THE ROLE OF STROMELYSIN1 (MMP3) IN ENDOTHELIAL-TO-MESENCHYMAL TRANSITION AND MYOFIBROBLAST DIFFERENTIATION

BY

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(Under the Direction of Somanath Shenoy)

ABSTRACT

Endothelial to mesenchymal transition (EndMT) and myofibroblast differentiation (FibroMF) occur in embryogenesis and adult pathologies such as the organ fibrosis. Stromelysin1, a matrix metalloprotease-3 (MMP3) is another molecule that has been indicated in vascular injury and organ fibrosis. There is a gap in knowledge on the specific role of stromelysin1 either in EndMT or in FibroMF. The objective of the current master of science research dissertation was to investigate the role of stromelysin1 in TGFβ2-induced EndMT and TGFβ1-induced FibroM. In our results, TGFβ2 treatment of endothelial cells (ECs) induced EndMT and increased expression of stromelysin1 and mesenchymal markers. Inhibition of stromelysin1 blunted TGFβ2-induced EndMT. In contrast, treatment of NIH-3T3 fibroblasts with TGFβ1 promoted FibroMF. Intriguingly, stromelysin1 inhibition in TGFβ1-stimulated myofibroblasts further exacerbated fibroproliferation with increased FibroMF marker expression. In conclusion, our study has identified that EndMT and FibroMF are reciprocally regulated by stromelysin1.

INDEX WORDS: EndMT; Myofibroblast; MMP3; Stromelysin1; TGFβ2; N-cadherin

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DEDICATION

This thesis wholeheartedly is dedicated to my family without whom it was impossible for me to complete my thesis work. A special feeling of gratitude to my parents Muslih and Hessia who have been a great source of inspiration and support. Literally, I am lucky to have you in my life. Also, I express my thanks to my sisters and my brothers. Last, but not least I would like to thank my professors and friends.

Thank you for always believing in me. Words cannot express how much I love you all.

This thesis is only the beginning of my journey.

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CHAPTER 1

1. Introduction and literature review

1.1. Endothelium and endothelial to mesenchymal transition (EndMT)

The endothelium is a monolayer of endothelial cells that forms the inner cellular lining of blood vessels such as veins and arteries and is the primary barrier that separates the organs and the blood (Feletou, 2011). The endothelium also plays a key role in the lymphatic system and is always in direct contact with the circulating blood and lymph (Pepper & Skobe, 2003). Under physiological conditions, the endothelium has an important role in controlling the flow of blood, platelet aggregation, regulation of inflammation, etc. (Yau, Teoh, & Verma, 2015). Endothelial cells control vascular tone by releasing vasodilatory factors such as a nitric oxide (NO), prostacyclin (PGI_2) and contracting factors such as vasoconstrictive factors such as endothelin-1 (ET-1) and thromboxane (TXA_2) (Sandoo, van Zanten, Metsios, Carroll, & Kitas, 2010).

Studies have shown that endothelial dysfunction contributes to atherosclerosis, stroke, various organ injuries, hypo- and hypertension and pulmonary arterial hypertension, etc. (Hadi, Carr, & Al Suwaidi, 2005). A lot of research was done to understand all the causes of endothelial dysfunction (Rajendran et al., 2013). It is apparent that endothelial dysfunction is related to a reduction in the levels of nitric oxide (NO) in blood vessel walls which, in turn, leads to vasoconstriction through a cascade of biological events (Rajendran et al., 2013). These functional abnormalities tend to promote several diseases; endothelial dysfunction is thought to be a major contributing factor for cardiovascular diseases (Barthelmes et al., 2017). The primary function of the endothelial cell-cell barrier is to maintaining the integrity of the blood-tissue barrier and

prevent any leakage between the cells (Sandoo et al., 2010). Junctional complexes such as the adherens junction (AJ) and tight junction (TJ) in the endothelium play a critical role in the molecular organization and in vascular homeostasis (Dejana, Orsenigo, Molendini, Baluk, & McDonald, 2009). Furthermore, Junctions in the endothelium regulate leukocyte recruitment into the inflamed area through opening and closing of endothelial cell-to-cell contacts (Wallez & Huber, 2008). Junctions complexes are essential in maintaining a functional endothelium (Hartsock & Nelson, 2008).

Endothelial-to-mesenchymal transition (EndMT) is a trans-differentiation process in which endothelial cells (ECs) lose their endothelial specific markers such as CD31, VE-Cadherin, eNOS, change their morphology, and acquire mesenchymal features characterized by the expression of mesenchymal markers such an N-Cadherin, alpha-smooth muscle actin (α-SMA) , fibronectin, and fibroblast-specific protein-1 (FSP-1) etc. (J. G. Cho, Lee, Chang, Lee, & Kim, 2018). Furthermore, one or more of the transcription factors such as Snail, Slug, ZEB-1, SIP-1, Twist, and LEF-1 are also involved in the loss of cell-cell contacts and EndMT, thus suppression of these transcription factors are critical in maintaining endothelial cell-cell contacts in (Lamouille, Xu, & Derynck, 2014).

1.2. Physiological and pathological role of EndMT

EndMT is essential in the embryonic stages, where it has been shown to occur in a subset of ECs during cardiogenesis and vasculogenesis (Zeng et al., 2013). During the embryogenesis, EndMT is critical for cardiac development particularly in the formation of the valves and septa of the adult heart. In addition, EndMT plays a pivotal role in the maturation of pulmonary veins and arteries and in angiogenic sprouting. Disruption of EndMT at this embryonic stage results in embryonic

lethality and EndMT is dormant in adults until pathological stimuli sets in to promote a cardiovascular disease condition (Medici, Munoz-Canoves, Yang, & Brunelli, 2016).

Growing evidence suggests that EndMT contributes to several pathological conditions. For example, EndMT is associated with fibrotic diseases in many organs through the generation of myofibroblasts, which is the main source of scar tissue following a tissue injury (Wynn, 2008). Additionally, EndMT has been reported in inflammatory conditions. Several other diseases associated with EndMT include, but not limited to, pulmonary hypertension (Qiao et al., 2014; Zhu et al., 2006), diabetic nephropathy (Liu, Zhang, Xu, Gao, & Yin, 2018), hypertrophic cardiomyopathy (Yoshimatsu & Watabe, 2011), inflammatory bowel disease (Yun, Kim, & Kim, 2019), acute lung injury (Sabbineni et al., 2019), human cerebral and orbital cavernous malformations (Takada, Hojo, Tanigaki, & Miyamoto, 2017), cerebral cavernous malformations (Maddaluno et al., 2013), and atherosclerosis (Kherbeck et al., 2013).

1.3. Molecular mechanisms regulating EndMT

Transforming growth factor-beta (TGFβ) has been implicated in inducing EndMT in several disease models (Pardali, Sanchez-Duffhues, Gomez-Puerto, & Ten Dijke, 2017). There is growing evidence for the predominance of TGFβ2 in inducing EndMT which was also confirmed in our preliminary data where we showed that TGFβ2 is more potent than the other isoforms TGFβ 1 and 3 (Sabbineni, Verma, & Somanath, 2018). EndMT has also been reported in Lipopolysaccharide (LPS)-induced lung injury and vascular remodeling (Suzuki et al., 2017). Interestingly TGFβ might also be necessary for LPS-induced injury through a paracrine effect (Sabbineni et al., 2019).

TGFβ2 has been shown to promote EndMT through Smad/Akt pathway (J. G. Cho et al., 2018). Akt1 has also been demonstrated to have a protective role to preserve barrier integrity

through molecular cross-talk with Src (Gao, Sabbineni, Artham, & Somanath, 2017). Loss of Akt1 leads to decreased basal and angiopoietin1-induced endothelial-barrier resistance and enhanced VEGF-induced endothelial-barrier breakdown (Gao et al., 2016). Additionally, Akt1 deficiency during the early stages of hypoxia will result in vascular injury and EndMT (Ranchoux et al., 2018). Our laboratory has shown that Akt1 deficiency results in increased stromelysin1 expression and activity involving FoxO activation thus reducing the Claudin-5 expression (Artham et al., 2019). This correlation between increased stromelysin1 expression and activity with exacerbated acute lung injury and EndMT-associated pathological pulmonary vascular remodeling is the basis for undertaking this study.

1.4. Myofibroblast differentiation

Myofibroblasts, the hallmark of fibrosis, are characterized by spindle-shaped cells There is a similarity between myofibroblasts and smooth muscle cells in that both of them express α-smooth muscle actin (αSMA) stress fibers, a feature that plays a key role in its contractile phenotype (Shinde, Humeres, & Frangogiannis, 2017). During tissue injury, fibroblasts differentiate into myofibroblasts (highly contractile cells) which are essential for wound healing by reducing the size of injury due to the presence of αSMA (α-smooth muscle actin) filaments in the cytoplasm of these cells (Li & Wang, 2011). Myofibroblasts exhibit increased αSMA expression and excess production, secretion, and assembly of the extracellular matrix (ECM) proteins (Kendall & Feghali-Bostwick, 2014). *α*SMA is the most commonly used molecular marker to study the myofibroblast phenotype (Sun, Chang, Reed, & Sheppard, 2016). The myofibroblasts secrete the ECM components and matrix metalloproteinases (MMPs) which mediate tissue remodeling post tissue/organ injury (Duarte, Baber, Fujii, & Coito, 2015). The major ECM proteins expressed by

myofibroblasts are collagen I, collagen III, glycoproteins; and proteoglycans (fibronectin, laminin, and tenascin) all of which contribute to tissue remodeling and wound healing (Bonnans, Chou, & Werb, 2014). Myofibroblasts can be derived from many sources such as resident fibroblasts, fibrocytes, vascular smooth muscle cells, endothelial cells, pericytes, and mesenchymal stem cells (Barron, Gharib, & Duffield, 2016). Many studies have shown that the primary growth factor that promotes fibroblast-to-myofibroblast (FibroMF) differentiation is TGFβ1 that involves the PI3K/Akt pathway (Abdalla, Goc, Segar, & Somanath, 2013; Abdalla et al., 2015; Goc, Sabbineni, Abdalla, & Somanath, 2015; Kulasekaran et al., 2009).

1.5. Physiological and pathological role of FibroMF differentiation

Myofibroblasts have been shown to be crucial in the process of wound healing and tissue remodeling (Darby, Laverdet, Bonte, & Desmouliere, 2014). During the wound healing, fibroblasts differentiate into myofibroblasts which help to contract the wound due to the presence of αSMA filaments in the cytoplasm of these cells (Li & Wang, 2011). When tissue is repaired myofibroblasts disappear by apoptosis (Li & Wang, 2011).

