Newt anterior gradient (nAG) protein, a salamander-derived protein, as an inhibitor of TGF-β signaling and fibrotic responses

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ABSTRACT

Background: Salamanders have the amazing ability to regenerate their limbs within 30 days when amputated. One of the key proteins responsible for this regeneration is the newt anterior gradient (nAG) protein. A previous study demonstrated that local injection of a recombinant nAG protein reduces hypertrophic scarring in a rabbit ear model. Fibrotic disorders of the skin such as scleroderma, hypertrophic scarring and keloids are characterized by excessive TGF-β action, leading to an increase in deposition of collagen and other extracellular matrix (ECM) components, resulting in functional impairment which is often debilitating. To date, the treatment options remain limited. We hypothesized that nAG protein would inhibit the TGF-β signaling pathway and fibrotic responses in skin fibroblasts of scleroderma patients and may therefore have therapeutic potential for the treatment of scleroderma. The aim of this study is to examine the effectiveness of nAG protein to inhibit fibrotic responses in scleroderma skin fibroblasts and study its regulation of the TGF-β signaling pathway.

Methods: Fibroblasts from lesional scleroderma patient skin were treated with nAG protein in doses of 0, 100 pM,1 nM and 10nM for 24 hrs and were then left untreated or treated with 20 pM of TGF-β. TGF-β-mediated pro-fibrotic responses were determined by measuring the levels of ECM (collagen III, Fibronectin), connective tissue growth factor (CTGF/CCN2) and alpha smooth muscle actin (α-SMA) protein production by Western blot and immunofluorescence. Also, gene expression at the mRNA level was determined by quantitative PCR. Activation of the TGF-β pathway was determined by measuring the TGF-β receptor 1 (ALK5) and phosphorylated Smad2/3 levels by using Western blot and immunofluorescence.

The ability of nAG protein to inhibit Smad3-driven transcriptional activity induced by the three TGF-β isoforms (-β1, -β2, -β3) was determined using a luciferase (CAGA12-lux) reporter assay. Cell migration was assessed using an in vitro wound healing (scratch) assay and finally colocalization of nAG protein with ALK5 was evaluated using confocal microscopy.

Results: The results show that treatment with nAG protein inhibits TGF-β-induced fibrotic responses as shown by a decrease in the levels of collagen III, alpha smooth muscle actin (α-SMA), connective tissue growth factor (CTGF/CCN2) and fibronectin in scleroderma patient skin fibroblasts. In addition, nAG protein treatment decreases the levels of ALK5 and phosphorylated Smad2/3, thus inhibiting the TGF-β pathway. Furthermore, nAG protein inhibited TGF-β1-dependent activation of Smad3-driven (CAGA12-lux) by 83% while it inhibited TGF-β2 and TGF-β3 activity by 30% and 50%, respectively. Cell migration is significantly inhibited with nAG protein treatment and confocal microscopy revealed that nAG protein and ALK5 receptors co-localize with each other.

Conclusion: Treatment with nAG protein leads to a decrease in ECM protein production and myofibroblast differentiation in scleroderma patient skin fibroblasts. This is associated with diminished TGF-β/Smad2/3 signaling, Smad3-driven transcriptional activity, ALK5 expression levels and TGF-β-induced cell migration, suggesting that the anti-fibrotic effects of nAG protein are mediated by inhibition of the canonical TGF-β signaling pathway. This research is anticipated to lead to the development of an injectable nAG protein-based antifibrotic agent for the treatment of fibrotic disorders such as scleroderma, hypertrophic scars and keloids.

CONTRIBUTION TO ORGINAL KNOWLEDGE

1. I have demonstrated that nAG protein significantly inhibits fibrotic responses in human scleroderma fibroblasts.

2. I have showed that the nAG protein suppresses the canonical TGF-β pathway.

3. I have demonstrated that nAG protein is a potent inhibitor of TGF-β induced cell migration.

4. I have demonstrated that nAG protein most effectively inhibits the responses induced by the TGF-β1 isomer, suggesting that nAG protein decreases fibrotic responses in a TGF-β1-isoform specific manner.

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RESULTS

Figure 1. **nAG protein inhibits TGF-β1-induced ECM protein production and myofibroblast differentiation.** Scleroderma patient fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of the indicated amounts of nAG protein for 24 hours. Cell lysates were analyzed by Western blot using antibodies to detect fibronectin, collagen type III, α-SMA (marker for myofibroblast differentiation), CTGF/CCN2 and α-tubulin as a loading control. Scleroderma fibroblast cells treated with only TGF-β1 (without nAG) showed a significant increase in fibrotic responses compared to the control group. TGF-b1-induced fibronectin, collagen III, α-sma and CTGF protein levels were all **decreased** when treated with **nAG** after 24 hours. Concentrations of **1 nM** and **10 nM** were most effective in inhibition while 0.1 nM (100 pM) was less effective.

Figure 2. nAG protein inhibits TGF-β1-induced ECM protein production and myofibroblast differentiation. After repeating the experiment shown in Figure 1 three times with fibroblasts from 3 different patients, densitometry of the western blot band intensities was performed using IMAGE J software and a statistical analysis was performed using a Student's t-test. This revealed a 50% decrease in fibronectin, collagen III, α-sma and CTGF protein when nAG was used compared to the TGF-β group alone (without nAG) (*$P < 0.05$).

Figure 3. nAG protein inhibits TGF-β1-induced fibronectin production in scleroderma fibroblasts. Scleroderma fibroblasts were treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 24 hours. Cells were fixed and stained with an anti-Smad2 antibody and detected by immunofluorescence. On Immunofluorescence 24 hours after treatment of scleroderma fibroblasts, fibronectin staining (green) was increased in the TGF-β group alone with a marked decrease in fibronectin staining when nAG at a concentration of 10 nM was added.

Figure 4. nAG protein inhibits TGF-β1-induced collagen type III mRNA expression in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated in the presence of 0, 0.1, 1.0 or 10 nM nAG protein for 24 hours. Total RNA was extracted and analyzed by qPCR to measure type III collagen mRNA and GAPDH (internal control). A Q-PCR for collagen III of scleroderma fibroblasts revealed an increase in collagen III mRNA when treated with TGF-β alone. This effect was inhibited when scleroderma fibroblasts were treated with nAG protein with a 50% reduction in collagen III mRNA at concentrations of 0.1 nM and 1.0 nM ($P < 0.05$). The 10 nM concentration of nAG did not reach significance.

Figure 5. nAG protein decreases TGF-β1-induced phosphorylation of Smad2 and Smad3 in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of 0, 0.1, 1.0 or 10 nM of nAG protein for 1 hour. Cell lysates were

analyzed by Western blot with antibodies to detect phosphorylated and total Smad2 and Smad3. Western blot after 1 hour of treatment with nAG revealed a marginal decrease in pSmad3 at a nAG concentration of 1 nM and a significant decrease in pSmad2 at concentrations of 1 nM and 10 nM.

Figure 6. nAG protein decreases ALK5 receptor levels in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of 0, 0.1, 1.0 or 10 nM nAG protein for 24 hours. Cell lysates were analyzed by Western blot using antibodies to detect ALK5 and α-tubulin (loading control). The TGF-β treated group without nAG protein increased the amount of ALK5 protein while nAG protein treatment at concentrations of 1 nM and 10 nM decreased the ALK5 levels to almost equivalent to the control group thus inhibiting the effect of TGF-β signaling. nAG protein at 100 pM did not decrease ALK5. After repeating the experiment 3 times with scleroderma fibroblasts from 3 different patients, densitometry of western blot band intensities revealed a 50 % reduction in ALK5 in the presence of nAG protein at concentrations of 1 nM and 10 nM (*$P < 0.05$)

Figure 7. nAG protein inhibits TGF-β1-induced migration in scleroderma fibroblasts. Scleroderma fibroblasts were scratched with a pipette tip and left untreated or treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 48 hours. At 0 hours, the wounds were equal in width in all three groups. 48 hours after wound creation the TGF-β group showed faster cell migration (wound closure). nAG treatment at a concentration of 10 nM decreased TGF-β1-induced migration (wound closure) to a level similar to the control group. Quantitation of results is presented in Figure 8.

Figure 8. nAG protein inhibits TGF-β1-induced migration in scleroderma fibroblasts. Scleroderma fibroblasts were scratched with a pipette tip and left untreated or treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 48 hours. Experiments were performed on fibroblasts from 3 different scleroderma patients. Densitometry was performed on western blot band intensities using Image J software. The nAG treatment group at a 10 nM concertation showed a significant inhibition of TGF-β1-induced cell migration ($P < 0.05$).

Figure 9. nAG protein inhibits TGF-β-induced Smad3-driven ($CAGA_{12}$-lux) activation in 293T cells. Using a luciferase assay with CAGA reporter cells with different isomers of TGF-β revealed nAG protein to be most effective in inhibiting TGF-β1 signaling by 83%. TGF-β2 and TGF-β3 isomer activity were also reduced by 30% and 50% respectively. All three isomer inhibitions were statistically significant ($P < 0.05$).

Figure 10. nAG protein colocalizes with ALK5 in scleroderma fibroblasts. Scleroderma fibroblasts were treated with 10 nM of nAG protein for 24 hours. Cells were fixed and

immunostained for nAG protein (green), ALK5 (red) and counterstained with DAPI (nuclear stain). Immunofluorescence confocal microscopy reveals colocalization of ALK5 (red) and nAG protein (green) shown by the yellow color in the merged image mostly within the cytoplasm.

ABBREVIATIONS

AEC	apical epidermal cap
AGR2	anterior gradient 2
ALK	activin receptor-like kinase
BMP	bone morphogenetic proteins
BrdU	bromodeoxyuridine
CCN2	connective tissue growth factor
COL1A1	collagen 1 antibody
COL3AI	collagen 3 antibody
Co-SMAD	receptor-regulated SMADS
CTGF	connective tissue growth factor
DMEM	Dulbecco's Modified Eagle's medium
EB	early bud
ECM	extracellular matrix
ED	early differentiation
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

ELISA	enzyme-linked immunosorbent assay
EMT	epithelial to mesenchymal transition
ERK	extracellular Signal-Regulated Kinase
FGF	fibroblast growth factor
GDF	growth and differentiation factors activins
GPI	glycosylphosphatidylinositol anchored protein
H&E	hematoxylin and eosin stain
IF	immunofluorescence
I-SMAD	antagonistic SMAD
JNK	Jun N-terminal kinases
LAP	latency-associated protein
LB	late bud
LTBP	latent binding protein
MAD	mothers against decapentaplegic
MAPK	K mitogen activated protein kinase
MIF	Mullerian inhibitory factor
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
MSV	murine sarcoma virus
nAG	newt anterior gradient
PAI	plasminogen activator inhibitor
PAL	palette
PD	positional identity
PKCδ	protein kinase C delta
PSMAD	phosphorylated SMAD
QPCR	quantitative polymerase chain reaction
Ras/MEK/ERK	MAPK/ERK kinase pathway
R-SMAD	receptor kinases
RT-PCR	real-time polymerase chain reaction
SEI	scar elevation index
SGF	sarcoma growth factor
Sma	small body size
SMAD	for SMA/MAD related
Sp1	specify protein 1
Src	proto-oncogene tyrosine-protein kinase Src

SSc	systemic sclerosis
TFP	three-finger protein
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
TIMP	tissue inhibitor of metalloprotease
α-sma	alpha smooth muscle actin

CHAPTER 1:
INTRODUCTION

INTRODUCTION

1.1 Rationale for the current study

Fibrotic disorders such as scleroderma are known to occur due to overproduction and excess deposition of collagen as well as other extracellular matrix (ECM) proteins. These disorders can affect any organ and are often debilitating. Some treatments do exist for fibrotic diseases however to date they are limited and further research is required to find new ways to treat fibrotic disorders (2).

