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THE ROLE OF MYOCARDIN IN THE PROGRESSION OF NON-SMALL CELL LUNG CANCER

By

SOROMIDAYO AKINSIKU

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in Biotechnology
Department of Cellular and Molecular Biology

Pierre Neuenschwander, Ph.D., Committee Chair

School of Medicine

The University of Texas at Tyler
May 2021

SIGNATURE PAGE

The University of Texas at Tyler
Tyler, Texas

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ABSTRACT

Lung cancer is the leading cause of cancer-related mortality in the world and NSCLC accounts for 85% of all lung cancer cases. The mainstay of treatment for patients with stage I, II and IIIA NSCLC is surgery, followed by post-operative cisplatin-based chemotherapy. Additional adjuvant therapy involving targeted tyrosine kinase inhibitors has been in use, however even for the targeted therapy, resistance eventually develops. Therefore, there is a need for identifying novel targets for this life-threatening disease. Given that preliminary studies in Ikebe lab revealed that myocardin knockdown significantly promoted caspase-3 degradation, in this study, using myocardin siRNA, we investigated the effect of the knockdown (KD) of myocardin gene on important apoptotic biomarkers such as PARP, caspase-3, and Bax; and on mitochondrial dysfunction involving respiration, and ROS formation. Our results indicated changes in the expression of multiple important apoptotic markers by myocardin knockdown in A549 NSCLC cells and myocardin knockdown was found to significantly decrease cell viability in A549 cells. Myocardin KD also showed a large, significant decrease in the maximal respiration of the cells and was found to significantly induce higher levels of ROS formation compared to the control. In summary, this study highlights myocardin as an important target in NSCLC progression and presents an interesting insight on the potential role of myocardin as a druggable target in lung adenocarcinoma. It also provides the basis for further investigation on its mechanism of apoptosis induction.

ACKNOWLEDGMENTS

I would like to express my heart-felt gratitude to the University of Texas at Tyler and Dr. Pierre Neuenschwander for creating this program. I have acquired enormous knowledge and skills in the field of biology and biotechnology within the past 21 months, more than I ever did in the past two decades of my life. My sincere thanks go to Dr. Mitsuo Ikebe, my supervisor, for his unwavering guidance during the course of this project and in my academic pursuits; to Dr. Sakai for his kindness and patience in teaching; to Dr. Sato for his readiness to assist at all times, and to all members of the Ikebe Laboratory for their support and assistance. I am also grateful to Reiko, for her genuine love, kindness, and welcoming disposition from the moment I stepped into the laboratory. A big thank you to Dr. Amy for her unwavering support to all my student-related affairs. I would also like to thank Dr. Torry Tucker for his support and interest in my academics, my applications, general wellbeing, and for believing in me.

I am highly grateful for my family, my parents, Mr., and Mrs. Akinsiku, especially my mom, who taught biology for many years, and had to cancel her plans for a master's degree when she found out she was pregnant with me; this is the degree you never had, mom. I am more than grateful to my siblings, Nimbe, Fiyin, Tomi and Sayo for their unwavering support, without whom I would not have been able to obtain this degree. Special thanks to a family friend, Dr. Fisayo who informed me first of this program and has shown enormous support throughout the course of study; thank you for all you do.

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LIST OF ABBREVIATIONS

A549: Adenocarcinomic Human Alveolar Basal Epithelial Cells

AKT3: Serine/Threonine-Protein Kinases 3

ATM: Ataxia-Telangiectasia Mutated

APC: Allophycocyanin

BAX: Bcl2- Associated X Protein

BAK: Bcl-2 Homologous Antagonist/Killer

BOK: BCL2 Family Apoptosis Regulator BOK

BCL2-XL: B-cell Lymphoma-Extra Large

ERBB4: Receptor Tyrosine-Protein Kinase 4

EGFR: Epidermal Growth Factor Receptor

CAS 3: Caspase-3

CCND1: Cyclin D1 Protein

CCNE4: Cyclin E4 Protein

CDKN2A: Cyclin Dependent Kinase Inhibitor 2a

CKD4: Cyclin-Dependent Kinase 4

CUL3: Cullin-3

FGR4: Fibroblast Growth Factor Receptor 4

HPMC: Human Peritoneal Mesothelial Cells

ICAD: Inhibitor of Caspase-Activated Deoxyribonuclease

KEAP1: Kelch-Like Ech-Associated Protein 1

KDR: Kinase Insert Domain Receptor

KRAS: Kirsten Rat Sarcoma Virus

LUAD: Lung Adenocarcinoma

LUSC: Lung Squamous Cell Carcinoma

LCC: Large Cell Carcinoma

MCL-1: Myeloid Cell Leukemia-1

MYOCD: Myocardin

NF1: Neurofibromin

NFE2L2: Nuclear Factor Erythroid 2-Related Factor 2

NSCLC: Non-Small Cell Lung Cancer

NTRK: Neurotrophic Tyrosine Receptor Kinase

PARP: Poly (ADP-Ribose) Polymerase

PTEN: Phosphatase and Tensin Homolog

RB1: Retinoblastoma Protein

ROS: Reactive Oxygen Species

SCLC: Small-cell Lung Cancer

siControl: universal control siRNA

siMyocardin: siRNA specific for Myocardin

STK11: Serine/Threonine Kinase 11

SRF: Serum Response Factor

INTRODUCTION

Lung cancer

Lung cancer is the leading cause of cancer-related mortality in the world and is traditionally subdivided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). This classification takes into account the differences in histopathology, pathophysiology, and therapeutic options of the two types of lung cancer. NSCLC presents greater resistance to chemotherapy compared to SCLC and SCLC progresses more quickly to metastasis (2). NSCLC accounts for 85% of all lung cancer cases and is further subdivided into three primary subtypes: lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC) and large cell carcinoma (LCC) (3). Well known mutated genes in lung cancer include KRAS, EGFR and TP53 (4), and Table 1 consists of some mutated genes in LUAD and LUSC reported in literature (3, 4).

The molecular landscape of NSCLC is highly heterogenous such that LUAD and LUSC, which account for 40% and 25-30% of all lung cancers respectively, have been found to present with vastly different molecular, clinical, and pathological features (2). Tobacco smoking is the most common etiology of lung cancer and is associated with the major histological subtypes. However, smoking is a more prevalent etiology of SCLC and LUSC than LUAD, and LUAD presents more commonly in patients lacking any history of smoking (3).

