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THE ROLE OF MTORC2 IN MESOMESENCHYMAL TRANSITIONING IN HUMAN PLEURAL MESOTHELIAL CELLS

by

PERPETUAL SERWAAH KYEI

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

Torry Tucker, Ph.D., Thesis Advisor

School of Medicine

The University of Texas at Tyler
April 2023

SIGNATURE PAGE

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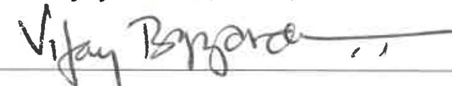

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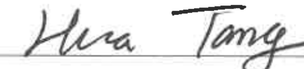

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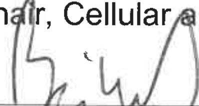

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TABLE OF CONTENTS

TABLE OF CONTENTS i
LIST OF FIGURES ii
LIST OF TABLES iii
ABSTRACT iv
INTRODUCTION 1
RESEARCH HYPOTHESIS 6
Experimental objectives 6
MATERIALS AND METHODS 7
Primary Pleural Mesothelial Cell Culture 7
Cell Treatments and Lysis 7
Gene Silencing 8
Western blotting 9
Quantitative PCR analysis 10
Streptococcus pneumoniae model of pleural fibrosis 12
Post Injury Evaluation 12
Immunohistochemistry 13
Microscopy 14
Statistical Analysis 14
RESULTS 15
Pharmacological Inhibition of mTOR c1 and c2 signaling blocks MesoMT 15
Rictor knockdown blocks MesoMT 20
Rictor Knockdown Enhances Apoptosis in HPMCS 22
Treatment with INK 128 reduces pleural injury in Strep injured mice. 24
Treatment with INK128 reduces MesoMT Markers in Vivo. 26
DISCUSSION 28
REFERENCES 33
VITA 37

LIST OF FIGURES

Figure 1. Blockade of mTOR signaling with INK128 blocks MesoMT……...17
Figure 2. Blockade of mTOR signaling with AZD8055 blocks MesoMT……18
Figure 3. Treatment with AZD 8055 reverses TGF-β induced MesoMT.......19
Figure 4. Rictor knockdown blocks MesoMT in HPMCs…...........................21
Figure 5. Rictor Knockdown Enhances Apoptosis in HPMCS..……………....23
Figure 6 Treatment with INK128 reduce injury in Strep injured mice...........25
Figure 7. Treatment with INK128 reduces MesoMT Markers in vivo………..27

LIST OF TABLES

Table 1. Primers and Probes used for RT-qPCR……….………......…..…....11

ABSTRACT

Pleural fibrosis can occur after empyema or complicated parapneumonic effusion as a result of tissue remodeling and excessive expression and deposition of extracellular matrix proteins. Pleural mesothelial cells transition into mesenchymal cells and acquire a profibrotic phenotype. This pathologic transition occurs through a process termed MesoMT and directly contributes to pleural fibrosis. MesoMT is regulated by diverse signaling pathways, including PI3K/AKT, which lie upstream of mTOR. However, the role of mTOR signaling in MesoMT is unknown. Using several mTOR inhibitors, we showed that inhibition of mTORC1/2 with INK128 and AZD8055 blocked and reversed TGF-β induced MesoMT. Targeted knockdown studies showed that mTORC2/Rictor pathway is indispensable for the Induction of MesoMT. Conversely, mTORC1/Raptor knockdown had no appreciable effect on MesoMT. Our *in vivo* analyses showed that INK128 treatment could attenuate pleural thickening injury and subsequent pleural fibrosis. Biomarkers of MesoMT, α-SMA and collagen were likewise reduced in INK128 treated mice compared to vehicle-treated mice. These studies suggest that mTORC2 may be an important therapeutic target for the treatment of PF.

INTRODUCTION

Fibrosis, the formation of fibrous tissue, is a pathological process which is often denoted by the excessive production of extracellular matrix (ECM) proteins resulting in the scarring and thickening of tissues [1]. This process can occur in numerous organs resulting in liver fibrosis, heart fibrosis, mediastinal fibrosis, bone marrow fibrosis, skin fibrosis and lung fibrosis [2]. Fibrosis is often initiated in response to chronic injury and/or persistent infection [3]. Pleural fibrosis (PF) occurs as a result of a variety of inflammatory disorders; complications from parapneumonic pleural effusions and tuberculous pleurisy, exposure to asbestos, as well as infections such as bacterial pneumonia [4]. Persistent pneumonia, which is the primary cause of PF, is defined as an acute inflammation of the lung parenchyma induced by viral, mycobacterial, fungal, or bacterial infection [5]. Pleural infections are mainly observed in children and senior citizens [6]. The mortality typically ranges from 7-30%. With the advancement of biomedical research and excellent healthcare practices, substantial improvement in clinical outcomes has been seen in the United States over the last fifty years [7]. However, critical cases of pneumonia can cause severe and persistent pleural injury. The development of inflammatory exudative pleural discharge creates an ideal microenvironment where PF can develop.

PF can result in respiratory impairment and subsequently respiratory failure. Numerous profibrotic mediators and activation of diverse signaling cascades play

critical roles in the pathogenesis of PF [8]. As PF progresses, it is characterized by notable tissue reorganization including increased pleural thickening and the pathological deposition of extracellular matrix proteins, such as collagen, fibronectin, and fibrin [9]. PF is also denoted by the appearance of the profibrotic myofibroblast in the pleural space [10]. Myofibroblasts are α-smooth muscle actin (αSMA) positive cells with numerous roles in the pathophysiological environment. Myofibroblasts facilitate wound contraction and thus are critical for normal wound healing and tissue repair. However, they typically disappear after the injury is resolved. In chronic injury states, these αSMA expressing cells persist and are associated with diverse types of organ fibrosis, including idiopathic pulmonary fibrosis. These cells also demonstrate enhanced expression of the ECM proteins associated with PF [11].

The source of the myofibroblasts found in PF remains under active investigation. The pleural space consists of a lining of mesothelial cells which can transition into myofibroblast in chronic injury and inflammatory conditions. Previous data from our laboratory and others have established that pleural mesothelial cells can contribute to the myofibroblast population via a process termed mesothelial mesenchymal transition (MesoMT) [12-13]. MesoMT can be triggered by numerous mediators in the inflammatory and repair processes, such as transforming growth factor (TGF)-β, factor Xa, thrombin, plasmin, and urokinase plasminogen activator (uPA) [14]. Through this process, relatively quiescent PMCs acquire a more fibrotic phenotype. Transitioning cells begin expressing αSMA and

increased levels of ECM proteins, such as collagen and fibronectin. These cells also drive fibrin deposition by increasing the expression of the plasminogen activator inhibitor (PAI)-1, which inhibits activators of plasminogen, uPA and tissue type plasminogen activator (tPA). Diverse signaling pathways are also reported to promote MesoMT, including PI3K/Akt, GSK-3β and NF-κB [15]. Previously we showed that the PI3K/Akt pathway is critical for the induction of MesoMT [16]. As such, we now propose to investigate the role of associated signaling pathways. In this study we investigated the contribution of the mammalian/mechanistic target of rapamycin (mTOR) axis. The aberrant activation of the mTOR pathway has been reported in various tumors and malignances [17]. Further, therapeutically targeting the PI3K/Akt/mTOR signaling pathway has been effective for the treatment of some solid malignancies [18]. The mTOR inhibitor, Everolimus is part of a combination therapy to treat hormone receptor-positive metastatic breast cancers.[19] In lung fibrosis, activation of mTORC1 has been reported to promote mechanisms that prolong activated lung fibroblast survival and excessive collagen expression. mTORC1 inhibitors successfully modulated the progression of bleomycin-induced pulmonary fibrosis in mice [20]. However, the role of the mTORC pathway in the progression of PF has not been investigated.