Myofibroblasts have been reported in many pathologic conditions, such as inflammation, fibrosis, vascular thickening, pulmonary hypertension, etc. (Kendall & Feghali-Bostwick, 2014). Myofibroblasts synthesize, express and/or release adhesion molecules such as the intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), which contributes to the recruitment of lymphocytes, neutrophils and inflammatory reactions (Smith, 1993). FibroMF is a crucial event in many diseases such as scleroderma (Dantas et al., 2015), cancer (Wurm, Tomasik, & Tesch-Romer, 2008), and fibrosis of the liver (Eulenberg & Lidbury, 2018), kidney (M. H. Cho, 2010), and lung (Abdalla et al., 2015). Myofibroblasts have also been implicated in

heart failure and myocardial infarction patients (Ma, Iyer, Jung, Czubryt, & Lindsey, 2017). Furthermore, recent studies indicate the contributions of the myofibroblasts in the growth and metastasis of epithelial tumors (Otranto et al., 2012).

1.6. Molecular mechanisms regulating Myofibroblast differentiation

Many studies have shown that the primary growth factor promoting FibroMF differentiation is TGFβ1, and the process involves canonical Smad (Biernacka, Dobaczewski, & Frangogiannis, 2011) and non-canonical Akt pathway (Abdalla et al., 2013; Abdalla et al., 2015). It has been demonstrated in our laboratory that Akt1 mediated alpha-smooth muscle actin expression involving myocardin and serum response factor expression is essential for FibroMF differentiation (Abdalla et al., 2013). Loss of Akt1 has a protective effect on pulmonary vascular pathologies and fibrosis in idiopathic pulmonary fibrosis (Abdalla et al., 2015; Huetsch, Suresh, Bernier, & Shimoda, 2016).

1.7. Stromelysin1 (Matrix metalloproteinase-3; MMP3)

Matrix metalloproteinase-3 (Stromelysin1), a secreted matrix-degrading enzyme plays an important role in both physiology and pathology (Nerusu et al., 2007). Enzymatic activity of the proteins of the matrix metalloproteinase (MMP) family are required during the developmental process and the normal physiology in several ways including, but not limited to (1) To degrade ECM molecules and allow cell migration, (2) To alter the ECM microenvironment and result in alteration in cellular behavior and (3) To modulate the activity of biologically active molecules by direct cleavage, release from bound stores, or the modulating of the activity of their inhibitors (Frantz, Stewart, & Weaver, 2010; Lu, Takai, Weaver, & Werb, 2011).

The primary role of stromelysin1 is to degrade the extracellular matrix (ECM) such as fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans (Lu et al., 2011). Moreover, stromelysin1 has also been shown to activate several growth factor peptides and kinases contributing to pathological phenomena such as epithelial to mesenchymal transition (EMT) (Kalluri & Weinberg, 2009). stromelysin1 is thought to be involved in wound repair, the progression of atherosclerosis, tumor initiation, arthritis, and metastasis.

1.8. Molecular mechanisms regulating stromelysin1 expression and activity

It was shown that LPS treatment increases the secretion of stromelysin1 *in vitro* (Artham et al., 2019). This is in agreement with our data in the mouse models where we observed an upregulation of stromelysin1 in LPS-induced acute lung injury model (Artham et al., 2019). Hypoxia, a very well-known inducer of vascular remodeling and EndMT *in vitro* and *in vivo* also has been demonstrated to enhance the secretion of stromelysin1 (Lamouille et al., 2014). Given that both EMT and EndMT are facilitated by the transcription factors ZEB1, TWIST, FoxC2, and Snail1, etc. (Lamouille et al., 2014), the biological similarities between the two phenomena, and the importance of stromelysin1 in regulating EMT, we speculate that stromelysin1 plays an important role in modulating EndMT and FibroMF as well.

The objective and central hypothesis

Endothelial-to-mesenchymal transition (EndMT), where endothelial cells (ECs) lose their specific markers such as CD31 and eNOS, change their morphology to acquire myofibroblast-like features gaining the expression of mesenchymal markers such as N-Cadherin and smooth muscle cell actin-α (αSMA). We recently showed that matrix metalloproteinase-3 (MMP3; stromelysin1) promotes Lipopolysaccharide (LPS)–induced exudative stage of acute lung injury in mice. However, there is a *gap in our knowledge* of the involvement of MMP3 in promoting EndMT. We seek to address this gap and investigate how targeting MMP3 could inhibit EndMT *in vitro*.

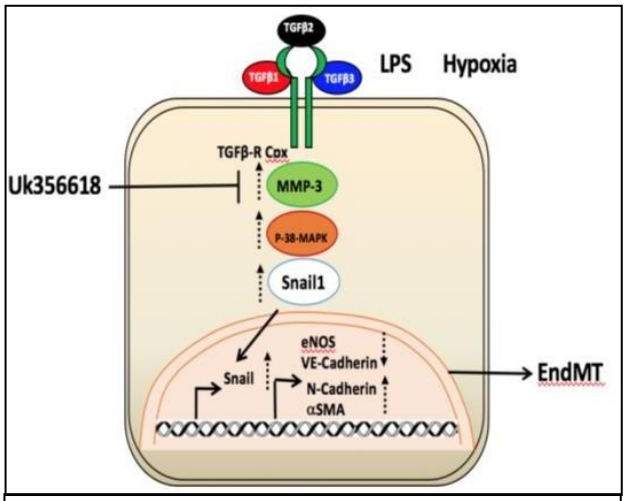

Figure 1: Diagram showing the hypothesis to be tested that MMP3 is involved in the promotion of EndMT and pathological vascular remodeling and that pharmacological inhibition of MMP3 will inhibit TGFβ2 induced EndMT, and vascular remodeling.

The **objective** of the current study is to investigate the role of MMP3 in the regulation of TGFβ2 induced EndMT *in vitro.*

Our **central hypothesis** is that activation of MMP3 is necessary to promote EndMT, pathological vascular remodeling. The *rationale* for the study is that upon study completion, we will be able to determine the role of MMP3 in EndMT and target EndMT by using MMP3 inhibitor UK 356618.

Specific Aims

Aim 1: Test the hypothesis that MMP3 is necessary for the TGFβ2-induced EndMT *in vitro*: Human microvascular ECs (HMECs) will be subjected to treatment with TGFβ2 (1 ng/ml) in the presence or absence of a stromelysin1 inhibitor (UK 356618) for 3 days, and subjected to Western blot analysis for changes in the expression of eNOS, N-cadherin, αSMA, stromelysin1, and transcription factors Snail, and FoxC2. Cells will also be analyzed microscopically for any morphological changes

Aim 2: Test the hypothesis that MMP3 activity modulates TGFβ1-induced myofibroblast differentiation *in vitro*: NIH3T3 fibroblasts will be subjected to treatment with TGFβ1 (1 ng/ml) in the presence or absence of a stromelysin1 inhibitor (UK 356618) for 3 days, and subjected to Western blot analysis for changes in the expression of N-cadherin, αSMA, stromelysin1, and transcription factors Snail, and FoxC2. Cells will also be analyzed microscopically for any morphological changes.

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Chapter 2

Distinct effect of stromelysin1 inhibition on endothelial-to-mesenchymal transition and myofibroblast differentiation

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Abstract

Endothelial-to-mesenchymal transition (EndMT) and fibroblast-to-myofibroblast (FibroMF) differentiation are frequently reported in organ fibrosis. Stromelysin1, a matrix metalloprotease-3 (MMP3) has been indicated in vascular pathologies and organ injuries that often lead to fibrosis. In the current study, we investigated the role of stromelysin1 in EndMT and FibroMF differentiation, which is currently unknown. In our results, whereas TGFβ2 treatment of endothelial cells (ECs) induced EndMT and increased expression of stromelysin1 and mesenchymal markers such as α-Smooth muscle actin (αSMA), N-cadherin and activin linked kinase-5 (ALK5), inhibition of stromelysin1 blunted TGFβ2-induced EndMT. In contrast, treatment of NIH-3T3 fibroblasts with TGFβ1 promoted FibroMF differentiation accompanied by increased expression of αSMA, N-cadherin and ALK5. Intriguingly, stromelysin1 inhibition in TGFβ1-stimulated myofibroblasts further exacerbated fibroproliferation with increased FibroMF marker expression. Gene Expression Omnibus (GEO) data analysis indicated increased stromelysin1 expression associated with EndMT and decreased stromelysin1 expression in human pulmonary fibrosis fibroblasts. In conclusion, our study has identified that EndMT and FibroMF differentiation are reciprocally regulated by stromelysin1.

Keywords: EndMT; myofibroblast; stromelysin1; MMP3, fibrosis

Introduction

Many recent studies have indicated endothelial-to-mesenchymal transition (EndMT) (Pardali, Sanchez-Duffhues, Gomez-Puerto, & Ten Dijke, 2017; Yu et al., 2014) and fibroblast-to-myofibroblast (FibroMF) differentiation (Weiskirchen, Weiskirchen, & Tacke, 2019) in vascular injuries, tissue remodeling, and organ fibrosis. Research in our laboratory has demonstrated the integral role of Akt1 in FibroMF differentiation (Abdalla, Goc, Segar, & Somanath, 2013; Goc, Sabbineni, Abdalla, & Somanath, 2015), pulmonary fibrosis (Abdalla et al., 2015), pulmonary vascular injury (Artham et al., 2019) and EndMT associated pulmonary vascular remodeling (Sabbineni et al., 2019). Whereas Akt suppression exacerbated the vascular injury and lung edema in a mouse model of lipopolysaccharide (LPS)-induced acute lung injury (Artham et al., 2019), it promoted EndMT and vascular remodeling in a SUGEN-hypoxia model of pulmonary hypertension (Sabbineni et al., 2019) and cardiac fibrosis (Ma, Kerr, Naga Prasad, Byzova, & Somanath, 2014). Interestingly, whereas TGFβ1, the primary inducer of FibroMF activated Akt in NIH 3T3 cells and human fibroblasts, treatment with LPS (Artham et al., 2019) or TGFβ2 (Gao, Sabbineni, Artham, & Somanath, 2017; Sabbineni et al., 2019; Sabbineni, Verma, & Somanath, 2018), two primary inducers of EndMT resulted in suppression of Akt activity indicating reciprocal regulation of FibroMF differentiation and EndMT by Akt.