The mainstay of treatment for patients with stage I, II and IIIA NSCLC is surgery, followed by post-operative cisplatin-based chemotherapy. Despite the reported therapeutic benefits of this adjuvant therapy, relapses with metastatic disease have been reported to occur. The high mortality rate of NSCLC is also fueled by the late-stage diagnosis of most cases (5). Recently, lung cancer treatment has evolved over the decades from the traditional non-targeted therapy involving the use of cytotoxic agents to a personalized medicine approach in which genetic alterations in each patient's tumor informs the use of targeted therapies (3). Additional adjuvant therapy involving targeted tyrosine kinase inhibitors such as Osimertinib when coupled with surgical resection, and atezolizumab coupled with several rounds of chemotherapy following surgical resection have been associated with an additional 2-year disease-free survival rate in patients with stage IB to IIIA NSCLC. Most stage III NSCLC are considered unresectable, although surgery is considered in some cases. And in peculiar cases, a combination of chemotherapy, radiation, and targeted immunotherapy such as durvalumab correlated with a 4-year survival rate (5). However, even for the targeted therapy, resistance eventually develops. Therefore, there is a need for identifying novel targets for this life-threatening disease.

Myocardin

The differentiation of cells into various phenotypes involves the interaction of various signaling pathways to ensure a coordinated and specific gene expression as required for cellular homeostasis (6). This process usually requires cell lineage restricted DNA

transcription factors and co-regulators such as transcription co-activators, DNA-binding proteins, and transcription repressors, in response to growth signals and developmental cues to modulate the process of genome transcription which leads to the differentiation and growth of cell (7). Muscle genes are frequently characterized by the presence of cis-acting sequence called the CArG box (CC(A/T)6GG, a binding site for Serum Response Factor (SRF) belonging to the family of MADS transcription factor. SRF contains a MADS domain which functions in the recruitment of transcriptional co-factors that modulate DNA binding affinity, gene specificity and the transcriptional process (8).

Myocardin was first identified by Wang et. al., (2001) as an important co-activator of SRF in myocardial cell differentiation. It is a member of a family of nuclear proteins called SAP (SAF-A/B, acinus, PIAS) which function in various aspects of chromatin remodeling and modulation of transcription. Myocardin was reported to be a transcriptional activator of the promoter regions of genes such as SM22, atrial natriuretic factor, myosin light chain-2V and myosin heavy chain genes in an SRF-dependent manner (8). Initially described to be expressed in embryonic smooth and cardiac muscle cell differentiation and being subsequently restricted to the myocardium, myocardin has been reported to be expressed in visceral and vascular smooth muscles tissues to the same degree as in cardiac cells, and its function in the modulation of smooth muscle cell differentiation has also been established (9). In the differentiation of smooth and cardiac muscle cells, myocardin is an important co-activator for SRF in its interaction with the CArG box found in promoter regions encoding for myogenic, myofibrillar, cytoskeletal and contractile proteins in these

cells (7, 10, 11). Though the expression of myocardin has been established in the cardiac tissues and smooth muscle cells, myocardin mRNA was found lacking in skeletal muscle, both *in vivo* and *in vitro* (8, 12). In smooth muscle cells, myocardin is a known regulator of several marker proteins such as calponin, α-SMA, SM22, H-caldesmon and smooth muscle myosin heavy chain that are important for smooth muscle differentiation (9).

Myocardin in Cancer

Alterations in the endogenous level of myocardin expression has been linked to the development of pathophysiological conditions in cancer, heart failure, diabetes and acute vessel disease (6). Evidence of possible tumor suppressive activities of myocardin in cancers such as nasopharyngeal carcinoma (13), and fibrosarcoma cell lines (14), has been reported in literature. In human uterine leiomyosarcoma cell lines, myocardin was found to be significantly downregulated and its overexpression resulted in significantly decreased cell proliferation, it was found to exert an indirect tumor suppressor effect via the activation of p21, a growth inhibitor in a SRF-binding CArG-dependent manner (15), and via the modulation of contractile genes such as CNN1, a tumor suppressor (16).

Conversely, as with the complex nature of diseases, elevated expression of myocardin has been reported in tumor cells and pathophysiological conditions. Myocardin was found to be highly expressed and upregulated in human retroperitoneal leiomyosarcoma tumors and myocardin KD correlated with decrease in cell migratory ability of these cells and decrease

Table 1: Common Genetic Alteration in Lung Cancer.
The following genes have been reported in literature for LUAD and LUSC.

NSCLC cancer subtype	Cell proliferation	Cell cycle regulation	DNA repair
LUSC	PTEN, TP53, NFE2L2, AKT3	CDKN2A	NFE2L2, KEAP1, CUL3
LUAD	EGFR, KRAS, ERBB4, KDR, STK11, NF1, FGFR4, NTRK	CDKN2A, CCND1, CKD4, CCNE4	KEAP1, ATM

Govindan et al recently identified 26 significantly mutated genes consisting of ERB44, KDR, FGR4, NTRK (oncogenes) and TP53, NF1, STK11, RB1, ATM and APC (tumor suppressors) in a pool of LUAD patients[3].

mesenchymal transition of Human Pleural mesothelial cells when stimulated with TGF-β. Also, it was found that myocardin knockdown results in the reversal of Mesothelial to mesenchymal transition of HPMCs and prevents pleural fibrosis (17). In another recent study, the knockdown of myocardin in cardiomyocytes was found to induce subendothelial necrosis and the upregulation of Nix, a death gene (18).

Few studies on the role of myocardin in lung cancer exist in literature. Recent findings by Tong et al (2019) demonstrated that myocardin is overexpressed in and promoted TGFβ-mediated epithelial to mesenchymal transition and invasion of non-small cell lung cancer, A549 cells (19). Interestingly, recent findings at the Ikebe laboratory (2021) have revealed that the knockdown of myocardin may induce apoptosis in A549 cells, a NSCLC cell line. And put together, these findings raise the possibility that myocardin may play an important role in the progression of non-small cell lung cancer.

Apoptosis

Apoptosis is one of the types of programmed cell death which is a process that is highly regulated, and is required for the maintenance of cellular homeostasis and removal of dying cells (20). It plays an important role in embryonic development and in the immune system reaction to invading pathogens (21). It is characterized by cytoskeletal breakage, plasma membrane blebbing, DNA fragmentation, chromatin condensation and cell shrinkage (22, 23). Dysfunctions in the process of apoptosis is implicated in some diseases like

autoimmune disorders, ischemic heart disease, neurodegenerative diseases and cancer (21). Apoptosis functions to destroy metastatic cells in cancer and inhibit their proliferation. Invasion and metastasis are only successful when cells circumvent the apoptotic mechanism in the cells.