mTOR has two distinct complexes, complex 1 (C1) and 2 (C2). The mTORC1 complex contains the regulatory-associated protein of mTOR (RAPTOR), which is encoded by the RPTOR gene, MTOR, MLST8, DEPTOR and other associated proteins. [21] MTORC1 responds to signals from growth factors, certain amino

acids, insulin, and phosphatidic acid. MTORC1 also plays a central role in the production of nucleotides, lipids and protein needed for cell growth. Activation of the mTORC1 complex promotes phosphorylation of p70-S6 kinase 1 (P70S6K) and 4E-BP1. P70S6K phosphorylates the S6 ribosomal protein and upregulates translation [22]. 4E-BP1 is a binding protein that binds to eIF4E to inhibit the eIF4F translation initiation complex. The mTORC1 signaling pathway is directly regulated by activation of PI3K/AKT by mediators such as insulin and TGF-β. PI3K activation consequentially leads to the inhibition of TSC1/2 which indirectly activates mTORC1 via activation of Rheb. [22]

The mTORC2 complex is composed of the rapamycin-insensitive companion of mTOR (RICTOR), MTOR, MLST8, MSIN1 and other associated proteins. mTORC2 and its related signaling pathway has not been extensively studied and mapped as the MTORC1 [23]. mTORC2 activation leads to phosphorylation of AKT at serine 473, which subsequently enhances the signaling of the mTORC1 complex. mTORC2 also activates the serum and glucocorticoid-regulated kinase (SGK1), which phosphorylates and activates n-myc downstream regulated gene 1 (NDRG1). [24] Activation of mTORC2 has also been reported to regulate the function of the p90 ribosomal S6 kinase (RSK), another modulator or ribosome activity and translation.[25] The mechanism(s) of mTORC2 activation remain unclear.

Studies have established that PI3K/AKT signaling is critical for TGF-β mediated MesoMT [26, 31]. Nevertheless, the specific role of the mTOR

complexes, particularly mTORC2, in the progression of MesoMT has not been studied. In this study, we determined if the activation of the mTORC2 signaling pathway is critical for myofibroblast differentiation in HPMCs.

RESEARCH HYPOTHESIS

Previous studies from our laboratory have established that the activation of the PI3K/AKT signaling is critical for TGF-β mediated Meso - mesenchymal transition. The central hypothesis of this thesis is that inhibition of the PI3K/Akt effector, mTOR can block and reverse the process of MesoMT. mTOR has two distinct complexes. We suggest that these complexes have distinct effects on MesoMT and the progression of pleural fibrosis.

Experimental objectives

Objective 1: Determine the role of mTOR in the myofibroblast differentiation of HPMCs. Pharmacological inhibitors of mTOR will be evaluated for their ability to regulate MesoMT phenotype in HPMCs.

Objective 2: Determine the role of specific mTOR complex; C1 or C2 pathway, in mesomesenchymal transitioning in HPMCs. This will be accomplished through gene silencing. Raptor and Rictor targeting siRNA will be utilized to knockdown mTORC1 and mTORC2, respectively, and their distinctive contributions to MesoMT progression will be assessed.

Objective 3: Determine the role of mTOR in a pre-clinical model of pleural fibrosis. Pleural injury will be induced in mice with *S. pneumoniae* followed by a subsequent administration mTOR inhibitor INK128 to the test group. Pleural tissues will be collected to analyze markers of MesoMT and to assess pleural fibrosis.

MATERIALS AND METHODS

Primary Pleural Mesothelial Cell Culture

Human pleural mesothelial cells (HPMCs) were cultured in a humidified incubator at 37°C in 5% CO_2/95% air. HPMCs were isolated from pleural fluid obtained from hospital patients suffering from congestive heart failure. HPMCs were cultured and maintained in BEGM (containing the BulletKit minus epinephrine and retinoic acid, Lonza, Basel, Switzerland) containing 3% fetal bovine serum (Gibco), 2% antibiotic-antimycotic (Lonza) and 1% GlutaMAX (Invitrogen). Only cells with a calretinin positivity of >85% via FACs analysis were used for these studies.

Cell Treatments and Lysis

HPMCs were serum starved for 16-24 hours in serum-free RPMI-1640 (SFM) containing GlutaMAX. Cells were used either in blockade or reversal analyses. Cells were treated with TGF-β (5 ng/ml R&D, Minneapolis MN) and pan mTOR inhibitors. Cells were treated for 24 hours for RNA expression and for 48 hours for protein analysis. For blockade studies, HPMCs were treated with different doses of mTOR inhibitors prior to the treatment with TGF-β. mTORC1/2 signaling pathways were blocked with the inhibitors INK128 (200 – 1 nM - Chemietek) and AZD8055 (1 - 0.1μM, Selleckchem) for 24 hours. The media was removed, cells

washed with PBS, and SFM and inhibitors replaced. Cells were then treated with TGF-β in the presence or absence of mTOR inhibitors and allowed to incubate for 24 hours or 48 hours.

In reversal studies, serum-starved HPMCs were treated with TGF-β for 24 hours prior to the introduction of mTOR inhibitors to the cells. Cells were cultured in the presence of mTOR inhibitors for another 24 hours (RNA) or 48 hours (protein) and then prepared for RNA or protein analyses. The conditioned media was collected, and the cells washed with PBS. For both reversal and blockade studies, controls were treated with neither inhibitors nor MesoMT inducing agonists.

After treatment, cells were lysed with 150 μL PBX-100 (PBS with 1% Triton-X 100) which contained protease and phosphatase inhibitors. The cells were collected using a cell scraper and pipette. Cellular lysates were incubated on ice for 30 minutes. Lysates were then centrifuged at 13,000 rpm for 15 minutes at 4°C and the supernatant collected. The protein concentration of the lysate was determined via BCA analysis, as previously described [29].

Gene Silencing

HPMCs were plated on coated 6-well CellBIND culture dishes in BEGM medium prior to transfection. HPMCs were transfected with control siRNA, Raptor siRNA and Rictor siRNA (Table 1, Sigma-Aldrich) using Lipofectamine 3000 or

RNAiMAX, as previously described.[24] Silencing RNAs were diluted in serum-free RPMI 1640 medium (200 μL) for a final concentration of 200 nM and Lipofectamine 3000 (Invitrogen) (23 μL in 600 μL serum-free RPMI 1640 medium) in separate tubes. Cells were incubated and maintained in transfection mixture for 6 hours before changing to fresh BEGM medium to achieve knockdown of target proteins. Serum-starved cells were treated with TGF-β to induce MesoMT. Controls were untransfected HPMCs and cells transfected with non-specific control siRNA in the presence and absence of agonist.

Western blotting

For immunoblot analysis, HPMCs were lysed as stated above, cleared and their concentrations were determined through BCA assay, as previously described [25-26]. Lysate and conditioned medium were incubated at 95°C, resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane using the Trans-Blot Turbo Blotting System (Bio-RAD). Resolved lysates and condition media were probed for changes in α-SMA (Cat. No. MAB1420; R&D), collagen 1 (1310-08, Southern Biotech, Birmingham AL), phosphorylated Akt (Ser473, Cat. No. 4060 and Thr308, Cat. No. 13038, Cell Signaling, Danvers MA), total Akt (Cat. No. 4685, Cell Signaling), total SGK (Cat No. 9644, Cell Signaling), phosphorylated and total NDRG expression (5482 and 9485, Cell Signaling). All antibodies were used at a

concentration of 1:1000. GAPDH and β-actin were used as loading controls (1:10,000). The membrane was developed using a ChemiDoc XRS+ (Bio-Rad)

Quantitative PCR analysis

After cell treatments, total RNA was isolated using RNA Extraction Kit (Qiagen) and transcribed into cDNA. Changes in collagen 1, α-SMA, fibronectin, and PAI-1 gene expression were determined by qPCR analyses using a Bio-Rad CFX Touch system (Table 1) as previously described [30].

	Source	Sequence or Identifier	Fluorophore
αSMA	BioRad	qHsaCIP0028813	FAM
Col-1	BioRad	qHsaCEP005010	Tex 615
PAI-1	BioRad	qHsaCEP0050406	Cy5.5
FN	BioRad	qHsaCEP0050873	Cy5
GAPDH	BioRad	qHsaCEP0041396	HEX
RICTOR	Sigma	F: CGAUCAUGGGCAGGUAUUA R: UAAUACCUGCCCAUGAUCG	n/a
RAPTOR	Sigma	F: CUAGUCUGUUUCGAAAUUU R: AAAUUUCGAAACAGACUAG	n/a

Table1; Primers and Probes used for RT-qPCR.

