# TALE OF TWO ALKs: ALK1 AND ALK5 IN TGFβ1-INDUCED MYOFIBROBLAST DIFFERENTIATION

BY

**ASMA GAH**

**(Under the Direction of Somanath Shenoy)**

## ABSTRACT

Because TGFβ type-1 receptors are the primary mediators in the initiation of myofibroblast differentiation and tissue fibrosis, it appears that which TGFβ type-1 receptor present in the fibroblasts at a given time will determine the course of myofibroblast development and fibrosis. Literature suggests TGF-β type-1 receptors, activin linked kinases 1 and 5 (ALK1 and ALK5) are involved in promoting TGFβ effects in physiology and pathology. However, the exact TGFβ type-1 receptor involved in myofibroblast differentiation is still unknown. Our results show that ALK1 is significantly reduced in TGFβ1 treated fibroblasts with a significant increase in ALK5 expression. Elevated αSMA and Akt pathway in TGFβ1-induced fibroblasts was reduced after the treatment with ALK5 inhibitor. Our data provides the novel molecular insight on the pathogenesis of myofibroblast differentiation and provides much valuable information to develop suitable drugs to prevent fibrosis by pharmacologically targeting the ALK5 receptor.

**INDEX WORDS:** Myofibroblast; ALK5; αSMA; TGFβ1; N-cadherin

# TALE OF TWO ALKs: ALK1 AND ALK5 IN TGFB1-INDUCED MYOFIBROBLAST DIFFERENTIATION

BY

ASMA GAH

PharmD, Umm Al-Qura University, Saudi Arabia

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2019

TALE OF TWO ALKs (ALK1 AND ALK5) IN TGFB1-INDUCED MYOFIBROBLAST DIFFERENTIATION

BY

ASMA GAH

PharmD, Umm Al-Qura University, Saudi Arabia

**Major Professor:** **Somanath Shenoy, Ph.D., FAHA**

**Committee:** **Susan Fagan, Pharm.D., BCPS, FCCP**

**S. Priya Narayanan, Ph.D.**

Electronic Version Approved:

Susanne Barbour
Dean of the Graduate School
The University of Georgia
August 2019

## DEDICATION

I would like to dedicate this thesis to the Almighty Allah for everything in my life, for the guidance, strength, and power of the mind. I would also like to wholeheartedly dedicate this thesis to my beloved parents for being my main source of inspiration to pursue my dream of studying in the US, and for providing moral, spiritual, emotional and financial support to be a better pharmacist.

I am grateful to thank my sisters, Shaima and Sumaya, and my brothers for supporting me in every decision I have made, and for always be on my side on every step in my life.

I would like to thank all my friends specially Alaa Majeed, Lojain Habash and Nada Bima who guided me and supported me when I was at my lowest.

## ACKNOWLEDGMENTS

Several guiding hands have supported me during my two-year journey, that my success would not have been possible without them. First, I am greatly thankful to my advisor, Dr. Somanath Shenoy, for being my guardian in the instruction and training. Thank you for being such a great mentor who could find every potential skill to be improved in every student. I thank you for your patience, caring and kind words that helped me to continue and never give up. Thank you for motivating me to develop interest in research, and for having faith on me. I have gained a lot of knowledge and skills that will help me to be a better researcher in the future.

I would like to thank all members of Dr. Shenoy's laboratory, Ahlam, Abdulrahman, Arti, Rana and especially Mir, who was a great participant and helper at every step in my research work. He helped me focus and cheered when my experiments failed. I am grateful to my committee members, Drs. Fagan and Narayanan for their great support, constructive criticism and invaluable advice throughout the course. I gained lots of invaluable knowledge and basic laboratory skills that are required to obtain my thesis from Dr. Sabbineni and Dr. Segar who taught me unique valuable lab techniques in order to handle my experiments easily.

Finally, a special thanks to my batch mates and co-pilots in this journey, Ahlam, Mohammed, and Waleed for all their support in overcoming all the obstacles on my path to completion. Thanks for always being there when I needed a helping hand.

I am sincerely grateful for being a part of the Clinical and Experimental Therapeutics (CET) family. I would like to thank all the staff and students at the UGA-CET program and the Charlie Norwood VA Medical Center for their continued support.

**Table of Contents**

# CHAPTER 1

## 1. Introduction and literature review

### 1.1. Fibrosis and epidemiology

Tissue fibrosis is a phenomenon associated with the pathology and progress of many diseases such as idiopathic pulmonary arterial hypertension (iPAH), bronchitis, cardiac fibrosis, cirrhosis, and benign prostatic hyperplasia, etc. Fibrosis is characterized by the overgrowth and scarring of granulation tissue, the end result of chronic inflammation induced by persistent tissue injury (Wynn, 2008). It is estimated that 45% of worldwide deaths are reported to be caused by organ fibrosis (Wynn, 2004). Tissue fibrosis has very limited treatment options and hence high mortality (Craig, Zhang, Hagood, & Owen, 2015). Thus, organ fibrosis limits patient life expectancy as a result of the lack of treatments mainly due to the uncertainties in the molecular mechanisms and due to the lack of effective early screening methods. Research efforts have been oriented to the pathobiology of tissue fibrosis as it has been shown that myofibroblast differentiation is crucial for its pathogenesis (Wunderlich, 1969).

### 1.2. Fibroblast-to-Myofibroblast (FibroMF) differentiation

Any fibrotic disease is characterized by the presence of myofibroblasts, which cause tissue remodeling and progressive fibrosis (Falke, Gholizadeh, Goldschmeding, Kok, & Nguyen, 2015). Myofibroblasts can be derived from a large variety of cell types such as fibrocytes, pericytes, and endothelium through epithelial-mesenchymal transition (EMT) (van der Vusse, Kalkman, & van der Molen, 1974). In normal condition, a fibroblast is characterized by the presence of fibronectin only, which plays important roles in cell adhesion, migration, growth, and differentiation (Falke

et al., 2015). On the other hand, the fibroblast is also characterized by the absence of filamentous-actin, alpha-smooth muscle Actin (αSMA) and extra domain A fibronectin (ED-A fibronectin) (Falke et al., 2015). After tissue injury that causes mechanical stress to the fibroblast, it transforms into proto-myofibroblast that produces ED-A fibronectin, contains stress fibers and focal adhesions, but does not produce αSMA yet, which characterize the immature myofibroblast (Falke et al., 2015; Gabbiani, 2003). Mature myofibroblasts display excessive production of αSMA in addition to the presence of ED-A fibronectin and F-actin. The fibroblast to protomyofibroblast transition is reversible, but once it becomes myofibroblast, it cannot be reversible (Falke et al., 2015). This transition from fibroblasts to myofibroblasts is mainly stimulated by Transforming growth factor beta (TGF-β), that modulates the fibro-contractive changes in many fibrotic diseases such as liver cirrhosis and renal fibrosis (Gabbiani, 2003).

### 1.3. The physiological role of Myofibroblast differentiation

Myofibroblasts play an important role in normal wound healing (Darby, Laverdet, Bonte, & Desmouliere, 2014). After tissue injury, wound healing process proceeds in three phases in order to restore the injured tissue. In the inflammatory phase, damaged capillaries trigger multiple chemokines, platelets, fibrin and fibronectin to fills in the lesion (Gabbiani, 2003). The second phase is the proliferative phase, where angiogenesis occurs. In this phase, new capillaries are produced to deliver nutrients to the wound in addition to the proliferation of fibroblasts. Finally, the regeneration phase, including maturation, scar formation, and re-epithelialization (Darby et al., 2014). In this stage, fibroblasts start to synthesize extracellular matrix (ECM) components, EDA fibronectin, and αSMA, which are essential for myofibroblasts differentiation (van der Vusse et al., 1974). MF differentiation is a key process during normal physiological wound healing

but can be dysregulated in fibrotic diseases via its cellular pathways such as OXPHOS pathway (Lei, Lerner, Sundar, & Rahman, 2017).

### 1.4. Pathological role of MF in various diseases

During normal wound healing, myofibroblasts are disappeared via apoptosis, leaving minimum tissue scar. However, in fibrotic diseases, myofibroblasts fail to undergo cell death, persist and apoptosis is inhibited leading to pathology and scarring such as hypertrophic scarring (Darby et al., 2014; Gabbiani, 2003). Furthermore, dysregulation in the healing process can also affect FibroMF differentiation leading to tissue fibrosis. Persistent inflammation phase can impair the development of granulation tissue which results in the chronic wound and excessive scarring (Darby et al., 2014). Although almost every cell type can be compromised in the fibrogenesis, pulmonary fibrosis is the most devastating, progressive and fatal fibrotic disease used to illustrate many key points (Ludwig, 1968). In a broader context, other diseases are associated with FibroMF differentiation such as cardiac failure, atherosclerosis, and asthma (Ludwig, 1968).

### 1.5. Myofibroblast and molecular mechanisms regulating MF

The word ‘myofibroblast’ was first initiated years ago for fibroblastic cells located within granulation tissue characterized by the presence of cytoplasmic microfilament bundles and focal adhesions (van der Vusse et al., 1974). It contains actin, myosin, and αSMA that give the cell the ability to synthesize extracellular matrix component and the force for the contraction of granulation tissue (Gabbiani, 2003). Myofibroblasts can be generated from a variety of sources including fibroblasts, (EMT), endothelial-mesenchymal transition (EndMT), Mesenchymal stem cells (MSC) and from bone marrow-derived mesenchymal precursors (fibrocytes) (Ludwig, 1968). EMT can contribute to organ fibrosis through the local formation of interstitial myofibroblasts from organ epithelium (Thiery, Acloque, Huang, & Nieto, 2009). EMT can be triggered by

different mediators such as TGF-β superfamily, wingless-type MMTV integration site family members (WNTs), epidermal growth factor (EGF), fibroblast growth factor (FGF) (Thiery et al., 2009). Moreover, EndMT also plays an important role in the development of fibrotic diseases, and can also be induced by TGF-β1 in a canonical dependent manner (Zeisberg et al., 2007). Studies have also shown the involvement of EndMT in vascular remodeling, renal fibrosis and many others (Ludwig, 1968). In addition, reactive oxygen species (ROS), which derived from the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase family, is considered a major inducer for FibroMF differentiation and involved in the pathogenesis of cardiovascular and kidney diseases (Barnes & Gorin, 2011). A recent study has demonstrated that NAD-dependent deacetylase sirtuin-3 (SIRT3) has a critical role in the pathogenesis of pulmonary fibrosis induced by TGFβ1 and/or bleomycin-induced mouse model (Bindu et al., 2017).