The signaling cascade involved in the process of apoptosis is traditionally divided into two: the intrinsic/mitochondria apoptotic pathway and the extrinsic apoptotic pathway. However, cytoplasmic granule toxin, perforin, and serine protease, granzyme A have been shown to induce apoptosis of target cells via a caspase-independent manner, when secreted by exocytosis, thus characterizing a third pathway (24). In addition, a caspase-dependent component of the perforin/granzyme pathway exist which is mediated by granzyme B (25). In the extrinsic pathway, death ligands such as such as Fas ligand and TNF-α bind to the special domains called death domains of transmembrane receptors from the tumor necrosis factor family (TNF) on the cell surface leading to the formation of death-inducing signaling complex (DISC) and the subsequent activation of procaspase-8 which in turn activates caspase 3. T-cell mediated cytotoxicity which employs the Perforin/granzyme B pathway in the elimination of virus-infected cells leads to the activation of caspase 10 and converges with the intrinsic and extrinsic pathway at the execution pathway involving the activation of caspase-3 (21, 26).

Intrinsic Apoptotic Pathway

The events which mediate the intrinsic pathway are initiated in the mitochondria and the stimuli that trigger the process, unlike the extrinsic pathway, are non-cytoplasmic related signals. The intrinsic pathway is also called the mitochondria pathway because it plays a major role and is the site of pro-apoptotic and anti-apoptotic proteins interaction, as well the initiation of the proteolytic cascade of caspase activation. The process of apoptosis in this pathway is regulated by a family of Bcl2 proteins which are divided into antiapoptotic protein such as bcl2, BclXL, MC-1 and proapoptotic proteins such as Bax, Bak and Bok which to regulate the process of life and death for the cell depending on the conditions (27). In normal healthy cells, the antiapoptotic proteins inhibit the proapoptotic proteins, however, negative stimuli such as oxidative stress, DNA damage and growth factor deprivation causes the Bcl2 proteins containing one bcl2 homology domain, called BH3-only proteins such as bid to either inhibit the antiapoptotic proteins or bind directly to proapoptotic proteins like Bax leading to its activation, thus favoring the process of apoptosis (28). Bax /Bak play crucial roles in the regulation of apoptosis, Bax, which is domicile in the cytosol in healthy cells becomes translocated to the mitochondria during apoptosis to undergo oligomerization alongside Bak, upon bid binding to Bcl2. This process drives mitochondria permeability transition as Bax imbeds itself tightly into the mitochondria, an event which induces the formation of mitochondria outer membrane pore and the subsequent of release of cytochrome c, an essential component of the mitochondria electron transport chain into the cytosol (27-29). Here, cytochrome c, forms an apoptosome

complex with Apoptotic protease activating factor (Apaf1) and procaspase-9 leading to the activation of proteolytic enzyme caspase 9 by dimerization. Activated caspase 9 then binds and cleaves downstream executioner caspases 3, 6 and 7 which are so-called because their activation involves proteolytic events which leads to the cleavage of various regulatory and structural proteins, thereby mediating the morphological changes such as membrane blebbing, nuclear condensation and DNA fragmentation that accompany apoptosis (30). Procaspase-3, a proenzyme of caspase (32kDa) consists of a p17, p13 subunits and p3 pro-domain which upon activation by caspase-9 becomes the active caspase-3 form. This active form has been reported to be less stable than the proenzyme forma and rapidly degraded by a mechanism involving its own catalytic activity, which arises possibly as a cell regulatory means of preventing prolonged expression of the destructive signaling of proteolytic activity during programmed cell death (20). Several studies have established caspase-3 as the main effector caspase in the process of apoptosis, and activated caspase 3 is responsible for the cleavage of many proteins during apoptosis including nuclear antiapoptotic proteins poly(ADP-ribose)polymerase protein-1 PARP-1, and ICAD (inhibitor of caspase-activated DNase) (20, 21, 26, 31). PARP-1 is the most ubiquitous and founding member of the PARP family which functions in repairing single-stranded breaks in DNA. As a substrate of activated caspase-3, it is cleaved to 89kDa and 24kDa subunits, thereby losing its activity and furthering the process of apoptosis (32, 33).

Mitochondria Bioenergetics and Apoptosis

Mitochondria bioenergetics is altered during apoptosis and is characterized by respiratory rate suppression, drop in membrane potential and a slight decrease in cytosolic pH (34). In addition to cellular stress, hypoxia, UV, drugs, heat shock and development cues, Reactive Oxygen Species (ROS) have also been implicated in the induction of apoptosis. Intracellular ROS produced from the leakage in the Electron Transport Chain (ETC) causes oxidative damage of mitochondrial proteins and malfunction of the ETC leading to the release of cytochrome c. Conversely, the loss of cytochrome c can cause a further increase in ROS levels of the mitochondria due to alterations in the ETC (35). Mitochondria undergo damages and loss of several functions during apoptosis which includes the loss of maintenance of calcium ion homeostasis, fragmentation of the mitochondria membrane, remodeling of the cristae, formation of superoxide due to the loss of cytochrome c, and distortion in membrane potential required to generate ATP (36). This, in addition to its role as the main site of pro and anti-apoptotic factors, makes the investigation of bioenergetic events in the mitochondria a crucial part of studies involving apoptosis.

Given that preliminary studies in Ikebe lab revealed that myocardin knockdown significantly promoted caspase-3 degradation, in this study, using myocardin siRNA, we described effect of the knockdown of myocardin gene on important apoptotic biomarkers such as PARP, caspase-3, and Bax; and on mitochondrial dysfunction involving respiration and ROS formation associated with the process of apoptosis.

RESEARCH HYPOTHESIS

The overall hypothesis of this research is that the myocardin signaling pathway plays an important role in the progression of NSCLC. The knockdown of myocardin induces apoptosis and decreased metabolic cell viability of NSCLC A549 cells. Decreased myocardin levels also alters mitochondrial bioenergetics, thus triggers mitochondria/intrinsic apoptosis pathway.

MATERIALS AND METHODS

Cell Culture and Transfection

Non-small cell cancer cell (A549) cells were cultured and maintained in FK-12 media (Gibco) containing 10% Fetal Bovine Serum and Penicillin-Streptomycin at 37°C in 5% CO_2. Transfection was done with FuGENE SI® Transfection Reagent following the manufacturer's protocol and using 10nM myocardin siRNA (pool of three siRNAs :5'-GGAUCAGACUCGAAGUGAAtt-3', 5'-GGUUUACACUCUUCUGAUAtt-3' and 5'-CGAAGAGCCUCACUUUGAUtt-3') and siControl (Universal negative control 2; sigma). Transfection reagent (7.5µL) and siMyocardin/siControl (5µL) was diluted with 117.5 and 120µL DMEM (serum free) media respectively and then mixed and allowed to incubate for 10 minutes before addition to the cells in a dropwise manner in 6-well plates.