Streptococcus pneumoniae model of pleural fibrosis

Streptococcus pneumoniae (*S. pneumoniae* strain D39, National Collection of Type Cultures) was used to induce pleural injury. C57Bl/6 mice were infected with *S. pneumoniae* on day 0 through an intrapleural injection of 1.8×10^8 cfu of *S. pneumoniae*. Antibiotic treatment (ampicillin, 100 mg/kg) was initiated 4 hours after infection and administered daily by IP injection for 4 days. For mTOR inhibitor studies, 0.5 mg/kg of mTOR inhibitor; INK128 or vehicle control, dimethyl sulfoxide (DMSO), in a volume of 40 μL was administered once daily by intraperitoneal injection. For subacute studies, treatment began on day 3 and ended on day 7. In chronic studies, treatment began 7 days after infection and ended on day 14. Vehicle controls mice received intrapleural injections of DMSO as previously described.[28]

Post Injury Evaluation

On the seventh day post-administration of inhibitors, mice were weighed. Mice were intubated with a 20 g x 1" cannula and pulmonary function of the mice was assessed using a flexiVent system (ScireQ). Anesthesia was maintained using isoflurane in the ventilation system. The GE Explore Locus Micro-CT Scanner was used to take CT scans to determine lung volumes and 3D image construction during full lung inflation. After analysis, mice were sacrificed, and the lungs together with other body organs were collected.

Immunohistochemistry

Lung tissues from control and test group mice were processed using a Tissue Tek II Tissue Processor. Tissues were embedded in paraffin using a Microm paraffin dispensing console and chilled to allow for hardening. Tissue sections were cut (5 μm) and fixed to positively charged histology slides (StatLab, McKinney, TX).

Slides were incubated at 60°C for 30 minutes to melt paraffin and allow tissue to adhere to slides. Slides were deparaffinized by passing slides through ClearRite and rehydrated through a series of decreasing percentages of ethanol washes. The tissue sections were then decloaked using the Biocare Medical Decloaking Chamber. Tissue sections were then stained for αSMA and collagen. Appropriate isotype matched mouse and rabbit IgG were prepared to set background fluorescence. Primary antibody was added to tissues and incubated overnight at 4°C. The appropriate secondary antibody containing buffer was applied and incubated for staining. Results were visualized using a confocal microscope. Collagen staining was also assessed using Trichrome staining, as previously described [27]. Lung histology and morphometry were determined by trichrome staining.

Microscopy

Light microscopy was employed to analyze lung morphology and to measure the pleural thickening of the lungs in mice. Phase contrast microscopy was used to document morphological variations in treated HPMCs. Confocal microscopy was used to visualize immunofluorescence staining of tissue slides. Confocal microscopy was used to record the immunofluorescence staining for α-SMA and collagen. Confocal images were acquired from a field of view at 0.4 um z-axis increments using the LSM 510 Meta confocal system.

Statistical Analysis

Data was analyzed using GraphPad Prism software and all data is expressed as means ±SEM. Two-tailed paired t test or one-way way analysis of variance (ANOVA) using Tukey's multiple comparison test, as appropriate, was used to analyze statistical significance between the treatment groups and P value of < 0.05 will be considered statistically significant.

RESULTS

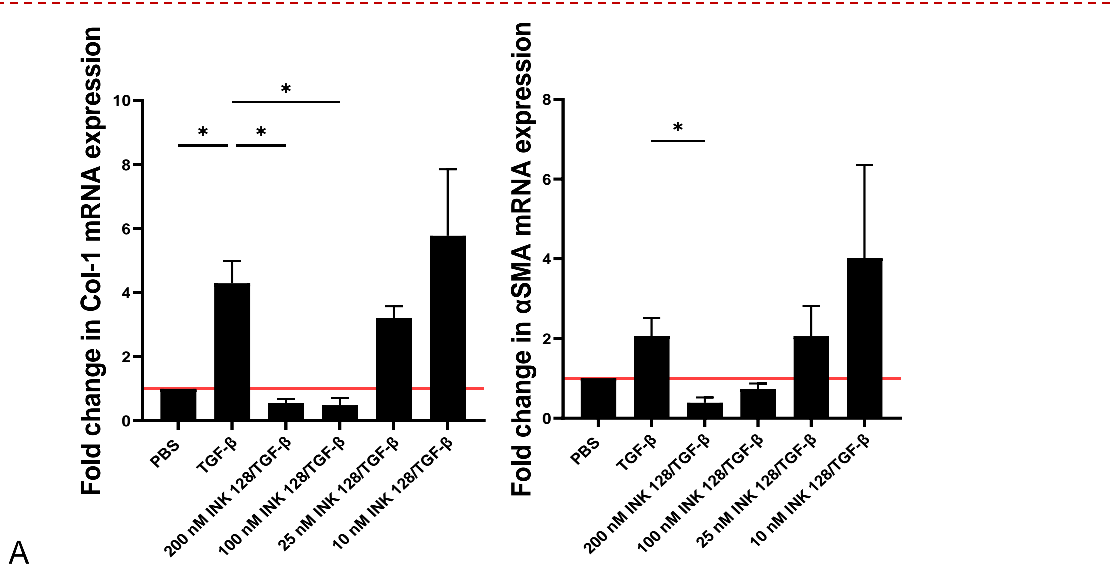
Pharmacological Inhibition of mTOR c1 and c2 signaling blocks MesoMT

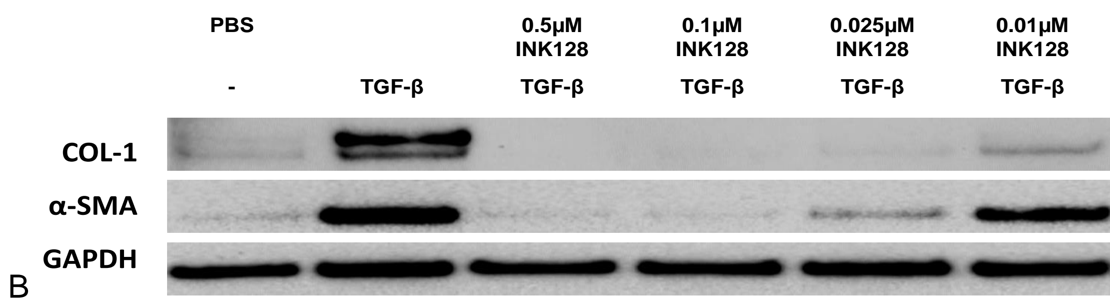
To determine if mTOR plays a role in MesoMT, pharmacological inhibitors were used to block mTORC1/2 activation. For these studies HPMCs were pretreated with various doses of the mTORC1/2 inhibitor INK128 (500 - 10nM) in serum free conditions for 16-24h. HPMCs were then treated with TGF-β to induce MesoMT. As previously reported, TGF-β increased αSMA and collagen 1 mRNA expression in HPMCs (Figure 1A) [14]. Conversely, cells treated with TGF-β in the presence of INK128 demonstrated reduced expression of αSMA and collagen mRNA levels (Figure 1A). Similar results were demonstrated in immunoblotting analyses as INK128 pretreatment dramatically reduced TGF-β mediated increases in αSMA and collagen 1 protein levels (Figure 1B)

To confirm the role of mTORC1/2 activation in MesoMT, complimentary analyses with a separate mTORC1/2 inhibitor were performed. For these studies HPMCs were pretreated with various doses of the mTORC1/2 inhibitor AZD8055 (1μM – 0.1μM) in serum free conditions for 16-24 hours. HPMCs were then treated with TGF-β to induce MesoMT. Similar to the INK128 studies, AZD8055 appreciably blocked TGF-β mediated increases in α-SMA and collagen mRNA expression compared to levels in positive control cells (Figure 2A). Similarly, western blotting analyses performed in parallel showed a reduction of α-SMA and Col-1 levels in AZD8055-treated cells in the presence of TGF-β compared to TGF-

β treatment alone (Figure 2B). This data suggests that activation of the mTORC1/2 pathways are critical for the induction of MesoMT.