Transforming growth factor-β (TGF-β) is a multifunctional growth factor that is considered to be one of the most potent profibrotic stimulus to fibroblasts causing an increase in ECM production and thus worsening of the fibrotic condition (3). Moreover, extensive evidence has shown that the canonical TGF-β pathway is critically involved in the pathogenesis of fibrosis (4).

Unlike humans, salamanders have the amazing ability to regenerate their limbs completely when amputated and their wounds heal with very little inflammation and never leads to scar formation (5). During salamander limb regeneration, a protein named newt Anterior Gradient (nAG) is secreted from the Schwann cells of the regenerating nerve as well as the wound epidermis. This protein was found to play a key role in proliferation and differentiation of the stem cells at the amputation site. In the absence of nAG protein, amputation of a limb leads to fibrosis and regeneration failure in salamanders. This emphasizes the importance of nAG protein in the regeneration and healing process (1).

In previous studies, nAG showed great antifibrotic potential when transfected in human fibroblasts. Furthermore, injection of nAG recombinant protein decreased formation of hypertrophic scarring in a rabbit ear model (6, 7).

Taking into account the previously described antifibrotic effects nAG had on normal human fibroblasts, the current thesis investigates the effects of nAG recombinant protein on scleroderma patient skin fibroblasts. The goal was to obtain insights on the effects of this protein on scleroderma fibroblasts and how it regulates the canonical TGF-β pathway. This research is anticipated to lead to the development of an injectable nAG protein-based antifibrotic agent for the treatment of fibrotic disorders such as scleroderma.

1.2 Scleroderma is a fibrotic disease

Scleroderma, also known as systemic sclerosis (SSc) is an autoimmune connective tissue disease that causes sclerotic changes in the skin and can also affect many other organs. The two main types of scleroderma are the localized form (localized scleroderma) which affects mainly the skin of the face, arms or hands and the systemic form (diffuse scleroderma) which affects larger areas of the skin and one or more internal organs. Morbidity and mortality of this disease is high and treatments such as vasodilators and immunosuppressants are mainly aimed for symptomatic relief. Further research is needed to obtain better therapeutic options to target the pathology behind scleroderma (8).

The pathogenesis of scleroderma is due to abnormal immune activation, vascular dysfunction and overproduction and excess deposition of ECM such as collagen (9). Overactivation of lesional fibroblasts plays an important role in the fibrotic process and a

comparison between normal fibroblasts with SSc fibroblasts *in vivo* revealed SSc fibroblasts to have elevated expression of collagen as well as fibronectin (3).

Interestingly, the amount of TGF-β, an important factor that increases fibrosis, produced by SSc fibroblasts has been shown to be equal to the amount produced in normal fibroblasts *in vitro* (10). However the key receptors involved in the TGF-β signaling pathway , both TGF-β receptor I (TGF-βRI) and TGF-β receptor II (TGF-βRII) expression, were shown to be elevated in dermal fibroblasts of skin sections from patients with SSc in comparison to normal fibroblasts (11), which could explain the increase in collagen production of SSc fibroblasts. SSc fibroblasts were found not only to have an increase in TGF-β receptor levels but also showed an increase in both protein and mRNA levels of SMAD3 as compared to healthy controls (12). Levels of phosphorylated SMAD2/3 and their nuclear localization were also elevated suggesting an increase in the activation of the canonical TGF-β pathway (12). It is therefore anticipated that developing specific therapeutic agents to block the canonical TGF-β pathway may lead to novel agents for the treatment of scleroderma patients.

1.3 Transforming growth factor-β in fibrosis

TGF-β is a member of the TGF-β superfamily, which is comprises as many as 100 different proteins that share at least one region of similar amino acid sequence (13). The family includes TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins and Mullerian inhibitory factor (MIF). These different cytokines have several unique roles such as that of BMP-2 and BMP-7 in inducing cartilage and bone formation (14). TGF was initially named sarcoma growth factor (SGF) when it was first discovered in 1980 in cells transformed by murine sarcoma virus (MSV) (15). It was later found to be composed of

two factors - TGF-α and TGF-β. TGF-α was discovered to compete for binding to the epidermal growth factor receptor (EGFR), however TGF-β did not (16).

TGF-β is an extracellular protein that is synthesized and secreted by many parenchymal cell types, and is also produced by infiltrating cells such as lymphocytes (e.g. T cells), monocytes/macrophages, and platelets (17, 18). The three isoforms in mammals are TGF-β1,-β2 and -β3. Both TGF-β1 and -β2 promote fibrosis when unchecked. However, it is still unclear if TGF-β3 has a pro or anti-fibrotic effects (or both) (19).

TGF-β1 is the most studied of the three TGF-β isoforms and is important in processes involved in tissue development and wound healing such as cell proliferation, angiogenesis, cell cycle progression and cell migration. It has also been shown to promote tumorigenesis in the later stages of tumor development (13, 20). It has also been identified as playing a crucial role in the pathogenesis of fibrotic disorders such as, hypertrophic scars, keloids and scleroderma by stimulating excessive synthesis of collagens, fibronectin, proteoglycans, plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloprotease-1 (TIMP-1) as well as inducing connective tissue growth factor (CTGF/CCN2) and fibroblast to myofibroblast trans-differentiation (4, 13).

TGF-β1 is 25 kDa homodimeric protein and is secreted by cells as a latent, inactive complex containing two proteins, the mature TGF-β dimer and the latency-associated protein (LAP). This complex has been named small latent TGF-β. A third protein called latent TGF-β binding protein (LTBP) which binds to LAP has also been found to form even bigger complexes called large latent TGF-β complexes which are secreted more rapidly. LTBPs mediate the association of the latent form of TGF-β1 with the ECM and serve to store TGF-β in the ECM.

The active form of TGF-β is generated as a result of dissociation of LAP from the mature TGF-β by several different factors such as proteases, integrins, pH, and reactive oxygen species. Once activated the TGF-β ligand will initiate the canonical TGF-β signaling pathway by binding to its high-affinity cell surface receptors (21, 22).

1.4 Canonical TGF-β signaling pathway

1.4.1 TGF-β receptors

The two main receptors involved in the canonical TGF-β pathway are TGF-β receptor II (TβRII) and TGF-β receptor I (TβRI), also known as ALK5. These two receptors are structurally similar transmembrane serine/threonine kinases but type I receptor has a Gly/Ser-rich region immediately upstream from the intracellular kinase domain (23). The active form of TGF-β displays a high affinity for TβRII and does not interact with the isolated ALK5. Once TGF-β binds to TβRII, a constitutively active kinase, it trans-phosphorylates ALK5 leading to the formation of a heterotetrameric complex of two type I and two type II receptors. Signaling is achieved only when there is a formation of this complex (24). Once the TGF-β signaling pathway is triggered by the ligand with subsequent receptor phosphorylation, a group of proteins called SMADs become activated and carry the signal to the nucleus.

Endoglin, betaglycan and CD109 are three TGF-β co-receptors that do not have an intrinsic signaling function but rather regulate TGF-β signaling by different mechanisms. Betaglycan, a trans-membrane proteoglycan, binds to all three TGF-β isomers and forms a betaglycan/TGF-β/TβRII complex which facilitates TGF-β binding to TβRII and this is most evident with the TGF-β2 isomer. Betaglycan not only concentrates TGF-β on the cell surface but

also stabilizes TGF-β in a conformation that is optimal for binding to the signaling receptors (25).

Endoglin is a transmembrane glycoprotein that binds with TGF-β1 and TGF-β3 isomers, but unlike betaglycan, does not bind to TGF-β2. Complexes between endoglin and TGF-β receptors have been observed. However, their role in the signaling pathway is still unclear; and it has been found that overexpression of endoglin decreases rather than enhances TGF-β responses (25, 26). In contrast, studies have also revealed an important profibrotic role of endoglin in SSc fibroblasts via the ALK1/SMAD1 signaling pathway (27).

CD109 is a novel TGF-β co-receptor that our group has recently identified (28-30). CD109 is a GPI-anchored protein that binds the TGF-β1 isoform with high affinity, but has lower affinity for the other isoforms in cell-based assays. On the membrane, CD109 forms a heteromeric complex with the TGF-β receptors and negatively regulates TGF-β signaling and responses in skin cells (28-30). In addition, CD109 promotes TGF-β receptor internalization into caveolae and promotes TGF-β receptor degradation (31, 32). Moreover, CD109 antagonizes TGF-β-induced epithelial-mesenchymal transition (EMT) and ECM production via both Smad and MAP kinases pathways (33), establishing CD109 as an important modulator of TGF-β responses.

1.4.2 SMAD proteins

The name SMAD is a combination of both the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from the gene *Sma* for small body size) (34, 35). SMADs fall into three subfamilies: receptor-activated SMADs (R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5, SMAD8), which become phosphorylated by the

type I receptors; a common mediator SMAD (Co-SMAD: SMAD4), which forms a complex with activated R-SMADs; and inhibitory SMADs (I-SMADs: SMAD6 and SMAD7), which are induced by TGF-β family members (36). The R-SMADs involved in the canonical TGF-β signaling pathway are SMAD2 and SMAD3.

After ALK5 is phosphorylated, SMAD2 and SMAD3 in the cytoplasm transiently associate with the receptor. This interaction with the TGF-β receptor complex phosphorylates these SMADs, and once phosphorylated, they dissociate to now bind with SMAD4 to be carried into the nucleus where SMAD2 and SMAD3 regulate gene expression (37) (Fig 1) . SMAD2 was found to be associated with cellular migration and proliferation while SMAD3 is known to control the production of ECM such as collagen (38).

