial cells, and pericytes; each of them plays essential roles in physiological and pathological conditions. The main cellular component of the myocardium is cardiomyocytes (CM), a contractile, excitable heart cell making up cardiac muscle and driving continuous heart beating, carrying out the cardiac function of the heart by effectively executing the contraction-relaxation cycle. Morphology, gene expression, and function of ventricular CMs are different from atrial CMs.

As illustrated in Fig. 1, in human beings and many other animals, cardiomyocytes are the first cells to terminally differentiate, thus making the heart one of the first organs to form in a developing fetus. The postnatal cell cycle withdrawal of CMs shortly after birth and most CMs in adult hearts are terminally differentiated postmitotic cells exhibiting minimal regenerative potential. The low turnover rate of cardiomyocytes is problematic because the heart has insufficient regenerative capacity after injury or in diseased states. For example, a variety of stimuli, including systemic or regional anoxia or hypoxia caused by acute or chronic myocardial ischemia, or neurohumoral and mechanical triggers caused by pressure overload, could induce ventricular cardiomyocyte death through pathways such as necrosis, apoptosis, and possibly excessive autophagy, resulting in loss of cardiomyocytes in the heart, leading to acute or chronic heart failure. Thus, it is imperative to better understand the pathogenesis of various cardiac
disorders to promote cardiomyocyte growth and survival as an effective way not only to treat but prevent the effects of cardiac injury.

*Figure 1: Cell cycle activities in fetal, neonatal, and adult mammalian cardiomyocytes.*

In development, the ballooning of the chamber and the further growth of the heart are accomplished by fetal cardiomyocyte proliferation. After birth, cardiomyocytes gradually stop proliferating and exit the cell cycle (G0/G1 phase). Initially, this arrest is reversible, as newborn cardiomyocytes can re-enter the cell cycle and proliferate upon injury. Yet during postnatal development, cardiomyocytes undergo one more incomplete cycle, resulting in binucleated or polyploid neonatal cardiomyocytes. In the case of cell cycle re-entry of adult cardiomyocytes, such as during pathological hypertrophy in contrast to physiological hypertrophy (e.g., sports and pregnancy), cells undergo polyploidization, endomitosis, and polynucleation but not cell division. This is a common feature of heart failure.


**1.1.2 Cardiomyocyte Hypertrophy and Death in Heart Diseases**

Currently, cardiovascular diseases (CVD) account for more than one-third of human mortality and is one of the leading causes of death worldwide. Various causes induce cardiac damage and dysfunction by affecting cardiomyocytes' growth and survival. Due to the unique character of cardiomyocytes, cardiomyocytes respond to the stimuli distinctively from other cells in the cell growth and survival. For example, in the presence of physiological or pathological hemodynamic stress, the heart develops hypertrophy, in which cardiomyocyte is characterized by increased cell size, instead of an increase in cell number, due to the limited regenerative capability. In addition, since the heart has minimal repair capacity, it is thus
susceptible to various stressors leading to cardiomyocyte death, both progressive and acute, which is a hallmark of various cardiac diseases, including myocardial infarction (MI), ischemia/reperfusion (IR) and hypertrophic heart failure (HF).

Physiological hypertrophy of the heart, such as a result of exercise or pregnancy, is an adaptive response to hemodynamic stress, which is believed to be reversible and contributesto the improvement of cardiac function. However, in pathological stress conditions, such as hypertension and valvular disease, pathological hypertrophy develops, which is characterized by an excessive increase in ventricular dimensions, accompanied by myocardial dysfunction and fibrosis. Myocardial supply-demand mismatch occurs, secondary to the increased myocardial oxygen consumption of the hypertrophic heart, which further increases cell death, eventually leading to the deterioration of cardiac output. Thus, hypertrophy is an independent risk factor for CVD, including arrhythmias, myocardial infarction, cerebrovascular events, and sudden death. Thus, pathological ventricular hypertrophy is a predictor of cardiovascular morbidity and mortality.

Myocardial ischemia, acute or chronic, is the most common cause of cardiomyocyte death. The disruption in blood flow leading to deficient oxygen supply to cardiac tissue induces cardiac injury, resulting in irreversible cell necrosis and apoptosis by oxidative stress, calcium overload, ER stress, and mitochondrial dysfunction, leading to heart failure. Thus, there is an urgent need to understand the underlying mechanisms of cell death and develop a strategy to promote cardiomyocyte survival under these diseases.

1.2 Valosin-Containing Protein (VCP) Regulates Cardiac Cell Growth and Survival

Previous studies from my mentor, Dr. Qiu’s lab, have identified VCP as a key
downstream mediator of cardiomyocyte growth and survival, although the mechanisms are yet unknown\(^1\). This novel gene was shown to be delicately involved in numerous known cell growth and survival mechanisms. More importantly, it selectively ameliorated the diseased state with little to no effects at baseline \(^2\). Our study seeks to further characterize the cardioprotective mechanisms of VCP, which may lead to a better therapeutic avenue for patients with heart diseases.