Because mTORC1/2 inhibition significantly blocked induction of MesoMT, we next determined whether this inhibition could reverse established MesoMT. For these studies, serum starved HPMCs were treated with TGF-β for 16-24 h. Cells were then treated with various doses of AZD8055 (1 – 0.1 μM) in the presence of TGF-β and incubated for another 24-48h. Like the blockade studies, AZD8055 reduced TGF-β mediated expression of αSMA and collagen 1 mRNA (Figure 3A). Similar results were observed in western blotting analyses, as cells treated with AZD8055 after TGF-β stimulation showed a reduction of α-SMA and Col-1 proteins compared to TGF-β treatment alone (Figure 3B). This data suggests again that activation of the mTORC1/2 pathways are critical for induction of MesoMT.





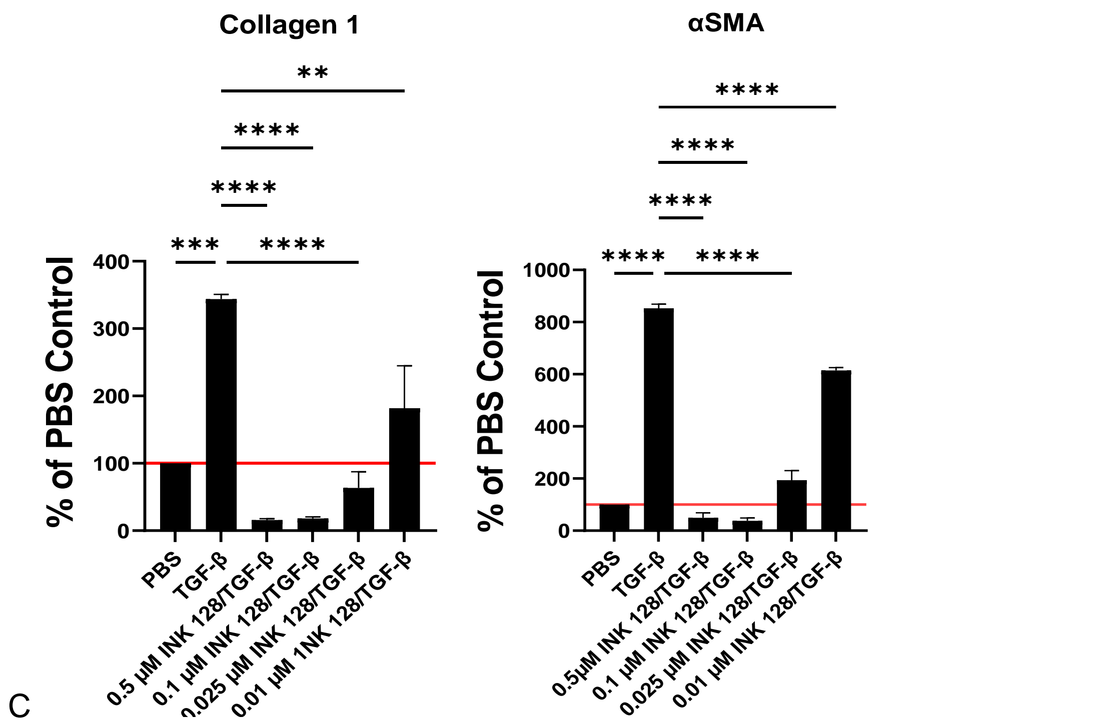
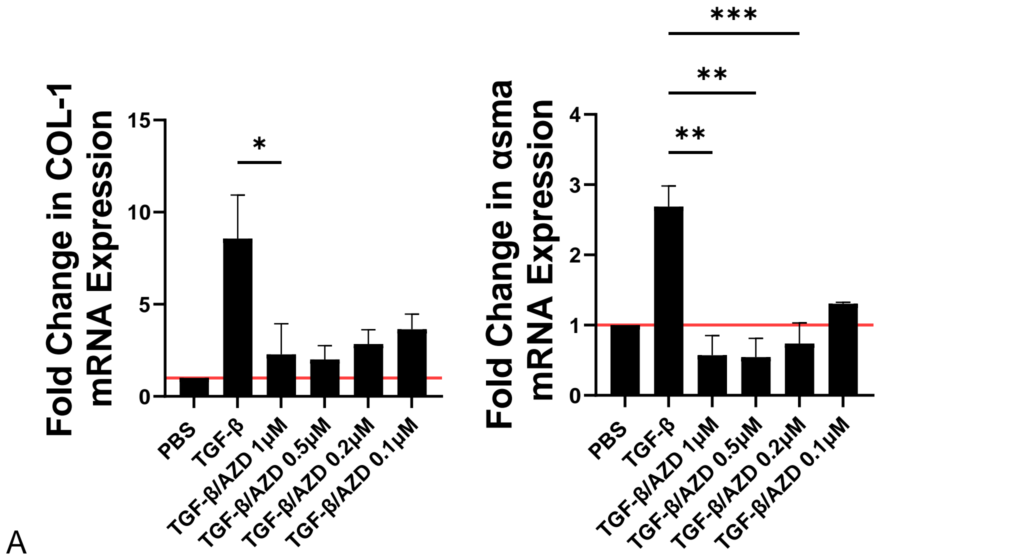
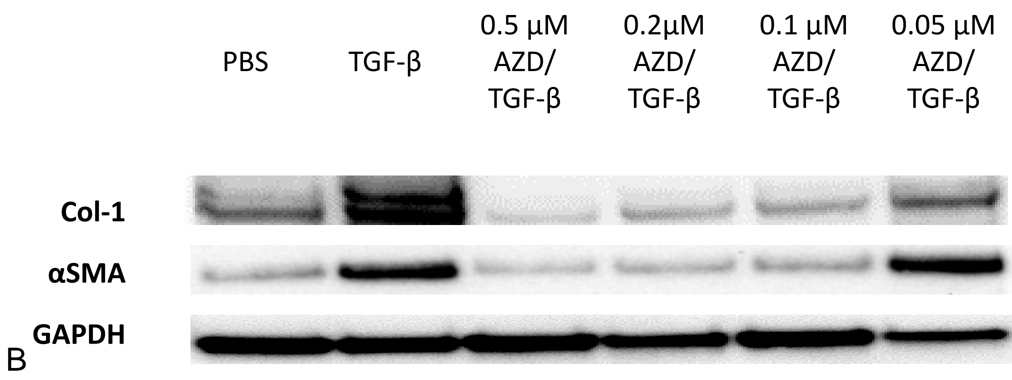


Figure 1: Blockade of mTOR signaling with INK128 reduces TGF-β induced MesoMT in a dose dependent manner. HPMCs were treated with the mTOR inhibitor INK128 prior to treatment with TGF-β. A) mTOR inhibition resulted in a dose dependent reduction of αSMA and collagen (Col-1) mRNA levels compared TGF-β treated only cells. B) Blockade of mTOR activation resulted in a dose dependent reduction of αSMA and collagen protein expression compared to TGF-β treated cells. C). Densitometric analyses of αSMA and collagen protein expression quantified from the blots. *, $p<0.05$, **, $p<0.01$, ***$p<0.001$, ****$p<0.0001$, n=3