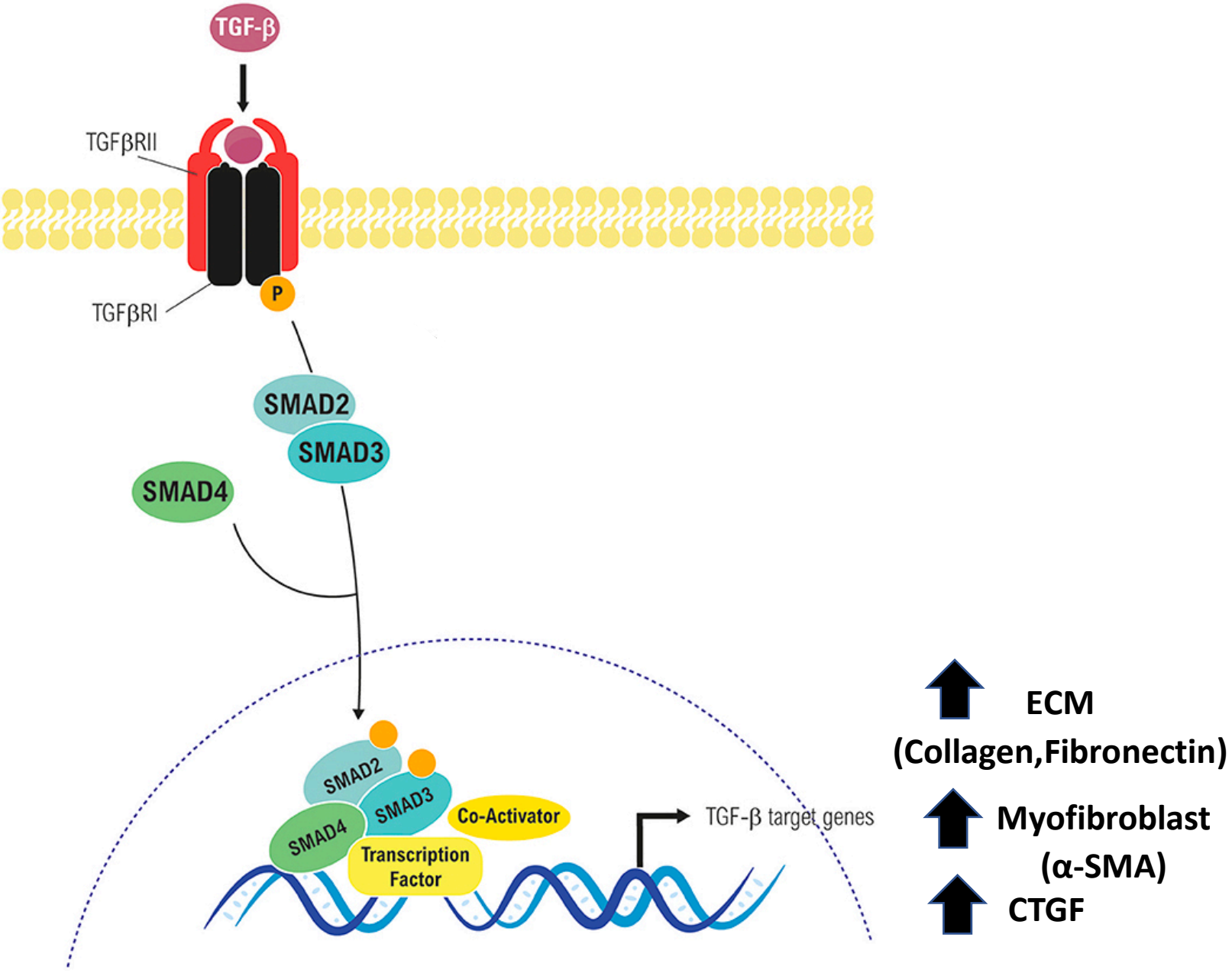


Figure 1. The canonical TGF-β pathway. TGF-β once activated forms a complex with TβRII on the cell surface. TβRII, a constitutively active kinase, then phosphorylates and activates TβRI (ALK5), which in turn phosphorylates SMAD2/3 (to form pSmad2/3). pSMAD2/3 form a complex with SMAD4 and enters the nucleus to regulate gene expression resulting in the increase in the amount of ECM, myofibroblast differentiation (a-SMA expression) and CTGF/CCN2.

1.5 Hallmarks of the Fibrotic Process

1.5.1 Extracellular matrix (ECM) production

The extracellular matrix (ECM) is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins. It provides biochemical and structural support to the surrounding cells. ECM is a highly dynamic structural network that is constantly undergoing remodeling; and deregulation of the ECM can lead to several pathological conditions (39, 40).

Excess ECM deposition such as the elevated expression of collagen types I, III, VI, VII, fibronectin, glycosaminoglycans, as well as the elevated expression of tissue inhibitor of metalloproteinases (TIMPs) and intercellular adhesion molecules have all been demonstrated when comparing scleroderma fibroblasts to healthy fibroblasts. TGF-β is a key mediator of tissue fibrosis and signaling through the canonical TGF-β pathway has been shown to increase ECM synthesis. SSc fibroblasts have been shown to be more sensitive to the actions of TGF-β leading to a hyper-fibrotic response (41).

Collagen is the most abundant fibrous protein within the interstitial ECM in all animals and is also present in pericellular matrices like the basement membrane. Its function is to provide strength and support to many tissues in the body, including cartilage, bone, tendon and skin. There are five common types of collagen (I-V), where collagen type I is the most abundant and collagen type III being the second most abundant (42). Collagen types I and III present in the skin are found to be increased in scleroderma patients (43). Fibroblasts of SSc patients also demonstrate an increase in collagen synthesis and this is further increased by adding TGF-β to the scleroderma fibroblasts in culture (44, 45).

Fibronectin is an important part of the ECM that has critical biologic activities, such as cell-to-cell adhesion, cell-to-ECM adhesion, migration and differentiation, maintenance of cellular structure and shape, wound healing, and blood coagulation (46). The structure of fibronectin has collagen binding domains which serve as the extracellular links to translate the intracellular actin-myocin contraction into an extracellular matrix contraction which occurs during wound contraction (47, 48). Increased production and deposition of fibronectin have been reported in both fibroblasts and skin sections of scleroderma patients (43, 49). SMAD3-dependent CTGF/CCN2 acts as important mediators of fibronectin gene expression in primary normal human skin dermal fibroblasts (46). Other pathways such as protein kinase C delta (PKCδ) and the Jun N-terminal kinases (JNK) pathways are also involved in fibronectin synthesis (50, 51).

1.5.2 Connective tissue growth factor

CTGF/CCN2 is a 38 kDa cysteine rich peptide that was found to be induced by TGF-β and modulates fibroblast cell growth and ECM secretion and its expression correlates with the degree of fibrosis (52). CTGF/CCN2 mRNA expression was found to be increased in several fibrotic disorders including scleroderma, keloids, and hypertrophic scars; while almost absent from healthy unaffected skin (53-55).

In SSc patients CTGF gene transcription is increased due to a substantially high CTGF promoter activity. The CTGF promoter contains a SMAD3 binding site that can be activated via the canonical TGF-β pathway. Excess CTGF expression, even in the absence of TGF-β stimulation, has been observed in in SSc fibroblasts, which could explain the persistence of fibrosis in these patients (56). It has been proposed that TGF-β, through the canonical pathway,

acts synergistically with the Ras/MEK/ERK pathways along with specificity protein 1 (Sp1) to induce CTGF expression (57, 58).

In addition to SMAD3, SMAD1 signaling also plays a pivotal role in the process of fibrosis and it has been proposed that CTGF/CCN2 contributes to the pro-fibrotic TGF-β signalling through a CCN2/αvβ3 integrin/Src/Smad1 axis and CTGF/CCN2 is indispensable in the activation of this pathway (59, 60) (Fig 2).

One of the features of skin fibrosis is the ability of dermal pericytes to differentiate into myofibroblasts and CTGF/CCN2 appears to be required for this process (60). CTGF/CCN2 also binds with fibronectin via the receptors integrins α4β1, α5β1 and syndecan 4 to potentiate the ability of fibroblasts to spread on fibronectin and matrix organisation. Furthermore, loss of endogenous CTGF/CCN2 resulted in a delay in α smooth muscle actin stress fiber formation, and reduced ERK phosphorylation (61).

Another study showed that CTGF/CCN2 binds with TGF-β through a binding site in the amino-terminal Von-Willebrand factor domain of CTGF and assists in presenting TGF-β to the high-affinity TβRII; thus increasing TGF-β signaling (62). Inhibiting CTGF was associated with a marked reduction in the number of myofibroblasts in hypertrophic scars, decreased transcription of TIMP-1 and a reduction in collagen I and III (63, 64).

TGF-β alone seems to be responsible for the initiation of fibrosis but co-application of CTGF causes the fibrotic response to TGF-β in the skin to persist (65).

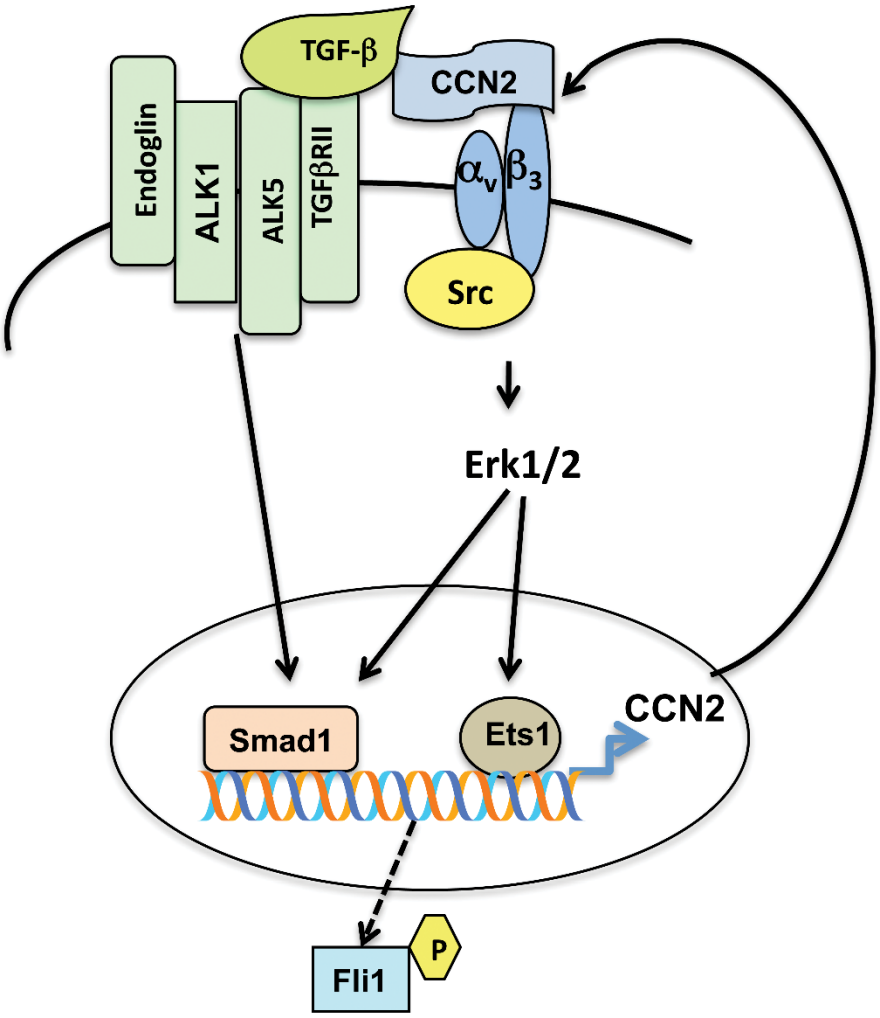


Figure 2. A proposed model for the positive feedback loop between TGF-β/Smad1 and CCN2 pathway. Taken from Nakerakanti et al 2011 (66).

1.5.3 Myofibroblast differentiation & cell migration

Fibroblasts are mesenchymally-derived spindle shaped cells that synthesize collagen to provide structural support to organs and tissues of the body, and they are also vital for wound healing. A myofibroblast is a contractile cell that is in between a fibroblast and a smooth muscle cell in phenotype. Myofibroblasts express α-smooth muscle actin (α-sma) which is important in the contractility of the cell and causes wound contraction (67). There are multiple origins of myofibroblasts, which include, resident fibroblasts that underwent myofibroblast transition, pericytes which express Sox2, precursors of fibroblasts in the blood named fibrocytes, and epithelial cells that undergo epithelial to mesenchymal transition (EMT).