### 1.6. TGFβ: the master regulator of fibrosis

TGFβ receptor is a serine/threonine kinases receptor activated via binding to the prototypical ligand of the TGFβ superfamily (Massague, 1998). TGFβ superfamily consists of 34 family members, but the most common studied members are TGFβ1, TGFβ2 and TGFβ3 (Biernacka, Dobaczewski, & Frangogiannis, 2011; Massague, 1998). It is believed that TGFβ1 is involved in the pathogenesis and development and metastatic cancer via suppressing the immunity and modulating angiogenesis and cell apoptosis. During the early stages of tumorigenesis, TGFβ functions as an anti-proliferative agent that suppresses the tumor. On the other hand, TGFβ act as a tumor promoter helping in metastatic progression (Chaudhury & Howe, 2009). Activation of the heteromeric complex that forms the serine/threonine kinases receptors of TGFβ Type I and types II is the first step toward fibrosis. When TGFβ type II receptor is bound to its ligand, the type I receptor is then trans-phosphorylated and thus activated. This activation will further promote

downstream signaling such as Smad2/3 and 1/5 phosphorylation leading to nuclear changes (Rahimi & Leof, 2007).

### 1.7. Activin linked kinases or Activin receptor-like kinases: ALK1 and ALK5

It is known that TGFβ to signal via ALK receptors (de Kroon et al., 2015). There are seven known mammalian type I receptors, activin receptor-like kinase or ALKs, ALK 1-7 (Rahimi & Leof, 2007). SMAD pathways are important in myofibroblast differentiation and fibrosis and regulated through ALKs receptors. SMAD2 and SMAD3 proteins (SMAD2/3) are phosphorylated by ALK5, while ALK1 induces SMAD1, SMAD5 and SMAD8 proteins (SMAD1/5/8) phosphorylation (Nakao et al., 1997). It is reported that the two ALKs ALK5 and ALK1 pathways have opposite effects on endothelial cells. ALK1 stimulates EC migration and proliferation, and ALK5 inhibits these processes (Goumans et al., 2002). Nevertheless, it is still the unknown exact mechanistic role of ALK1 and ALK5 in myofibroblast differentiation and fibroblast.

### 1.8. The physiological role of ALK1 and ALK5

During normal cell function, cell migration and proliferation are inhibited by ALK5 signaling via Smad2/3, however, ALK1 signaling via Smad1/5 promotes these events (Goumans et al., 2002). In addition, it is reported that ALK1 modulates ALK5 signaling in the normal activation phase of angiogenesis, which increases vascular permeability, including tip/stalk cell selection, migration, and proliferation (Goumans et al., 2002). ALK1 plays an important role in stabilizing the binding of the BMP10 prodomain (Roman & Hinck, 2017). Additionally, inhibiting ALK5 cause an increase in the inhibition of Claudin-5 and inhibits expression of adhesion molecules (Watabe et al., 2003). ALK5 signaling, via Gadd45b, was shown to plays an important role in mediating neural plasticity and functional recovery after cerebral ischemia (Zhang et al.,

2019). Although it was shown that both ALK1 and ALK5 are involved in the cellular mechanism of cell function, it is still not clearly understood the which ALK is involved in the pathology and physiology of cell function.

### 1.9. Pathological role of ALK1 and ALK5 in various diseases

ALK1 and ALK5 have been addressed in many various diseases. ALK1 mutation in humans causes vascular dysplasia disease called Osler-Rendu-Weber syndrome (Curado et al., 2014). A Defect in ALK1 signaling may also cause autosomal dominant vascular disorder, hereditary hemorrhagic telangiectasia (HHT) (Kim, Peacock, George, & Hughes, 2012). It was also demonstrated that the number, migration and dendritic arborization of newborn neurons have been reduced in genetic deletion of ALK5 (He et al., 2014). A study has shown that ALK1 and ALK5 imbalance is involved in the development of pulmonary artery hypertension (Lu, Patel, Harrington, & Rounds, 2009). Another study has shown that abnormal TGFβ/ALK5 signaling is crucial in mediating idiopathic pulmonary arterial hypertension iPAH development (Thomas et al., 2009). ALK1 is reported to be associated with coronary artery atherosclerotic lesions development in the human endothelium, neointima, and media (Yao, Zebboudj, Torres, Shao, & Bostrom, 2007). An in vivo and in vitro study has demonstrated that ALK5 gene deficiency in chondrocytes leads to the development of osteoarthritis (OA) (Wang et al., 2017).

### 1.10. Molecular mechanisms regulating ALK5 expression

ALK5 is the main TβR-1 that mediates most cellular responses. When the ligand binds to TβRII and activates it, it will consequently phosphorylate TβR1 /ALK-5 and activate the downstream signaling. Smad2 and Smad3 are then activated and associated with the co-Smad (Smad4) in order to translocate to the nucleus for gene transcription (Itakura & Carlson, 1975). ALK5 activity is required for EMT in Endocardial Cells (Arnold, Stewart, & Aguilar, 1990).

### 1.11. Scientific Premise, objective and central hypothesis

Pulmonary fibrosis is a devastating, severe, and progressive lung disease characterized by an increasing cause of morbidity and mortality worldwide with limited therapeutic treatment that has a slight improvement in patient's quality of life. Akt1 (protein kinase Bα) plays an important role in regulating pulmonary fibrosis process. Based on that, we have demonstrated that Akt mediates myofibroblast differentiation through myocardin and serum response factor (Abdalla, Goc, Segar, & Somanath, 2013) and that pharmacological inhibition of AKT, using triciribine, has a beneficial effect in treating pulmonary fibrosis through reversing TGFβ-induced pulmonary fibrosis in mice (Abdalla et al., 2015). Studies on endothelial cells have demonstrated the opposing roles of TGF-β type-1 receptors (ALK1 and ALK5) in physiology and pathology (Goumans et al., 2002). However, there is a *gap in knowledge* on the exact molecular mechanisms and the specific role of ALK1 and ALK5 in myofibroblast differentiation and pulmonary fibrosis, thus preventing further therapeutic development.

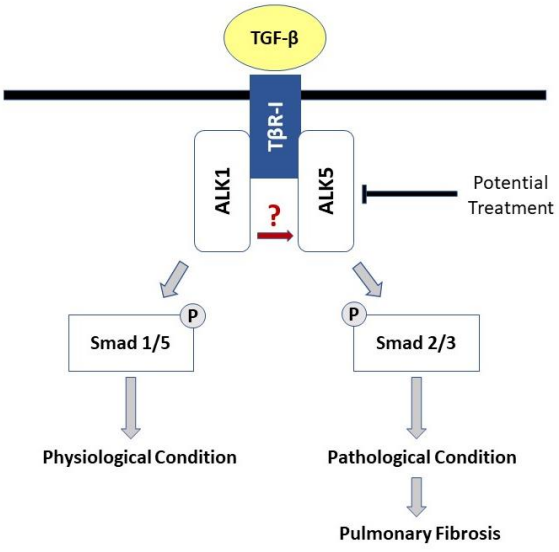



**Figure 1:** Diagram showing the activation pathways of ALK1 & ALK5 in physiological and pathological conditions, and that specific inhibition of ALK5 will inhibit TGFβ-induced myofibroblast differentiation and organ (E.g. Pulmonary) fibrosis.

Our *long-term goal is* to develop suitable therapeutics for treating pulmonary fibrosis. *The object of the current proposal* is to identify the mechanism by which the switch occurs from normal fibroblast to myofibroblast through changing the expression of TGF-β receptors type-1 (ALK1 and ALK5). *Our hypothesis* is that specific inhibition of ALK5 will inhibit TGFβ-induced myofibroblast differentiation and pulmonary fibrosis. The *rationale* for the proposed research is

that by identifying the expression changes in TGF-β receptors type-1 ALK1 and ALK5 in physiology and pathology, we will be in a position to develop therapeutics to treat pulmonary fibrosis. In addition to our supportive preliminary data, we are prepared to conduct this research as we have all the required equipment and materials needed to test our hypothesis and to obtain the definitive outcomes.

### 1.12. Innovation/Impact

The idea of this proposal is *novel* as the molecular regulator of transforming early fibroblast into myofibroblast such as ALK1 and ALK5 is not clear and well-studied. Therefore, the *impact* includes identifying the exact mechanism involved in FibroMF differentiation which will enable us to develop suitable drug targets (ALK5, in our proposal) in the treatment for organ fibrosis.

## SPECIFIC AIMS

We propose to test our hypothesis by pursuing the following *specific aims*:

**Aim 1: To determine the effect of ALK1 and ALK5 on FibroMF differentiation.** We *hypothesize* that ALK1 is highly expressed in normal fibroblasts compared to ALK5 with a high ALK1 to ALK5 ratio. In pathology, ALK1 to ALK5 ratio is reduced due to increased expression of ALK5 and/or reduced expression of ALK1.

**Aim 2: To identify the effect of inhibiting ALK5 on TGFβ1-induced FibroMF differentiation.** We *postulate* that the inhibition of ALK5 signaling pathway will abrogate TGFβ-induced FibroMF differentiation *in vitro*. Using the inhibitors of various intracellular signaling molecules, we will determine the candidate responsible for TGFβ1-induced ALK5 expression.

**References**


Abdalla, M., Goc, A., Segar, L., & Somanath, P. R. (2013). Akt1 mediates alpha-smooth muscle actin expression and myofibroblast differentiation via myocardin and serum response factor. *J Biol Chem, 288*(46), 33483-33493. doi:10.1074/jbc.M113.504290

Abdalla, M., Sabbineni, H., Prakash, R., Ergul, A., Fagan, S. C., & Somanath, P. R. (2015). The Akt inhibitor, triciribine, ameliorates chronic hypoxia-induced vascular pruning and TGFbeta-induced pulmonary fibrosis. *Br J Pharmacol, 172*(16), 4173-4188. doi:10.1111/bph.13203

Arnold, M. W., Stewart, W. R., & Aguilar, P. S. (1990). Rectocele repair. Four years' experience. *Dis Colon Rectum, 33*(8), 684-687.