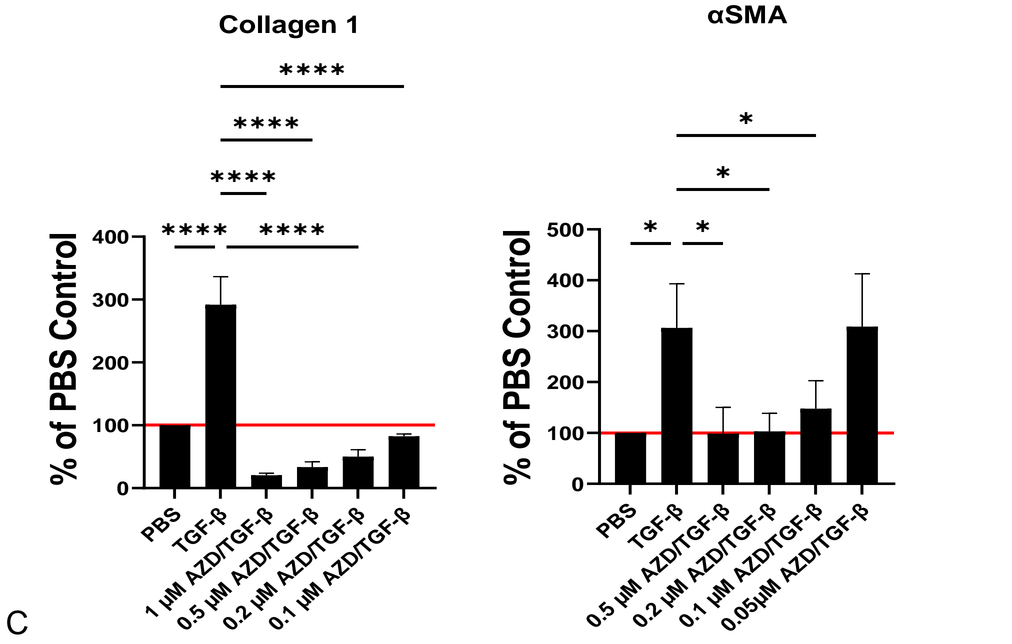


Figure 2: Blockade of mTOR signaling with AZD8055 reduces TGF-β induced MesoMT in a dose dependent manner. HPMCs were treated with mTOR inhibitor AZD8055 prior to treatment with TGF-β. A) mTOR inhibition resulted in a dose dependent reduction in the levels of αSMA and collagen mRNA compared to PBS only cells and TGF-β treated only cells. B) Blockade of mTOR activation resulted in a reduction of αSMA and collagen protein expression compared to PBS only cells and TGF-β treated only cells. C). Densitometric analyses of αSMA and collagen protein expression quantified from the blots. *, $p<0.05$, **, $p<0.01$, *** $p<0.001$, **** $p<0.0001$ n=3

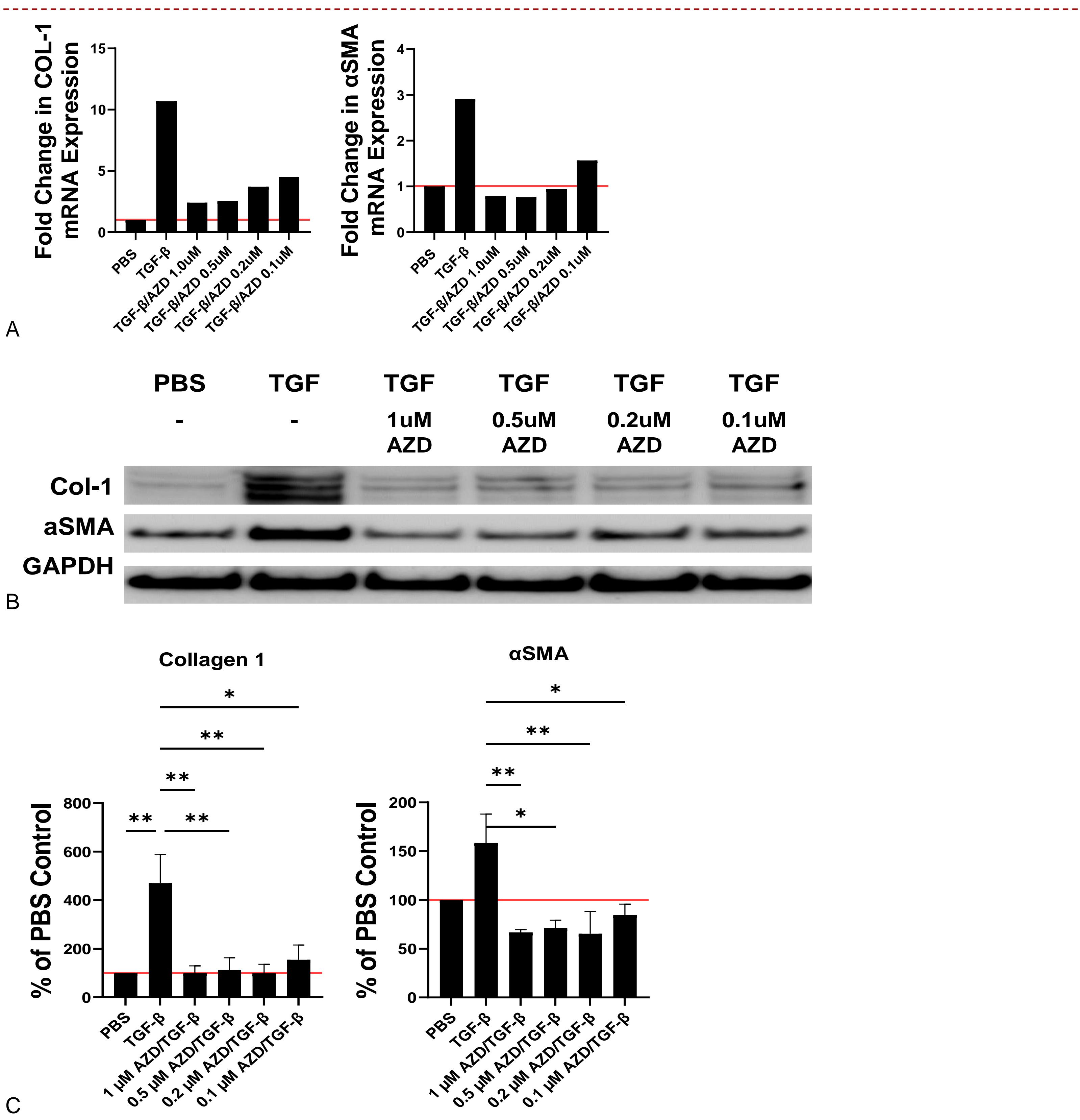


Figure 3: Treatment with AZD8055 reverses MesoMT induction. TGF-β treated HPMCs were treated with the mTOR inhibitors AZD8055. A) mTOR inhibition resulted in a dose dependent reduction of αSMA and collagen mRNA levels compared TGF-β treated only cells. B) Treatment with AZD8055 TGF-β mediated induction of αSMA and collagen protein expression compared TGF-β treated only cells in a dose dependent manner. C.αSMA and collagen protein expression quantified from the blots. *, $p<0.05$, **, $p<0.01$ A; n=1 B;N=3

Rictor knockdown blocks MesoMT

Having determined that mTORC1/2 inhibition effectively blocked and reversed TGF-β mediated MesoMT, next we sought to determine which mTOR complex was responsible and indispensable for the induction and progression of MesoMT. To address this gap in knowledge, HPMCs were transfected with control siRNA, RAPTOR siRNA or RICTOR siRNA. Cells were then treated with TGF-β to induce MesoMT. Changes in MesoMT marker expression were then assayed using both qPCR and Western blotting analyses. RNA analyses showed that RICTOR siRNA significantly reduced RICTOR expression by greater than 90% compared to levels in control siRNA cells. TGF-β mediated induction of αSMA and collagen were reduced in Rictor siRNA transfected HPMCs compared to control siRNA cells. (Figure 4A). While RAPTOR expression was significantly reduced by targeting siRNA, RAPTOR knockdown had no effect on TGF-β mediated MesoMT. Similar results were observed in western blotting, which showed significant reductions of α-SMA and collagen protein expression in cells transfected with RICTOR siRNA (Figure 4B). RAPTOR knockdown did not appear to affect the progression of MesoMT. These studies strongly support our hypothesis that mTOR complex 2/Rictor is critical for the induction of MesoMT.