Quantitative PCR

Total RNA was isolated using the Pure Link™ RNA Mini Kit according to the manufacturer's protocol. RT-qPCR of up to one (1) µg of total RNA was then performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and qPCR and analysis was done using TaqMan Gene Expression Assay (Applied Biosystems) and QuantStudio 6 Flex. TaqMan probes for Myocd (Hs01122781_m1) and GAPDH (Applied Biosystems).

Western Blotting

The cells were washed twice with PBS, collected, centrifuged, treated with 20% TCA for ten minutes and washed twice with water. Sodium bicarbonate (5mM) was added and incubated for 5 minutes followed sonication at 10% duty cycle and output 2 for 5 seconds and quantified using Modified Lowry Method (37). The samples were then treated with 2.5X SDS buffer (0.5M Tris-HCL pH 6.8; glycerol; 0.5g SDS; 0.25% Bromophenol Blue; and 5% 2-mecaptoethanol) appropriately, boiled for ten minutes, and centrifuged for two minutes at 12000 rpm. Based on the Laemmilli discontinuous buffer system (38) , the samples were run on 6%-18% polyacrylamide gradient gel at 180V for 55 minutes. Gel transfer to nitrocellulose membrane was done using the semi-dry transfer for 30 minutes at 20V. The membranes were washed with TBST (containing 0.05% Tween-20) and blocked for 1hr on a rotor at room temperature with 5% skimmed milk. The blots were incubated with Antibodies Anti-Beta-actin (A1978, sigma), anti-PARP (9542T, Cell signaling), anti-BAX (2772S, Cell signaling), anti-Caspase 3(ab90437, Abcam) 1:1000 dilutions with 1X BSA in TBST solution overnight at 4°C. The blots were washed twice 15 minutes each and incubated with peroxidase-conjugated rabbit and mouse antibody (in 5% skimmed milk) for 90 minutes. After washing twice, the blots were developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized with Molecular Image ChemiDoc XRS+ (Bio-Rad).

Measurement of Mitochondrial respiration

Mitochondria oxygen consumption rate was measured using the Agilent Seahorse XFp Cell Mito Stress Test kit on the Seahorse XFp Extracellular Flux Analyzer following the manufacturer's protocol using A549 cells that had been transfected with siMyocardin and control for 5 days. Reagents: Oligomycin, 1.5µM, FCCP (1µM) and Rotenone/Antimycin A (1µM) were employed.

Cell normalization was done using the Crystal Violet Staining method (39).

Measurement of Mitochondrial ROS Formation

Mitochondria ROS formation was measured as described (40). Myocardin knockdown and control cells were starved overnight, stimulated with TGF-β, seeded at 12,000 cells per well in a 96-well plate 48hrs before the experiment and incubated in F-12K (FBS free) media. The cells were then washed twice with PBS and incubated with 100µL 100mM hydrogen peroxide (216763, sigma) in F-12K media for 2 hours at 37°C. The cells were washed twice with PBS and 100µL of 5µM MitoSox™ Red mitochondrial superoxide indicator in FluoroBrite DMEM (with 5mM HEPES) was added and the fluorescence read at 585nm for 75minutes.

Cell Viability Assay

MTT Assay was used for the determination of cell viability (41). Myocardin knockdown and control cells were seeded 2 X 10^4 cells/mL into 96 well plates.

Cells were starved overnight and 100µL DMSO, 25Mm Glutamine, 1mM NAC, 0.1Mm Hydrogen peroxide and 10ng/mL TGFβ were added for 24 and 48 hours.

MTT reagent was then added and incubated for 4 hours. The supernatant was removed, and the formazan crystals were dissolved in MTT solvent. The OD at 565 nm after completely dissolving formazan crystals.

Statistical Analysis

All Data were presented as mean ±SEM. An independent Student's t-test was used for direct comparisons between two groups.

RESULTS

The Effect of Myocardin on Apoptotic Markers

To investigate the role of myocardin in the progression of apoptosis, we first examined the effect of myocardin gene silencing using the specific siRNA. Since the efficacy of gene silencing is influenced by the time after administration of the siRNA, we examined the effect on myocardin mRNA expression by using qPCR technique. Figure 1 shows the effect of myocardin specific siRNA on the expression of myocardin mRNA at 3 days and 5 days, respectively after siRNA transfection. The expression of myocardin mRNA was significantly reduced by siRNA for both 3 days (79.3 +/- 0.02 % decrease of the control) and 5 days (85.7 +/- 0.037 % decrease of the control) after the transfection. Using this myocardin specific siRNA, we examined the effect of myocardin knock-down (KD) on the expression of apoptosis marker proteins.

Figure 2 shows the effect of myocardin KD on the protein expression of PARP and caspace-3 in A549 cells revealed by western blotting analysis. Activated caspase-3 is an established marker of apoptosis, and cleaves PARP, an antiapoptotic protein which is important in DNA repair, a process which promotes apoptosis (42). Myocardin KD notably increased the degradation of procaspase-3 and PARP, suggesting that myocardin KD facilitates apoptosis (Fig. 2). Bax is a key regulator of mitochondrial pathway of apoptosis. During apoptosis, it oligomerizes at the mitochondrial outer membrane and produces a pore that facilitates the release of mitochondrial components including cytochrome-c (27). The

overexpression of Bax is thought to promote apoptosis (see introduction). As shown in Fig. 2B, western blot analysis revealed a notable increase in Bax protein expression by myocardin KD. These results indicated the changes in the expression of multiple important apoptotic markers by myocardin knockdown in A549 cells. The normalized ratio of caspase/ □-actin, PARP/□-actin and Bax□□-actin protein expression were presented in figure 2c and 2d.

A.