During EMT, decreased expression of E-cadherin occurs. E cadherin is known to be responsible for maintaining the lateral contacts of epithelial cells via adherens junctions and for cell-cell adhesion. When E-cadherin is decreased this allows the keratinocytes to become mobile and several growth factors such as epidermal growth factor (EGF), Fibroblast growth factor (FGF), CTGF and TGF-β, increase expression of transcription factors (SNAIL,Slug,TWIST) and this results in keratinocyte to myofibroblast transition (68, 69). TGF-β directly increases the motility of cells; but cell migration was found to be induced via both SMAD dependent and SMAD independent pathways (ERK, JNK, p38) (70, 71).

In normal wound healing, myofibroblasts disappear from the site of injury; however, in fibrosis they persist. During fibrosis such as in scleroderma, an increased number of fibroblasts migrate towards the lesion and cells called myofibroblasts develop. The accumulation and persistence of a large number of myofibroblasts are responsible for the uncontrolled increase production and deposition of ECM in scleroderma. TGF-β induces the increased population of myofibroblasts seen in the skin of SSc patients (60, 67, 72, 73).

1.6 Salamander limb regeneration

Urodeles are a group of vertebrates that have the amazing ability to perfectly regenerate various tissues after amputation or injury, completely scar free. Two commonly studied urodeles in the lab are axolotl and newts which can regenerate body parts (limbs, tails heart, spinal cord) and heal their wounds without fibrosis throughout their entire lives. Among all of the complex structures that the salamanders can regenerate, limb regeneration has been the best described and studied. Such studies will aid us in understanding why humans form scar tissue instead of perfect regeneration and this may lead to the development of novel medical treatments (74).

Following amputation, the stages of limb regeneration comprise of two major phases, known as the preparation phase and the re-development phase. In the preparation phase, epidermal cells migrate from the amputation stump to cover the wound and form the apical epidermal cap (AEC). Cells at the amputation stump then begin to lose their differentiated characteristics and become de-differentiated. These de-differentiated cells migrate beneath the AEC and form a mound of mesenchymal stem cells called the blastemal, which starts to proliferate. The blastemal cells originate from fibroblasts and other surrounding cells. However, fibroblasts were the cells that were shown to be essential for growth control and pattern formation in the blastema (75). It was found that during the early stages of this phase, a strong expression of TGF-β is present in the blastema, possibly to increase mesenchymal cell migration towards the blastema. It has been proposed that TGF-β and the canonical pathway, specifically via SMAD2, is important in this phase of limb regeneration (76). In a study to analyze the role of TGF-β signaling in salamander limb regeneration, an ALK5 inhibitor (SB-431542) was used. SB431542 blocked limb regeneration and prevented blastema formation, establishing that TGF-β is essential in the early preparation phase of limb regeneration (77). However, in the same study

TGF-β was found to be barely visible in the re-development phase; suggesting that although TGF-β is essential in the early stages of limb regeneration, during the later stages TGF-β is almost non-existent in the re-development phase. In the re-development phase the blastemal cells are re-differentiated to regenerate the lost parts and a new limb is developed (78).

1.6.1 newt Anterior Gradient protein (nAG) &Prod 1

At approximately 5-7 days after amputation in salamanders, nAG protein is secreted from the Schwann cells that make up the nerve sheath of the regenerating axons. At around 10-12 days post amputation, the nAG protein is expressed in glands in the dermis underlying the wound epithelium and also by glands under the skin just proximal to the amputation plane. nAG was found to act directly on blastemal cells by stimulating their proliferation and promoting entry of these cells into the S phase (79). nAG secretion was found to be nerve-dependent. After denervation of the brachial plexus of amputated limbs of newts, there is no nAG secretion from Schwann cells (80). This results in failure of limb regeneration; and instead results in fibrosis of the stump despite the presence of an initial cohort of blastemal cells. The addition of nAG protein to these denervated limbs was able to rescue the regeneration process which continued to the digit stage. The amount of muscle, however, was reduced in denervated limbs despite the addition of nAG protein suggesting that perfect muscle regeneration is not possible by substituting nAG alone (1).

The nAG protein is a ligand for a receptor named Prod1. Prod1 is a glycosylphosphatidylinositol (GPI) anchored protein present on the cell surface and is a member of the three-finger protein (TFP) superfamily. It was identified by the use of retinoic acid and screening cDNA of newt blastemal cells. Retinoic acid was found to increase Prod 1 expression and is responsible for positional identify. It is expressed in a gradient along the Proximo-distal axis of the regenerating limb. The more proximal the amputation, the higher the degree of expression of Prod 1; and the opposite applies. For example, a blastema formed at the level of the wrist will have lower Prod 1 expression and result in regeneration of only the missing hand whereas amputation at the shoulder level will have higher Prod 1 expression and result in

regeneration of the entire limb. This is known as positional or proximo-distal (PD) identity (81-83).

In addition to positional identity, Prod 1 was found to act in cell-cell interactions, to increase matrix metalloproteinase 9 (MMP9) expression involved in the breakdown of ECM through activation of epidermal growth factor receptor (EGFR) signaling and phosphorylation of ERK1/2 , and to induce blastemal proliferation when bound to its ligand nAG(84) (79, 85).

MMP9 is essential in preventing scar formation during the regenerative process and this might explain why in the absence of nAG protein the limb stump heals by fibrosis (86).

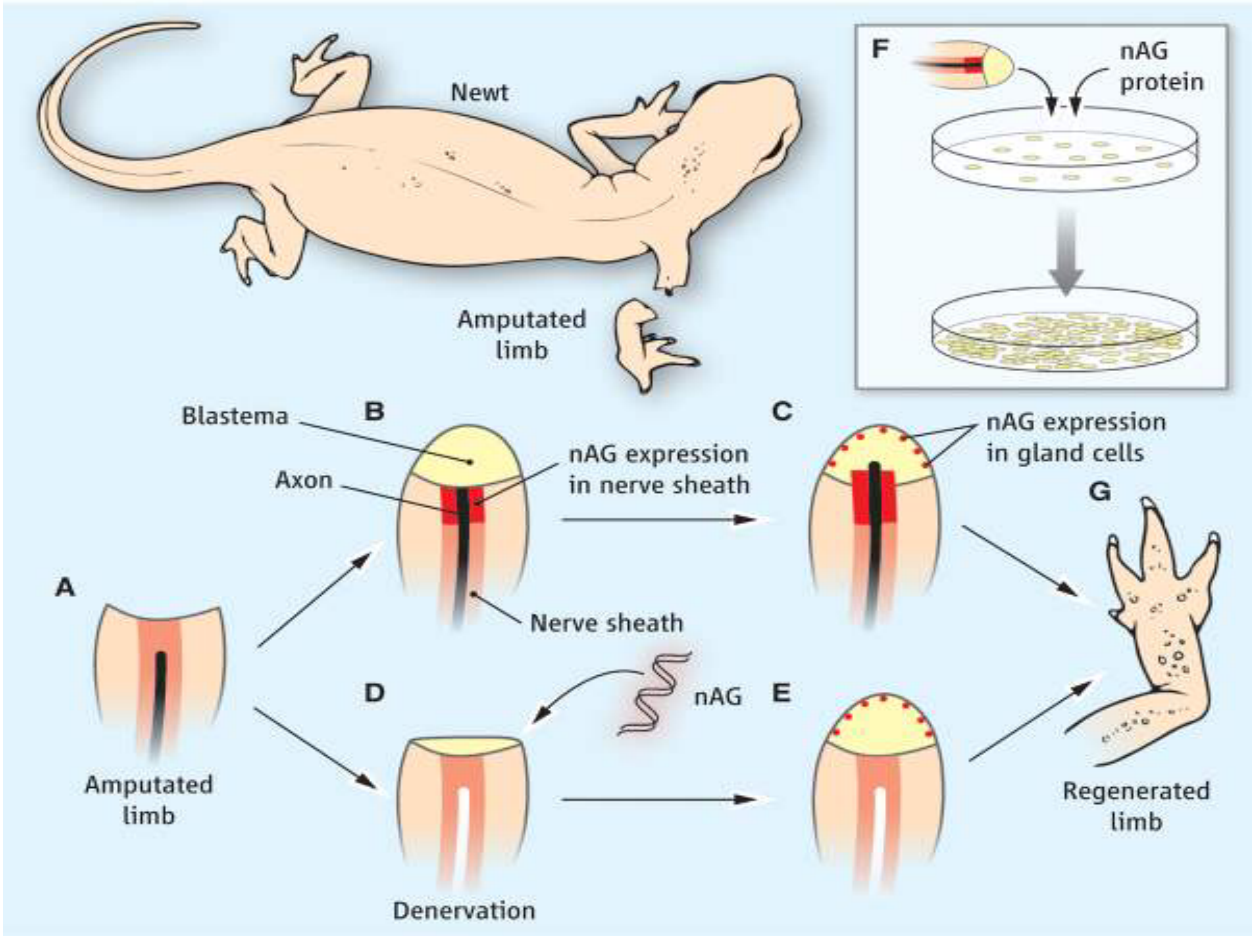


Figure 3. Regeneration of salamander limbs requires concomitant regeneration of the severed nerves. A single protein, nAG, can substitute for the regenerating nerve cells (1).

1.7 Human homologs of nAG & Prod 1

The mammalian homolog of newt Anterior Gradient (nAG) in mammals is anterior gradient 2 (AGR2). AGR2 is 69% similar at an amino acid level to nAG and unlike its salamander counterpart, AGR2 is retained in the endoplasmic reticulum and is not secreted (87). This protein plays a role in cell migration, cellular transformation and metastasis and as a p53 inhibitor. AGR2 is upregulated in certain cancers such as breast adenocarcinomas, pancreatic and gastrointestinal cancers (88). Knockout AGR2 mice show an imbalance in differentiated cell types of the gastrointestinal tract and defects in mucous production (89). A GPI-anchored AGR2 receptor was identified named C4.4A belonging to the same three finger protein superfamily as Prod 1. The interaction of AGR2 with C4.4A has been shown to increase the aggressiveness of cancer cells by increasing tumor growth and metastasis (90). The nAG protein in salamanders stimulates proliferation of stem cells, while the AGR2 in humans stimulates cancer cell proliferation and they both seem to act via indirectly stimulating epidermal growth factor receptor (EGFR) signaling (84, 91). Interestingly, AGR2 induced pancreatic tissue regeneration in a pancreatitis mouse model by promoting EGFR delivery to the cell surface. EGFR signaling then can stimulate DNA synthesis and cell proliferation and this resulted in recovery from the pancreatitis and tissue regeneration (92).

There is no known homolog for the nAG receptor Prod 1. Although it was initially thought that the human homolog of Prod 1 was CD59; further studies revealed only a 22% sequence identity between the two proteins; and Prod 1 was never found in mammals. The absence of this receptor may explain the lack of limb regenerative capabilities in humans (79, 82).

1.8 Previous on nAG research

1.8.1 Overexpression of nAG in human fibroblasts

In 2013 a nAG gene was designed for optimal production in human cells. nAG mRNA and protein sequences were obtained from the NCBI nucleotide database of the *notophthalmus virdescens (*newt) anterior gradient protein (7). The 166-amino acid sequence of the protein was then entered into DNA 2.0 Gene Designer Software and the software was set to codon-optimize the protein sequence to homo sapiens for efficient expression in human cells. A V5 peptide was added to the sequence for later detection of the protein using anti-V5 antibody. This was the first nAG plasmid that was designed to be expressed in human cells (7).