Recent reports from other laboratories also indicate the crucial role of the Akt-mTOR pathway in pulmonary fibrosis (Lawrence & Nho, 2018) that subsequently progresses to lung cancer (Tzouvelekis et al., 2019). Intriguingly, in an adenovirus TGFβ-induced model of pulmonary fibrosis, pharmacologically targeting Akt pathway using triciribine was observed to have a beneficial effect in halting the disease progression (Abdalla et al., 2015). As of today, there

is no knowledge of how Akt reciprocally regulates EndMT and FibroMF differentiation, which is a critical problem that limits the use of Akt inhibitors in the treatment of organ injury and fibrosis.

Stromelysin1, also known as matrix metalloprotease-3 (MMP3) was identified as a primary candidate in endothelial cells (ECs) causing endothelial-barrier breakdown and pulmonary edema in mice lacking Akt1 in ECs (Artham et al., 2019). The loss of EC Akt1 in mice also resulted in EndMT (Sabbineni et al., 2019), which is also associated with LPS-induced acute lung injury (Suzuki et al., 2016). Interestingly, Akt1 inhibition in TGFβ1-treated NIH 3T3 fibroblasts suppressed FibroMF differentiation associated with reduced stromelysin1 expression (Abdalla et al., 2013; Abdalla et al., 2015). However, there are uncertainties and a gap in our literature on the precise role of stromelysin1 in fibrosis (Giannandrea & Parks, 2014) and whether EndMT and EMT indeed contribute to the myofibroblast population in organ fibrosis (Medici & Kalluri, 2012; Pardali et al., 2017).

In the current study, we used human microvascular endothelial cells (HMECs) and NIH 3T3 fibroblasts, two cell lines extensively used in fibrosis research to investigate the effect of pharmacological inhibition of stromelysin1 on EndMT and FibroMF differentiation. Our study revealed a distinctly different effect of stromelysin1 activity suppression on TGFβ-induced EndMT and FibroMF differentiation *in vitro*, indicating the complexity of stromelysin1 signaling and cellular differentiation in organ fibrosis.

2. Materials and methods

2.1. Cell culture

Human dermal (Telomerase-immortalized) microvascular ECs (HMEC) (CRL-4025; ATCC, Manassas, VA) were maintained in EC Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD) and 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 and TGFβ2 were obtained from R&D Systems (Minneapolis, MN) and were reconstituted according to the manufacturer's protocol. HMECs or NIH 3T3 fibroblasts were treated with 5 ng/ml doses of TGFβ2 and TGFβ1, respectively, in 5% serum-containing medium for 72 hours. The growth factors were replenished every 24 hours. The dose of stromelysin1 inhibitor (UK356618, Tocris, Minneapolis, MN) was determined based on the IC50 values, its dose-dependent cytotoxicity, and our previous study (Artham et al., 2019).

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 TGFβ2 or TGFβ1, respectively for 72 h in the presence and absence of 20 μM stromelysin1 inhibitor UK356618 during the last 24 hours. 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-488 or 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.

2.3. Cell migration assay

Cell migration (monolayer scratch healing) assay was performed as explained previously (Goc, Abdalla, Al-Azayzih, & Somanath, 2012). Cells were grown on 6-well plates until reaching 60-70% confluence. Scratch was made in the cell monolayer using 1ml pipette tip followed by a one-time wash with 1X PBS. Cells were incubated with 5 ng/ml TGFβ2 or vehicle (PBS) in DMEM containing 5% FBS for 24 hours in the presence and absence of stromelysin1 inhibitor 20 μM UK356618. Images of scratches were taken immediately after scratching (0 hours) and 24 hours after treatment. The rate of migration was measured as a percentage of scratch filling using the equation ([1-T24/T0] X 100), where T24 is the area at the end point (24 hours) and T0 is the area measured immediately after making the scratches.

2.4. MTT assay

Cell proliferation and viability were determined as previously published from our laboratory using the MTT assay (Al-Azayzih, Missaoui, Cummings, & Somanath, 2016). Cells were seeded in 48-well cell culture plates at 5×10^4 cells/ml and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Cells were treated with 20 μM stromelysin1 inhibitor UK356618 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).

2.5. 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 et al., 2018). Antibodies used include stromelysin1, N-cadherin and GAPDH from Cell Signaling Technology (Danvers, MA), αSMA and β-actin from Sigma (St. Louis, MO) and ALK5 antibodies from Abcam (Cambridge, MA). Band densitometry was done using NIH Image J software.

2.6. ***Stromelysin1 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 "stromelysin1/MMP3 and mesenchymal transition", and "stromelysin1/MMP3 and Fibrosis/myofibroblast". The search resulted in six datasets that were performed during the last decade. The gene expression levels of stromelysin1 were downloaded for different groups in all studies. The gene expression was compared between control and EndMT/FirbroMF group using student t-test and/or one-way ANOVA (for dose effects). The mean $\pm$ SD were presented for those studies with at least a sample size of $\geq$3.

2.7. 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. TGFβ2-induced EndMT and endothelial expression of mesenchymal markers were inhibited by stromelysin1 activity suppression.

To determine whether stromelysin1 expression was modulated in EndMT, we treated HMECs with EndMT inducing TGFβ2 for 72 hours. Our results indicated a significant increase in the expression of stromelysin1 with TGFβ2 stimulation (Figure 1A), which was accompanied by a significant increase in the expression of mesenchymal markers such as αSMA (Figure 1B), N-cadherin (Figure 1C) and TGFβ type I receptor, ALK5 (Figure 1D). Interestingly, TGFβ2-induced increased expression of stromelysin1 and the mesenchymal markers were attenuated by co-treatment with stromelysin1 inhibitor UK356618 during the 48-72 hours of TGFβ2-induced EndMT.

3.2. TGFβ2-induced loss of actin stress fibers in HMECs was blunted by strmelysin1 activity suppression

Next, we determined whether the promotion of EndMT and cell motility by TGFβ2 stimulation was associated with reduced actin stress fiber, which is an indicator of increased Rac and P21 activated kinase signaling (Kichina, Goc, Al-Husein, Somanath, & Kandel, 2010; Somanath & Byzova, 2009) and reduced RhoA activity (Abdalla et al., 2013). Staining of TGFβ2-stimulated HMECs with FITC-labelled phalloidin that binds to actin stress fibers indicated loss stress fibers in HMECs treated with TGFβ2 for 72 hours (Figure 2A), which resulted in increased cell migration

in an HMEC monolayer scratch assay (Figure 2B). Interestingly, TGFβ2-induced loss of actin stress fibers, as well as increased cell migration, was mitigated by co-treatment with stromelysin1 inhibitor UK356618 during the 48-72 hours of TGFβ2-induced EndMT (Figure 2A-B). As per our previous reports, TGFβ2-induced EndMT was associated with some cell death (Sabbineni et al., 2019; Sabbineni et al., 2018), which surprisingly was even higher in combination with UK356618 treatment (Figure 2C). In the absence of TGFβ2, UK356618 treatment had a minimal but significant effect on reducing EndMT marker expression (Figure 2D-F).

3.3. TGFβ1-induced FibroMF differentiation and fibroblast expression of mesenchymal markers were inhibited by stromelysin1 activity suppression.

To determine whether stromelysin1 expression was modulated in FibroMF differentiation, we treated NIH 3T3 fibroblasts with FibroMF-inducing TGFβ1 for 72 hours. Our results indicated a non-significant increase in stromelysin1 with TGFβ1 stimulation (Figure 3A), which was accompanied by a significant increase in the expression of mesenchymal markers such as αSMA (Figure 3B), N-cadherin (Figure 3C) and TGFβ type I receptor, ALK5 (Figure 3D). Interestingly, TGFβ1-induced increased expression of the mesenchymal markers was attenuated by co-treatment with stromelysin1 inhibitor UK356618 during the 48-72 hours of TGFβ1-induced FibroMF differentiation.

3.4. TGFβ1-induced actin stress fiber formation in NIH 3T3 fibroblasts was blunted by strmelysin1 activity suppression

Next, we determined whether the promotion of FibroMF differentiation by TGFβ1 stimulation was associated with increased actin stress fiber, which is indicative of increased RhoA activity (Abdalla et al., 2013). Staining of TGFβ1-stimulated NIH 3T3 fibroblasts with Alexa555-labelled phalloidin that binds to actin stress fibers indicated increased stress fibers in NIH 3T3 fibroblasts treated with

TGFβ1 for 72 hours (Figure 4A). Interestingly, TGFβ1-induced increased actin stress fibers was mitigated by co-treatment with stromelysin1 inhibitor UK356618 during the 48-72 hours of TGFβ1-induced FibroMF differentiation (Figure 4A). As per our previous reports, TGFβ1-induced FibroMF differentiation was associated with increased cell survival (Abdalla et al., 2013; Abdalla et al., 2015), which was significantly inhibited by treatment with UK356618 (Figure 4B). In the absence of TGFβ1, UK356618 treatment had only minimal changes in the αSMA and N-cadherin expression but significantly reduced ALK5 expression (Figure 4C-D).

3.5. Stromelysin1 gene expression was enhanced in EndMT associated with hypoxia-induced pulmonary vascular remodeling.

GEO datasets retrieved from the studies over a decade with the search terms 'stroemelysin1 and EndMT' were analyzed to confirm a positive correlation between stromelysin1 gene expression and EndMT as observed from the *in vitro* results. Whereas one of the studies (GEO ID: GDS252/98833_at) indicated increased stromelysin1 expression in hypoxia-induced pulmonary hypertension associated EndMT (Figure 5A), the study had a smaller sample size to derive any significant conclusion. Interestingly, another study (GEO ID: GDS252/98833_at), which is a model of EMT that share similar features of EndMT (Gao et al., 2018) indicated a significant time-dependent increase in stromelysin-1 expression in response to TGFβ in A549 lung carcinoma cell line (Figure 5B) indicating a positive correlation between stromelysin expression and mesenchymal transition.

3.6. GEO analysis show increased stromelysin1 in fibroblasts and lung tissues treated with TNFα or bleomycin, but reduced stromelysin expression in IPF fibroblasts.