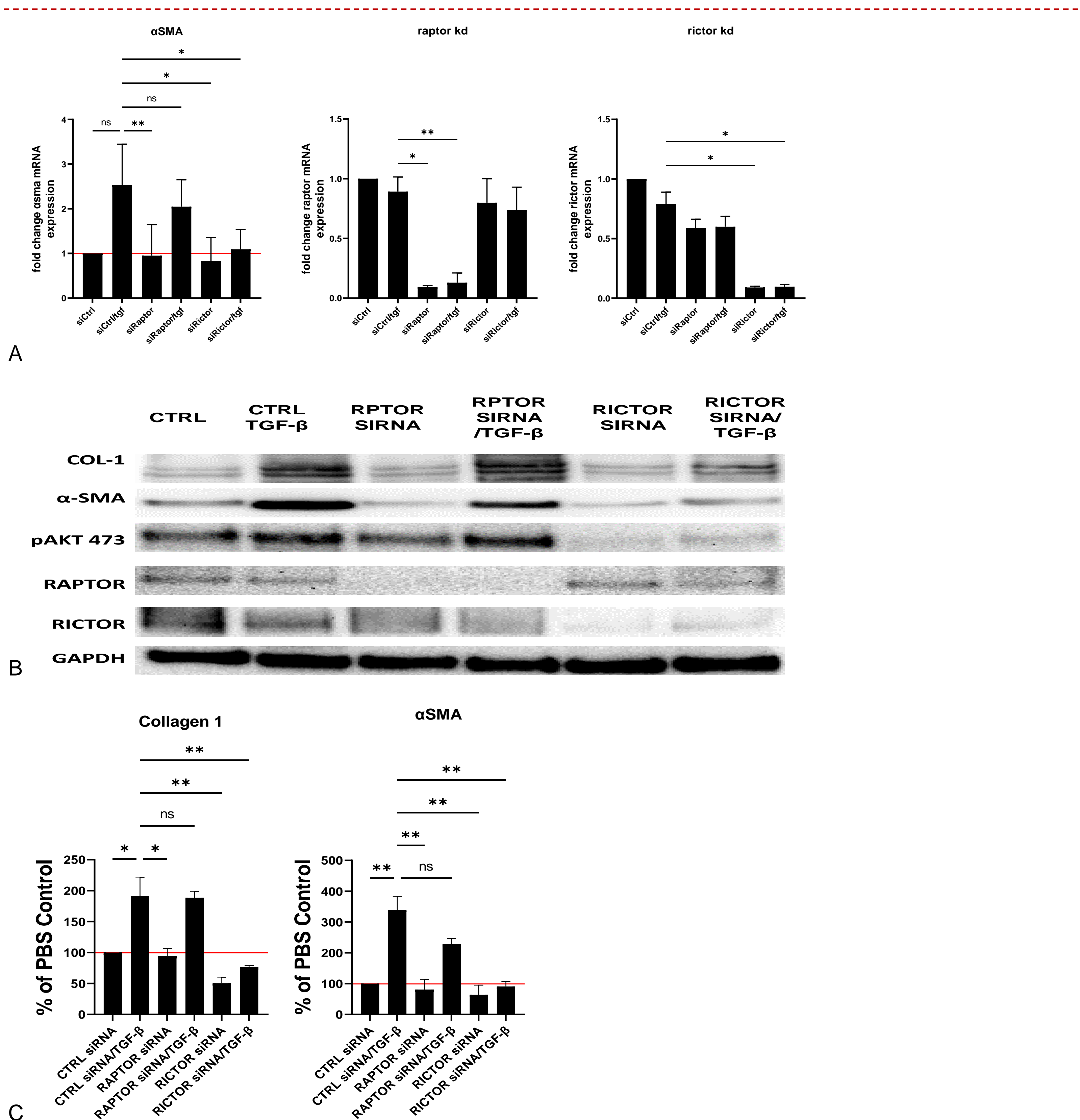
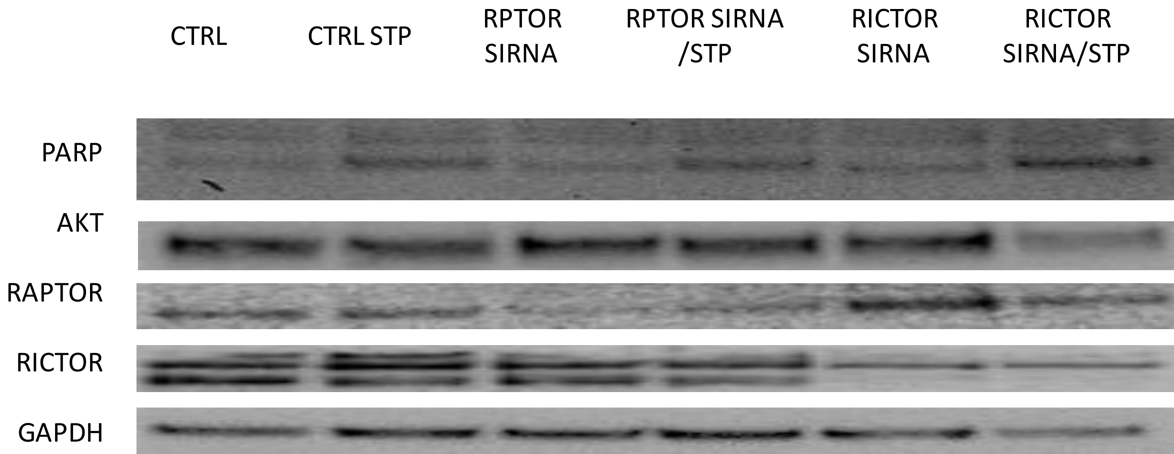


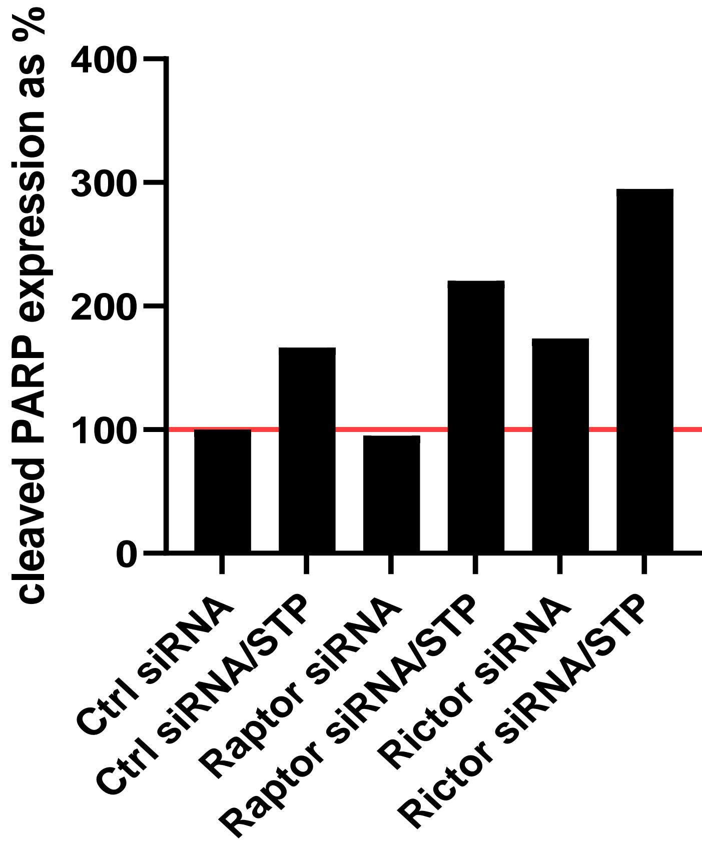
Figure 4: Rictor knockdown blocks MesoMT. HPMCs were transfected with CONTROL siRNA, RAPTOR siRNA or RICTOR siRNA and treated with TGF-8 to induce MesoMT. Cells were assayed using qPCR and Western blotting. A) RNA analyses showed that RICTOR siRNA reduced RICTOR expression resulting in lower levels of a-SMA mRNA expression in HPMCS treated with TGF-8 compared to control siRNA and RAPTOR siRNA treated cells. B) western blotting showed a reduction of a-SMA and collagen levels in cells transfected with RICTOR siRNA suggesting that the mTOR complex 2 is critical for the induction of MesoMT*, p<0.05, **, p<0.01 N=3

Rictor Knockdown Enhances Apoptosis in HPMCS

During MesoMT, the profibrotic myofibroblast persists and resist normal cell death. Having determined that RICTOR knockdown effectively attenuated the induction and progression of MesoMT, we next sought to determine if Rictor knockdown could reduce resistance to apoptosis associated with myofibroblast differentiation. HPMCs were treated with control, Rictor (C2) and Raptor (C1) siRNA. Cells were then treated with staurosporine (1μM) for 3 hours to induce apoptosis (Figure 5). The apoptosis associated biomarker cleaved PARP was immunoblotted. Rictor and Raptor protein levels were also determined. The knockdown of Rictor and thus mTORC2 appreciably upregulated the cleavage of PARP.