Barnes, J. L., & Gorin, Y. (2011). Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. *Kidney Int, 79*(9), 944-956. doi:10.1038/ki.2010.516

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-beta signaling in fibrosis. *Growth Factors, 29*(5), 196-202. doi:10.3109/08977194.2011.595714

Bindu, S., Pillai, V. B., Kanwal, A., Samant, S., Mutlu, G. M., Verdin, E., . . . Gupta, M. P. (2017). SIRT3 blocks myofibroblast differentiation and pulmonary fibrosis by preventing mitochondrial DNA damage. *Am J Physiol Lung Cell Mol Physiol, 312*(1), L68-L78. doi:10.1152/ajplung.00188.2016

Chaudhury, A., & Howe, P. H. (2009). The tale of transforming growth factor-beta (TGFbeta) signaling: a soigne enigma. *IUBMB Life, 61*(10), 929-939. doi:10.1002/iub.239

Craig, V. J., Zhang, L., Hagood, J. S., & Owen, C. A. (2015). Matrix metalloproteinases as therapeutic targets for idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol, 53*(5), 585-600. doi:10.1165/rcmb.2015-0020TR

Curado, F., Spuul, P., Egana, I., Rottiers, P., Daubon, T., Veillat, V., . . . Genot, E. (2014). ALK5 and ALK1 play antagonistic roles in transforming growth factor beta-induced podosome formation in aortic endothelial cells. *Mol Cell Biol, 34*(24), 4389-4403. doi:10.1128/MCB.01026-14

Darby, I. A., Laverdet, B., Bonte, F., & Desmouliere, A. (2014). Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Investig Dermatol, 7*, 301-311. doi:10.2147/CCID.S50046

de Kroon, L. M., Narcisi, R., Blaney Davidson, E. N., Cleary, M. A., van Beuningen, H. M., Koevoet, W. J., . . . van der Kraan, P. M. (2015). Activin Receptor-Like Kinase Receptors ALK5 and ALK1 Are Both Required for TGFbeta-Induced Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells. *PLoS One, 10*(12), e0146124. doi:10.1371/journal.pone.0146124

Falke, L. L., Gholizadeh, S., Goldschmeding, R., Kok, R. J., & Nguyen, T. Q. (2015). Diverse origins of the myofibroblast-implications for kidney fibrosis. *Nat Rev Nephrol, 11*(4), 233-244. doi:10.1038/nrneph.2014.246

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol, 200*(4), 500-503. doi:10.1002/path.1427

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., & ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J, 21*(7), 1743-1753. doi:10.1093/emboj/21.7.1743

He, Y., Zhang, H., Yung, A., Villeda, S. A., Jaeger, P. A., Olayiwola, O., . . . Wyss-Coray, T. (2014). ALK5-dependent TGF-beta signaling is a major determinant of late-stage adult neurogenesis. *Nat Neurosci, 17*(7), 943-952. doi:10.1038/nn.3732

Itakura, C., & Carlson, H. C. (1975). Pathology of spontaneous hemorrhagic enteritis of turkeys. *Can J Comp Med, 39*(3), 310-315.

Kim, J. H., Peacock, M. R., George, S. C., & Hughes, C. C. (2012). BMP9 induces EphrinB2 expression in endothelial cells through an Alk1-BMPRII/ActRII-ID1/ID3-dependent pathway: implications for hereditary hemorrhagic telangiectasia type II. *Angiogenesis, 15*(3), 497-509. doi:10.1007/s10456-012-9277-x

Lei, W., Lerner, C., Sundar, I. K., & Rahman, I. (2017). Myofibroblast differentiation and its functional properties are inhibited by nicotine and e-cigarette via mitochondrial OXPHOS complex III. *Sci Rep, 7*, 43213. doi:10.1038/srep43213

Lu, Q., Patel, B., Harrington, E. O., & Rounds, S. (2009). Transforming growth factor-beta1 causes pulmonary microvascular endothelial cell apoptosis via ALK5. *Am J Physiol Lung Cell Mol Physiol, 296*(5), L825-838. doi:10.1152/ajplung.90307.2008

Ludwig, W. M. (1968). The importance of gastroscopic photography in the managenment of giant gastric ulcer. *Gastrointest Endosc, 15*(1), 43-45.

Massague, J. (1998). TGF-beta signal transduction. *Annu Rev Biochem, 67*, 753-791. doi:10.1146/annurev.biochem.67.1.753

Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., . . . ten Dijke, P. (1997). TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J, 16*(17), 5353-5362. doi:10.1093/emboj/16.17.5353

Rahimi, R. A., & Leof, E. B. (2007). TGF-beta signaling: a tale of two responses. *J Cell Biochem, 102*(3), 593-608. doi:10.1002/jcb.21501

Roman, B. L., & Hinck, A. P. (2017). ALK1 signaling in development and disease: new paradigms. *Cell Mol Life Sci, 74*(24), 4539-4560. doi:10.1007/s00018-017-2636-4

Thiery, J. P., Acloque, H., Huang, R. Y., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell, 139*(5), 871-890. doi:10.1016/j.cell.2009.11.007

Thomas, M., Docx, C., Holmes, A. M., Beach, S., Duggan, N., England, K., . . . Budd, D. C. (2009). Activin-like kinase 5 (ALK5) mediates abnormal proliferation of vascular smooth muscle cells from patients with familial pulmonary arterial hypertension and is involved in the progression of experimental pulmonary arterial hypertension induced by monocrotaline. *Am J Pathol, 174*(2), 380-389. doi:10.2353/ajpath.2009.080565

van der Vusse, G. J., Kalkman, M. L., & van der Molen, H. J. (1974). 3Beta-hydroxysteroid dehydrogenase in rat testis tissue. Inter- and subcellular localization and inhibition by cyanoketone and nagarse. *Biochim Biophys Acta, 348*(3), 404-414. doi:10.1016/0005-2760(74)90220-3

Wang, Q., Tan, Q. Y., Xu, W., Qi, H. B., Chen, D., Zhou, S., . . . Chen, L. (2017). Cartilage-specific deletion of Alk5 gene results in a progressive osteoarthritis-like phenotype in mice. *Osteoarthritis Cartilage, 25*(11), 1868-1879. doi:10.1016/j.joca.2017.07.010

Watabe, T., Nishihara, A., Mishima, K., Yamashita, J., Shimizu, K., Miyazawa, K., . . . Miyazono, K. (2003). TGF-beta receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J Cell Biol, 163*(6), 1303-1311. doi:10.1083/jcb.200305147

Wunderlich, F. (1969). The macronuclear envelope of Tetrahymena pyriformis GL in different physiological states. I. Quantitative structural data. *Exp Cell Res, 56*(2), 369-374.

Wynn, T. A. (2004). Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol, 4*(8), 583-594. doi:10.1038/nri1412

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol, 214*(2), 199-210. doi:10.1002/path.2277

Yao, Y., Zebboudj, A. F., Torres, A., Shao, E., & Bostrom, K. (2007). Activin-like kinase receptor 1 (ALK1) in atherosclerotic lesions and vascular mesenchymal cells. *Cardiovasc Res, 74*(2), 279-289. doi:10.1016/j.cardiores.2006.09.014

Zeisberg, E. M., Tarnavski, O., Zeisberg, M., Dorfman, A. L., McMullen, J. R., Gustafsson, E., . . . Kalluri, R. (2007). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med, 13*(8), 952-961. doi:10.1038/nm1613

Zhang, K., Zhang, Q., Deng, J., Li, J., Li, J., Wen, L., . . . Li, C. (2019). ALK5 signaling pathway mediates neurogenesis and functional recovery after cerebral ischemia/reperfusion in rats via Gadd45b. *Cell Death Dis, 10*(5), 360. doi:10.1038/s41419-019-1596-z

**CHAPTER 2**

# Regulation of TGFβ type-I receptor (ALK1 and ALK5) expression in TGFβ1-induced myofibroblast differentiation

***Gah, Asma et al*, Submitted to journal *Life Sciences*, June 20, 2019**

## Abstract

Fibroblast-to-myofibroblast (FibroMF) differentiation is crucial for organ fibrosis. Although transforming growth factor-β (TGFβ) is the primary mediator of FibroMF differentiation, the type-I receptor (TGFβRI) responsible for this has not yet been confirmed. In the current study, we investigated the ALK1 and ALK5 expressions in TGFβ1-stimulated NIH 3T3 fibroblasts to compare with the data from the gene expression omnibus (GEO) repository. In our results, whereas TGFβ1 treatment promoted FibroMF differentiation accompanied by increased ALK5 expression and reduced ALK1 expression. TGFβ1 promoted FibroMF differentiation and increased expression of α-smooth muscle actin (αSMA) and ALK5 were inhibited by co-treatment with ALK5 inhibitor SB431542. GEO database analysis indicated increased ALK5 expression and reduced ALK1 expression in fibrotic compared to a normal mouse or human tissues correlating to organ fibrosis progression. Finally, the inhibitors of Akt, mTOR, and β-catenin suppressed TGFβ1-induced ALK5 expression indicating that the Akt pathway is involved in ALK5 expression regulation and fibrosis promotion.



**Keywords:** ALK1; ALK5; TGFβ1; myofibroblast; fibrosis

## 1. Introduction

Fibroblast-to-myofibroblast (FibroMF) differentiation is the hallmark of organ fibrosis (Weiskirchen, Weiskirchen, & Tacke, 2019). The integral role of TGFβ1-Akt1 pathway in FibroMF differentiation (Abdalla, Goc, Segar, & Somanath, 2013; Goc, Sabbineni, Abdalla, & Somanath, 2015) and pulmonary fibrosis (Abdalla et al., 2015) has been demonstrated in our laboratory. Akt1 gene deletion conferred a protective effect in ischemia-induced cardiac fibrosis (Ma, Kerr, Naga Prasad, Byzova, & Somanath, 2014). Pharmacological inhibition of the Akt pathway using triciribine in an adenovirus TGFβ-induced model of pulmonary fibrosis inhibited fibrosis and prevented vascular rarefaction (Abdalla et al., 2015). An essential step in the TGFβ1/Akt1-induced FibroMF differentiation is the activation of TGFβ receptors (Morikawa, Derynck, & Miyazono, 2016; Wynn, 2008). Specifically, the identity of the type-I TGFβ receptor (TGFβRI) involved in the pathological FibroMF differentiation has not been confirmed.

Activin linked kinases, ALK1 and ALK5, are the two predominantly expressed and most studied TGFβRI in fibroblasts (Akhurst, 2017). An early study has reported that ALK1 and ALK5 can form receptor complexes upon TGFβ stimulation and promote fibrogenesis by producing the extracellular matrix (ECM) (Pannu, Nakerakanti, Smith, ten Dijke, & Trojanowska, 2007), indicating that cooperation between ALK1 and ALK5 may be necessary for tissue remodeling and wound healing. A role for ALK1 has been implicated in vascular pathologies such as hereditary hemorrhagic telangiectasia independent of ALK5 and TGFβRII (Park et al., 2008) and in TGFβ1-induced developmental angiogenesis (Oh et al., 2000). An elevated ALK1/ALK5 ratio has been reported in osteoarthritis in humans and mice (Blaney Davidson et al., 2009). The studies indicate that ratio ALK1/ALK5 modulates connective tissue growth factor in hepatocytes (Gressner et al.,

2009; Weng et al., 2007). ALK1 and ALK5 have also been reported to regulate leptin expression in mesenchymal stem cells via two independent mechanisms (Zeddou et al., 2012). The importance of ALK1/ALK5 ratio in FibroMF differentiation and fibrosis needs to be investigated.