GEO datasets retrieved from the studies over a decade with the search terms 'stroemelysin1 and fibrosis' were analyzed to confirm a negative correlation between stromelysin1 gene expression

and FibroMF differentiation as observed from the *in vitro* results. One of the studies (GEO ID: GDS5261/A_23_P161698) showed increased stromelysin1 expression associated with TNFα-induced inflammatory response in IMR-90 (Human fetal lung fibroblast) cells (Figure 6A). Another two studies (GEO ID: GDS251/98833_at 8 and GDS1492/1418945_at) showed similar effects in a mouse model of bleomycin-induced fibrosis challenging our data from the in vitro studies (Figure 6B-C). Intriguingly, a study conducted in human idiopathic pulmonary fibrosis fibroblasts, but not in systemic sclerosis fibroblasts, revealed reduced stromelysin1 similar to our *in vitro* results in NIH 3T3 cells. This suggests that the mechanisms of fibrosis with bleomycin and systemic sclerosis as compared to other forms of idiopathic pulmonary fibrosis may be different, particularly with respect to the expression and role of stromelysin1.

4. Discussion

Until a decade ago, ECs were considered terminally differentiated cells that cannot undergo further differentiation (Kovacic & Boehm, 2009). Recent advancements in the EC biology and TGFβ growth factor signaling have identified EndMT as a process by which endothelial cells shun their characteristic features and acquire mesenchymal markers such as N-cadherin, αSMA, Snail1, FoxC2, TWIST, ZEB, vimentin, etc. (Cho, Lee, Chang, Lee, & Kim, 2018). EndMT was initially identified in the embryonic development where cell differentiation is rampant and contributes to the development of various internal organs (Medici & Kalluri, 2012). This process, however, is quiescent in adult tissues that get initiated only in the pathological conditions (Dejana, Hirschi, & Simons, 2017). Recent studies have identified the specific role of EndMT in various pathological conditions such as pulmonary hypertension (Qiao et al., 2014; Zhu et al., 2006), diabetic nephropathy (Liu, Zhang, Xu, Gao, & Yin, 2018), hypertrophic cardiomyopathy (Yoshimatsu &

Watabe, 2011), inflammatory bowel disease (Yun, Kim, & Kim, 2019), acute lung injury (Sabbineni et al., 2019), human cerebral and orbital cavernous malformations (Maddaluno et al., 2013; Takada, Hojo, Tanigaki, & Miyamoto, 2017), and atherosclerosis (Kherbeck et al., 2013).

Akin to EndMT, FibroMF differentiation occur in physiology and in tissue repair process such as the wound healing (Li & Wang, 2011; Patel, Baz, Wong, Lee, & Khosrotehrani, 2018). However, uncontrolled FibroMF differentiation leads to fibrosis, which in the advanced stages is irreversible, which is a hallmark of pulmonary, liver, kidney and cystic fibrosis, etc. (Weiskirchen et al., 2019). Both EndMT and FibroMF differentiation are regulated by TGFβ signaling (Pardali et al., 2017; Wynn, 2008). Whereas TGFβ1 is the predominant isoform involved in FibroMF differentiation (Abdalla et al., 2013), EndMT is reliant on TGFβ2 stimulation (Sabbineni et al., 2019; Sabbineni et al., 2018). PI3-Kinase Akt pathway, regulated by TGFβ, has been identified to be central to both EndMT (Sabbineni et al., 2019) and FibroMF differentiation (Abdalla et al., 2013; Abdalla et al., 2015). Whereas TGFβ1 activates Akt in fibroblasts to promote αSMA expression via myocardin and serum response factor (Abdalla et al., 2015), TGFβ1 and TGFβ2 inhibited Akt in ECs promoting EndMT involving FoxC2 (Sabbineni et al., 2019). Interestingly, TGFβ1 stimulation of epithelial cells also inhibited the Akt pathway in promoting EMT (Gao, Alwhaibi, et al., 2017). Since many researchers believe that EndMT and EMT, in addition to FibroMF differentiation, are two major sources of myofibroblasts in a fibrotic tissue (Wynn, 2008), this reciprocal regulation of cellular differentiation by TGFβ-Akt signing in these cell types is intriguing. However, TGFβ signaling is well-known for its cell and context-specific effects, which are often paradoxical in nature (Biernacka, Dobaczewski, & Frangogiannis, 2011).

Matrix protein degrading MMPs are important in organ fibrosis (Duarte, Baber, Fujii, & Coito, 2015; Giannandrea & Parks, 2014; Pardo, Cabrera, Maldonado, & Selman, 2016), and are

suggested for therapeutic development (Craig, Zhang, Hagood, & Owen, 2015). Mice deficient in stromelysin1 has been demonstrated to be resistant to bleomycin-induced pulmonary fibrosis (Yamashita et al., 2011). In our studies, both LPS treatment and Akt1 gene deletion in ECs resulted in increased stromelysin1 expression and activity (Artham et al., 2019). *In vivo*, LPS-induced ALI, as well as EC-specific gene deletion in mice, resulted in FoxO1/3a-mediated increase in stromelysin1 expression and activity, which was attenuated by co-treatment with stromelysin1 inhibitor UK356618 (Artham et al., 2019). Studies from other laboratories have demonstrated that EndMT occurs during the LPS-induced ALI (Suzuki et al., 2017; Suzuki et al., 2016). These studies suggested a potential role for stromelysin1 in FibroMF differentiation and EndMT that needed further investigation.

In the current study, 72-hour stimulation with TGFβ2 increased stromelysin1 and mesenchymal marker expression in ECs. However, a significant change in stromelysin1 expression was not observed in TGFβ1-stimulated NIH 3T3 fibroblasts. Co-treatment with stromelysin1 inhibitor at 48-72-hours of TGFβ2 stimulation blunted the increased mesenchymal marker expression and cell migration indicating the involvement of stromelysin1 in EndMT. On the other end, co-treatment of NIH 3T3 fibroblasts with stromelysin1 inhibitor at 48-72-hours of TGFβ1 stimulation further increased the mesenchymal marker expression indicating that stromelysin1 resists FibroMF differentiation and fibrosis. Intriguingly, whereas TGFβ2 stimulation of ECs reduced actin stress fibers promoting cell migration, TGFβ1 stimulation of fibroblasts promoted stress fiber formation reducing its motility and promoting contractility, a characteristic of myofibroblasts. Interestingly, these changes in the actin cytoskeletal remodeling were mitigated by co-treatment with stromelysin1 inhibitor. Although surprising, the fact that TGFβ1 activates Akt in fibroblasts and TGFβ1/2 inhibit Akt in ECs, this paradoxical regulation of cell

differentiation by stromelysin1 in ECs and Fibroblasts is mechanistically explained. Such differential regulation of styromelysin1 expression in ECs and NIH 3T3 fibroblasts has also been previously demonstrated in our laboratory (Abdalla et al., 2015; Artham et al., 2019).

One deficiency in our study is the lack of an *in vivo* animal model to prove the concept that stromelysin1 activity suppression will inhibit EndMT but promote FibroMF differentiation. Hence, we analyzed the data on stromelysin1 expression in studies involving cells, animals, 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 stromelysin1 expression correlating with EndMT and increased inflammation. Stimulation of fibroblasts with TNFα *in vitro* as well as bleomycin-induced lung injury and fibroproliferation in mice expressed increased stromelysin1 expression. Although intriguing, this association was not observed in human systemic sclerosis fibroblasts, a disease pathologically similar to the bleomycin-induced mouse model, suggesting that increased stromelysin1 expression observed in the bleomycin and TNFα-induced studies could be because of the inflammatory response rather than the fibrotic response. This is further supported by the fact that IPF fibroblasts expressed significantly lower levels of stromelysin1 compared to normal fibroblasts. These findings, however, raises questions on the current dogma that EndMT and EMT contribute to the myofibroblast pool in fibrosis (Pardali et al., 2017). Future research will explain if changes in the repertoire of cell surface receptors during cell differentiation is the underlying reason for such complexity in the signaling pathways.

In summary, our results demonstrate for the first time that although stromelysin1 expression increases in EndMT, its inhibition exacerbates TGFβ1-induced FibroMF differentiation with no significant effects on the normal ECs and fibroblasts. Future studies are warranted to

expand our knowledge on the specific effects of stromelysin1 activity regulation on EndMT and FibroMF differentiation associated diseases and to identify the signaling partners of stromelysin1 in the process. Nevertheless, our study indicates the need for caution while considering stromelysin1 as a target to treat organ fibrosis.

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Conflict of interest

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

Figure legends

Figure 1: Stromelysin1 activity suppression inhibits TGFβ2-induced EndMT. (A) Representative Western blot images and bar graph with band densitometry analysis indicating increased stromelysin1 expression in ECs with TGFβ2 treatment accompanied by increased expression of mesenchymal markers such as α-SMA **(B)**, N-cadherin **(C)** and ALK5 **(D)**, all of which were significantly inhibited by co-treatment with stromelysin1 inhibitor UK356618. Data are shown as Mean ± SD.

Figure 2: Stromelysin1 inhibition reverses TGFβ2-induced loss of actin stress fibers in ECs. (A) Confocal images of phalloidin-stained EC-monolayers treated in the presence and absence of TGFβ2 and/or stromelysin1 inhibitor UK356618 showing loss of actin stress fibers with TGFβ2 treatment and restoration of actin stress fibers upon co-treatment with stromelysin1 inhibitor. **(B)** Bar graph showing increased migration of ECs with TGFβ2 treatment, which is significantly suppressed by co-treatment with stromeelysin1 inhibitor. **(C)** Histogram showing reduced viability of ECs upon co-treatment of UK356618 with TGFβ2 treatment. **(D)** Representative Western blot images and bar graph with band densitometry analysis indicating changes in the expression of N-cadherin expression in ECs treated with UK366618 and other mesenchymal markers αSMA **(E)** and ALK5 **(F)**. Data are shown as Mean ± SD.

Figure 3: Stromelysin1 activity suppression exacerbates TGFβ1-induced FibroMF. (A) Representative Western blot images and bar graph with band densitometry analysis showing stromelysin1 expression in NIH 3T3 fibroblasts with TGFβ1 treatment accompanied by increased expression of mesenchymal markers such as ALK5 **(B)**, α-SMA **(C)** and N-cadherin **(D)**, all of

which were significantly inhibited by co-treatment with stromelysin1 inhibitor UK356618. Data are shown as Mean ± SD.

Figure 4: Stromelysin1 inhibition reverses TGFβ2-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 and/or stromelysin1 inhibitor UK356618 showing increased actin stress fibers with TGFβ1 treatment and further exacerbation upon co-treatment with stromelysin1 inhibitor. **(B)** Histogram showing increased viability of NIH 3T3 fibroblasts with TGFβ1 treatment, which was blunted by co-treatment with UK356618. **(C-E)** Representative Western blot images and bar graph with band densitometry analysis indicating the expression levels of ALK5, N-cadherin and αSMA in NIH 3T3 cells with UK356618 in the absence of TGFβ1. Data are shown as Mean ± SD.