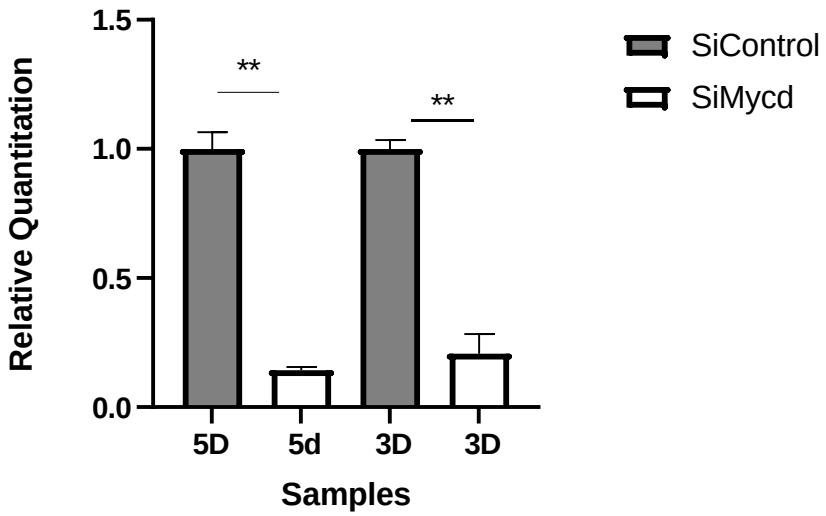


Figure 1: Effect of Myocardin Specific siRNA on Myocardin mRNA Expression in A549 Cells. **(A).** qPCR analysis of myocardin knockdown using siRNA 5- and 3-days post-transfection is shown. RNA was isolated from cells and subject to RT-PCR and then real-time qPCR. **, $p < 0.0036$ and 0.0033 respectively. siControl is siRNA for universal negative control and siMycd is siRNA specific for myocardin.

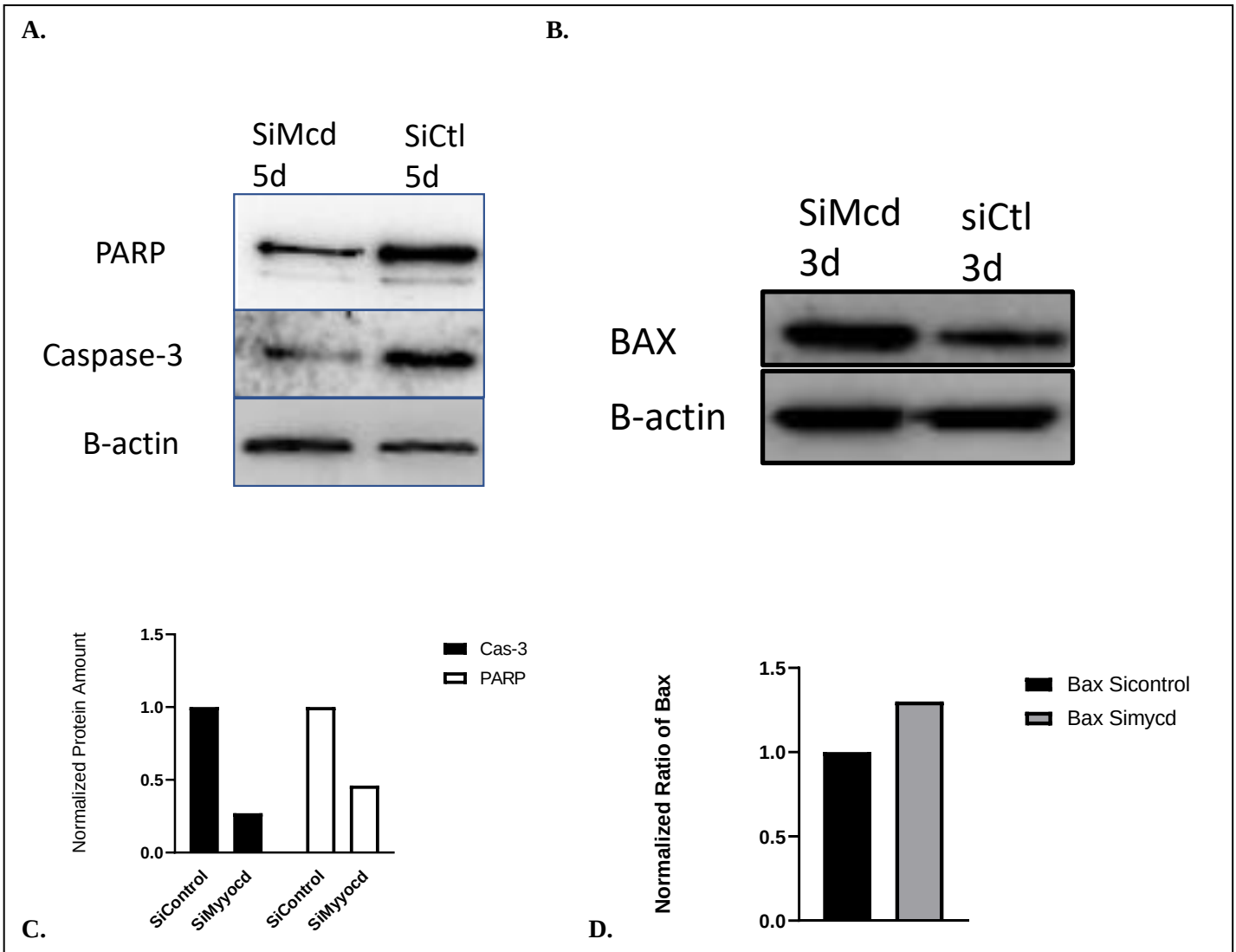


Figure 2: Myocardin Gene Silencing Influences the Protein Amount of Apoptosis Markers.
A. Effect of myocardin gene silencing on PARP and caspase 3. Western blotting analysis of A549 cells 5 days post-transfection with siMyocardin and siControl for caspase-3 and PARP. B-actin was used as loading control. **(B).** Effect of myocardin gene silencing on Bax protein levels. Western blotting analysis of A549 cells 3 days post-transfection with siMyocardin and siControl for Bax protein. β-actin was used as loading control. **C.** Normalized ratio of Cas-3 and PARP are presented in bar graphs after image analysis and D. Normalized ratio of Bax

The Effect of Myocardin gene silencing on the Viability of Lung adenocarcinoma cells (A549)