1.2.1 Molecular Character of VCP and its Biological Roles

VCP/p97 (also called Cdc48 in yeast and plants) is a hexameric ATPase protein belonging to type II AAA (ATPases associated with various cellular Activities) ATPase family and is ubiquitously expressed in all cells, \(^2\) and responsible for mediating protein homeostasis\(^3\). Its structure, shown in Fig. 2, contains a conserved N-terminal domain (1-187) followed by two copies of the AAA domains, D1 (209-460) and D2 (481-761) and a C terminal domain (762-806)\(^4\). By interacting with adaptor proteins, VCP is involved in a number of cellular activities, including cell cycle control, transcriptional regulation, apoptosis, protein degradation, and stress responses \(^5\)-\(^12\). It plays a major role in the endoplasmic reticulum- associated degradation where it extracts ubiquitinated proteins from the ER and maintains their misfolded state until proteasomal proteins can degrade them. Increased expression of VCP correlates with growth and survival of cancer cells\(^13\)-\(^15\). Specific genetic mutations of VCP are associated with multisystem disorders in muscle, brain and bone and with other diseases such as Alzheimer’s disease, Parkinson’s, and Amyotrophic Lateral Sclerosis (ALS) \(^16\)-\(^19\), though the underlying mechanisms are not fully understood. Inhibition of VCP activity, therefore, has a range of consequences for the cell, beginning with the accumulation of misfolded, polyubiquitinated
proteins and culminating in apoptosis, often triggered by ER stress and the unfolded protein response \(^3,20\).

**Figure 2: Structure of the AAA-ATPase Cdc48p/VCP.**

Domain structure of Cdc48p/VCP. Cdc48p/VCP consists of four domains, the N-terminal domain (N), the ATPase domains D1 and D2 comprising the motifs Walker A, Walker B and the 'second region of homology' (SRH) motif, and the C-terminal domain (C). In Cdc48p/VCP the major ATPase activity is located in the D2 domain, whereas the D1 domain is responsible for oligomerization. The N-terminal domain is involved in the binding of substrates and cofactors.


### 1.2.2 Identification of VCP in the Heart.

VCP was first identified VCP in the heart when it was shown that it acts as a novel downstream effector of the cardioprotective signaling mechanism conferred by Hsp22 \(^1\). Initial studies *in vitro* showed that VCP represents the link between Hsp22-mediated activation of Akt and nuclear factor-kappa B (NF-κB)-induced expression of iNOS in cardiac myocytes, thereby playing a central role in the mechanisms of cardiac cell survival promoted by Hsp22. 2-dimensional electrophoresis followed by mass spectrometry was used to isolate and identify potential proteins which interact with Hsp22 and Akt in the peri-nuclear area (Fig. 3).

**Figure 3: Identification of VCP in the heart.**
Proteins were first separated based on their isoelectric points, and then further separated based on their size. Proteins that were differentially expressed in Wild-type (WT) and Hsp22 Transgenic (TG) samples were marked, cut out of the gel, and then identified by mass spectrometry. Importantly, VCP was upregulated in Hsp22 samples compared to WT.

Coimmunoprecipitation was then used to determine whether there were any interactions between VCP, Akt, and Hsp22. Remarkably, the data revealed that VCP interacts with both Hsp22 and Akt, suggesting that they form a complex.

### 1.2.3 Cardiac Protection of VCP in Pathological Conditions.

VCP has been proven to be crucial to various cellular processes such as membrane fusion, transcriptional activation, and cell cycle progression \(^{21}\), as VCP is abundantly present in cardiac tissue, and its role in various heart diseases in being continuously established. Imbalance in protein homeostasis is a hallmark of many cardiac diseases like myocardial infarction, heart failure, and diabetic cardiomyopathy. \(^{22}\) As one of VCP’s primary roles is maintaining proteostasis, it is thought that VCP may play a protective role in cardiovascular pathological conditions.

**A. VCP Protects Cardiomyocytes Against Induced Cell Death and Hypertrophy In Vitro.**

Previous studies showed that overexpression of VCP protected isolated cardiomyocytes against apoptosis as measured by TUNEL, *in vitro* (Fig. 4)\(^1\). Lizano et al\(^1\) showed that VCP activates NF-kB, AKT and STAT3 and increases iNOS expression in a dose-response manner. VCP overexpression reduced chelerythrine apoptosis of cardiomyocytes by 50%, and these effects
were eliminated by the addition of the Akt inhibitor, NF-kB inhibitor STAT3 inhibitor, and iNOS inhibitor. Thus, VCP protects cardiomyocytes against apoptotic death by promoting STAT3/AKT/NF-kB/INOS signaling. Other studies have shown that overexpression of VCP reduces cardiac hypertrophy in vitro. ²

![Figure 4: VCP resists cell death in isolated cardiomyocytes.](image)

Apoptosis measured by TUNEL in vehicle-treated (open bars) vs. chelerythrine-treated (closed bars) cardiac myocytes, upon over-expression of VCP in the presence of NF-kB inhibitor (SN50), pan-NOS inhibitor (L-NNA), and specific iNOS inhibitors (AG, 1400W) compared with vehicle. *p <0.05 vs. same group without chelerythrine; #P<0.05 vs. corresponding β-Gal control.