Figure 5: GEO analysis show increased stromelysin1 gene expression in EndMT. (A) Bar graph showing increased stromelysin1 expression associated with the pulmonary vascular remodeling in a mouse model of hypoxia-induced pulmonary hypertension. **(B)** Bar graph showing increased stromelysin1 expression associated with hypoxia-induced EMT of the human lung adenocarcinoma cell line at 8-10 hours post hypoxia *in vitro*. Data are shown as Mean ± SD.

Figure 6: GEO analysis show increased stromelysin1 in fibroblasts and lung tissues treated with TNFα or bleomycin and reduced stromelysin in IPF fibroblasts. (A) Bar graph showing increased stromelysin1 expression associated with TNFα-induced inflammatory response in IMR-90 (Human fetal lung fibroblast) cells. **(B and C)** Bar graphs of two different studies showing increased stromelysin1 expression associated with bleomycin-induced mouse lung injury and fibrosis. **(D)** Bar graph showing reduced stromelysin1 in IPF fibroblasts, but not systemic sclerosis fibroblasts compared to normal fibroblasts. Data are shown as Mean ± SD.

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Figure 1

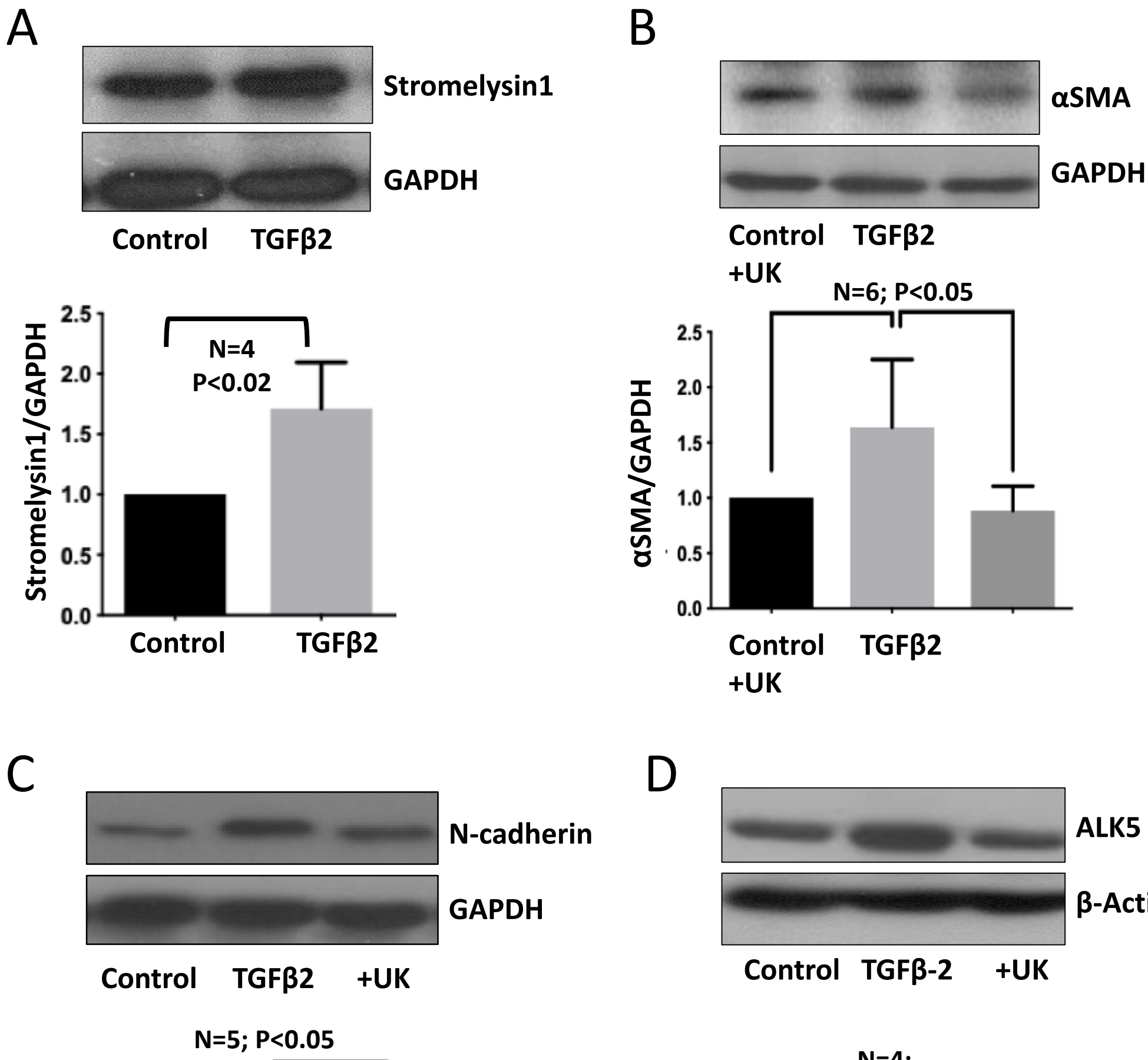

A
Stromelysin1
GAPDH
Control
TGFβ2
Stromelysin1/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
N=4
P<0.02
Control
TGFβ2
B
αSMA
GAPDH
Control
+UK
TGFβ2
N=6; P<0.05
αSMA/GAPDH
Control
TGFβ2
+UK
C
N-cadherin
GAPDH
Control
TGFβ2
+UK
N=5; P<0.05
N-cadherin/GAPDH
Control
TGFβ2
+UK
D
ALK5
β-Actin
Control
TGFβ-2
+UK
N=4;
P<0.001
ALK5/β-actin
Control
+UK
TGFβ2

Figure 2

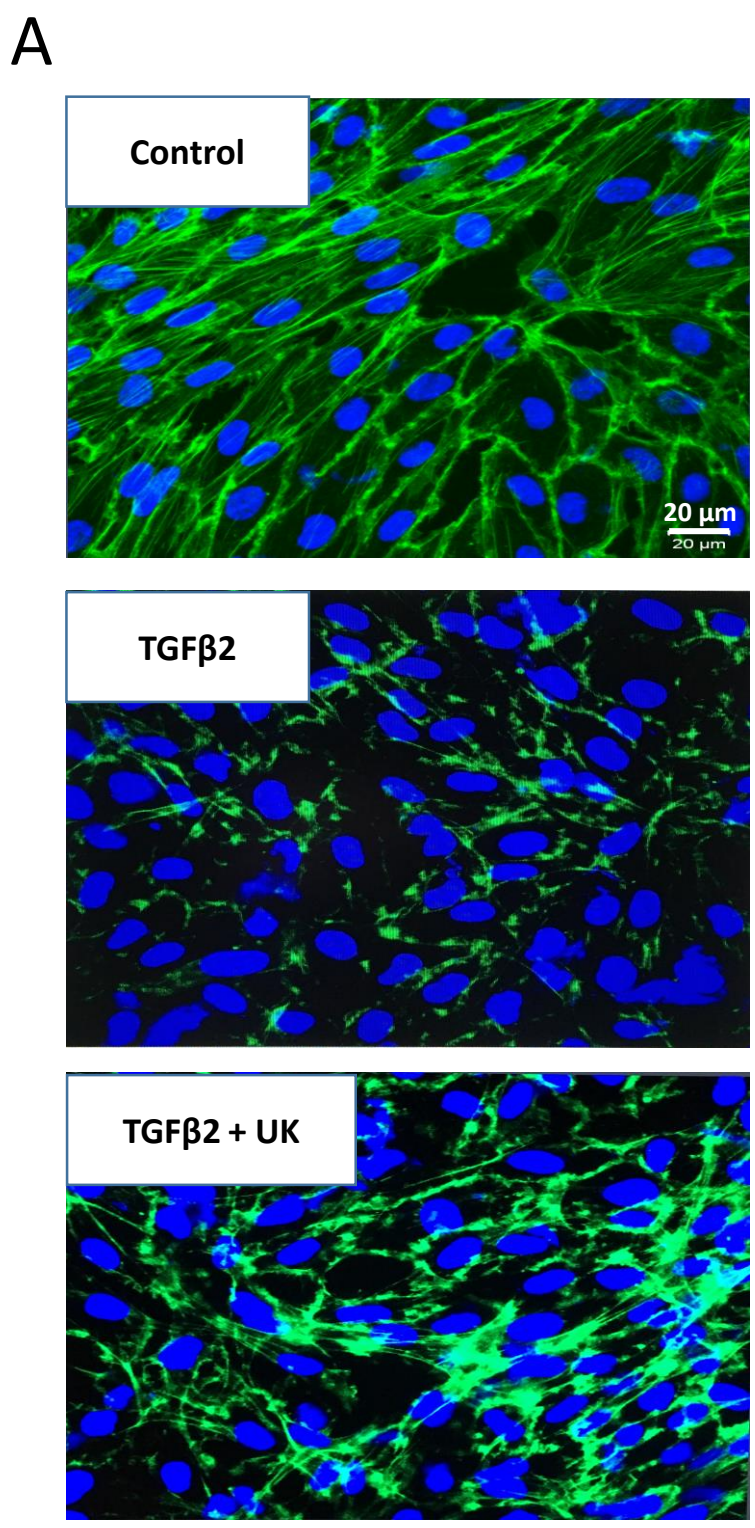

A
Control
20 µm
TGFβ2
TGFβ2 + UK
B
N=3; P<0.001
Rate of migration
100
80
60
40
20
0
Control
TGFβ2
+UK
C
N=28; P<0.001
Viability %
250
200
150
100
50
0
Control
TGFβ2
+UK
D
N-cadherin
GAPDH
N=3; P<0.05
N-cadherin/GAPDH
1.5
1.0
0.5
0.0
Control
UK
E
αSMA
GAPDH
N=3; P<0.05
αSMA/GAPDH
1.5
1.0
0.5
0.0
Control
UK
F
ALK5
β-Actin
N=3; NS
ALK5/β-actin
1.5
1.0
0.5
0.0
Control
UK