The nAG plasmid was then transfected into primary human fibroblasts. Western blot analysis of the transfected cells revealed nAG protein expression in human fibroblast with a molecular weight of 18.9 kDA. Importantly, the results showed that expression of nAG had an inhibitory effect on proliferation of transfected human fibroblasts. In addition, the expression of nAG protein in these cells suppresses basal and TGF-β-induced expression of collagen I and III. This suppression was shown to be due to a dual effect of nAG both by decreasing collagen synthesis and by increasing collagen degradation. Interestingly, gelatin zymography showed that nAG increased pro-MMP-2 by 37% with an 85% increase in the active form of MMP-2 in the transfected fibroblasts. Pro-MMP-1 levels showed a 53-fold increase in transfected fibroblasts and a 4-fold increase when fibroblasts were treated with recombinant nAG protein (7). This was the first experiment that revealed nAG's antifibrotic potential demonstrated by the increase in MMPs which degrade collagen, decrease in fibroblast proliferation and decrease in collagen I

and III with a more pronounced effect on collagen III (7). This is relevant because collagen III overproduction was found to be a hallmark of pathological fibrosis such as in hypertrophic scars (93).

1.8.2 Injection of nAG in hypertrophic scar model

In another study, the same authors showed the ability of nAG protein to prevent the formation of hypertrophic scars (6). Five rabbits were used in a hypertrophic scar ear model where a 7-mm punch biopsy was created down to bare cartilage on the ventral surface of the ear. Daily injections of 100 µl of either 100 nM nAG solution or saline alone were done starting after wound epithelization on day 14 and ending after hypertrophic scar formation at day 27. Scar elevation index at day 28 after wounding was found to be significantly lower in the nAG group compared to the control group. Histological analysis revealed the nAG group to have thinner, more arranged collagen fibers as compared to the thick, dense and disorganised collagen fibers of the scars of the control group 28 days after wounding. Real-time PCR showed a 95% decrease in collagen I mRNA, and a 48 % decrease in collagen III mRNA in the nAG group scars compared to the control group. The explanation of why in rabbits, collagen I is more inhibited, while in humans, collagen III is more inhibited by nAG, may be due to the different composition proportion of collagen I to collagen III between human skin and rabbit skin. Collagen I is the most abundant collagen in normal human skin while collagen III is the most abundant in normal rabbit skin (94).This experiment displayed the potent antifibrotic effects of nAG recombinant proteins on hypertrophic scars (6).

1.8.3 Injection of nAG in a distal digit amputation model

In a third study, the same authors added both human optimized nAG and its receptor Prod-1 plasmids to mice amputated digits (87). Using adult mice, the central three digits of one hind foot limb were amputated at the level of the base of the distal phalanx and was left open. Co-transfection by direct injection of 50 µl of DNA plasmid solution (containing 100 µg of nAG and 100 µg of Prod-1) into the digital footpad opposite the base of the central (third) digit was performed 1 day before amputation and twice weekly until tissue collection. Control groups were injected with 50 µl of saline without plasmids. Tissue was collected at 14 days for histologic evaluation and 28 days for histology, immunohistochemistry and Quantitative PCR. On gross appearance of the amputation stumps at 28 days, the nAG group mice had less swollen digits and a more tapered appearance as compared to control mice. At 14 days, hematoxylin and eosin (H&E) staining revealed that the nAG group stumps showed immature bone proximally and cartilage differentiation distally whereas control group stumps showed only fibrous tissue. At 28 days, the nAG group showed mature bone as compared to the control group which showed woven bone formation. Staining for osteocalcin, a marker for new bone formation and osteoblast activity, revealed the nAG group stumps to have mature bone with cortex delineation and a medullary cavity, while the control stumps showed scattered staining mixed with fibrous tissue at 28 days. Q-PCR showed that osteocalcin mRNA was increased by 21-fold, and cartilage specific markers collagen X and Indian hedgehog were increased by 2.377 and 1.34 times respectively in the nAG group as compared to the control group 28 days after amputation. This study demonstrated that the addition of nAG and its receptor Prod-1 led to faster mature bone regeneration and a significantly higher osteoblast activity after digit amputation (95).

CHAPTER 2:
MATERIALS AND METHODS

MATERIALS & METHODS

2.1 Antibodies used

Purified mouse Anti-Fibronectin (610078) was obtained from BD Biosciences (Mississauga, ON). TGFβ RI Antibody (V-22): sc-398 and COL3A1 Antibody (B-10): sc-271249 were both from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370, Anti-rabbit IgG, HRP-linked Antibody #7074 and Anti-mouse IgG, HRP-linked Antibody #7076 were purchased from Cell Signaling Technology (Danvers, Massachusetts). Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 were bought from Thermo Fisher Scientific (Waltham, Massachusetts). Finally, Anti-CTGF antibody (ab6992), Anti-alpha smooth muscle Actin antibody (ab5694), Anti-alpha Tubulin antibody [DM1A] - Loading Control (ab7291), Anti-Smad3 (phospho S423 + S425) antibody [EP823Y] (ab52903), Anti-Smad3 antibody (ab55480), Anti-Smad2 (phospho S255) antibody [EPR2856(N)] (ab188334), Anti-Smad2 antibody [7A5] (ab71109) and Anti-V5 tag antibody [SV5-Pk1] (ab27671) were all obtained from Abcam (Cambridge, Massachusetts).

2.2 Cell culture & treatment

Scleroderma patients were diagnosed according to the classification criteria of the American College of Rheumatology (96). Punch biopsies were taken from scleroderma skin lesions of the arm with informed consent and fibroblasts were isolated as previously described (97). Briefly, biopsies were incubated in 0.5% dispase (Invitrogen, Carlsbad, CA, USA) overnight at 4°C, and the dermis was separated from the epidermis. The dermis was incubated with 0.1% collagenase (Invitrogen) overnight and the released fibroblasts were cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. Experiments were performed using fibroblasts between passages 3 and 6. Once the cells reached 80% confluence in a T75 flask the cells were washed with PBS and trypsinized and a manual cell count was done. The cells (1 x 10^5 cells) were then plated in each well of a six well plate in 2 ml of DMEM with penicillin and FBS in each well and were left for 24 hours. The medium was then discarded and after the cells were washed with PBS, 2ml of serum free DMEM was added to each well and the cells were kept in the incubator for 24 hours to undergo serum starvation.

Human recombinant nAG protein was synthesized by Genscript Company (Piscataway, NJ, USA) as described in the previous chapter and the aforementioned papers (6, 7). 1 mg of nAG protein powder was dissolved in 1 ml PBS to give a concentration of 52.91 µM. Stock concentrations of 10 µM, 1 µM and 100 nM nAG solution were made by dilution in serum free DMEM. 2 µl of 400 nM TGF-β1 provided by (Genzyme Corporation, Framingham, MA, USA) was dissolved in 40 ml serum free DMEM to give a final concentration of 20 pM.

The 6 well plate was divided into six groups with 2 ml of medium. A control group with only serum free DMEM, secondly a group with only TGF-β1 (20 pM), and the remaining groups with

TGF-β1 (20pM) in addition to nAG recombinant protein in the final concentrations of 100pM, 1 nM, 10 nM and 100 nM respectively. After treatment, the cells were maintained at 37°C in a humidified incubator under 5% CO2/air for either 24 hours for ECM measurements or for 1 hour for phosphorylated SMAD measurement.

2.3 Western blot analysis

Cell lysis was done by adding 100 µl of 1X Laemmli buffer in each well. The cell lysates were collected from each group and heated for 10 minutes at 100°C. The samples were then mixed by vortex and by pipetting. An SDS-polyacrylamide gel was made and 20 µl of cell lysate from each sample was loaded per well. After separation by electrophoresis the gel was transferred to a nitrocellulose membrane (Fisher Scientific). This membrane was then blocked with Tris-buffered saline-Tween 20 (TBST) containing 5% milk for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The membrane was washed with TBST 3 times for ten minutes each and were incubated with the secondary antibody using either Anti-rabbit IgG, HRP-linked Antibody or Anti-mouse IgG, HRP-linked Antibody (Cell Signaling) accordingly for 2 hours at room temperature. The primary antibodies were diluted in 5% milk with TBST using the following ratio concentrations: Purified mouse Anti-Fibronectin (1:1000) (BD Biosciences). TGFβ RI Antibody (1:500), COL3A1 Antibody (1:200) (Santa Cruz Biotechnology), Phospho-p44/42 MAPK (Erk1/2) (1:1000) (Cell Signaling Technology), Anti-CTGF/CCN2 antibody (1:500), Anti-alpha smooth muscle Actin antibody (1:1000), Anti-alpha Tubulin antibody (1:4000), Anti-Smad3 (phospho S423 + S425) antibody (1:500), Anti-Smad3 antibody (1:500), Anti-Smad2 (phospho S255) antibody (1:500) and Anti-Smad2 antibody (1:500) (Abcam). The secondary antibodies, Anti-rabbit IgG, HRP-linked Antibody and Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology) were both diluted in a ratio of

(1:3000). The signals on the membranes were detected using enhanced chemiluminescence (ECL) system (Millipore, Canada). Alpha Tubulin (Abcam) was detected afterwards as a loading control.

2.4 Quantitative Polymerase Chain Reaction (qPCR) studies

Cells from six-well plates were extracted with TRIzol™ reagent (Invitrogen). Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was performed using oligonucleotide primers made by Thermofisher for human COL3A1 (forward primer, 5- TGGTCTGCAAGGAATGCCTGGA -3; reverse primer, 5- TCTTTCCCTGGGACACCATCAG -3; human COL1A1 (forward primer, 5- GATTCCCTGGACCTAAAGGTGC -3; reverse primer, 5- AGCCTCTCCATCTTTGCCAGCA -3); GAPDH (forward primer, 5- GTCTCCTCTGACTTCAACAGCG -3; reverse primer, 5- ACCACCCTGTTGCTGTAGCCAA -3). Quantitative PCR reaction was performed using DyNAmo HS SYBR Green qPCR Kit(Thermo Fisher Scientific) with an initial denaturation step of 15 min at 95°C, followed by 40 cycles of denaturation at 94°C (10 s), annealing at 60°C (30 s), and extension at 72°C (30 s) and analyzed in the Applied Bioscience's Step-one real-time instrument. Quantification of target mRNA was carried out by comparison of the number of cycles required in order to reach the reference and target threshold values (ΔΔ CT method).

2.5 In vitro wound healing assay

An in vitro wound healing (scratch) assay was used in order to assess the effect nAG had on cell migration Scleroderma fibroblast cells were seeded at a density of $6x10^5$ cells/well into Costar® 6 Well Clear TC-Treated Multiple Well Plates (Product #3516, Corning Inc, USA) and cultured for ~24 hours or until the cells had reached ~90% confluency. Cells were then pre-incubated with serum-free medium (SFM) for 24 hours to inhibit cell proliferation. The monolayer of fibroblast cells was scratched across the centre with a sterile 200 µl pipette tip to create a cell-free line. The culture medium was aspirated and washed three times to remove cellular debris. The culture plates were replenished with serum free DMEM in the absence or presence of 50pm TGF-β1 as well as 10 nM concentration of nAG protein. Samples were taken at the beginning (time 0) and at 48 h. Photographs were taken immediately at (0 h) and 48 h after the scratch and Image-J software was used to measure the width of the wound area. The experiments were repeated 3 times. Cell migration was expressed as a percentage of the scratch area filled by migrating cells at 48 h post scratch: migration rate = (T0 hr scratch width – T48 hr scratch width)/T0 hr scratch width) ×100%.