B. VCP Protects Ischemia-Reperfusion Injury in the Mouse Heart In Vivo.

Ischemia/Reperfusion (IR) is a cardiovascular disease characterized by the deficiency of oxygen supply to the heart (ischemic phase) followed by restoration of regular blood flow (reperfusion phase), both of which cause irreparable damage to cardiac tissue. Ischemic preconditioning, a process by which the myocardium produces endogenous nitrous oxide (NO) and regulates contraction/dilation, is known to protect the myocardial tissue from extensive damage caused by IR injury. ²³ Inducible NO synthase (iNOS) is the main mediator in NO synthesis, making it a vital part of ischemic preconditioning.
Based upon results obtained in vitro, the physiological relevance of VCP was tested in vivo by generating a cardiac-specific VCP TG mouse, overexpressing VCP by 3-4 folds. These TG mice and their WT littermates were anesthetized, and the left anterior descending arteries occluded for 45 minutes to induce ischemia followed by 24 hours of reperfusion (Fig. 5). The areas at risk (AAR) of infarct were determined using retrograde injection of Alcian blue through the aorta which highlights areas of reduced blood flow, and the infarcts measured after 15-minute incubation of Triphenyl tetrazolium chloride (TTC). Although the areas at risk were similar, as shown in Fig. 5, VCP overexpression reduced infarct size by 50% compared to WT, highlighting VCP’s cardioprotective role24.

Figure 5: Overexpression of VCP reduces myocardial infarct size in TG mice. (a) Schema of the ischemia/reperfusion protocol. (b) Representative staining of left ventricle by Alcian blue and Triphenyl Tetrazolium Chloride (TTC). (c) Quantitation of AAR/LV and IS/AAR in WT and TG mouse hearts (n = 5 for TG and 7 for WT group). Data are the mean ± SEM. **p = 0.0006 vs. WT. from Lizano et al 2017
C. VCP Protects Pressure Overload-Induced Cardiac Hypertrophy and Apoptosis. VCP also protects the heart from pressure overload-induced hypertrophy (Fig. 6). VCP TG mice and their WT littermates were anesthetized, and their transverse aortas isolated. Transverse aortic constriction (TAC) was performed by tying a 27-gauge needle to the aorta and then removing the needle, leaving behind the knot. Sham-operated mice did not receive the constriction. Hearts were excised and measured, and cardiomyocyte size determined by wheat germ agglutinin (WGA) staining (Fig. 6A). As expected, WT mice that underwent TAC experienced cardiac hypertrophy unlike WT sham mice as evidenced by increased left ventricular weight compared to tibia length (Fig. 6B), heart weight compared to body weight (Fig. 6C), and cross-sectional area (Fig. 6D). WGA staining revealed that the cardiomyocytes were enlarged in WT TAC hearts compared to sham control (Fig. 6D). Importantly, VCP TG TAC mice did not show significant differences to sham controls. In fact, VCP TG TAC hearts exhibited similar measurements to both VCP TG sham controls and WT sham controls, highlighting VCP’s cardioprotective abilities while not affecting cardiomyocyte size at baseline. Further experiments showed that TAC-induced cell apoptosis was significantly attenuated in the TAC TG mice compared to TAC WT mice, as measured by TUNEL assay (Fig. 6E). Thus, VCP promotes cell growth and survival under pathological conditions in TG mice.

Additionally, several regulatory mechanisms have been implicated in pressure overload-induced cardiac hypertrophy. Two major mechanisms are mechanical stretch-induced and neurohumoral stimulation-induced hypertrophic and gene responses in cardiomyocytes. VCP
appears to play a role in both. Firstly, mechanical stretch-induced reprogramming of fetal genes, a known mechanism of LVH, is inhibited by VCP, thus attenuating the cardiac response to pressure overload. Secondly, in AngII-induced hypertrophic cardiomyocytes, VCP is downregulated in a dose- and time-dependent manner, and VCP overexpression prevented AngII-induced alterations. ²

Figure 6: VCP reduces hypertrophy during pressure overload.
A. Examples of WT and VCP TG heart mass and cardiomyocyte size stained by wheat germ agglutinin (WGA) in Sham and Transverse Aortic Constriction (TAC) animals. B. Left ventricles (LV) weight/tibial length ratio (TL). C. Heart weight/body weight ratio (HW/BW). D. The cross-sectional area (CSA) of cardiomyocytes. E. Quantitative analysis of apoptotic cardiomyocytes by TUNEL assay. * P < 0.01 vs. sham, # P < 0.01 vs. WT TAC mice. n = 4–5/group. Data are shown as mean ± SEM, two-way ANOVA was used.

Source: Zhou N, Ma B, Stoll S, Hays TT, Qiu H. The valosin-containing protein is a novel repressor of cardiomyocyte hypertrophy induced by pressure overload. Aging Cell. 2017;16(5):1168-1179

1.2.4 Potential Molecular Mechanisms Underlying VCP-Mediated Cardiomyocyte Growth and Survival

A. VCP Regulates ER/Mitochondrial Functions. The endoplasmic reticulum (ER) is the largest organelle of the cell and is responsible for protein synthesis and folding, post-translational modifications and regulating transmembrane proteins. The sarcoplasmic reticulum (SR) is a morphologically distinct variation of the ER present especially in muscle cells and is specialized for Ca2+ release to aid in muscle
VCP IN CARDIOMYOCYTE SURVIVAL AND GROWTH

contraction. VCP is a ubiquitin-selective protein and plays an essential role in maintaining ER integrity by interacting with E3 ubiquitin ligases such as ERAD-associated HRD (HRD1) and glycoprotein78 (gp78). VCP is responsible not only for the regulation of the activity of various factors, but also the delivery of misfolded proteins for proteasomal degradation. The ER interacts with the mitochondria, and VCP plays a key role in extracting misfolded proteins from the mitochondria for mitochondria-associated degradation and in mitochondrial autophagy. Thus, due to the essential roles it plays in cellular functions, impairment of VCP activity can lead to ER and mitochondrial dysfunction, as illustrated in Fig. 7.