In the current study, we used NIH 3T3 fibroblasts that have been extensively utilized in fibrosis research to determine the importance of ALK1 and ALK5 in TGFβ1-induced FibroMF differentiation *in vitro*. Our study revealed an ALK1/ALK5 ratio is reduced in TGFβ-stimulated myofibroblasts. Analysis of data from the Gene Expression Omnibus (GEO) repository on pulmonary fibrosis and liver fibrosis in humans and mice further supported our *in vitro* findings that ALK1 levels are reduced and ALK5 levels are elevated in myofibroblasts and fibrotic tissue. Whereas ALK5 suppression by SB431542 inhibited TGFβ1-induced FibroMF differentiation, TGFβ1-induced ALK5 expression was reduced by treatment with the inhibitors of Akt, mTOR, and β-catenin. Altogether, our study shows that reduced ALK1/ALK5 ratio is an indicator of FibroMF differentiation and tissue fibrosis and that targeting ALK5 is beneficial in the treatment of organ fibrosis.

## 2. Materials and methods

### *2.1. Reagents and Cell culture*

NIH 3T3 fibroblasts (ATCC, Manassas, VA) were cultured in DMEM medium. All cultures were maintained in a humidified 5% CO2 incubator at 37 ℃ and routinely passaged when 80– 90% confluent. TGFβ1 was obtained from R&D Systems (Minneapolis, MN) and were reconstituted according to the manufacturer's protocol. NIH 3T3 fibroblasts were treated with 100 pM dose of TGFβ1, in 5% serum-containing medium for 72 hours. The growth factor was replenished every

24 hours. The dose of ALK5 inhibitor (2.5, 5, 10, and 20 µM SB431542, Selleckchem, Houston, TX) was determined based on the IC50 values, its dose-dependent cytotoxicity, and our previous studies (Abdalla et al., 2013; Abdalla et al., 2015; Goc et al., 2015). The doses of Akt inhibitor (10 nM Triciribine) (Abdalla et al., 2013; Abdalla et al., 2015; Gao, Alwhaibi, et al., 2017), mTOR inhibitor (25 nM rapamycin) (Abdalla et al., 2015), β-catenin (10 µM ICG-001) (Gao et al., 2016), FoxO (10 µM AS-1842856) (Artham et al., 2019), Src (10 µM PP2) (Gao, Sabbineni, Artham, & Somanath, 2017), and MEK/ERK (10 µM PD-98059), purchased from Selleckchem, Houston, TX or Calbiochem, Burlington, MA, were determined from our previous publications.

### *2.2. Immunocytochemistry*

Immunofluorescence staining was performed as described previously (Gao, Alwhaibi, Artham, Verma, & Somanath, 2018). Briefly, HMECs and NIH 3T3 were plated on 8-well chamber slides. After reaching 70% confluence, cells were treated with TGFβ1 for 72 hours in the presence and absence of 20 µM ALK5 inhibitor (SB431542). Next, cells were fixed with 4% paraformaldehyde in 1× PBS followed by permeabilization with 0.1% Triton X-100 in 1× PBS. The nonspecific staining was blocked with 2 % BSA for 1 hour at room temperature. The fixed and permeabilized cells were incubated with Alexa-555 labeled phalloidin for 40 minutes (1:1000 dilution) and washed. The slides were mounted with Vectashield (Vector Laboratories, PA), and imaged by a Zeiss confocal imaging microscope (Carl-Zeiss Microscopy, LLC, Thornwood, NY).

### *2.3. MTT assay*

Cell proliferation and viability were determined as previously published from our laboratory using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Al-Azayzih, Missaoui, Cummings, & Somanath, 2016). Cells were seeded in 48-well cell culture plates at $5 \times 10^4$ cells/ml and incubated at 37 °C in a 5% $CO_2$ incubator for 24 h. Cells were treated with 20

μM ALK5 inhibitor (SB431542) and DMSO (vehicle) and were incubated for 24 hours. MTT was added at this time point, at a final concentration of 0.25 mg/ml and plates were incubated at 37 °C. Non-reduced MTT and media were aspirated after 2 h and replaced with DMSO to dissolve the MTT formazan crystals. Plates were shaken for 15 min and absorbance was read at 590 nm using a Biotek plate reader (Biotek, Winooski, VT).

### *2.4. Western blot analysis*

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA) with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay from Bio-Rad (Hercules, and CA). Western blot analysis was performed as described previously (Goc et al., 2013; Sabbineni, Verma, & Somanath, 2018). Antibodies used include N-cadherin and GAPDH from Cell Signaling Technology (Danvers, MA), αSMA and β-actin from Sigma (St. Louis, MO), and ALK1 and ALK5 antibodies from Abcam (Cambridge, MA). Band densitometry was done using NIH Image J software.

### *2.5. ALK1 and ALK5 gene expression analysis from pre-clinical studies.*

Gene Expression Omnibus (GEO) is a public database repository of high throughput gene sequencing and microarrays that allows users to analyze the expression profiles of the gene(s) of interest from previously performed pre-clinical studies (Wilhite & Barrett, 2012). We searched GEO datasets using the keywords “ALK1 and Fibrosis/myofibroblast”, and “ALK5 and Fibrosis/myofibroblast”. The search resulted in several datasets that were performed during the last decade. The gene expression levels of ALK1 and ALK5 were downloaded for different groups in all studies. The gene expression was compared between control and FibroMF group using student t-test. The mean $\pm$ SD were presented for those studies with at least a sample size of $\geq$3.

### *2.6. Statistical Analysis*

All the data are presented as Mean + SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample t-test (dependent t-test) and/or further confirmed with non-parametric test Wilcoxon signed rank test. For all other analysis, Student's two-tailed t-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 and SPSS 17.0 software.

## 3. Results

### *3.1. TGFβ1-induced FibroMF differentiation is accompanied by reduced ALK1/ALK5 ratio.*

To determine whether ALK1 and ALK5 expression ratio were modulated in FibroMF differentiation, we treated NIH 3T3 cells with TGFβ1 for 72 hours. Our results indicated a significant increase in the expression of ALK5 and a significant decrease in the expression of ALK1 (Figure 1A-B). A higher ALK1/ALK5 ratio was observed in normal NIH 3T3 fibroblasts compared to TGFβ1-induced NIH 3T3 myofibroblasts (Figure 1C), indicating a pathological role for ALK5, but not ALK1 in FibroMF differentiation.

### *3.2. ALK1 gene expression was decreased in human idiopathic pulmonary fibrosis tissues.*

GEO datasets retrieved from the studies over a decade with the search terms 'ALK1 and myofibroblast/fibrosis' were analyzed to confirm a positive correlation between ALK1 gene expression and fibrosis as observed in our *in vitro* results. Whereas one study (GEO ID: GDS1252/125,37) indicated decreased ALK1 expression in human idiopathic pulmonary fibrosis tissues compared to normal lung tissues (Figure 2A), a second study (GEO ID:

GDS4580/226950_at) indicated a significant decrease in ALK1 expression in rapidly progressing idiopathic pulmonary fibrosis tissues compared to normal as well as slowly progressing idiopathic pulmonary fibrosis tissues (Figure 2B). These analyses indicated decreased ALK1 expression in organ fibrosis.

### *3.3. ALK5 gene expression was increased in human hepatitis and mouse bleomycin-induced pulmonary fibrosis tissues.*

GEO datasets retrieved from the studies over a decade with the search terms 'ALK5 and myofibroblast/fibrosis' were analyzed to confirm a positive correlation between ALK5 gene expression and FibroMF differentiation as observed from the *in vitro* results. Whereas one study (GEO ID: GDS4389 / 224793_s_at) showed increased ALK5 expression associated alcoholic hepatitis in human (Figure 2C), another study in mice with bleomycin-induced pulmonary fibrosis indicated increased ALK5 expression in the fibrotic lung compared to normal lung (Figure 2D). Together, these data from clinical and preclinical studies indicated increased ALK5 expression and a decreased ALK1/ALK5 expression ratio in organ fibrosis.

### *3.4. TGFβ1-induced FibroMF differentiation and fibroblast expression of mesenchymal markers were inhibited by ALK5 inhibition.*

To determine whether ALK5 expression modulation has a prominent role in FibroMF differentiation, we treated NIH 3T3 fibroblasts with FibroMF-inducing TGFβ1 for 72 hours in the presence and absence of ALK5 inhibitor SB431542. Treatment of normal fibroblasts with ALK5 inhibitor had no significant effect on αSMA expression (Figure 3A-B). However, co-treatment with ALK5 inhibitor SB431542 significantly attenuated TGFβ1-induced αSMA expression and phosphorylation of pro-fibrotic Akt phosphorylation (Figure 4A-B) with no changes in the total

Akt levels (Figure 4C) indicating ALK5 inhibition although does not affect normal fibroblasts, does inhibit TGFβ1-induced FibroMF differentiation.

### *3.5. TGFβ1-induced actin stress fiber formation in NIH 3T3 fibroblasts was blunted by ALK1 inhibition by SB431542.*

Next, we determined whether the promotion of FibroMF differentiation by TGFβ1 stimulation was associated with increased actin stress fiber formation co-regulated by Akt and RhoA signaling pathways (Abdalla et al., 2013). TGFβ1-stimulated NIH 3T3 fibroblasts exhibited increased actin stress fibers compared to NIH 3T3 fibroblasts treated with vehicle (PBS) (Figure 5A). Interestingly, TGFβ1-induced increased actin stress fibers was blunted by co-treatment with ALK5 inhibitor SB431542 (Figure 5A). Whereas cell survival/proliferation was increased in TGFβ1-stimulated myofibroblasts as measured by the MTT assay, the effect was significantly attenuated by co-treatment with ALK5 inhibitor SB431542 (Figure 5B).

### *3.6. Inhibition of Akt, mTOR, and β-catenin, but not FoxO, Src, and ERK inhibit TGFβ1-induced increased ALK5 expression in NIH 3T3 fibroblasts.*

In order to identify the upstream regulator of ALK5 expression in TGFβ1-treated NIH 3T3 fibroblasts, we performed co-treatment with the specific inhibitors of Akt, mTOR, β-catenin, FoxO, Src, and ERK pathways and determined their effects on TGFβ1-induced ALK5 expression. Our analysis indicated that TGFβ1-induced ALK5 expression in NIH 3T3 fibroblasts was significantly reduced upon co-treatment with mTOR inhibitor rapamycin, Akt inhibitor triciribine or β-catenin inhibitor ICG-001 but not with FoxO inhibitor AS-1842856, Src inhibitor PP2 or ERK inhibitor PD-98059 (Figure 6A-B).