2.6 Luciferase reporter Assay

In order to assess the specificity of nAG protein's action on different TGF-β isomers, CAGA reporter cells were used in a luciferase assay. The cells contain a luciferase gene driven by a promoter containing Smad3 (CAGA) binding sites (98). The CAGA cells were plated in 24 well plates and incubated for 24 hours with DMEM and FBS. The cells were then cultured in serum free medium for 24 hours and then treated with TGF-β1 (100 pM), TGF-β2 (100 pM) or TGF-β3 (100 pM) +/- nAG protein (10nM) and incubated for 24 hours. Afterwards the cells were washed with PBS 2 times and 100 µl of Passive Lysis Buffer was added to each well and plates shaken for 20-30 min on ice. Cell lysates were collected in 1ml tubes and Centrifuged for 10 min (13000 RPM). 45 µl of each sample with a 5 µl of Assay Buffer were added to a 96 well plate. 15 ml of 0.1M KH2PO4 and 120 µl of Luciferin was used to read the plate with the Illuminometer.

2.7 Immunofluorescence & Confocal microscopy studies

Scleroderma fibroblast cells were fixed in 4% paraformaldehyde (w/v) for 15 min, and permeabilized in PBS/0.3% Triton X-100 for another 15 min. Cells were washed with PBS, and blocked in 2% BSA for 1 h. Primary antibodies against, Purified mouse Anti-Fibronectin (610078) BD Biosciences (Mississauga, ON), TGFβ RI Antibody (V-22): sc-398 Santa Cruz Biotechnology (Santa Cruz, CA), Anti-Smad3 (phospho S423 + S425) antibody [EP823Y] (ab52903) and Anti-V5 tag antibody [SV5-Pk1] (ab27671) for nAG staining from Abcam (Cambridge, Massachusetts) were added to cells at 1:250 dilution in 2% BSA and incubated overnight at 4˚C. Cells were washed with PBS and labeled for 1 h with fluorophore-conjugated secondary antibodies (1:500 dilution; Alexa Fluor 594 and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 Thermo Fisher Scientific (Waltham, Massachusetts)). Cells were washed with PBS and the slides were mounted on coverslips with

DAPI-containing mounting medium ProLong® Gold Antifade Mountant with DAPI (P36935) (Thermofisher). Cells were visualized using a LSM780 confocal microscope to analyze nAG colocalization with TGF-βR1.

CHAPTER 3:
RESULTS

RESULTS

3.1 nAG decreases ECM protein production and myofibroblast differentiation in scleroderma patient skin fibroblasts

To measure the possible antifibrotic effects nAG protein has on scleroderma fibroblasts, components of the extra cellular matrix (collagen III, fibronectin), connective tissue growth factor (CTGF) a key mediator of fibrosis and Alpha- smooth muscle actin (α-sma) a marker for myofibroblasts were measured via western blot for protein levels 24 hours after treatment. All of these components have proven to be important in the fibrotic process and are activated by TGF-β signaling. Results shown in Figure 1 indicate that the TGF-β treated group without nAG treatment showed increased protein production of fibronectin, collagen III, α-sma and CTGF as compared to the control group without TGF-β or nAG which confirmed that the TGF-β1 was stimulating the fibrotic process.

The remaining groups containing scleroderma fibroblasts treated with both TGF-β1 (20 pM) and nAG protein in concentrations of 0.1 nM, 1 nM and 10 nM revealed a decrease in protein production of collagen III, fibronectin, CTGF and α-sma. The 1 nM and 10 nM concentrations of nAG treatment were found to be the most effective at inhibiting the fibrotic responses of TGF-β1 with the 0.1 nM nAG concentration being the least effective (Fig 1). The experiment was then repeated three times with fibroblasts from three different patients and a densitometric analysis done on western blot band intensities using ImageJ revealed a protein reduction of up to 50% for all the measured fibrotic responses when nAG protein was added in concentrations of 1 nM and 10 nM compared to the group treated with TGF-β1 alone without nAG ($P<0.05$) (Fig 2). The 0.1 nM nAG treatment group did not significantly reduce the protein

production except in the case of fibronectin with a 50% reduction ($P<0.05$). Similar results were obtained for fibronectin using immunofluorescence microscopy (Fig, 3).

To determine if nAG inhibits collagen III at the mRNA level, a qPCR was done after 24 hours from treatment. After repeating the experiment three times with fibroblasts from three different patients the data was analyzed using Graphpad Prism. The results indicate that the TGF-β treated group without nAG treatment show increased collagen III mRNA levels as compared to the control group without TGF-β or nAG. nAG treatment at concentrations of 0.1 nM and 1 nM significantly decreased the amount of collagen III mRNA by 50% ($P<0.05$) (Fig 4) . nAG concentrations of 10 nM did not reach statistical significance.

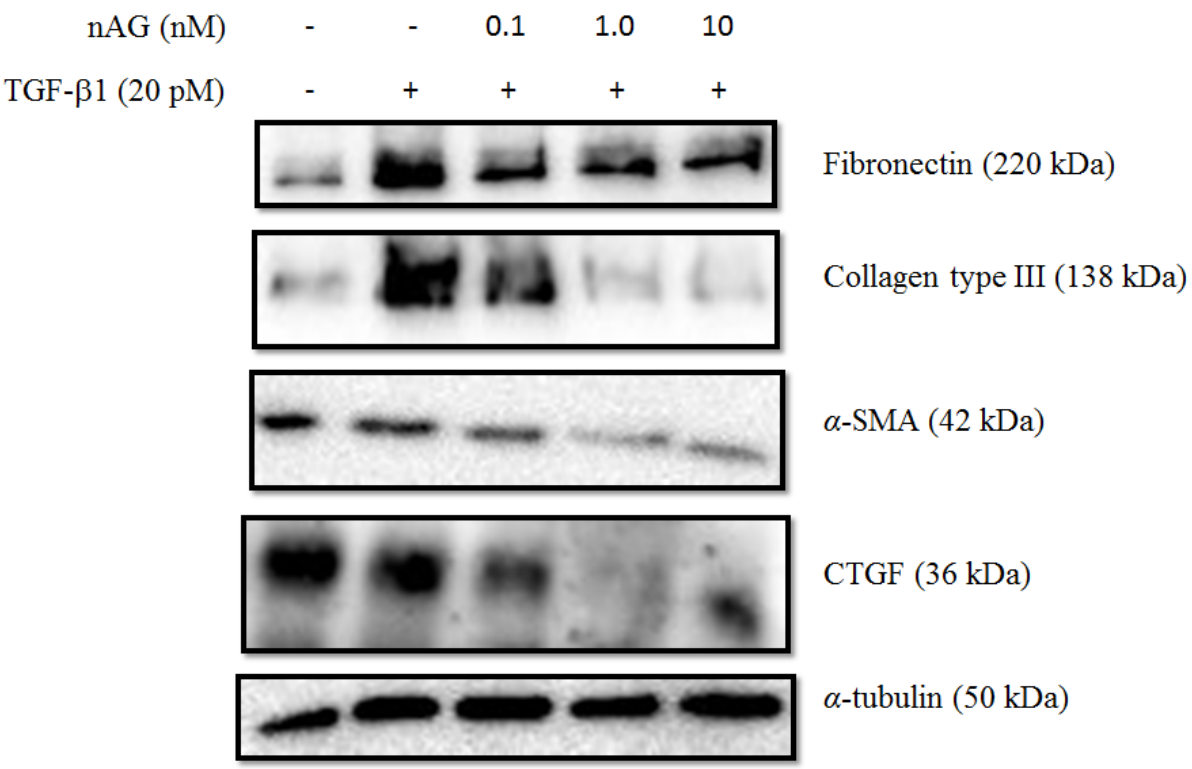


Figure 1. **nAG protein inhibits TGF-β1-induced ECM protein production and myofibroblast differentiation.** Scleroderma patient fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of the indicated amounts of nAG protein for 24 hours. Cell lysates were analyzed by Western blot using antibodies to detect fibronectin, collagen type III, α-SMA (marker for myofibroblast differentiation), CTGF/CCN2 and α-tubulin as a loading control. Scleroderma fibroblast cells treated with only TGF-β1 (without nAG) showed a significant increase in fibrotic responses compared to the control group. TGF-b1-induced fibronectin, collagen III, α-sma and CTGF protein levels were all **decreased** when treated with **nAG** after 24 hours. Concentrations of **1 nM** and **10 nM** were most effective in inhibition while 0.1 nM (100 pM) was less effective.