![Figure 7: VCP and ER/Mitochondria.](image)

Summary of the common features related to VCP in different diseases. Despite the fact that the cofactors and the regulatory signalling pathways underlying the pathogenesis of these disorders may vary, VCP could promote stress-induced misfolded protein degradation and maintain calcium homeostasis, which subsequently helps resist ER/mitochondrial stress-induced cell damage and death. As the increase of VCP in cancer cells would protect cells against death, the deficiency of VCP in neurodegenerative disorders and heart diseases would induce cell damage and death, due to the loss of these functions. (The red arrows represent the promotive effects and green arrows stand for the inhibitory/suppressive effects).


Studies have shown that VCP regulation of mitochondrial activity was dependent on
iNOS, with VCP overexpression protecting against IR injury by promoting iNOS activity and in turn improving mitochondrial function. However, VCP inhibitor KUS121 increases ATP production, reduces ER stress, improves mitochondrial function, and attenuates IR injury. These contradictory results might be due to varied method of activation and inhibition of VCP activity and supports evidence that the effect of VCP on iNOS is dose dependent.

**B. VCP Regulates mTOR Signaling in the Heart.** Multiple signaling pathways have been found to be involved in cardiac hypertrophy and survival under pathological conditions. The mechanistic target of rapamycin (mTOR), a key nutrient/energy/redox sensor and controller of protein synthesis, has been demonstrated as one of the critical signaling mechanisms involved in cardiac growth, based on the strong experimental evidence that inhibitors of mTOR, such as rapamycin, reduced pathological hypertrophy. mTOR is an atypical serine/threonine protein kinase that executes many cellular functions through interaction with various adaptor proteins to form two complex multiprotein complexes, mTORC1 and mTORC2. The mTOR pathway plays a vital role in regulating cellular homeostasis and stress response. mTORC1 is a master regulator of proteinsynthesis, and cell growth and proliferation, while mTORC2 regulates cell survival and polarity.

Through loss of function studies of various mTOR pathway components, animal models have indicated that mTOR is critical to embryonic cardiovascular development and has a hand in key cellular processes required for regular postnatal growth and maintenance of cardiac function. It has been studied that mTOR mainly exerts its function mainly through the formation of the protein complexes. So far, mTORC1 is known to constitute the proteins Raptor, mLST8, PRAS40, DEPTOR, and Tt1/Tel2, while mTORC2 contains mTOR, scaffold
protein Rictor, SIN1, PROTOR, mLST8, DEPTOR and Tt1/Tel2. Additionally, the Akt and AMPK pathways are the most well characterized regulators of mTORC1. Similarly, for mTORC2, Akt that is phosphorylated at serine 473 (pAKT S473) is the best-known substrate.\textsuperscript{34}

mTOR knockout mice show massive apoptosis, fibrosis, autophagy, mitochondrial dysfunction and sarcomere disarray, finally resulting in death 8 weeks after tamoxifen-induced gene deletion. \textsuperscript{40} Inducible cardiac-specific mTOR deletion in adulthood leads to chamber dilation and wall thinning, eventually leading to cardiac dysfunction and heart failure.

The previous studies in my mentor’s lab \textsuperscript{41} showed that VCP plays a dual role in the regulation of mTOR – it activates the survival-promoting mTORC2 by repressing stress induced-growth promoting mTORC1 (Fig. 8). During pressure-overload, VCP reduction was accompanied by the increase in phosphorylation of Akt at threonine 308 (pAKT T308) and elevated mTORC1 activity in LVH in TAC mice. Overexpression of VCP in the TAC VCP TG mice suppressed this pathway. Akt is a kinase protein and VCP, which lacks any phosphorylation abilities, has been studied to promote Akt phosphorylation under physiological conditions. Additionally, VCP is activated by Akt in persistent hypoxic stress conditions, and Akt works downstream of mTORC2. In VCP TG mice, mTORC2 is upregulated by VCP overexpression, which in turn promotes Akt phosphorylation. Thus, interactions between Akt and VCP is both direct and indirect, and given the key role Akt plays in cellular processes, better understanding of these interactions could help further reveal the role of VCP in cardiac hypertrophy. \textsuperscript{22}
Figure 8: Overexpression of VCP significantly repressed the transverse aortic constriction (TAC)-induced activation of the AKT/mTORC1/S6K pathway.

(A) The protein level of VCP in the hearts of WT and VCP TG mice at baseline and after 2-week TAC. (B) Western blots of proteins of PI3K, AKTs, and mTORC1 signalling in the LV tissues after 2-week TAC compared to sham in both WT and VCP TG mice. GAPDH is a loading control for total protein. *P < 0.01 vs. sham, #P < 0.01 vs. WT TAC mice. n = 4/group. Data are shown as mean ± SEM, two-way ANOVA was used.


Additionally, VCP suppressed mTORC1 only under the stress of TAC but not at baseline physiological conditions. Thus, the selective effects of VCP in regulating mTORC1 and mTORC2 is unique compared to other regulators of the mTOR pathway and highlights the clinical significance of VCP due to the ability to affect pathological conditions without impairing physiological growth and function. (Fig. 9)
Figure 9: An illustration of the mechanism by which valosin-containing protein (VCP) protects left ventricular hypertrophy (LVH) against pressure overload.