A





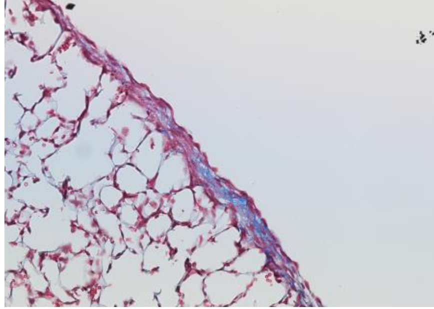
B

Figure 5: Rictor Knockdown Enhances Apoptosis in HPMCS. HPMCs were treated with Control, Rictor (C2) and Raptor (C1) siRNA. Cells were treated with staurosporine for three hours to induce apoptosis. The levels of apoptosis associated biomarker (PARP) was determined. Rictor and Raptor protein expression was also determined. Cells were treated with STP for 3 hours for protein analysis. N=1

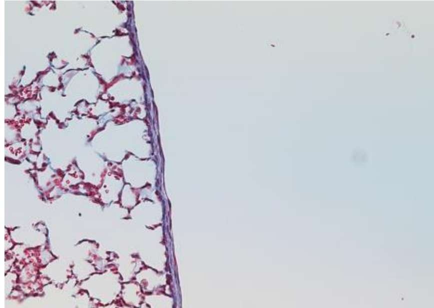
Treatment with INK 128 reduces pleural injury in Strep injured mice.

Having determined that mTORC1/2 inhibition effectively blocked and reversed MesoMT in HPMCs *in* vitro, we next sought to determine the effectiveness of mTORC1/2 pharmacological intervention in a mouse model of *S. pneumonia* induced pleural injury. Mice were intrapleurally administered 1.8E8 cfu of *S. pneumoniae* to initiate injury. Antibiotics were administered daily for the first 4 days post injury. Mice were then treated with DMSO (vehicle) or INK128, 7 days after induction of pleural injury. Lung tissues were then collected, fixed, and sectioned. Tissue morphometry was next determined from trichrome stained tissue sections (Figure 6). Vehicle treated *S. pneumoniae* injured mice demonstrated pronounced pleural thickening at 14d (≅ 30 μm). Conversely, INK128 treatment significantly reduced *S. pneumoniae* mediated pleural thickening (≅ 17 μm), when compared to vehicle treated mice. These data show that pleural injury progression is attenuated by mTORC1/2 inhibition.

STREP/DMSO



STREP/INK128



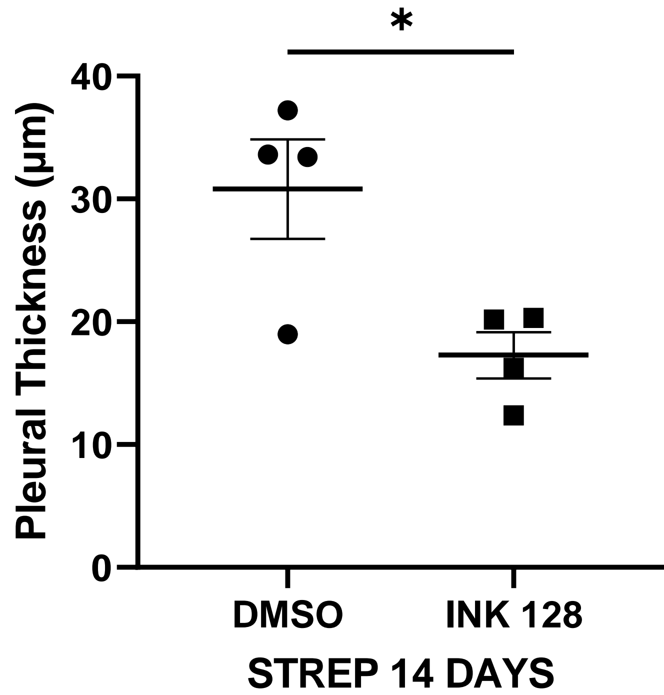


Figure 6: Treatment with INK128 reduced injury in Strep injured mice. Mice with *S. pneumoniae* induced pleural injury were treated with DMSO (vehicle), or INK128, 7 days after induction of injury. Lung images show a reduction in the pleural thickening in mice treated with INK128 when compared to vehicle (DMSO)-treated mice. *, $p<0.05$, ** n=3

Treatment with INK128 reduces MesoMT Markers in Vivo.

Having observed a reduction in pleural thickening in INK128 treated mice compared to vehicle treated mice, we next determined the effects of mTOR inhibition on the progression of MesoMT *in vivo*. Lung tissue sections from *S. pneumoniae* infected mice treated with vehicle or INK128 were immunostained for the MesoMT markers α-SMA and collagen. These sections were then imaged by confocal microscopy (Figure 7). Vehicle treated mice demonstrated increased expression of αSMA and collagen 1 in the pleural mesothelium. By contrast, pleural tissues from INK128 treated mice showed reduced α-SMA and collagen expression when compared to vehicle treated mice. These data suggest that INK128 treatment reduced the progression of *S. pneumoniae* induced MesoMT. Further, the reduced pleural thickening observed in the INK128 mice is likely due to attenuation of MesoMT.