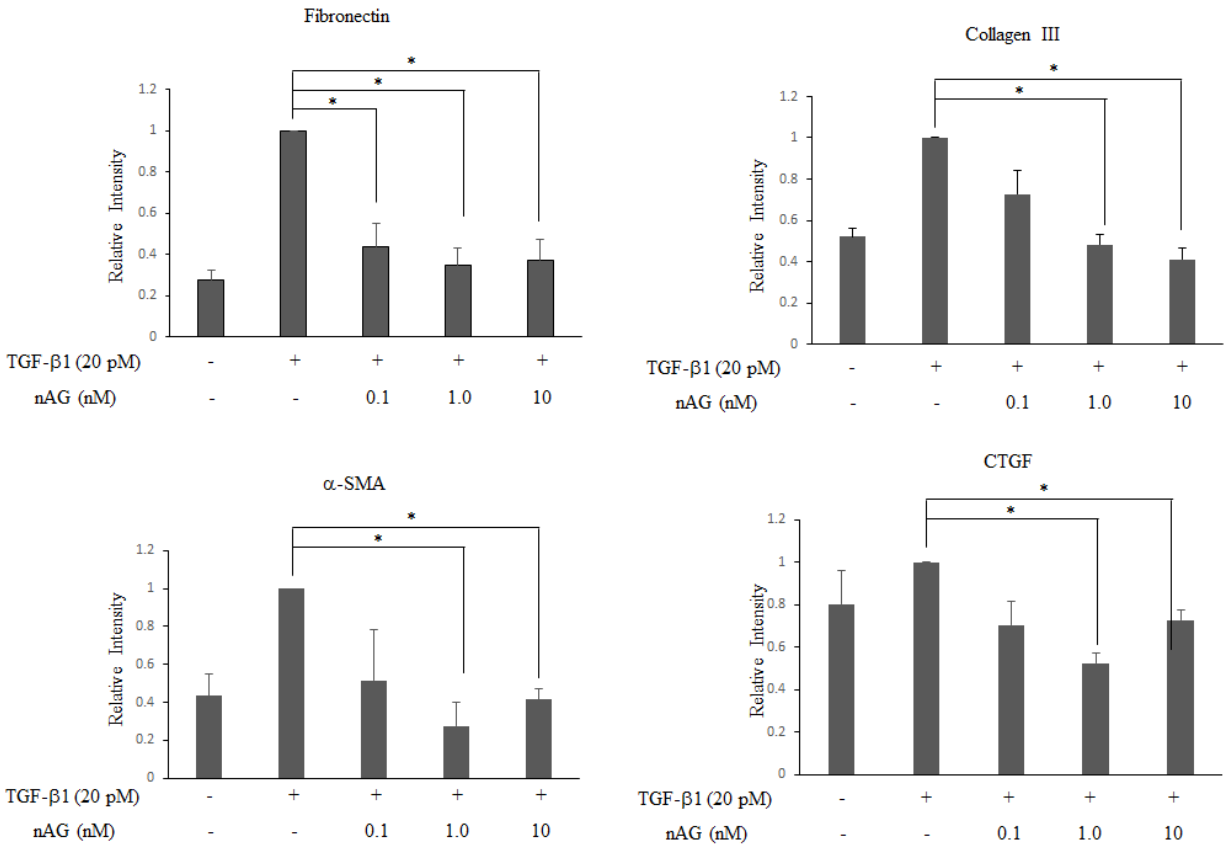
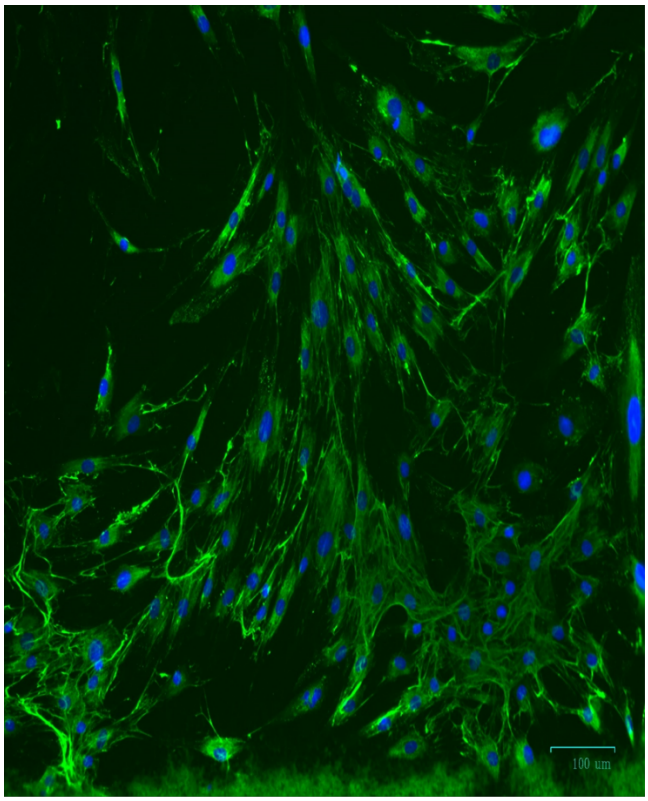
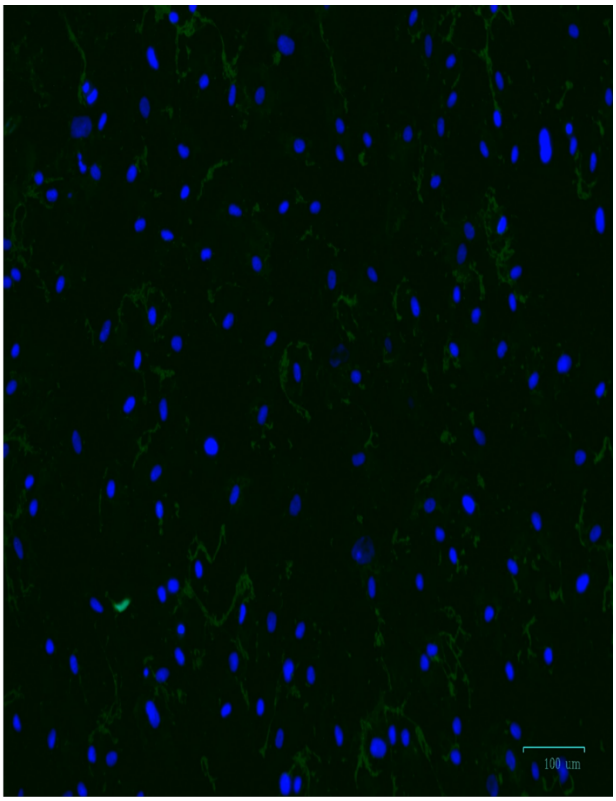


Figure 2. nAG protein inhibits TGF-β1-induced ECM protein production and myofibroblast differentiation. After repeating the experiment shown in Figure 1 three times with fibroblasts from 3 different patients, densitometry of the western blot band intensities was performed using IMAGE J software and a statistical analysis was performed using a Student's t-test. This revealed a 50% decrease in fibronectin, collagen III, α-sma and CTGF protein when nAG was used compared to the TGF-β group alone (without nAG) (*$P < 0.05$).



FIBRONECTIN TGF



FIBRONECTIN TGF + nAG

Figure 3. nAG protein inhibits TGF-β1-induced fibronectin production in scleroderma fibroblasts. Scleroderma fibroblasts were treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 24 hours. Cells were fixed and stained with an anti-Smad2 antibody and detected by immunofluorescence. On Immunofluorescence 24 hours after treatment of scleroderma fibroblasts, fibronectin staining (green) was increased in the TGF-β group alone with a marked decrease in fibronectin staining when nAG at a concentration of 10 nM was added.

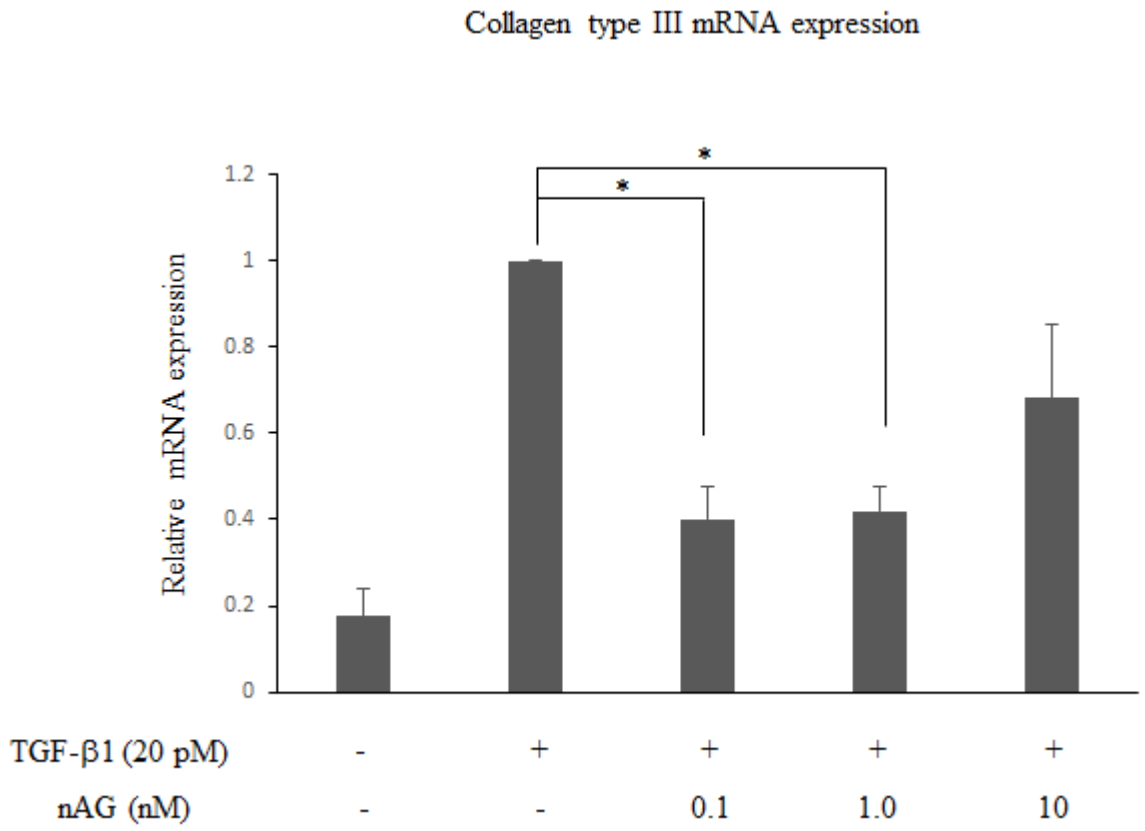


Figure 4. nAG protein inhibits TGF-β1-induced collagen type III mRNA expression in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated in the presence of 0, 0.1, 1.0 or 10 nM nAG protein for 24 hours. Total RNA was extacted and analyzed by qPCR to measure type III collagen mRNA and GAPDH (internal control). A Q-PCR for collagen III of scleroderma fibroblasts revealed an increase in collagen III mRNA when treated with TGF-β alone. This effect was inhibited when scleroderma fibroblasts were treated with nAG protein with a 50% reduction in collagen III mRNA at concentrations of 0.1 nM and 1.0 nM ($P < 0.05$). The 10 nM concentration of nAG did not reach significance.

3.2 nAG decreases ALK5 receptor & phospo SMAD 2/3 levels in scleroderma fibroblasts

To measure the effect nAG protein has on the canonical TGF-β pathway, TGF-β Receptor 1 (also known as ALK5) protein levels were measured via western blot 24 hours after treatment as well as phosphorylated SMAD2 and phosphorylated SMAD3 after 1 hour of treatment. The results revealed that the TGF-β group without nAG treatment showed increased protein production of ALK5 and PSMAD 2 and PSMAD 3 compared to the control group without TGF-β or nAG.

PSMAD2 levels were decreased at nAG concentrations of 1 nM and 10 nM whereas PSMAD 3 protein was only marginally decreased at a nAG concentration of 1 nM (Fig 5). TGF-β Receptor 1 (ALK5) protein levels however were significantly reduced at nAG concentrations of 1 nM and 10 nM (Fig 6). Western blot densitometry done using fibroblasts from three different patients revealed a significant 50% reduction of ALK5 receptors with the treatment of nAG protein ($P<0.05$) (Fig 6). The 0.1 nM concentration of nAG did not reduce the amount of PSMAD2/3 or ALK5 receptors.