Pressure overload reduces VCP expression in heart which activates mTORC2 and attenuates the inhibitive effect of VCP on AKT/mTORC1/S6K signalling, subsequently promotes the pro-growth pathway. VCP TG restores the TAC-suppressed VCP and represses the TAC-induced activation of AKT/mTORC1/S6K, fetal gene upregulation, and collagen synthesis and thus protects the heart against TAC-induced LVH.


Although great progress has been made in elucidating the complex regulation and function of mTOR signaling networks, many aspects of the pathophysiology of mTOR signaling in heart still remain unknown. For example, although the mTORC1 pathway has been extensively investigated, the regulation of mTORC2 is poorly understood, and the identified regulators of mTORC2 are few\(^3\). It is difficult to completely understand the full function of mTOR signaling in the heart without precisely ruling out the involvement of mTORC2 in these mechanisms. In addition, it has been shown that mTORC1 is not only responsible for the maladaptive cardiac hypertrophy but is also necessary for physiological cardiac growth. Thus, inhibition of mTORC1 not only attenuates the pathological response but may also affect its physiological effects\(^3\).
Furthermore, prolonged pharmacological treatment, such as rapamycin, disrupts both mTORC1 and mTORC2, causing unexpected side effects, which limit its use in human. Therefore, the approach which selectively inhibit the maladaptive functions of mTORC1 without impairing the normal functions of mTORC2 may lead to newer, safer therapies.

2. SPECIFIC AIMS

VCP has been known to interact with the mTOR pathway, and both play crucial roles in normal functioning of the heart and during cardiac pathologies. During certain cardiac pathologies, VCP levels are either increased or dramatically decreased, which contributes to cell death, and in turn, the mTOR pathway is affected. We are particularly interested in the regulation of VCP on mTOR signaling for the following reasons: 1) mTOR signaling is a well-known key pathway involved in cardiac growth and survival, but the mechanisms have not been fully understood. 2) The regulation of mTORC2 in the heart remains unclear despite its importance. Our preliminary data indicate that VCP is likely a mediator of mTORC2, which is not characterized yet; 3) VCP presents as a unique regulator of mTOR which differs from other mTOR inhibitors. The overall goal of the study is to determine how the proteins involved in the mTOR pathway are affected by differences in VCP levels in cardiomyocytes in vitro, to support in vivo studies observed in disease condition and in VCP TG mice.

Based on the previous findings, our hypothesis, as summarized in Fig. 10 is that VCP acts as a unique dual regulator of mTOR by selectively activating survival complex mTORC2 but inhibiting growth-promoting complex mTORC1.
Figure 10: Diagrammatic representation of the hypothesis that VCP plays a dual regulatory role in mTOR signaling. Red arrows show inhibitory effects while green arrows show promotive effects.

We proposed to test the hypothesis through the following two specific aims:

**Aim 1:** To determine how overexpression of VCP affects mTOR signaling in cardiomyocytes, in terms of the expression and activity of mTOR, its adaptor proteins and downstream signaling of mTORC1 and C2.

**Aim 2:** To determine whether VCP is necessary for the regulation of mTOR signaling by knocking down VCP in cardiomyocytes.

3. METHODS

3.1 Cell Culture

H9C2 cells are cardiomyocytes originally derived from embryonic rat ventricular tissue and iscommonly used in cardiovascular research. Cryopreserved H9C2 cell vials were rapidly thawed as per instructions for 2 minutes at 37°C and the contents quickly transferred to a 15mL tube and centrifuged at 900 rpm for 5 minutes at 22°C. The supernatant was discarded, and the
pellet resuspended in 2 mL of Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin and cultured in total 8-9mL of media in Corning 100mm dishes at 37°C in a humidified atmosphere of 5% CO2. Cells were fed every 3-4 days and sub-cultured at a 1:4 ratio when they reached >80% confluency. For the adenoviral infection, the cells were plated to achieve an average cell yield of 5.5x10⁶ cells/mL.

3.2 Construct of Adenovirus

A. VCP Adenovirus (Ad-VCP, Vector Biolabs), Fig. 11

Construct: Ad-GFP-h-VCP

Backbone: Adenoviral – Human Type 5 (dE1/E3)

Promoter: CMV

Transgene: human VCP Reporter/Tag: eGFP

PFU Titer: 4x10¹⁰ PFU/mL Control used: Ad-βGal

Figure 11: Backbone of Ad-VCP.
B. VCP shRNA silencing adenovirus (Ad-shRNA VCP), Fig. 12

Construct: Ad-h-VCP-shRNA

Backbone: Adenoviral – Human Type 5 (dE1/E3)

Promoter: U6

Reporter/Tag: eGFP

PFU Titer: 2.30x10^{10} PFU/mL

Control used: Ad-Scramble

![Diagram of Ad-U6-shRNA(Scramble)-GFP]

Figure 12: Backbone of Ad-siRNA-VCP.

3.3 Infection of Cultured Cells

For overexpression, H9C2 cells were infected with VCP adenovirus (Ad-VCP) conjugated to GFP (green fluorescent protein). The experiment was first optimized using different multiplicity of infection (MOI 0, 10, 20, 40, 100, 200) of the virus and 50 MOI was used for all consecutive experiments for 48 hours and was imaged after 24 hours to confirm successful infection. For knockout, the cells were infected with VCP shRNA adenovirus (Ad-shRNA VCP)
conjugated to GFP, and the experiment was optimized with various MOI as with the Ad-VCP. For all further experiments, 5 MOI of the virus was used for 4 days. Cells were imaged after 48 hours to confirm successful infection. Cells infected with Ad-CMV-BGal was used as control for overexpression and those infected with Ad-U6-Scramble were used as control for knockout. (Fig. 13)

Figure 13: Summary of experimental methods used for cell culture, infection and western blot.