## 4. Discussion

Physiological processes such as embryogenesis, tissue repair, and wound healing necessitate FibroMF differentiation (Li & Wang, 2011; Patel, Baz, Wong, Lee, & Khosrotehrani, 2018). Unlike the physiological processes where myofibroblasts recede after they are utilized, uncontrolled FibroMF differentiation develops irreversible fibrosis and scar formation in conditions such as pulmonary, kidney, and cystic fibrosis as well as hepatitis, etc. (Weiskirchen et al., 2019). TGFβ pathway, TGFβ1 in particular (Abdalla et al., 2013), is the primary driver of FibroMF differentiation (Pardali, Sanchez-Duffhues, Gomez-Puerto, & Ten Dijke, 2017; Wynn, 2008). TGFβ1-activated PI3-Kinase/Akt pathway and subsequent activation of myocardin and serum response factor transcription factors are crucial in αSMA synthesis and FibroMF differentiation (Abdalla et al., 2013; Abdalla et al., 2015).

TGFβ signaling is well-known for its cell and context-specific effects, which are often paradoxical in nature (Biernacka, Dobaczewski, & Frangogiannis, 2011). This complexity in the TGFβ signaling is offered by the presence of more than 20 TGFβ isoforms in its superfamily belonging to the different classes of TGFβs, activins, inhibins, bone morphogenic receptors, growth/differentiation factors, nodal, lefty, etc. (Weiss & Attisano, 2013). To complicate this further, TGFβ family members bind to a series of different receptors belonging to different classes (Heldin & Moustakas, 2016). Primarily, TGFβ receptors are broadly categorized into type-I, type-II and type-III receptors (Morikawa et al., 2016). Among these, type-III TGFβ receptors are not well characterized (Heldin & Moustakas, 2016; Morikawa et al., 2016). Upon binding of a growth factor to the TGFβ type-II receptor, the receptor is auto-phosphorylated subsequently trans-phosphorylating the TGFβ type-I receptor (Abdollah et al., 1997; Souchelnytskyi et al., 1997). The

activated type-I receptor then transduces the signals to induce myofibroblast marker expression through the phosphorylation and activation of the canonical pathway transcription factors known as Smads (Abdollah et al., 1997; Souchelnytskyi et al., 1997). Based on which ligand binds to which receptor and in what cell type or tissue, the effect of TGFβ pathway activation can be very unpredictable. Among the type-II receptors, TGFβRII binds to TGFβ1-3, and ActRII, ActRIIB, as well as BMPRII interact with other isoforms (Heldin & Moustakas, 2016). Intriguingly, the context-specific effects of TGFβ signaling, including the physiological versus pathological effects, is more reliant on the diversity of type-I receptors that the cells express (Roman & Hinck, 2017). Type-I TGFβ receptors include ALK1 that binds to TGFβ1-3 and growth differentiation factors and ALK2/3 that binds predominantly to BMP receptors (Heldin & Moustakas, 2016; Roman & Hinck, 2017). Whereas ALK4/7 are nodal receptors, ALK1 binds to TGFβ1-3 and BMP9/10 (Heldin & Moustakas, 2016; Roman & Hinck, 2017). The ratio between TGFβ type-I and type-II receptors often determines the physiological versus pathological effects of TGFβ signaling as described in the introduction section.

In the current study, the expression levels of ALK1 in the normal NIH 3T3 fibroblasts was significantly higher compared to the expression levels of ALK5. Interestingly, 72-hour stimulation of NIH 3T3 fibroblasts with TGFβ1 resulted in significantly increased ALK5 and reduced ALK1 expression compared to the normal NIH 3T3 fibroblasts. The overall ALK1/ALK5 ratio was higher in normal NIH 3T3 fibroblasts compared to TGFβ1-stimulated NIH 3T3 myofibroblasts. Whereas ALK5 inhibitor did not exhibit any effect on normal NIH 3T3 fibroblasts on αSMA expression indicating the low levels of ALK5 in normal fibroblasts, ALK5 inhibitor significantly reversed the TGFβ1-induced FibroMF differentiation, actin stress fiber formation and proliferation/viability. Co-treatment with the inhibitors of Akt, mTOR, β-catenin, FoxO, Src and

ERK signaling pathways revealed that TGFβ1-induced ALK5 expression was regulated by Akt/mTOR/β-catenin pathway.

In order to determine the clinical significance of our *in vitro* results, we analyzed the data on ALK1 and ALK5 expression changes in studies involving mice, and humans from the gene expression omnibus (GEO) repository. Analysis of several unpublished preclinical research data from the GEO repository indicated a significant increase in ALK5 expression in fibrotic conditions such as alcoholic hepatitis and bleomycin-induced pulmonary fibrosis. On the other end, reduced ALK1 expression was associated with idiopathic pulmonary fibrosis, particularly in the aggressive ones compared to the slow progressing fibrosis. Future research will inform us if such as a reduction in ALK1/ALK5 ratio is also applicable to other fibrotic diseases.

In conclusion, our study has demonstrated that although ALK1 expression is higher and ALK5 expression lower in normal fibroblasts, a ratio of ALK1/ALK5 is reduced upon TGFβ1-induced FibroMF differentiation. Increased ALK5 expression involved activation of the Akt1-mTOR-β-catenin pathway. Our study showed that such a correlation also exists in human and murine fibrotic conditions in the liver and lung.

## Acknowledgments

Funds were provided by the NHLBI grant R01HL103952, NCATS grant UL1TR002378, Wilson Pharmacy Foundation (intramural) and Translational Research Initiative grant (intramural) to PRS. Financial assistance provided by the Saudi Arabian Cultural Mission to AG is also acknowledged. This work has been accomplished using the resources and facilities at the VA Medical Center in Augusta, GA. The funders had no role in the study design, data collection, analysis, and decision to publish the data. The contents of the manuscript do not represent the views of the Department of Veteran Affairs or the United States Government.

## Conflict of interest

Authors declare that there are no financial or any other conflicts of interests exist.

**Figure legends**

**Figure 1: ALK1/ALK5 ratio is higher in normal compared to TGFβ1-induced NIH 3T3 myofibroblasts. (A-B)** Representative Western blot images and bar graphs with band densitometry analysis indicating increased ALK5 and reduced ALK1 expression in NIH 3T3 fibroblasts with TGFβ1 treatment. **(C-D)** Bar graphs indicating higher ALK1/ALK5 ratio in control NIH 3T3 fibroblasts compared to TGFβ1-induced NIH 3T3 myofibroblasts.

**Figure 2: GEO analysis show increased ALK5 and reduced ALK1 expression in human and mouse fibrotic lung and liver tissues. (A)** Bar graph showing reduced ALK1 expression in human idiopathic pulmonary fibrosis biopsies. **(B)** Bar graph showing reduced ALK1 expression in rapidly progressing human pulmonary fibrosis tissues compared to normal and slow-progressing pulmonary fibrosis tissues. **(C)** Bar graph showing increased ALK5 expression in human alcoholic hepatitis liver tissues. **(D)** Bar graph showing increased ALK5 expression in bleomycin-induced mouse pulmonary fibrosis samples. Data are shown as Mean $\pm$ SD.

**Figure 3: ALK5 inhibitor (SB431542) treatment has no significant effect on αSMA expression in normal NIH 3T3 fibroblasts. (A)** Representative Western blot images showing no significant changes in αSMA expression by treatment with ALK5 inhibitor in normal NIH 3T3 fibroblasts. **(B)** Bar graph with band densitometry analysis indicating no significant changes in αSMA expression by treatment with ALK5 inhibitor in normal NIH 3T3 fibroblasts. Data are shown as Mean $\pm$ SD.

**Figure 4: ALK5 inhibition by SB431542 suppressed TGFβ1-induced FibroMF differentiation via Akt inhibition. (A-C)** Representative Western blot images and bar graph with band densitometry analysis showing increased expression of myofibroblast marker αSMA accompanied by increased phosphorylation of Akt compared to total levels of Akt, respectively,

with a significant inhibition by co-treatment with ALK5 inhibitor SB431542. Data are shown as Mean ± SD.

**Figure 5: ALK5 inhibition reversed the TGFβ1-induced loss of actin stress fibers in ECs. (A)** Confocal images of phalloidin-stained NIH 3T3-monolayers treated in the presence and absence of TGFβ1 alone or in combination with ALK5 inhibitor SB431542 showing increased actin stress fibers with TGFβ1 treatment and inhibition of actin stress fibers upon co-treatment with ALK5 inhibitor SB431542. **(B)** Histogram showing increased viability of NIH 3T3 fibroblasts with TGFβ1 treatment, which is significantly inhibited by co-treatment with ALK5 inhibitor SB431542. Data are shown as Mean ± SD.

**Figure 6: Inhibition of Akt, mTOR, and β-catenin, but not FoxO, Src, and ERK inhibit TGFβ1-induced increased ALK5 expression in NIH 3T3 fibroblasts. (A)** Representative Western blot images showing changes in ALK5 expression in NIH 3T3 fibroblasts by treatment with TGFβ1 alone and in combination with one of the mTOR inhibitor rapamycin, Akt inhibitor triciribine, β-catenin inhibitor ICG-001, FoxO inhibitor AS-1842856, Src inhibitor PP2, and ERK inhibitor PD-98059. **(B)** Bar graph with band densitometry analysis indicating increased ALK5 expression in NIH 3T3 fibroblasts by treatment with TGFβ1 alone and significant inhibition upon co-treatment with mTOR inhibitor rapamycin, Akt inhibitor triciribine or β-catenin inhibitor ICG-001 but not with FoxO inhibitor AS-1842856, Src inhibitor PP2 or ERK inhibitor PD-98059. Data are shown as Mean ± SD.