Figure 3

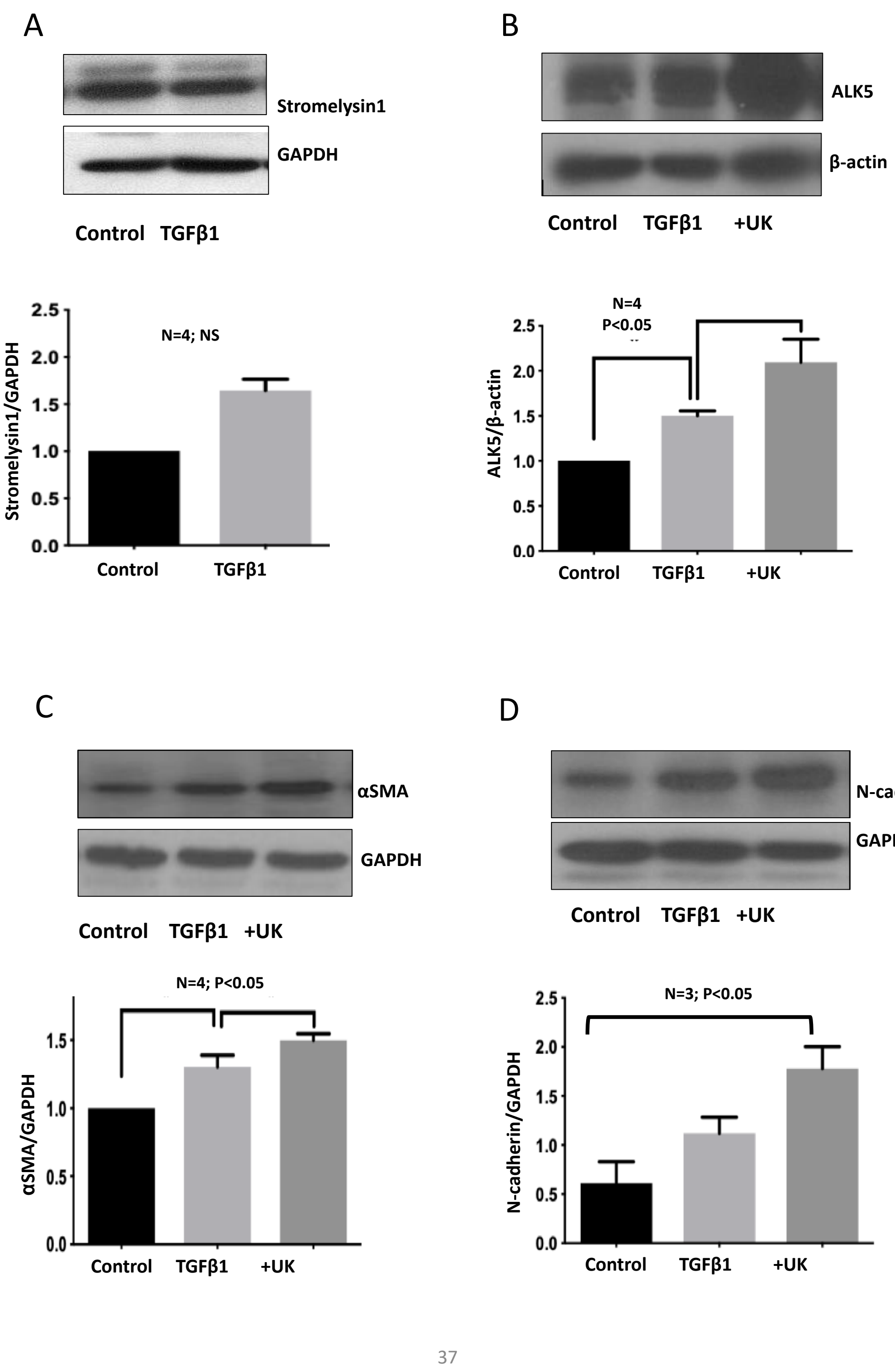
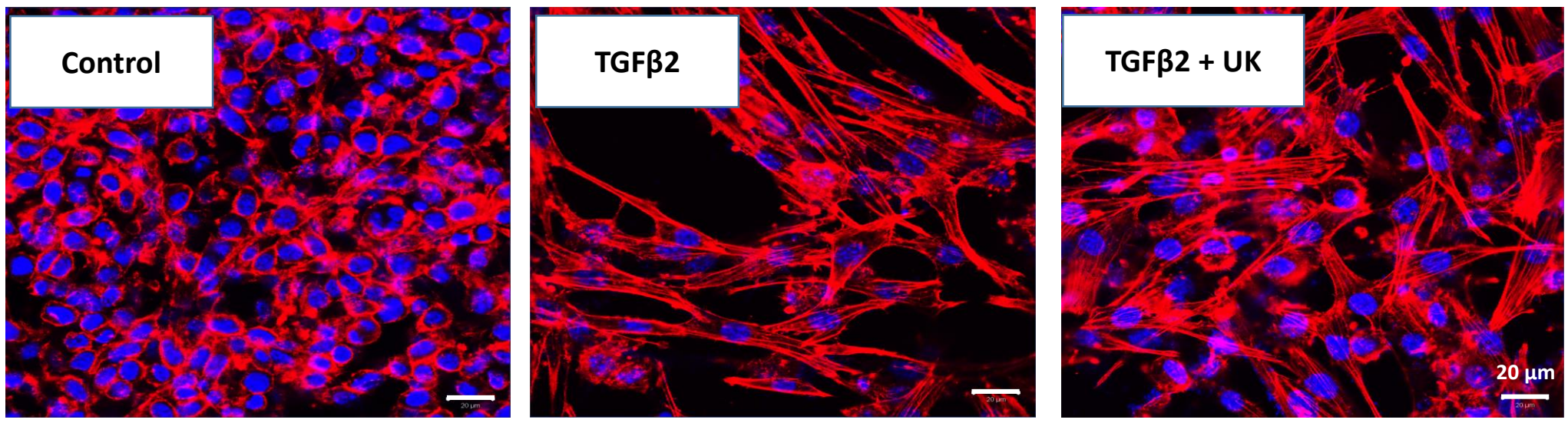

A
Stromelysin1
GAPDH
Control TGFβ1
N=4; NS
Stromelysin1/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
Control
TGFβ1
B
ALK5
β-actin
Control TGFβ1 +UK
N=4
P<0.05
ALK5/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
Control
TGFβ1
+UK
C
αSMA
GAPDH
Control TGFβ1 +UK
N=4; P<0.05
αSMA/GAPDH
1.5
1.0
0.5
0.0
Control
TGFβ1
+UK
D
N-cadherin
GAPDH
Control TGFβ1 +UK
N=3; P<0.05
N-cadherin/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
Control
TGFβ1
+UK

Figure 4

A



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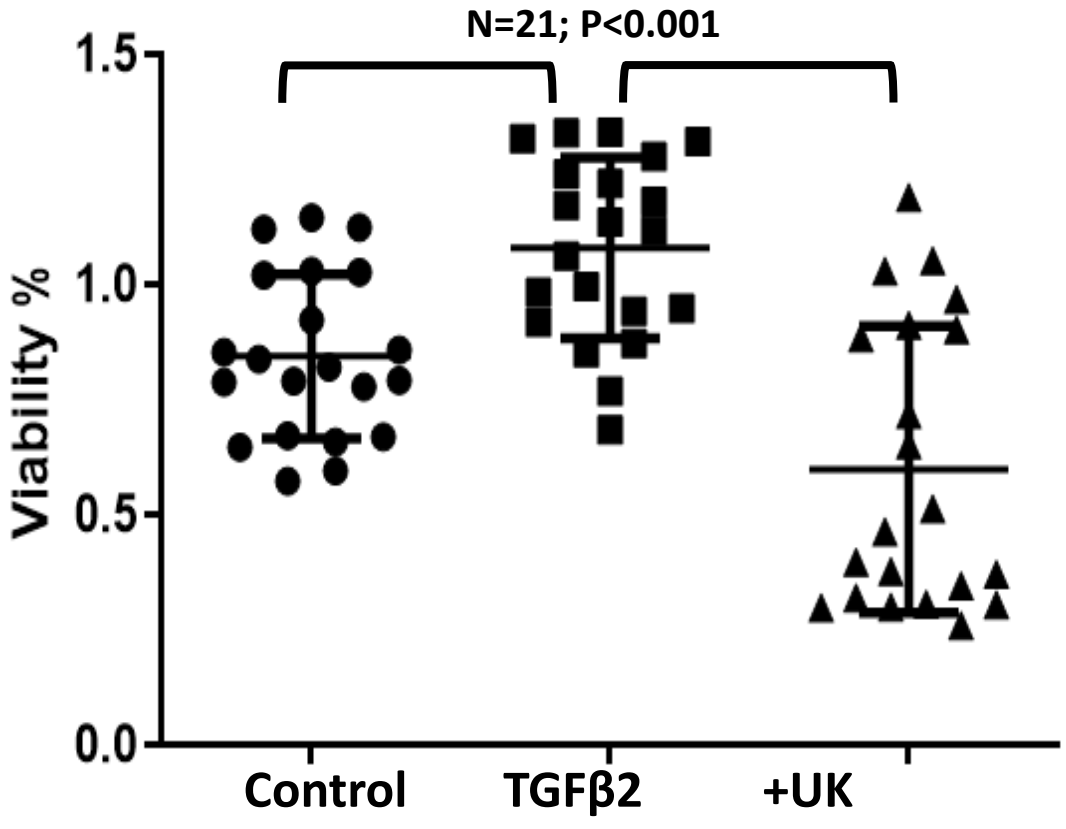
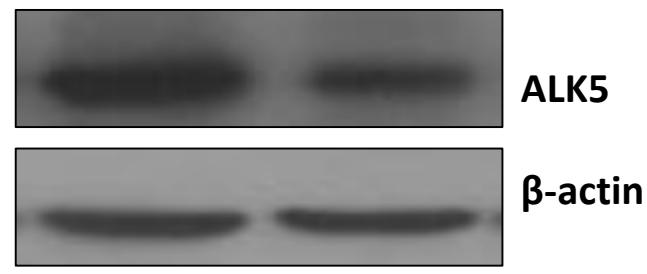
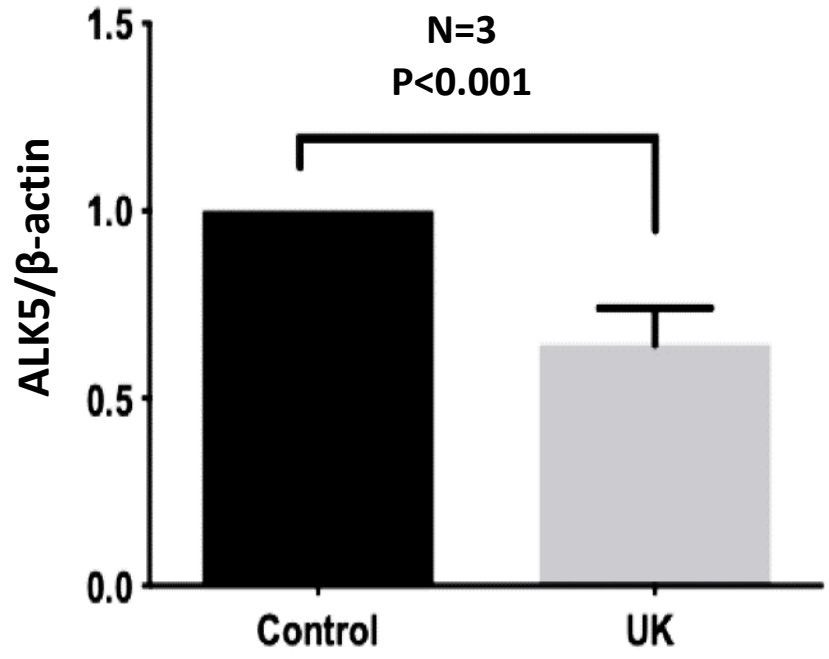
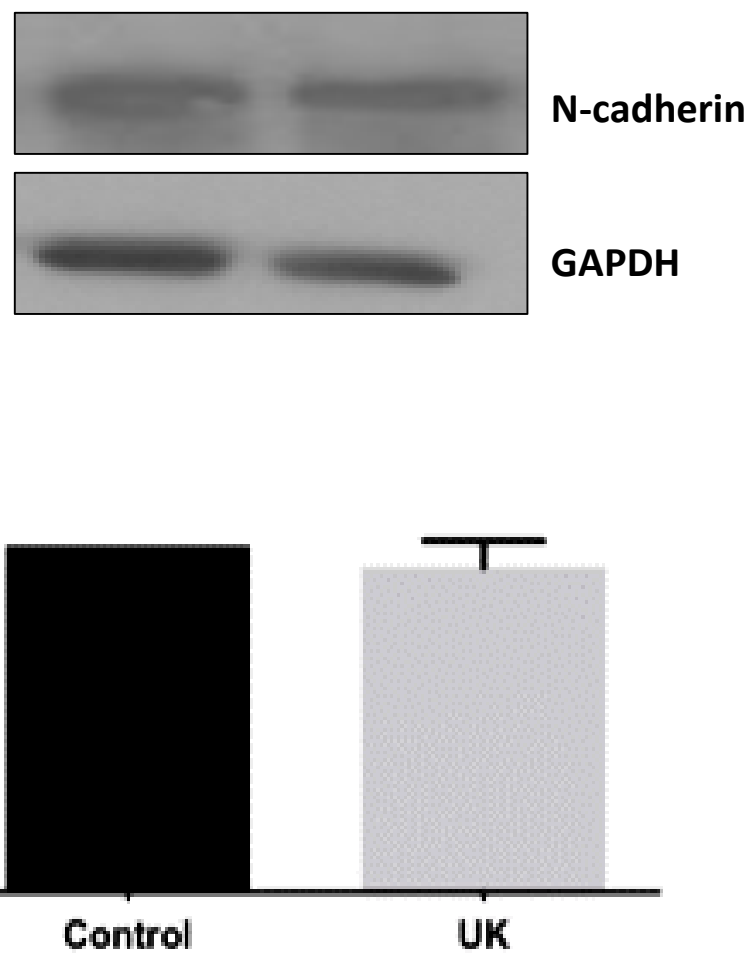