3.4 Western Blot

H9C2 cells were trypsinized, centrifuged at 900rpm for 5 min at 4°C and the pellet washed with 1mL cold PBS. After washing, the supernatant was discarded, and the pellet stored at -80°C until further use. The cells were then resuspended in 80ul of RIPA lysis buffer and kept on ice for 15-20 minutes. They were then centrifuged at 13000rpm for 15 min at 4°C and the supernatant was transferred to a new tube. Protein contents were measured using the Bradford assay with Bovine Serum Albumin (BSA) as the standard. Equivalent amounts of
protein (15ug) were calculated and made up to 20ul with LDS sample buffer, 10x reducing agent and double distilled (DD) water. The samples were denatured at 95°C for 5 minutes and then loaded onto a 10 well, 8% Novex Pre-cast Gel with 5ul of Precision Protein Plus molecular weight markers in the first well. The gel was run at 130V for 45 minutes and electrophoretically transferred to a Nitrocellulose membrane. After blocking with Intercept Blocking Buffer (Li-Cor) for 1 hour at room temperature, the membrane was incubated in the primary antibody at 4°C overnight. (Fig. 14)

Figure 14: Diagrammatic representation of Western blots and the proteins involved in mTOR signaling studied in this project.

The antibodies used were: VCP monoclonal antibody (1:5000), rabbit GaPDH (1:5000), rabbit mTOR mAb (1:3000), rabbit Raptor mAb (1:5000), rabbit Rictor (1:5000), rabbit pAkt T308 mAb (1:3000), and rabbit pAkt S473 (1:3000). Membranes were incubated with goat anti-mouse (imaged at 680nm) or donkey anti-rabbit (imaged at 720nm) secondary antibodies (1:10000) as appropriate for 1 hour at room temperature and imaged with the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

4. RESULTS
4.1 Optimization of Adenoviral MOI for VCP Knockdown

H9C2 cells were infected with different MOIs (0,10,20,40,100,200) of the Ad-siRNA-VCP virus and imaged to reflect GFP expression in cell. Images were taken after 48 hours, as shown in Fig. 15A, using the Li-Cor Odyssey System at 10x magnification. The increase in green as the MOI increases shows that more cells have successfully taken up the virus. However, confluency of the pictured culture was higher than desired, and fewer cells were seeded for the following experiments to achieve a final cell count of around 5.5x10^6 cells per 10cm^2 dish. A western blot was done (Fig. 15B) with VCP monoclonal antibody to show a dose-dependent decrease in VCP levels in the cells. As VCP expression was highly reduced for higher MOIs, an MOI of 5 was used for subsequent experiments.

A
Figure 15: Dose-response of Ad-siRNA-VCP in H9C2 cells.
A. Image of GFP in the infected cells with different MOI of virus. B. Western blot showing the VCP expression in the infected cells to optimize MOI of Ad-siRNA-VCP

4.2 Optimization of Adenoviral MOI for VCP overexpression

As with the Ad-siRNA-VCP viral infection, H9C2 cells were infected with different MOIs (0, 10, 20, 40, 100, 200) of the Ad-VCP virus and imaged after 48 hours (Fig. 16). Images were taken using the Li-Cor Odyssey system at 10x magnification to reflect GFP expression within the cells. 50 MOI of the virus was used for all succeeding experiments.
Figure 16: Optimization of Ad-VCP dose in H9C2 cells. Image of cells expressing different MOI of virus images under microscope 48 hours post transfection.

4.3 Western Blot to Confirm Functional Loss/Gain Model

A western blot was done using 8% Novex Pre-Cast Gel to confirm VCP knockdown and overexpression in H9C2 cells infected with Ad-shRNA-VCP and Ad-VCP respectively. Although Fig. 17 shows a clear decrease and increase in VCP expression upon knockdown and overexpression respectively, the control used in this experiment was uninfected cells, and thus, no substantial claims of knockdown/overexpression efficiency can be made.
Levels of VCP in H9C2 cells infected with Ad-siRNA-VCP and Ad-VCP were tested using a western blot. The control used here was uninfected H9C2 cells. GaPDH was used as loading control.

4.4 VCP and GaPDH Observed Only in the Controls

A western blot was run using the wet transfer method to reduce possibility of protein degradation and VCP and GaPDH protein levels were measured (Fig. 18). It was observed that while the two controls (Ad-BGal and Ad-Scramble) showed bands for both VCP and GaPDH, the lack of bands for GaPDH in samples (infected with Ad-siRNA-VCP and Ad-VCP) indicates issues arising either from experimental technique, or due to protein degradation (as samples were stored in -20°C for two weeks before the experiment was conducted).

Figure 17: Western blot showing VCP knockdown and overexpression.

Levels of VCP in H9C2 cells infected with Ad-siRNA-VCP and Ad-VCP were tested using a western blot. The control used here was uninfected H9C2 cells. GaPDH was used as loading control.

Figure 18: Western blot for functional loss/gain model validation.