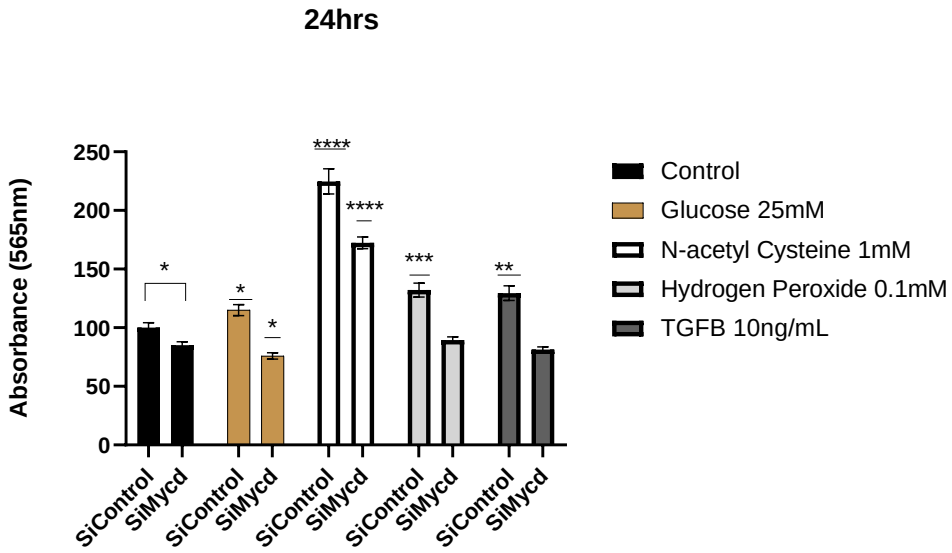
To further test the hypothesis of the contribution of myocardin to the process of apoptosis, we examined the effect of myocardin KD on the cell viability of A549. The cell viability test was done using the reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), a mono tetrazolium salt (*see METHODS*). A549 cells were treated with myocardin siRNA and control siRNA, respectively and the cells were subjected to cell viability assay at 5- and 6-days post-transfection. Modulators/reagents such as glutamine, which provides a source of carbon for cancer cell growth; N-acetylcysteine- a potent antioxidant which counters oxidative stress; hydrogen peroxide, an inducer of oxidative stress by the formation of reactive oxygen species; and TGF-β, a natural growth modulator of cells, were used to treat the cells following myocardin KD. Reagents such as glutamine 25mM, NAC 1mM, Hydrogen peroxide 0.1mM and TGFβ 10ng/mL were added 24 and 48hrs after serum starvation to modulate the level of cell viability. As shown in figure 3A and B, the level of metabolic viability for myocardin knockdown cells significantly decreased from 24hrs after treatment with the modulating reagents versus the siControl groups. In addition, in the 24 hours control group treated with DMSO only, myocardin knockdown was found to significantly decrease cell viability in A549 cells.

The Effect of Myocardin on Mitochondria Dysfunction

We next examined the effect of myocardin KD on mitochondrial function, as mitochondria is a critical player of the intrinsic apoptosis pathway (*see Introduction*) and Bax expression

was increased by MyoCD KD. Using Seahorse XFp Analyzer, myocardin knockdown was found to induce an increase in the basal respiration of the cells. However, myocardin KD showed a large, significant decrease in the maximal respiration of the cells (Fig. 4A). Consequently, a large decrease in the spare respiratory capacity was observed by myocardin KD (figure 3A and figure 4A). We also performed a qualitative analysis of proton extrusion into the extracellular medium per time alongside the OCR measurement using the SeaHorse Analyzer, this is related to glycolytic activity of the cells. The result suggested that myocardin KD elevated glycolytic process (figure 4B).

A.



B.

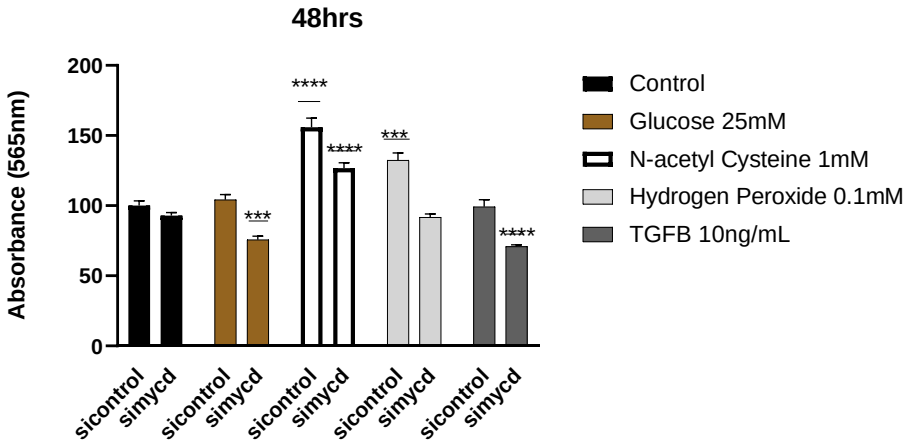


Figure 3: Myocardin Knockdown Reduces Metabolic Viability of A549 Cells. **(A).** MTT assay was used to evaluate the effect of myocardin knockdown on the viability of 5-day and 6-day post-transfected A549 cells (indicated by the absorbance at 565 nm); modulators such as glutamine 25mM, NAC 1mM, hydrogen peroxide 0.1mM and TGFβ 10ng/mL were added for 24hrs and **(B).** 48hrs after serum starvation. *, $p < 0.04$; **, p value < 0.0052; ***, p value < 0.0008; and ****, p value < 0.0001, and n=8

The Effect of Myocardin KD on Mitochondria Reactive Oxygen Species (ROS) Levels

The above results suggested that myocardin KD induces mitochondrial dysfunction. It has been known that ROS (reactive oxygen species) damages mitochondrial function (35), therefore we next asked whether the observed mitochondrial dysfunction is related to the increased ROS formation. To address this question, we investigated the effect of myocardin KD on ROS formation (Fig. 5). We employed MitoSOX as a probe for a quantitative analysis of the ROS formation (see METHODS). On mitochondria dysfunction, we performed a quantitative analysis of the ROS levels in siMyocardin A549 cells versus the control. Figure 5 shows the effect of myocardin in non-treatment (NT); and Hydrogen peroxide (0.1mM) treated groups, on ROS formation in the cells. Myocardin knockdown, in both the presence and absence of hydrogen peroxide, was found to significantly induce higher levels of ROS formation compared to the control.