## References


Abdalla, M., Goc, A., Segar, L., & Somanath, P. R. (2013). Akt1 mediates alpha-smooth muscle actin expression and myofibroblast differentiation via myocardin and serum response factor. *J Biol Chem, 288*(46), 33483-33493. doi:10.1074/jbc.M113.504290

Abdalla, M., Sabbineni, H., Prakash, R., Ergul, A., Fagan, S. C., & Somanath, P. R. (2015). The Akt inhibitor, triciribine, ameliorates chronic hypoxia-induced vascular pruning and TGFbeta-induced pulmonary fibrosis. *Br J Pharmacol, 172*(16), 4173-4188. doi:10.1111/bph.13203

Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., & Wrana, J. L. (1997). TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem, 272*(44), 27678-27685. doi:10.1074/jbc.272.44.27678

Akhurst, R. J. (2017). Targeting TGF-beta Signaling for Therapeutic Gain. *Cold Spring Harb Perspect Biol, 9*(10). doi:10.1101/cshperspect.a022301

Al-Azayzih, A., Missaoui, W. N., Cummings, B. S., & Somanath, P. R. (2016). Liposome-mediated delivery of the p21 activated kinase-1 (PAK-1) inhibitor IPA-3 limits prostate tumor growth in vivo. *Nanomedicine, 12*(5), 1231-1239. doi:10.1016/j.nano.2016.01.003

Artham, S., Gao, F., Verma, A., Alwhaibi, A., Sabbineni, H., Hafez, S., . . . Somanath, P. R. (2019). Endothelial stromelysin1 regulation by the forkhead box-O transcription factors is crucial in the exudative phase of acute lung injury. *Pharmacol Res, 141*, 249-263. doi:10.1016/j.phrs.2019.01.006

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-beta signaling in fibrosis. *Growth Factors, 29*(5), 196-202. doi:10.3109/08977194.2011.595714

Blaney Davidson, E. N., Remst, D. F., Vitters, E. L., van Beuningen, H. M., Blom, A. B., Goumans, M. J., . . . van der Kraan, P. M. (2009). Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J Immunol, 182*(12), 7937-7945. doi:10.4049/jimmunol.0803991

Gao, F., Alwhaibi, A., Artham, S., Verma, A., & Somanath, P. R. (2018). Endothelial Akt1 loss promotes prostate cancer metastasis via beta-catenin-regulated tight-junction protein turnover. *Br J Cancer, 118*(11), 1464-1475. doi:10.1038/s41416-018-0110-1

Gao, F., Alwhaibi, A., Sabbineni, H., Verma, A., Eldahshan, W., & Somanath, P. R. (2017). Suppression of Akt1-beta-catenin pathway in advanced prostate cancer promotes TGFbeta1-mediated epithelial to mesenchymal transition and metastasis. *Cancer Lett, 402*, 177-189. doi:10.1016/j.canlet.2017.05.028

Gao, F., Artham, S., Sabbineni, H., Al-Azayzih, A., Peng, X. D., Hay, N., . . . Somanath, P. R. (2016). Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover. *Cell Mol Life Sci, 73*(20), 3917-3933. doi:10.1007/s00018-016-2232-z

Gao, F., Sabbineni, H., Artham, S., & Somanath, P. R. (2017). Modulation of long-term endothelial-barrier integrity is conditional to the cross-talk between Akt and Src signaling. *J Cell Physiol, 232*(10), 2599-2609. doi:10.1002/jcp.25791

Goc, A., Al-Azayzih, A., Abdalla, M., Al-Husein, B., Kavuri, S., Lee, J., . . . Somanath, P. R. (2013). P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of transforming growth factor beta expression and enhanced matrix metalloproteinase 9 secretion. *J Biol Chem, 288*(5), 3025-3035. doi:10.1074/jbc.M112.424770

Goc, A., Sabbineni, H., Abdalla, M., & Somanath, P. R. (2015). p70 S6-kinase mediates the cooperation between Akt1 and Mek1 pathways in fibroblast-mediated extracellular matrix remodeling. *Biochim Biophys Acta, 1853*(7), 1626-1635. doi:10.1016/j.bbamcr.2015.03.016

Gressner, O. A., Lahme, B., Siluschek, M., Rehbein, K., Weiskirchen, R., & Gressner, A. M. (2009). Connective tissue growth factor is a Smad2 regulated amplifier of transforming growth factor beta actions in hepatocytes--but without modulating bone morphogenetic protein 7 signaling. *Hepatology, 49*(6), 2021-2030. doi:10.1002/hep.22850

Heldin, C. H., & Moustakas, A. (2016). Signaling Receptors for TGF-beta Family Members. *Cold Spring Harb Perspect Biol, 8*(8). doi:10.1101/cshperspect.a022053

Li, B., & Wang, J. H. (2011). Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability, 20*(4), 108-120. doi:10.1016/j.jtv.2009.11.004

Ma, L., Kerr, B. A., Naga Prasad, S. V., Byzova, T. V., & Somanath, P. R. (2014). Differential effects of Akt1 signaling on short- versus long-term consequences of myocardial infarction and reperfusion injury. *Lab Invest, 94*(10), 1083-1091. doi:10.1038/labinvest.2014.95

Morikawa, M., Derynck, R., & Miyazono, K. (2016). TGF-beta and the TGF-beta Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol, 8*(5). doi:10.1101/cshperspect.a021873

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., . . . Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A, 97*(6), 2626-2631. doi:10.1073/pnas.97.6.2626

Pannu, J., Nakerakanti, S., Smith, E., ten Dijke, P., & Trojanowska, M. (2007). Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. *J Biol Chem, 282*(14), 10405-10413. doi:10.1074/jbc.M611742200

Pardali, E., Sanchez-Duffhues, G., Gomez-Puerto, M. C., & Ten Dijke, P. (2017). TGF-beta-Induced Endothelial-Mesenchymal Transition in Fibrotic Diseases. *Int J Mol Sci, 18*(10). doi:10.3390/ijms18102157

Park, S. O., Lee, Y. J., Seki, T., Hong, K. H., Fliess, N., Jiang, Z., . . . Oh, S. P. (2008). ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. *Blood, 111*(2), 633-642. doi:10.1182/blood-2007-08-107359

Patel, J., Baz, B., Wong, H. Y., Lee, J. S., & Khosrotehrani, K. (2018). Accelerated Endothelial to Mesenchymal Transition Increased Fibrosis via Deleting Notch Signaling in Wound Vasculature. *J Invest Dermatol, 138*(5), 1166-1175. doi:10.1016/j.jid.2017.12.004

Roman, B. L., & Hinck, A. P. (2017). ALK1 signaling in development and disease: new paradigms. *Cell Mol Life Sci, 74*(24), 4539-4560. doi:10.1007/s00018-017-2636-4

Sabbineni, H., Verma, A., & Somanath, P. R. (2018). Isoform-specific effects of transforming growth factor beta on endothelial-to-mesenchymal transition. *J Cell Physiol, 233*(11), 8418-8428. doi:10.1002/jcp.26801

Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., & Heldin, C. H. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *J Biol Chem, 272*(44), 28107-28115. doi:10.1074/jbc.272.44.28107

Weiskirchen, R., Weiskirchen, S., & Tacke, F. (2019). Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications. *Mol Aspects Med, 65*, 2-15. doi:10.1016/j.mam.2018.06.003

Weiss, A., & Attisano, L. (2013). The TGFbeta superfamily signaling pathway. *Wiley Interdiscip Rev Dev Biol, 2*(1), 47-63. doi:10.1002/wdev.86

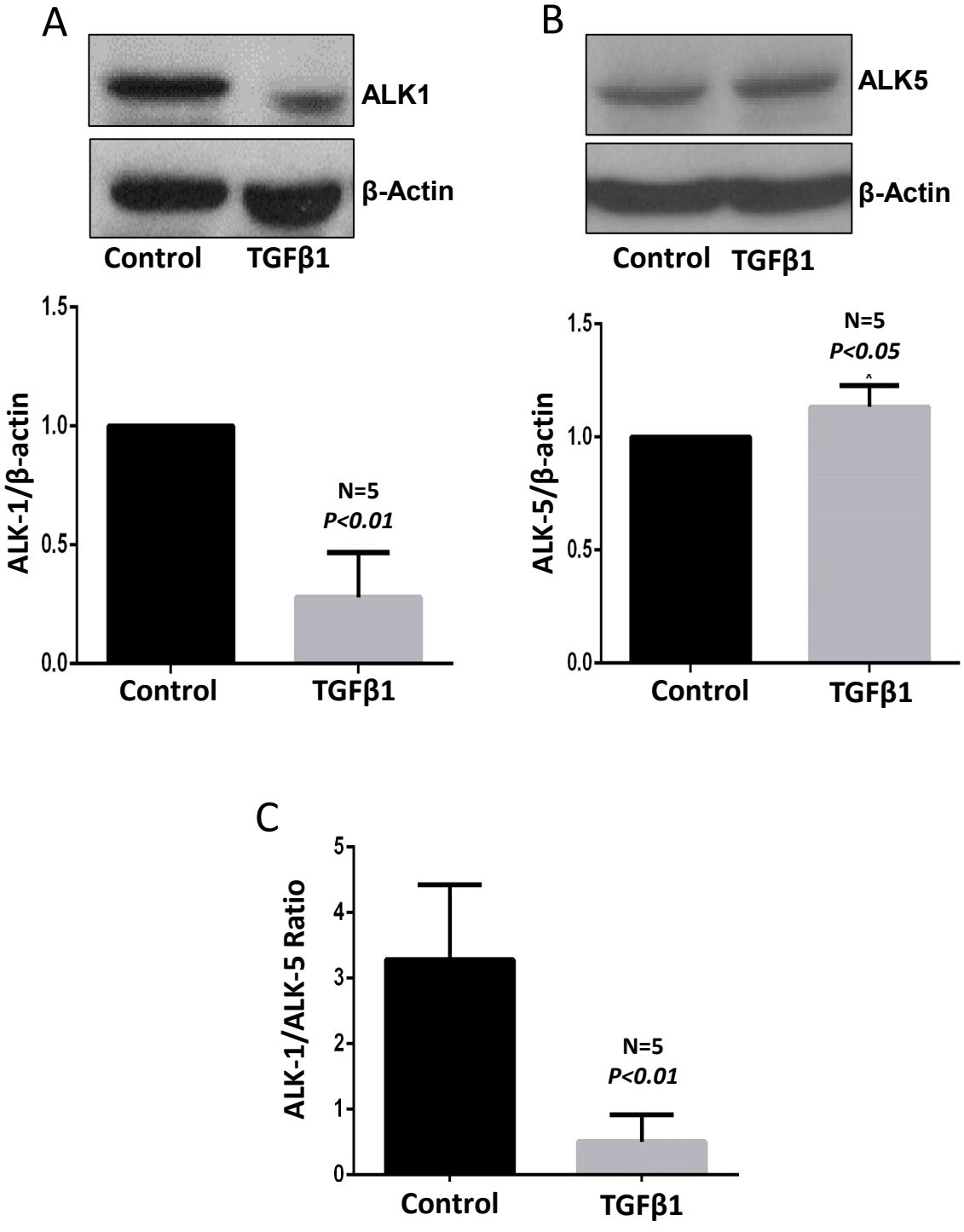
Weng, H. L., Ciuclan, L., Liu, Y., Hamzavi, J., Godoy, P., Gaitantzi, H., . . . Dooley, S. (2007). Profibrogenic transforming growth factor-beta/activin receptor-like kinase 5 signaling via connective tissue growth factor expression in hepatocytes. *Hepatology, 46*(4), 1257-1270. doi:10.1002/hep.21806

Wilhite, S. E., & Barrett, T. (2012). Strategies to explore functional genomics data sets in NCBI's GEO database. *Methods Mol Biol, 802*, 41-53. doi:10.1007/978-1-61779-400-1_3

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol, 214*(2), 199-210. doi:10.1002/path.2277