A. Western blot showing expression of VCP in cells. B. Western blot showing expression of GaPDH, commonly used as loading control.
4.5 Interaction Between VCP and Proteins Involved in mTOR Signaling

To determine whether any changes occurred in the levels of mTOR, Raptor (required for formation of mTORC1), and Rictor (required for formation of mTORC2) with VCP knockdown/overexpression, another western blot was run using 10% Novex Pre-Cast Gel followed by wet transfer in 4°C for 3 hours. While controls showed some expression of mTOR (Fig. 19A) and Raptor (Fig. 19B), this was consistent with the blots for VCP/GaPDH and thus, no substantial conclusion about interactions can be made. No bands were observed for Rictor (Fig. 19C).

A western blot was run using 10% Novex Pre-Cast gel followed by semi-dry transfer for 45 minutes, to determine levels of pAkt T308 (involved in the mTORC1 pathway) and pAkt S473 (involved in the mTORC2 pathway). pAkt has a molecular weight of around 56kDa. The blot for pAkt S473 (Fig. 19E) showed bands around 56kDa, but no definite conclusion can be made. On the other hand, for pAkt T308 (Fig. 19D), unspecific bands around 25kDa were observed and was possibly due to errors in sample loading and/or experimental techniques.
**Figure 19: Western blot showing expression of various proteins involved in the mTOR pathway.**
A. Membrane incubated with mTOR rabbit mAb. Bands were observed for controls (labeled 2 and 4) at 280 kDa. B. Membrane incubated with Raptor rabbit mAb, with bands observed at 149kDa for controls (labeled 2 and 4). C. Membrane incubated with Rictor rabbit mAb. D. Membrane incubated with pAkt T308 rabbit mAb, with unspecific bands observed at around 30 kDa. E. Membrane incubated with pAkt S473, bands observed at 56 kDa. All membranes were incubated with goat anti-rabbit secondary antibodies and imaged at 520nm.

5. DISCUSSION/TROUBLESHOOTING

The main aims of this study were to study the effects of VCP knockdown and overexpression on mTOR signaling pathway-related proteins in H9C2 cells by measuring protein levels in infected cells using the western blotting technique. The aims were based on the hypothesis that VCP plays a unique dual role in regulating mTOR by selectively inhibiting growth promoting complex mTORC1 while activating survival complex mTORC2, based on previous findings. By understanding the specific mechanisms involved in cardioprotection by VCP, strategies to prevent and treat various heart diseases can be formed.

The first part of the study was to optimize MOI of adenoviral infection for both VCP overexpression (Ad-VCP) and knockdown (Ad-siRNA-VCP) in order to establish conditions for accurate results. The process took longer than anticipated due to time constraints, and in the
future optimization will be further required to improve results. H9C2 cells were used as they are an established *in vitro* model for various cardiovascular diseases like ventricular hypertrophy and cardiomyopathies. These cells are commonly used in place of primary cardiomyocytes as they recapitulate hypoxic and hypertrophic environments. However, H9C2 cells deviate from cardiomyocytes in several ways, the primary being that their morphology and gene expression patterns are closer to resembling primary skeletal myoblasts than primary cardiomyocytes. Additionally, in response to hypertrophic stimuli, they do not show sarcomeric reorganization, nor do they beat spontaneously in culture. However, for this study, the characteristics of H9C2 cells are sufficiently similar to primary cardiomyocytes and thus are an effective model to use. The limitations of the model must still be taken into consideration. Further studies can use primary neonatal cardiomyocytes to study the molecular mechanisms involved in the interaction between VCP and the various proteins involved in the mTOR pathway.

For the western blot run to optimize MOI of Ad-siRNA-VCP virus required for adequate knockdown of VCP, a comparison can be made between images taken that show increasingly higher amounts of GFP and therefore higher percentage of infection and the VCP levels in the blot, showing that there is a dose-dependent decrease of VCP as MOI of virus used increases. However, an ideal experiment should have included controls like GaPDH or GFP in the same western blot membrane to confirm that the decrease in VCP levels was due to efficient knockdown and not due to differences in protein loading in the wells. Interestingly, Fig 14 shows a decrease in GaPDH levels in VCP overexpression. It is possible that VCP’s dose-dependent interaction with iNOS increased cellular toxicity and that VCP is no longer cardioprotective at a dosage of 50MOI of the Ad-VCP virus. Thus, future experiments will need to
try alternative loading controls while also better optimizing the dosage and duration of viral
infection.

The second part of the project focused on studying the interaction between VCP and
various proteins involved in the mTOR pathway. Akt is a downstream protein involved in the
pathway, with pAkt T308 being a substrate for mTORC1 and pAkt S473 for mTORC2. Although
bands were slightly visible for pAkt S473, it is possible that the membrane was cut too close to
the molecular weight of the protein to allow for acceptable conclusions to be drawn. For the
membrane blotted with pAkt T308 antibody, non-specific bands were observed around 25-
30kDa suggesting errors in experimental methods.

The other proteins tested were Raptor (involved in formation of mTORC1), Rictor (involved information of mTORC2) and mTOR. The first few western blots showed no results for
these proteins, possibly because the semi-dry transfer technique used was causing them to
degraded before they could be incubated with their respective antibodies. For the final blot, the
wet transfer technique was used so as to preserve the integrity of the proteins and yielded
better results. Clear bands were visible for Raptor and mTOR for both the controls Ad-Scramble
and Ad-βgal but were either faint or not visible for the two samples. When the same
membranes were incubated with VCP and GaPDH antibodies, a similar pattern was observed
thus suggesting that there were issues with sample loading and that the bands for the samples
were not representative of the actual protein levels in the cells. It is also possible that the
proteins in the samples degraded as they had been stored at -20°C for nearly two weeks before
the final experiment was conducted.