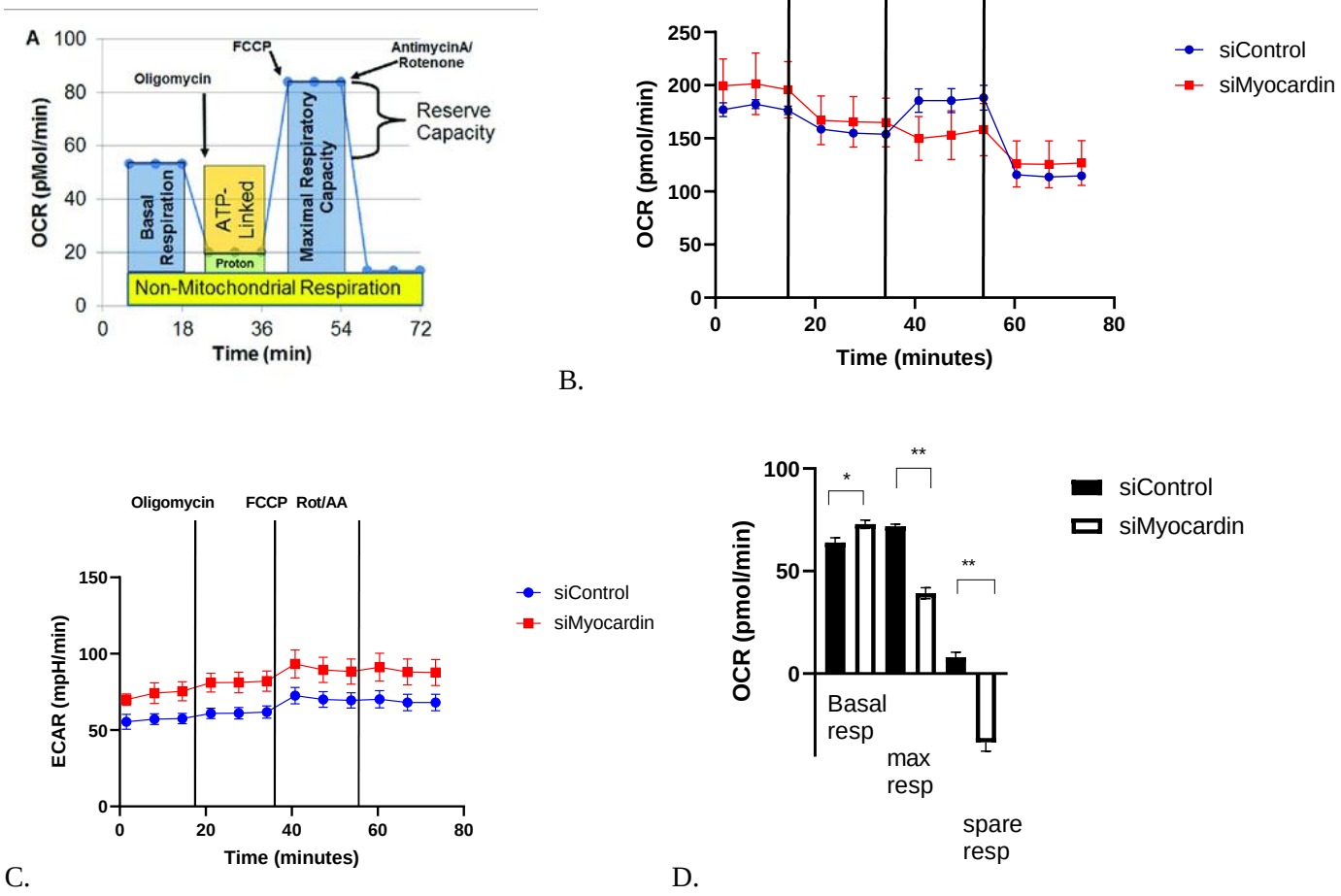


Figure 4: Myocardin Induces Mitochondria Dysfunction in A549 Cells. **A**. OCR (oxygen consumption rate) is used to measure mitochondria respiration. First, initial/basal respiration is measured, followed by the timed addition of oligomycin (complex V inhibitor) to determine the level of basal respiration linked to ATP-based respiration. The addition of FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore drives the respiration to the maximum level and maximal respiratory capacity is measured. Lastly, the addition of complexes III and I inhibitors stops the respiratory chain allowing the determination of non-mitochondrial related respiration and the spare respiratory capacity (1) **B.** Lung adenocarcinoma cells were treated with Oligomycin (1.5µM), FCCP (1µM) and Rotenone/Antimycin A (1µM) and the oxygen consumption rate was measured for both siMyocardin and siControl cells using SeaHorse Analyzer, n=3. OCR was normalized using crystal violet method following analysis. **C.** A qualitative analysis of extracellular acidification rate (ECAR) was done alongside the OCR measurement. Myocardin knockdown induced mitochondria dysfunction in terms of altered respiratory capacity, with an increased glycolytic rate. **D.** myocardin knockdown significantly altered basal, maximal and spare respiratory capacity of A549 cells, following treated with the aforementioned reagents. *, p value < 0.0329; and **, p value < 0.0027, and 0.0061 respectively and n=8

A.

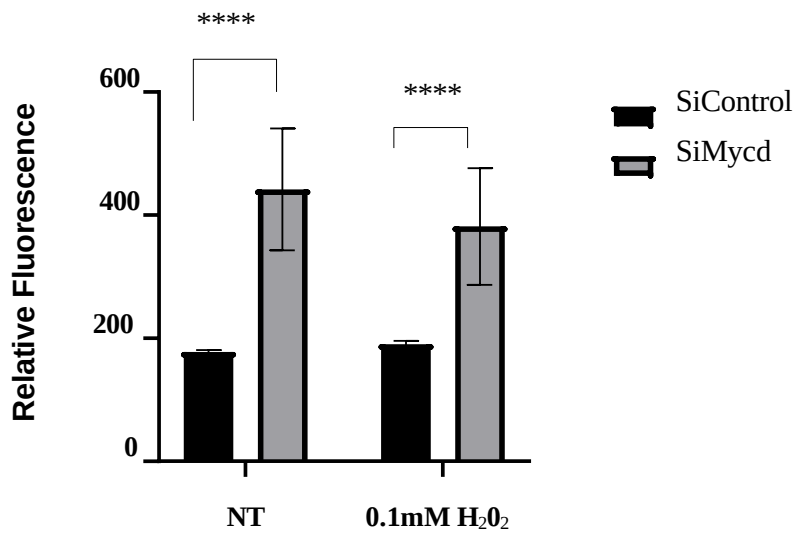


Figure 5: Myocardin Alters Mitochondria ROS levels. **A.** Mitosox was used to measure the fluorescence intensity of myocardin knockdown cells compared to the control, following treatment with hydrogen peroxide (0.1mM) and DMSO for non-treatment groups (NT). The Relative fluorescence is a direct measure of mitochondria ROS levels. p value < 0.0001; and ****, n=6

DISCUSSION

In the present study, we addressed our hypothesis that myocardin expression is possibly involved in the progression of apoptosis in lung adenocarcinoma cells, A549. The cleavage and activation of specific substrates determines unique cell fate and phenotypic changes that induce cell death via apoptosis, ferroptosis, necrosis, and necroptosis (43). Among various cell death pathways, cell apoptosis is differentiated by the activity of Bcl-2 proteins which leads to the activation of caspase-9 (28). Bcl2 proteins like Bax, and downstream effector and targets proteins like Caspase-3 and PARP have established roles in the intrinsic/mitochondrial apoptosis pathway, thus are definitive markers of this process of cell death.