C





D



E

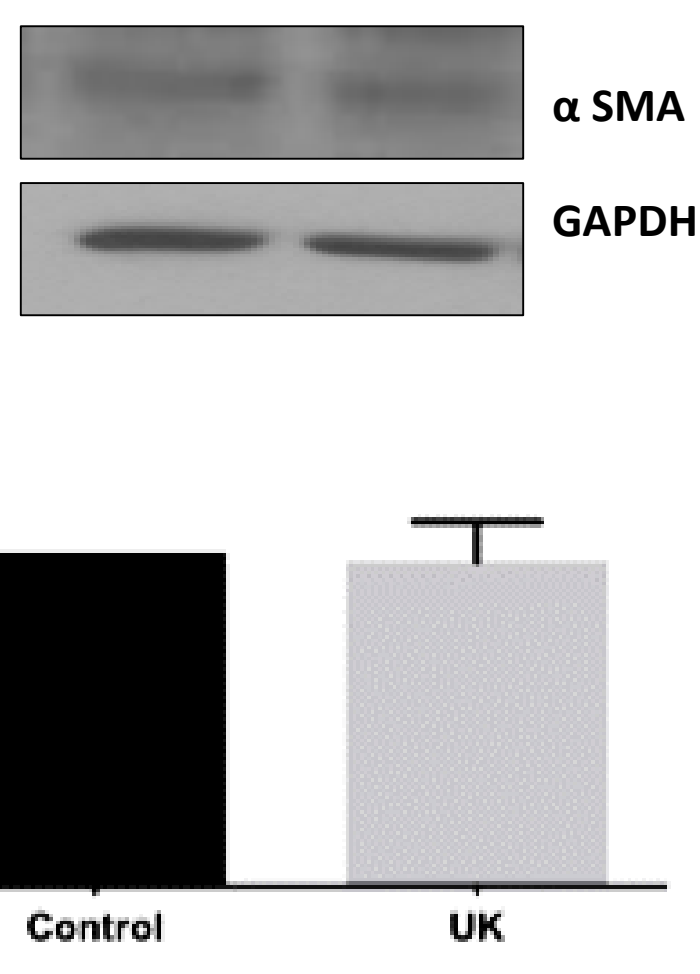
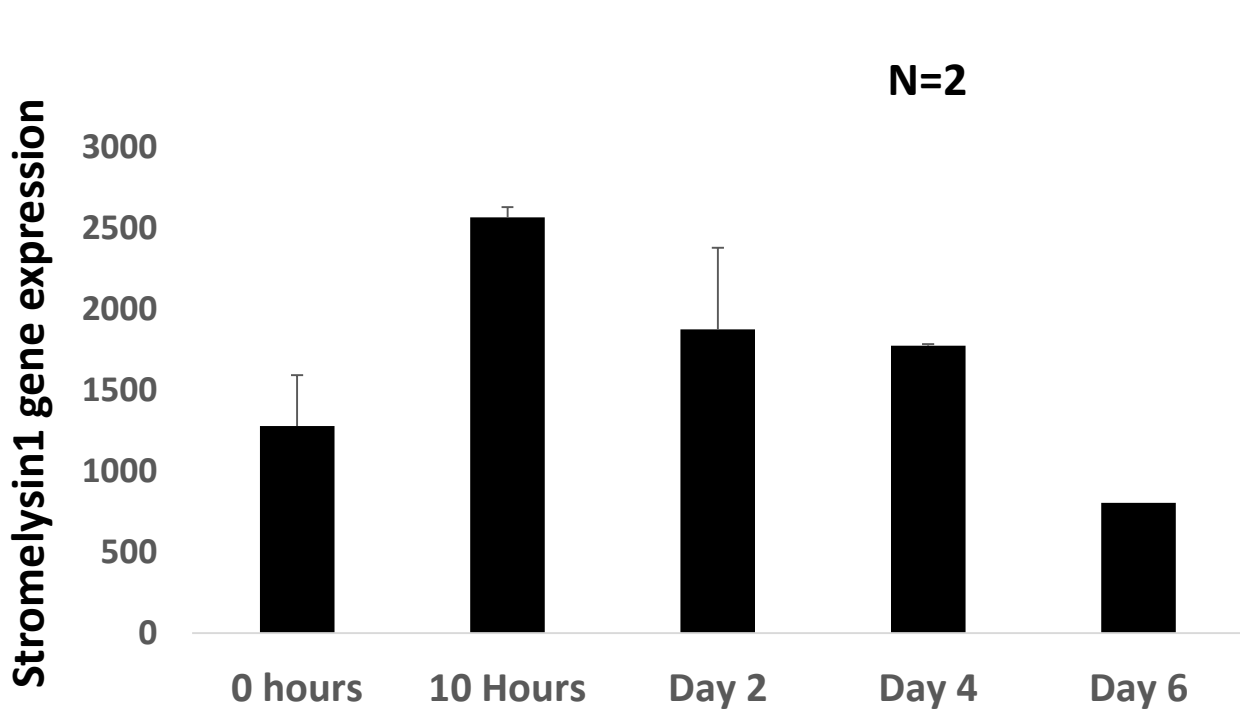