Thus, no evident conclusions can be made from this study, owing to time constraints
and errors in experimental technique. However, the project optimized the first step of the process and troubleshooting the issues faced later will be helpful in future endeavors. The project also highlighted the need to better understand the molecular mechanisms behind the role of VCP in cardioprotection and its interaction with various pathways like the mTOR pathway.

6. FUTURE DIRECTION

Better understanding the role of VCP and its involvement in cardioprotection is essential. In the future, elucidating these mechanisms is important, especially in the context of VCP’s interaction with the mTOR pathway which is the scope of this current project.

In the future, we will interrogate the molecular mechanisms underlying the selective effects of VCP on mTORC1 and mTORC2, in terms of the specific subcellular shuttling, protein-protein interaction and the crosstalk between mTORC1 and mTORC2. Secondly, we will determine the transcriptional mechanism by which VCP regulates mTOR signaling, e.g., VCP-mediated transcription factors (TFs) and gene networks/pathways. Additionally, as miRNA is a well-known, powerful negative regulator of gene expression, and has been linked to the regulation of mTOR in other tissues\textsuperscript{42}, we will also determine the VCP-regulated miRNAs. Furthermore, we will further define the domain-dependent regulatory mechanisms. Finally, we will elucidate the physiological relevance and the underlying mechanisms of VCP in the heart at baseline and under stress.

Based on our preliminary data and previous publications, we strongly believe that our proposed studies will elucidate the specific mechanisms involved in cardioprotection by VCP under cardiac stress which will provide a new strategy for preventing and possibly treating heart failure.
7. SUMMARY

Cardiovascular diseases are one of the leading causes of mortality around the world, and there is a constant need to better understand mechanisms involved so as to strategize new and more effective preventative and therapeutic solutions. Recent studies have highlighted the role of VCP in protecting the heart against various cardiomyopathies and injuries that are caused by cardiac stresses like ischemia and pressure overload, among others. Understanding this mechanism at the molecular level is imperative, along with elucidating the mechanisms with which VCP interacts with various key pathways in the heart.

The hypothesis of this project was that VCP plays an essential role in cardiomyocyte growth and survival by exerting a dual regulatory effect on mTOR signaling. Using a functional gain/loss strategy model, we aimed to better understand the interactions between VCP and some of the critical proteins involved in the mTOR pathway. By using recombinant adeno-viral systems, we successfully knocked down (using Ad-siRNA-VCP) and overexpressed VCP (using Ad-VCP) in H9C2 cells and confirmed the same using a western blot. Although no substantial conclusions can be made from the data obtained, these preliminary studies combined with previously published studies can be used to better understand the role of VCP in cardiomyocyte growth and survival through its interactions with mTOR.
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EXPERIENCE

Georgia State University, USA  
Graduate Research Assistant, Part-time  
August 2021- PRESENT  

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Duties: Assist postdoc (Dr. Xiaonan Sun) in various projects. Techniques used so far: PCR, Western blot, Cell culture, Adenoviral infection, Mouse studies (in training).

EDUCATION

Georgia State University, USA  
Master of Interdisciplinary Studies (M.I.S) Biomedical Science and Enterprise  
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University of Sheffield, UK  
Master of Science (M.Sc.) Stem cells and Regenerative Medicine  
September 2018- January 2020  

Result: Successful completion with Merit (GPA 3.75)

B.M.S College of Engineering, India  
Bachelor of Engineering (B.E) Biotechnology  
August 2014 - July 2018  

Result: First Class with Distinction (GPA 3.96)

SKILLS

Cell culture (including primary culture): counting, splitting, passaging, freezing, thawing

Immunological and cell-based assays like ELISA

Standard molecular biology techniques (Transfection, PCR, Western Blot)

Human embryonic stem cell culturing, maintenance and differentiation

Fluorescence-based live cell imaging

Flow cytometry, FACS Immunohistochemistry

Fluorescent Microscopy

Mouse models (currently in training)

Data Analysis: FlowJo, GraphPad Prism, ImageJ

Microsoft Office: Excel, Word, Powerpoint

Other software: Photoshop, Blender, Unity 3D-basics

PUBLICATIONS

“Heat Shock Protein 22 in Physiological and Pathological Hearts: Small Molecules, Large Potentials.”  

PROJECTS

● Masters’ Research Project: “Effects of Botulinum Neurotoxins X and D on Mast cell Mediator Release.”

PROJECTS
• Bachelors’ Research Project: “Purification of Methanol Tolerant Bacterial Lipases and their application in Biodiesel Production”.
• Participated in a project aimed at \textit{in-silico} identification of potential diagnostic biomarkers and therapeutic targets for lung squamous cell carcinoma. (Manuscript submitted)
• BIOMOD 2016: “In vivo medical diagnostic device.”

**CERTIFICATES AND INTERNSHIPS**

• “Health in Complex Humanitarian Emergencies”: Emory University by Coursera. (Certification). October 2017.
  “Science of Stem Cells”: American Museum of Natural History by Coursera (Certification), November 2020.
• Completed a two-month internship at Indian Institute of Science (IISc), Bangalore at the Department of Computational and Data Sciences. May-July 2017.
• Participated in BIOMOD – an annual biomolecular design competition hosted by Wyss Institute of Harvard University. October 2016.

**AWARDS**

Bronze team award; Best use of Game Engine: BIOMOD 2016

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