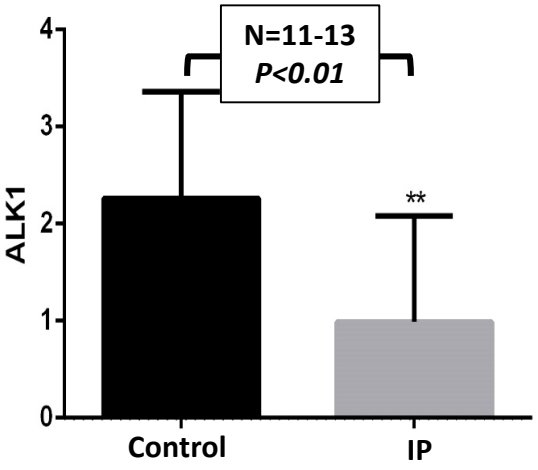
Zeddou, M., Relic, B., Malaise, O., Charlier, E., Desoroux, A., Beguin, Y., . . . Malaise, M. G. (2012). Differential signalling through ALK-1 and ALK-5 regulates leptin expression in mesenchymal stem cells. *Stem Cells Dev, 21*(11), 1948-1955. doi:10.1089/scd.2011.0321

# Figure 1


A
ALK1
β-Actin
Control
TGFβ1
B
ALK5
β-Actin
Control
TGFβ1
ALK-1/β-actin
1.5
1.0
0.5
0.0
N=5
P<0.01
Control
TGFβ1
ALK-5/β-actin
1.5
1.0
0.5
0.0
N=5
P<0.05
Control
TGFβ1
C
ALK-1/ALK-5 Ratio
5
4
3
2
1
0
N=5
P<0.01
Control
TGFβ1

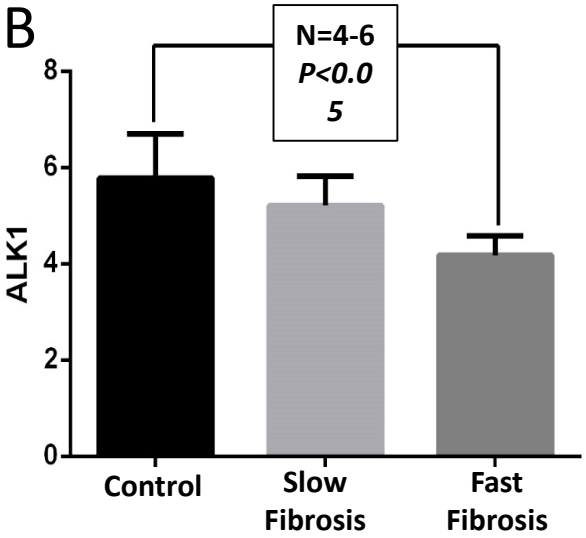
# Figure 2

A



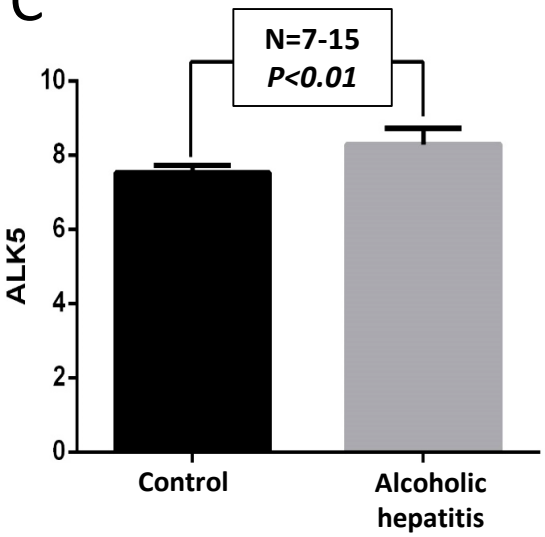

| | |
|---|---|
| **Profile** | GDS1252 / 125,37 |
| **Title** | Idiopathic pulmonary fibrosis |
| **Organism** | Homo sapiens |

B



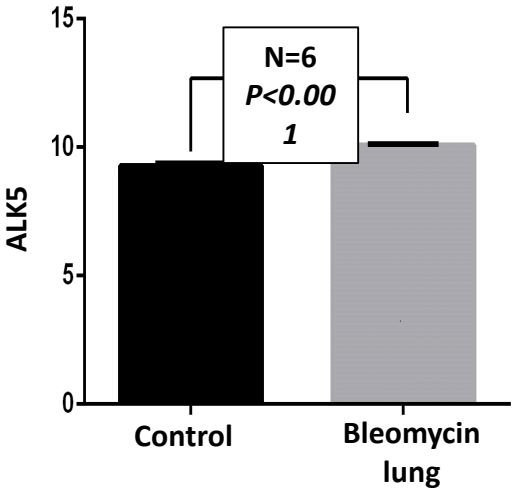

| | |
|---|---|
| **Profile** | GDS4580 / 226950_at |
| **Title** | Idiopathic pulmonary fibrosis: cultured lung fibroblasts |
| **Organism** | Homo sapiens |

C



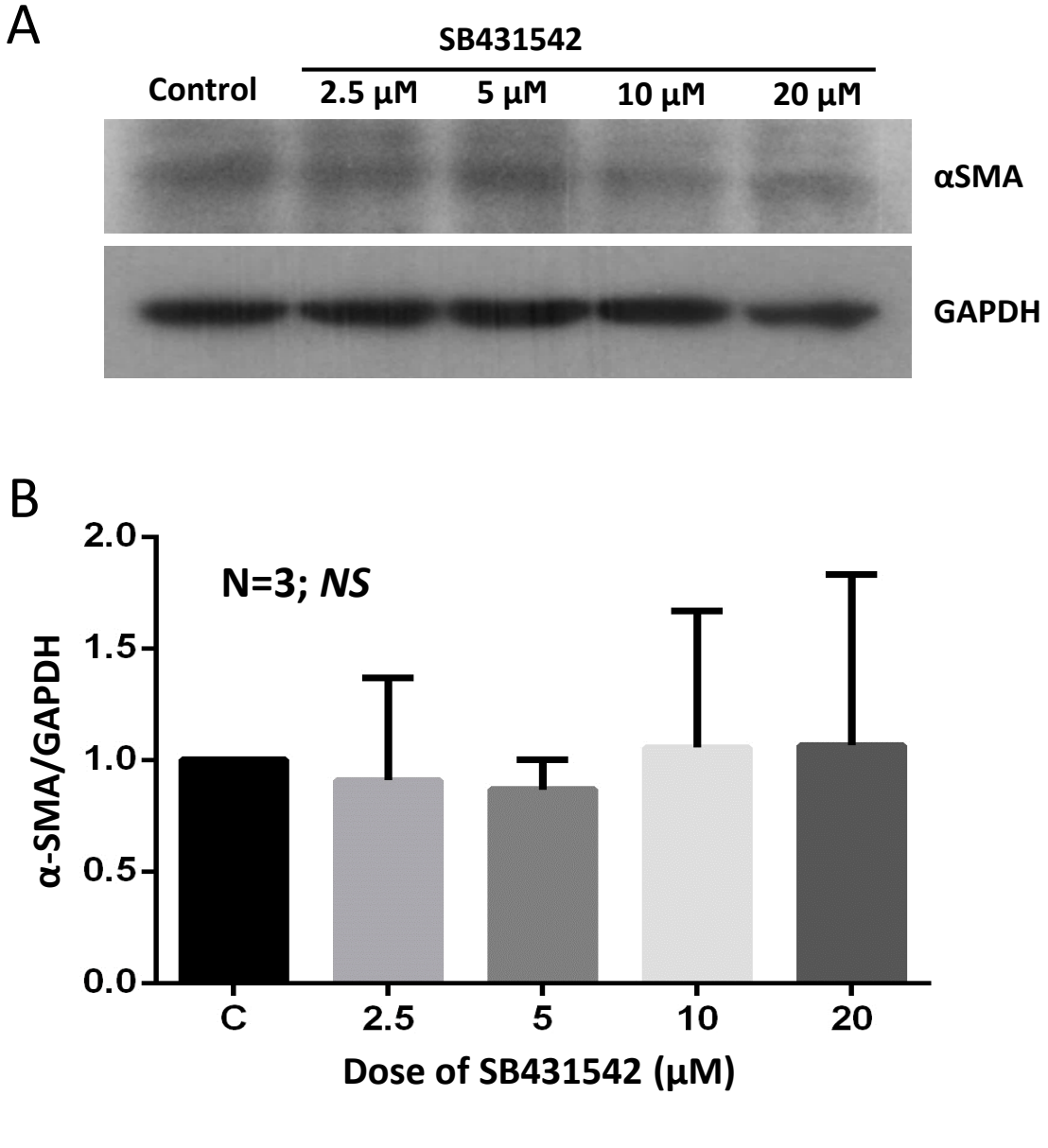

| | |
|---|---|
| **Profile** | GDS4389 / 224793_s_at |
| **Title** | Alcoholic hepatitis |
| **Organism** | Homo sapiens |