To address our hypothesis, we examined the effect of myocardin gene silencing on various aspects of apoptosis of A549. First, we investigated the effect of myocardin knock-down (KD) on the degradation of intrinsic apoptosis markers. Since caspases are responsible for various aspects of apoptosis processes such as the exposure of phosphatidylserine to outer leaf of plasma membrane, morphological changes that mediate the late-stage process of apoptosis like DNA fragmentation, membrane blebbing and formation of apoptotic vesicles, the probing of the effect of myocardin on caspase activity is considered important. Particular focus is caspase-3, one of the most characterized of the three executioner caspases (-3, -6 and -7) which are the substrates of upstream initiator caspases. While caspase-6 has a less defined role in apoptosis and caspase-3 and -7 share

proteolytic activity on downstream substrates, caspase-3 has been found to elicit a greater and more efficient activity on the target substrates than caspase-7 in the terminal stage of apoptosis (44). Therefore, we examined the effect of MyoCD KD on caspase 3 activity that is defined by the activation of procaspase 3 (35kDa) to the active form. Our result clearly demonstrated the increase in the degradation of full-length caspase 3 (Fig. 1b).

On the other hand, PARP, a nuclear protein is one of the earliest characterized of the hundreds of executioner caspases substrates and its degradation is a diagnostic indicator of apoptosis (42, 45). Therefore, the increase in the cleavage of PARP upon the knockdown of myocardin in A549 cells further indicated an induction of apoptosis with decreased expression of myocardin.

It has been known that the expression of pro-apoptotic Bcl2 protein, Bax increases during apoptosis. During apoptosis, the expressed Bax accumulates on mitochondrial outer membrane where it dimerizes to induces the mitochondria permeabilization with the consequent release of cytochrome c that further facilitates apoptosis (28). Therefore, increased expression of Bax by MyoCD KD found in this study also supports our view that myocardin KD facilitates apoptosis of A549 lung cancer cells.

In addition to the formation of the mitochondria outer membrane pore (MOMP) by oligomerized Bax/Bak in intrinsic apoptosis, other events like metabolic activity dysfunction, cristae remodeling, and fragmentation occur in the mitochondria (36). Dysfunction of the electron transport chain (ETC) can be a consequence of the formation

of MOMP which leads to the loss of cytochrome c from the inner mitochondria membrane space during apoptosis (46).

The effect of MyoCD KD on mitochondrial function was, therefore investigated using SeaHorse assay. Our investigation on the respiration rate given by the oxygen consumption rate capacity, which is a function of the oxidative phosphorylation and is contingent on the maintenance of the ETC integrity, revealed significant differences in mitochondrial activity between MyoCD KD cells and the control cells. The measured maximal respiration rate, induced with FCCP, a protonophore, was largely decreased in A549 MyoCD KD cells compared to the control. This suggests ETC disruption in mitochondria of MyoCD KD cells, and it is plausible that this is due to the loss of cytochrome c from the mitochondria via the MOMP.

Although the difference was marginal, there was an increase in basal respiration before the addition of stressors like oligomycin, a complex V inhibitor in the MyoCD KD cells. While the reason of this difference is unclear, it is plausible that some compensatory respiratory mechanism might be operating. It should be noted that MyoCD KD cells showed increased extracellular acidification rate based upon the qualitative analysis of the OCR indicating a greater reliance of energy production on glycolytic pathway during the process of apoptosis.

ROS has been implicated in the induction of intrinsic apoptotic pathway and ROS such as hydrogen peroxide and superoxide can disrupt the ROS equilibrium in the cell and induce cytochrome c release from the mitochondria, which disrupts the mitochondria

intramembrane potential and the significant loss of cytochrome c can in turn lead to increased ROS levels due to disruption of the ETC (35).

The investigation of ROS levels as a possible feature of myocardin-KD induced apoptosis, using a stimulant like H_20_2 which is known to increase ROS levels did not reveal a highly significant increase in ROS levels in the experimental group compared to the control. Interestingly, ROS level was highly significant in the non-treatment group. The reason for failure of augment induction of ROS levels by hydrogen peroxide could be the use of weak reagent as the concentration and incubation time was same as reported to induce ROS levels in literature (47-49). The increase in ROS levels by MyoCD KD suggests that increased ROS formation either mediates the process of myocardin-silencing induced apoptosis or is a consequence of this process. This study demonstrated that the downregulation of myocardin contributes to the induction of increased ROS formation in lung adenocarcinoma cells, A549.

It has been reported that myocardin is inactivated in some cancers and has been found to possess tumor suppressive roles (13-15). Myocardin has also been found to be less expressed in more proliferative and differentiated normal smooth muscle epithelioid cells. However, myocardin was also reported to increase TGFβ-induced metastasis and invasiveness in EMT in lung adenocarcinoma and increase cell migration of undifferentiated human retroperitoneal leiomyosarcoma (19, 50). Myocardin knockdown in cardiomyocytes induced apoptosis (18, 51). In light of these previous reports, the complex nature of diseases such as cancer provides the rationale for our findings on

myocardin KD-induced apoptosis and its potential role as a druggable target in un-metastasized lung adenocarcinoma. The exact mechanism of its induction of apoptosis, however, remains to be investigated.

Myocardin has been shown to interact with SMAD3/4 in a positive feedback loop in TGF-β induced EMT to promote EMT, cell migration and metastasis(19). The possible interaction of myocardin with SMAD3/4 may shed light in understanding the molecular basis of myocardin-induced apoptosis in lung adenocarcinoma. Additional study of the effect of myocardin KD on apoptosis in other NSCLC primary cell lines like NCI-H358 with KRAS mutation and NCI-H3255 with EGFR mutation (52) will be beneficial in establishing our findings in a mutation-dependent context. Investigations with primary cell cultures of lung adenocarcinoma may also be beneficial in overcoming genomic inconsistencies that may occur with the use of established cell lines. The effect of myocardin knockdown on cell migratory and metastasis abilities can also be performed to allow comparison with existing reports (53).

In summary, this study highlights myocardin as an important target in NSCLC progression and presents an interesting insight on the potential role of myocardin as a druggable target in lung adenocarcinoma. This also provides the basis for further investigation on its mechanism of apoptosis induction.

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VITA

After graduating with a Bachelor of Pharmacy degree at Nigeria's highest-ranking university, the University of Ibadan in 2016, Soromidayo gained work experience as a pharmacist and in cancer research for a few years before proceeding to the University of Texas at Tyler for her master's degree in the Biotechnology graduate program in August 2021. She performed her thesis work in the laboratory of Mitsuo Ikebe, PhD and received her Master of Science in Biotechnology degree in May, 2023.

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This thesis was typed by Soromidayo Akinsiku.