Figure 5

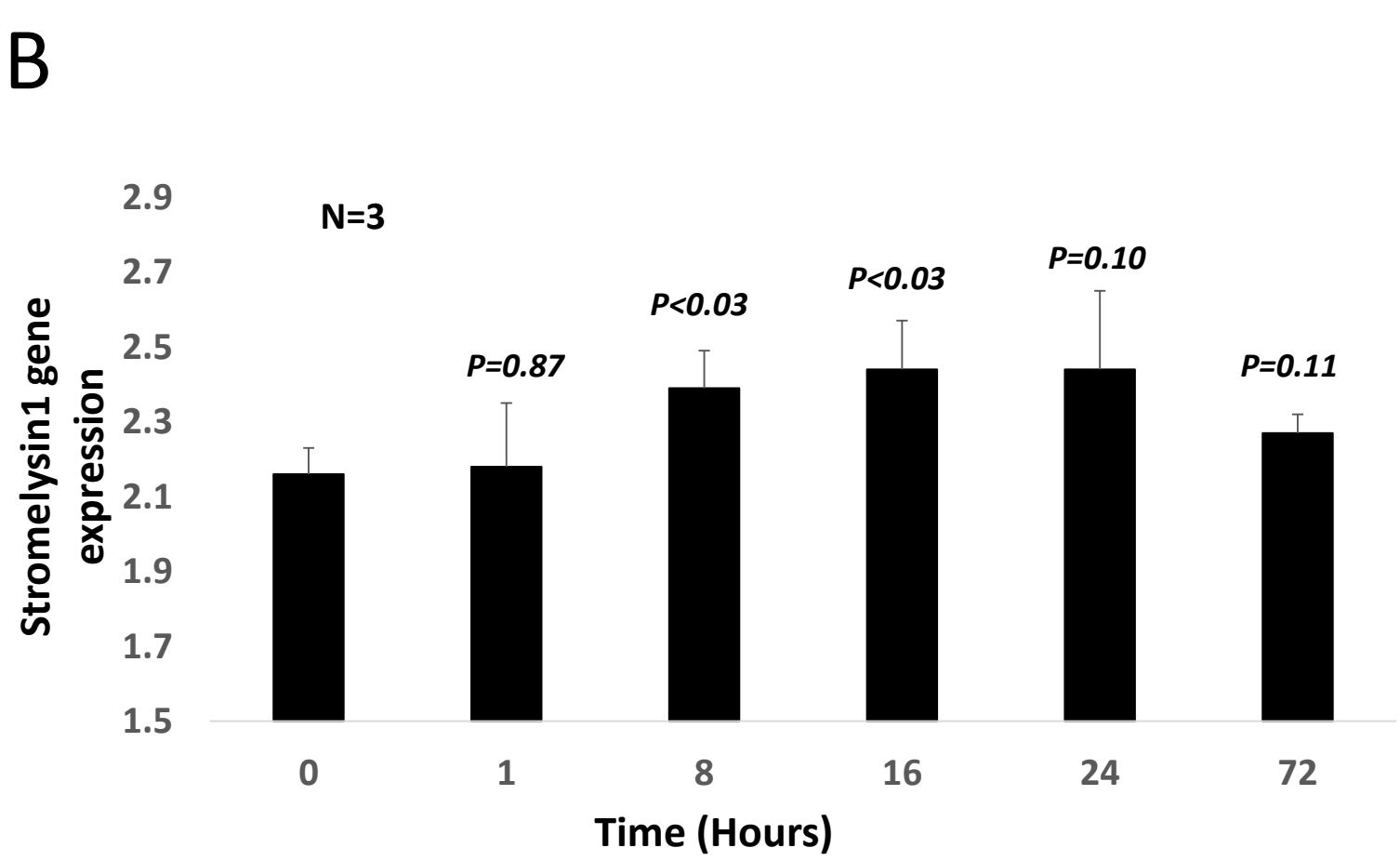
A



Profile	GDS252 / 98833_at
Title	Lung hypertension recovery (U74Av2)
Organism	Mus musculus

MMP3 gene expression in hypoxia-induced lung vascular remodeling

B



Profile	GDS3710 / 205828_at
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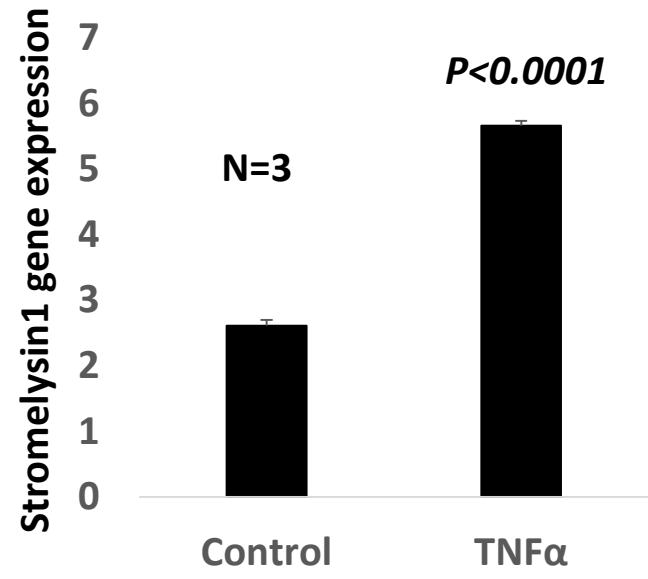
Organism	Homo sapiens

MMP3 gene expression in TGFβ-induced A549 lung adenocarcinoma cell EMT

Figure 6

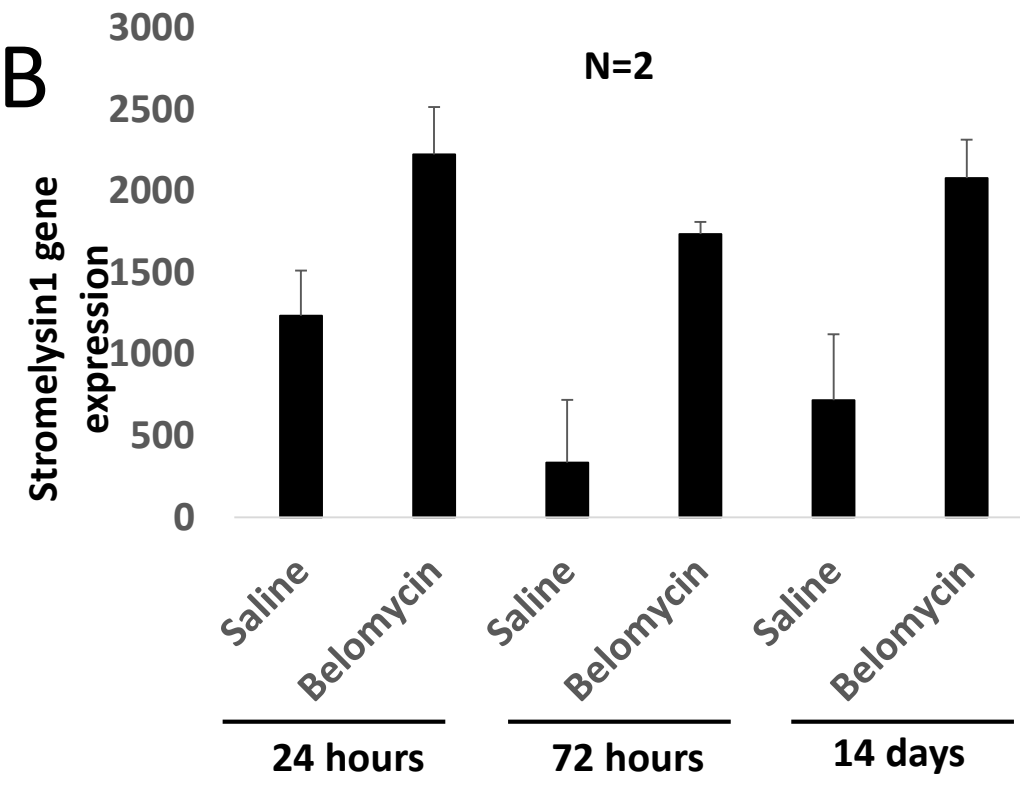
A



Profile	GDS5261 / A_23_P161698
Title	Interleukin 4, interleukin 13 and tumor necrosis factor-α effect on Matrigel-cultured IMR-90 lung fibroblast cells
Organism	Homo sapiens

MMP3 gene expression in TNFα-induced lung fibroblasts

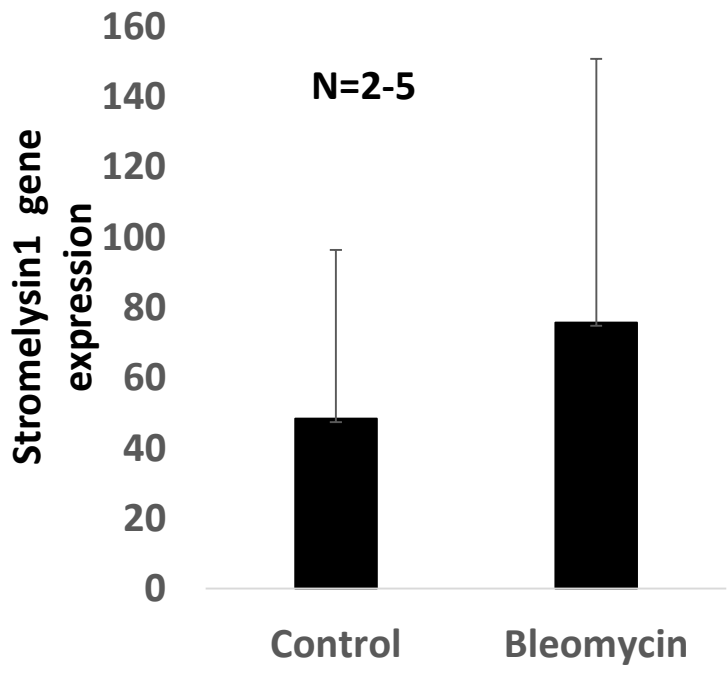
B



Profile	GDS251 / 98833_at
Title	Pulmonary fibrosis
Organism	Mus musculus

MMP3 gene expression in Normal vs. Fibrotic lung

C



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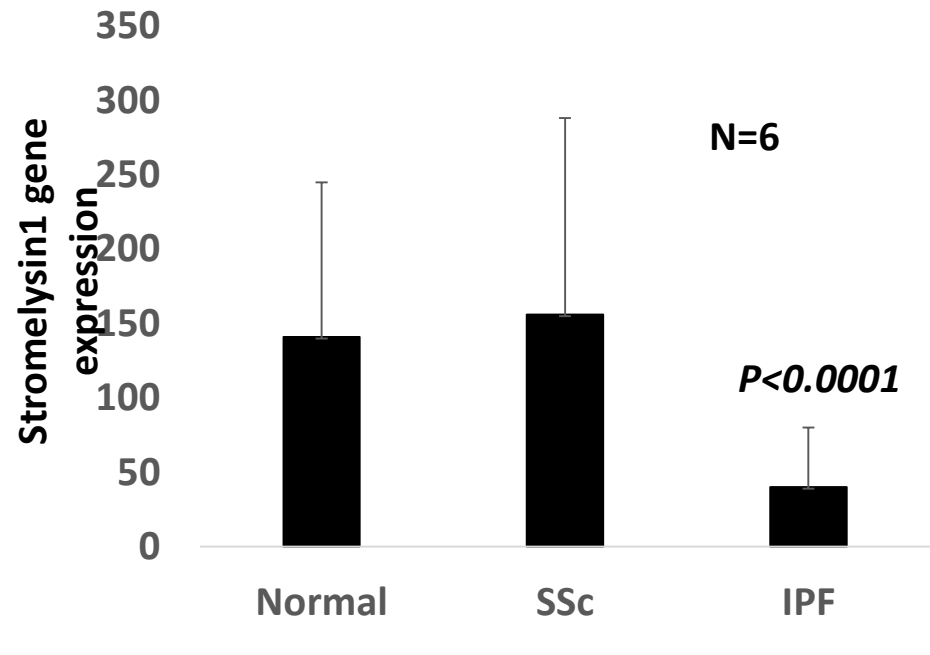
Title	Bleomycin effect on lungs: dose response and time course
Organism	Mus musculus

MMP3 gene expression in Normal vs. bleomycin-treated lungs

D



Profile	GDS1012 / 437_at
Title	Idiopathic and scleroderma-associated pulmonary fibrosis derived fibroblasts
Organism	Homo sapiens

MMP3 gene expression in Normal vs. IPF human fibroblasts