D

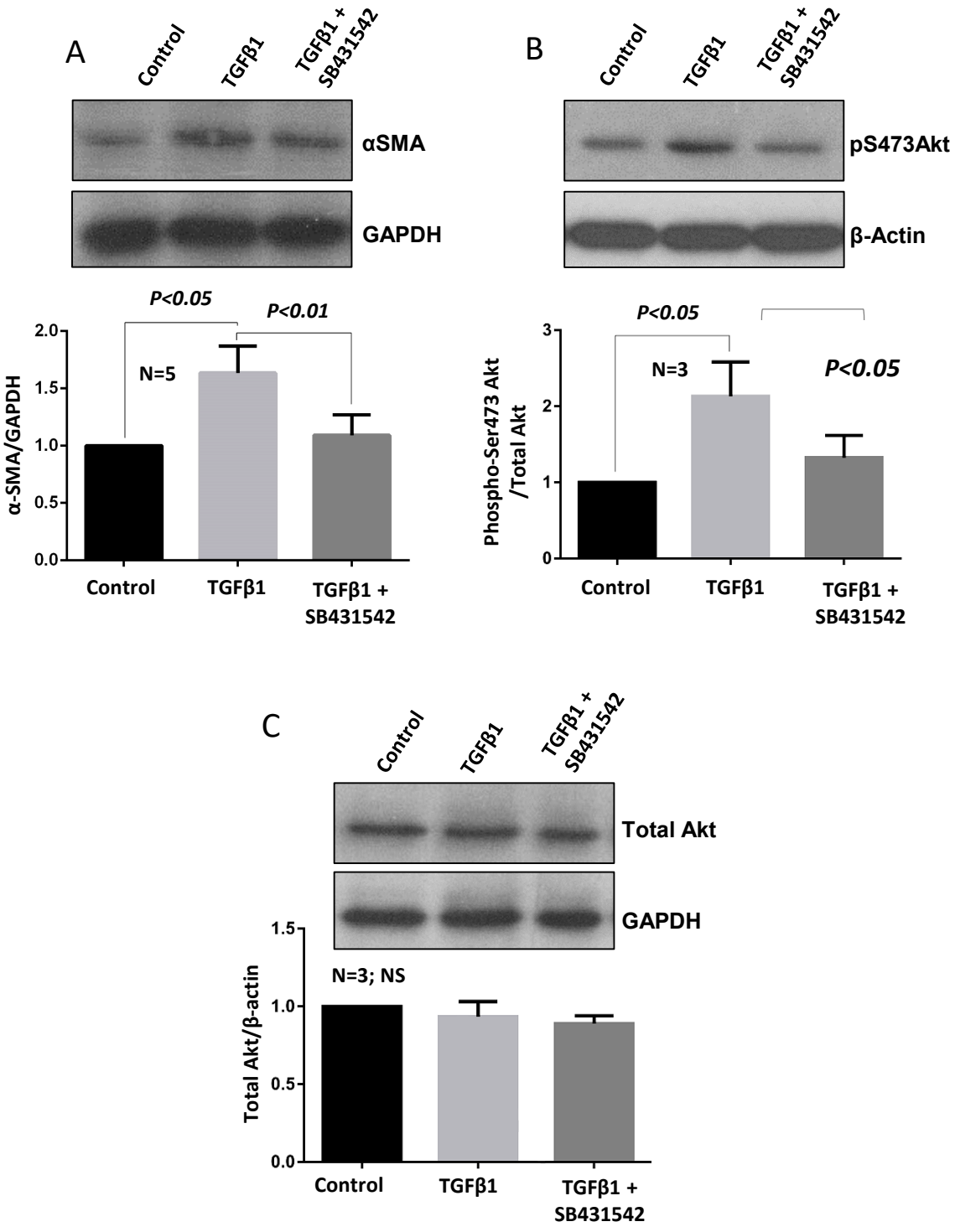



| | |
|---|---|
| **Profile** | GDS4902 / 1420895_at |
| **Title** | FIZZ2 deficient lung response to the fibrotic agent bleomycin |
| **Organism** | Mus musculus |

# Figure 3

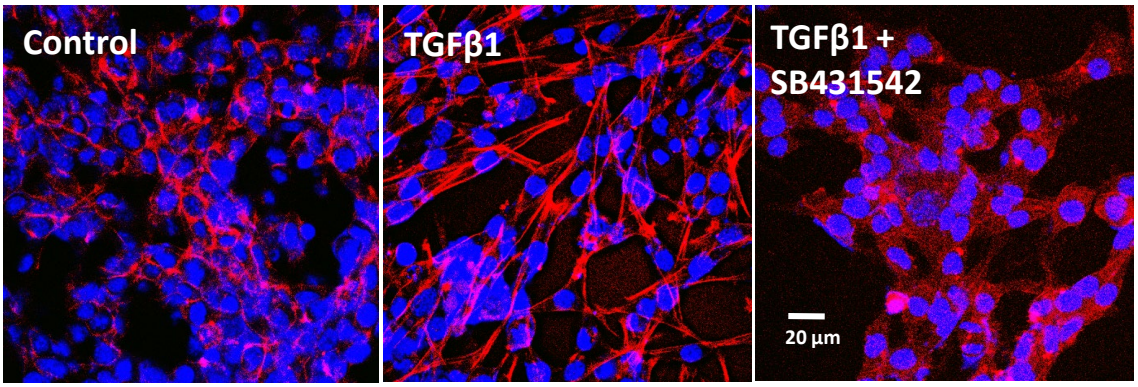

A
SB431542
Control
2.5 µM
5 µM
10 µM
20 µM
αSMA
GAPDH
B
N=3; NS
α-SMA/GAPDH
2.0
1.5
1.0
0.5
0.0
C
2.5
5
10
20
Dose of SB431542 (µM)

# Figure 4

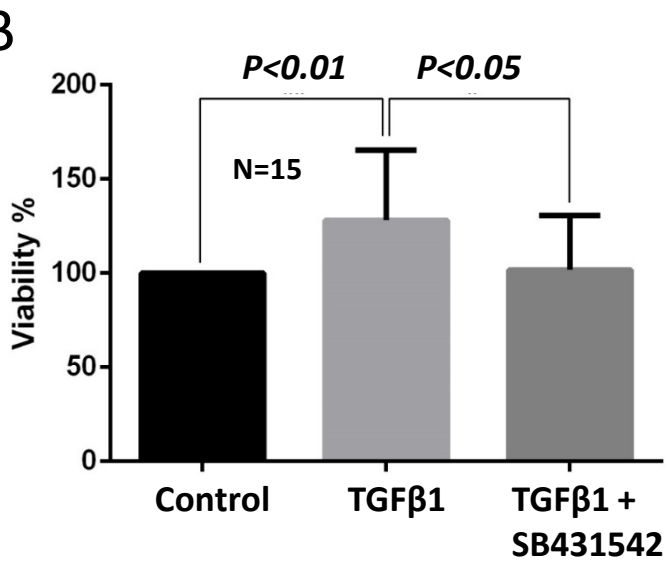

A
Control
TGFβ1
TGFβ1 + SB431542
αSMA
GAPDH
P<0.05
P<0.01
N=5
α-SMA/GAPDH
2.0
1.5
1.0
0.5
0.0
Control
TGFβ1
TGFβ1 + SB431542
B
Control
TGFβ1
TGFβ1 + SB431542
pS473Akt
β-Actin
P<0.05
N=3
P<0.05
Phospho-Ser473 Akt /Total Akt
3
2
1
0
Control
TGFβ1
TGFβ1 + SB431542
C
Control
TGFβ1
TGFβ1 + SB431542
Total Akt
GAPDH
N=3; NS
Total Akt/β-actin
1.5
1.0
0.5
0.0
Control
TGFβ1
TGFβ1 + SB431542

# Figure 5

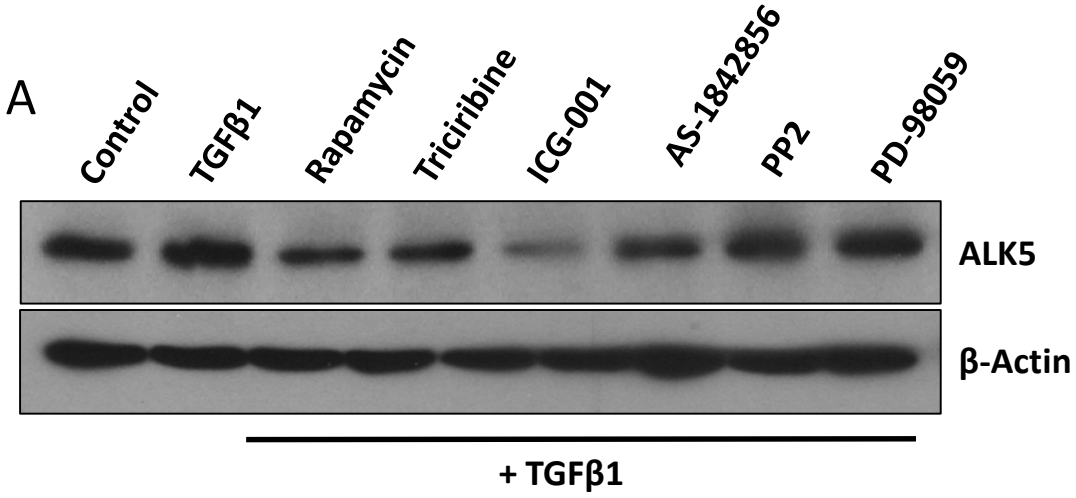
A

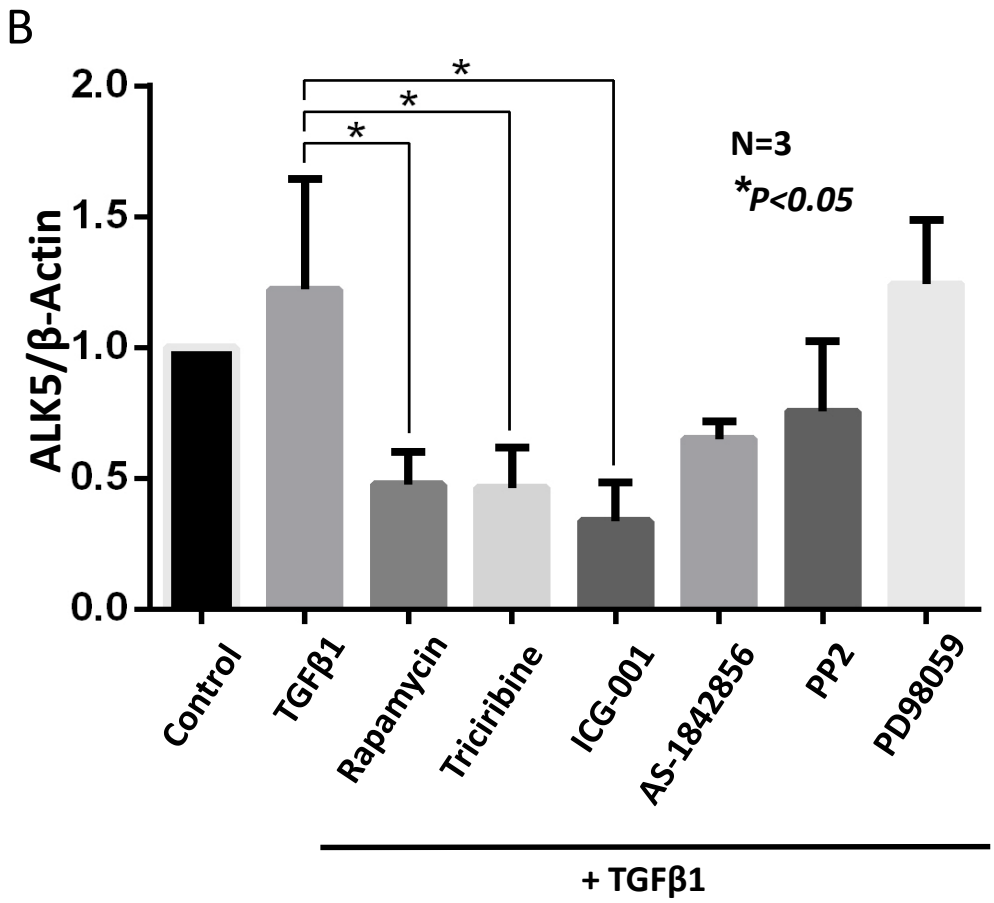

Control
TGFβ1
TGFβ1 +
SB431542
20 µm

B


P<0.01
P<0.05
N=15
Viability %
200
150
100
50
0
Control
TGFβ1
TGFβ1 +
SB431542

# Figure 6


A
Control
TGFβ1
Rapamycin
Triciribine
ICG-001
AS-1842856
PP2
PD-98059
ALK5
β-Actin
+ TGFβ1


B
ALK5/β-Actin
2.0
1.5
1.0
0.5
0.0
*
*
*
N=3
*P<0.05
Control
TGFβ1
Rapamycin
Triciribine
ICG-001
AS-1842856
PP2
PD98059
+ TGFβ1