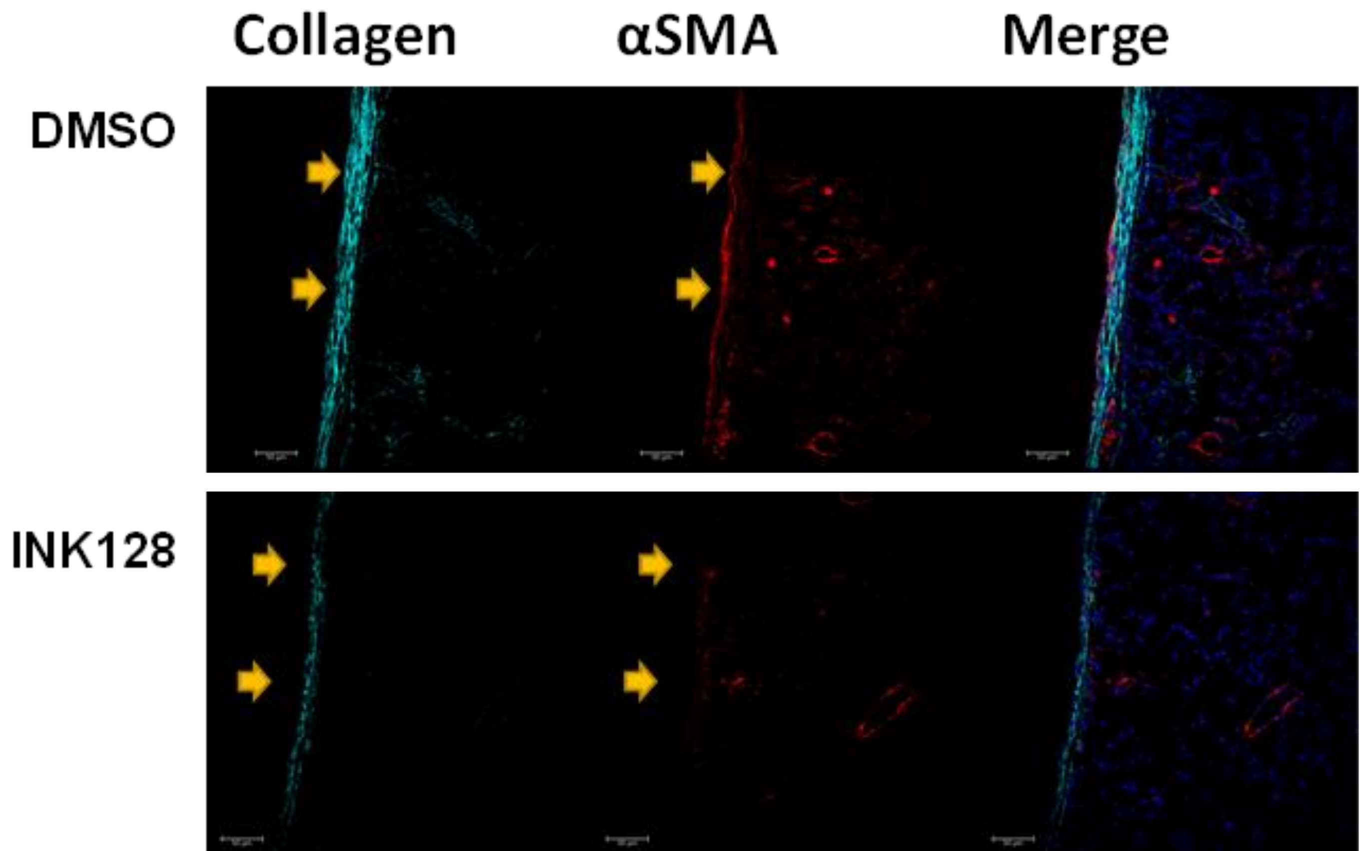


Figure 7: Treatment with INK128 reduced α-SMA and collagen expression *in vivo.* Lung tissues were collected *S. pneumoniae* injured mice treated with DMSO (vehicle) or INK128. Tissues were then fixed, processed and sectioned. Lung tissue sections were stained for α-SMA (red) and collagen (cyan) and imaged with confocal microscopy. Images show a reduction in the both collagen and αSMA expression in INK128 treated mice compared to vehicle-treated mice. Arrows indicate the pleural mesothelium. Bar- 50μm, n=3

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DISCUSSION

Pleural injury and subsequent fibrosis remain a substantial clinical concern. While progress has been made in understanding the mechanisms which drive pleural injury, there is a lack of a non-invasive, effective therapy for the treatment of pleural fibrosis [32]. As such, the need to identify and establish new more efficacious therapies capable of decelerating and reversing the growth of scar tissue in the pleural space remains. Previously, we have shown that the transitioning of pleural mesothelial cells into profibrotic mesenchymal cells is critical for the progression of pleural fibrosis [16]. Mediators such as TGF-β, plasmin, and thrombin promote the transition of human pleural mesothelial cells into profibrotic myofibroblast through the process termed as meso-mesenchymal transition (MesoMT). Thus, therapies targeting MesoMT could have the greatest potential for the treatment of pleural fibrosis.

Diverse signaling pathways have been reported to promote MesoMT. We previously reported that the activation of PI3K/Akt is critical for the induction of MesoMT [16]. This finding informed our rationale that associated pathways downstream of PI3K/Akt could likewise be critical for the induction and progression of MesoMT. One such pathway is mTOR. As such, we proposed to investigate the role of PI3K/Akt/mTOR signaling pathway in the pathogenesis of pleural fibrosis. mTOR signaling has been reported to be dysregulated in numerous disease states and malignancies [33].

The importance of mTOR activation in the induction and progression of MesoMT was established by our in vitro experiments which showed that pharmacological inhibition of mTOR blocked the induction of MesoMT. We also showed that the inhibition of mTOR was capable and effective in reversing and attenuating the progression of MesoMT. This finding suggests that the inhibition of mTOR is favorable as an effective therapy for MesoMT and pleural fibrosis.

mTOR has two distinct complexes: mTORC1 and mTORC2. Each complex has distinctive functions and thus are implicated in different physiological and pathophysiological states [34]. The activation of mTORC1 complex has been reported to be critical for the progression of pulmonary fibrosis and collagen expression.[35] While less is known about mTORC2, it has been reported to be critical for maintenance of the function of cochlear hair cells [36]. Thus, we sought to elucidate the role of mTOR signaling in the progression of MesoMT. Further, we wanted to determine which mTOR complex was critical for MesoMT.

Prior work in our lab had concluded that mTORC1 inhibition with the well characterized inhibitor rapamycin had no effect on TGFβ-mediated MesoMT. Further, there are no well-established mTORC2 inhibitors. As such, my studies focused on using the mTORC1/2 inhibitors INK128 and AZD8055. In these studies, we found that nanomolar concentrations of both inhibitors effectively blocked and reversed TGF-β mediated MesoMT. While rapamycin has been reported to affect mTORC2 at high doses, the doses we employed were confirmed to be specific for mTORC1 alone. Thus, our data suggest that mTORC2 may play a predominate

role in TGF-β mediated MesoMT. These initial studies focused on TGF-β as the principal driver of MesoMT; however other mediators are reported to induce MesoMT through diverse signaling pathways.[16] Accordingly, future studies will evaluate the effectiveness of these compounds on other mediators known to induce MesoMT, including Xa and thrombin. We predict that mTORC1/2 inhibition will effectively block the induction of MesoMT by other established mediators.

Because our inhibitors could not fully delineate the predominate mTOR complex, we next evaluated each complex independently. This was achieved through gene silencing. Small interfering RNAs for Raptor, which is specific to mTORC1, and Rictor, which is specific for mTORC2, were used. The data from these transfection studies suggest that mTORC2 activation alone is indispensable for the induction and progression of MesoMT. Knockdown of mTORC2 significantly downregulated TGF-β mediated MesoMT marker expression. Specifically, increased collagen deposition, which has been associated with pleural fibrosis progression and severity, was reduced in the Rictor siRNA treated HPMCs. The knockdown of mTORC1/Raptor had no significant effect on the induction and progression of MesoMT, specifically the expression of collagen nor αSMA. These results contrast with the work of Woodcock et al which reported that collagen expression in lung fibroblasts was mediated via activation of the mTORC1/Raptor/eif4f axis [35]. These findings suggest that differential pathway activation contributes to fibrosis in a cell type dependent manner. We also found that Rictor downregulation increased cellular susceptibility to apoptosis. This is an

important observation as resistance to apoptosis is a seminal reason myofibroblast populations persist in fibrosis. Future studies will evaluate this process with TGF-β treatment in Raptor and Rictor knockdown cells.

Due to the compelling *in vitro* results in human pleural mesothelial cells, we further evaluated the efficacy of INK128 in a preclinical model of pleural injury. Our mouse model of pleural injury induced by *Streptococcus pneumoniae* which evolves into fibrosis was utilized.[15] Our results show that treatment with INK128 resulted in a significant reduction in injury and pleural thickening as compared to infected mice which were treated with vehicle alone. Moreover, biomarkers of MesoMT, α-SMA and collagen were also appreciably reduced in INK128 treated mice. These results indicate that inhibition of mTOR attenuates the progression of pleural fibrosis resulting from *S. pneumoniae* infection. Additional experiments must be performed to determine if INK128 is capable of attenuating the progression of MesoMT and subsequent fibrosis in other PF mouse models.

Because INK128 is a small molecule inhibitor, the question remains whether the compound could have off-target effects, which indirectly affect the progression of MesoMT. To determine that the inhibition of mTOR is responsible for the observed effect, mice which are mTOR deficient in the mesothelium need to be generated to confirm the role of mTOR in MesoMT and the progression of pleural injury. Further studies are also needed to determine the mechanism by which mTOR inhibition blocked and reversed collagen and αSMA expression. Unfortunately, the mechanism of activation of mTORC2 is not clearly defined. Our

data suggests that mTORC2 activation is critical for the progression of MesoMT and thus it would be the most effective therapeutic target. This prompts further investigations into the activation and mechanism of action for mTORC2 in MesoMT and the evaluation of downstream effectors. Additionally, mTORC2 specific inhibitors should be developed and used in experiments juxtaposed with mTORC1 specific inhibitors, such as Rapamycin.

To conclude, our work has indicated that mTOR activation is critical for the induction and progression of MesoMT. We also showed that mTORC2 rather than mTORC1 is critical for MesoMT in HPMCs. This makes mTORC2 a very promising therapeutic target, which could be used in the treatment of pleural fibrosis and potentially other types of organ fibrosis.

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VITA

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This thesis was typed by Perpetual Serwaah Kyei