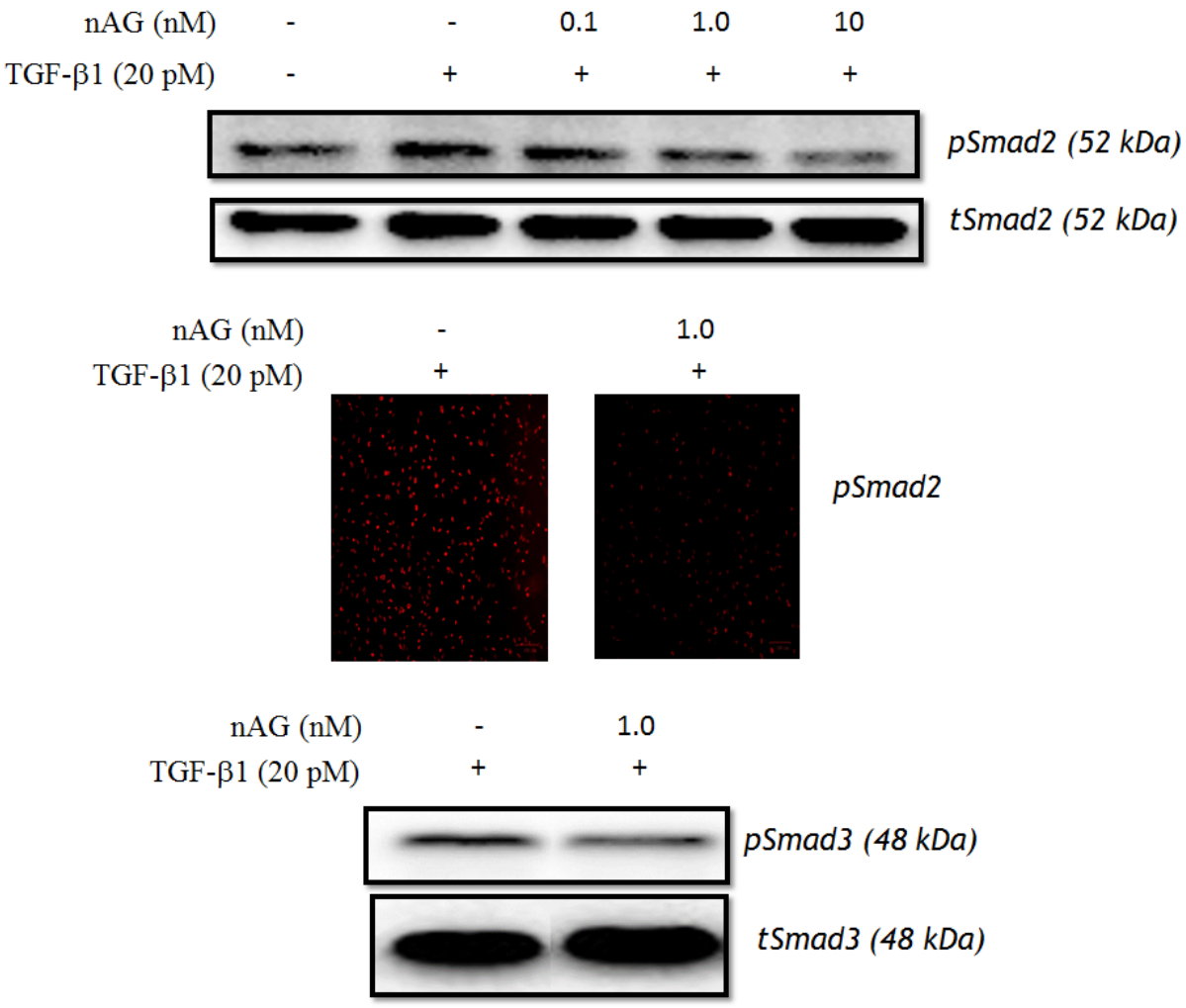


Figure 5. nAG protein decreases TGF-β1-induced phosphorylation of Smad2 and Smad3 in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of 0, 0.1, 1.0 or 10 nM of nAG protein for 1 hour. Cell lysates were analyzed by Western blot with antibodies to detect phosphorylated and total Smad2 and Smad3. Western blot after 1 hour of treatment with nAG revealed a marginal decrease in pSmad3 at a nAG concentration of 1 nM and a significant decrease in pSmad2 at concentrations of 1 nM and 10 nM.

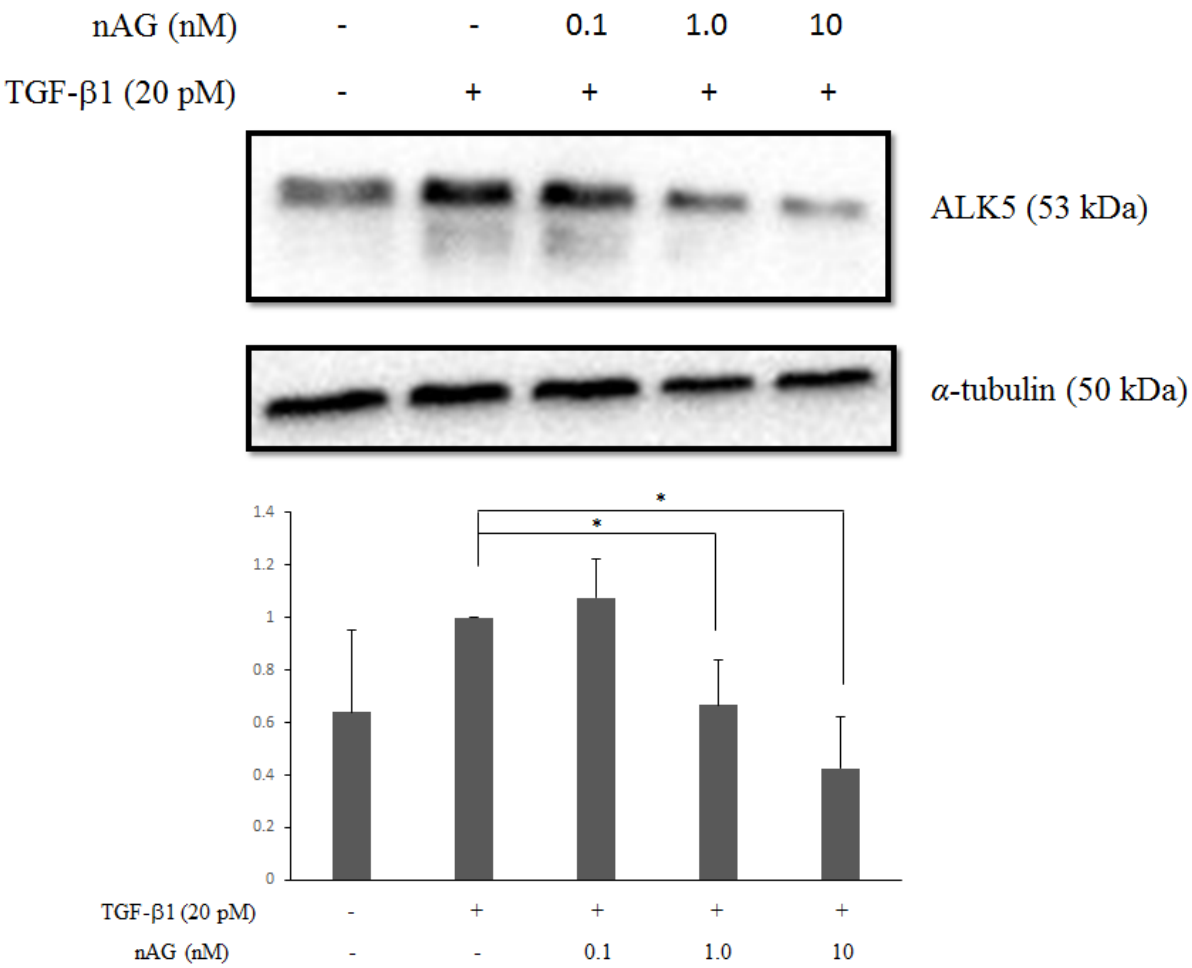


Figure 6. nAG protein decreases ALK5 receptor levels in scleroderma fibroblasts. Scleroderma fibroblasts were left untreated or treated with 20 pM TGF-β1 in the presence of 0, 0.1, 1.0 or 10 nM nAG protein for 24 hours. Cell lysates were analyzed by Western blot using antibodies to detect ALK5 and α-tubulin (loading control). The TGF-β treated group without nAG protein increased the amount of ALK5 protein while nAG protein treatment at concentrations of 1 nM and 10 nM decreased the ALK5 levels to almost equivalent to the control group thus inhibiting the effect of TGF-β signaling. nAG protein at 100 pM did not decrease ALK5. After repeating the experiment 3 times with scleroderma fibroblasts from 3 different patients, densitometry of western blot band intensities revealed a 50 % reduction in ALK5 in the presence of nAG protein at concentrations of 1 nM and 10 nM (*$P < 0.05$)

3.3 nAG decreases cell migration in scleroderma fibroblasts

TGF-β is known to increase cell migration. To assess the inhibition of TGF-β1 induced cell migration using nAG protein, an in vitro wound healing (scratch) assay was done. The results indicate that the TGF-β treated group without nAG treatment showed an increased number of cells within the created wound after 48 hours compared to the control group without TGF-β or nAG which confirmed that TGF-β1 was stimulating cell migration. nAG treatment at a concentration of 10 nM significantly decreased TGF-β1-induced cell migration ($P<0.05$). The rate of cell migration of the 10 nM nAG group was very similar to the control group without TGF-β stimulation thus almost completely inhibiting the effect TGF-β has on cell migration (Fig 7,8) .

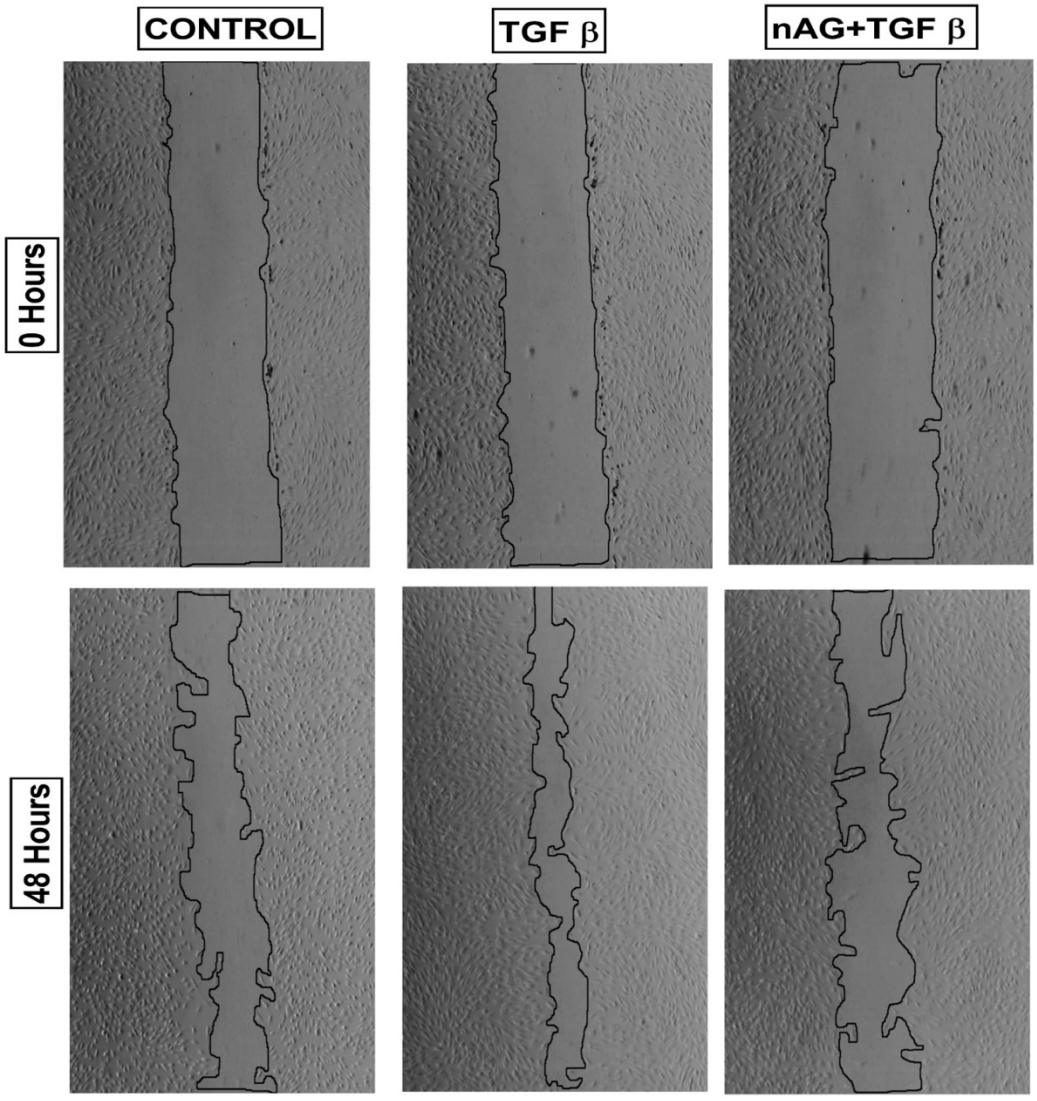


Figure 7. nAG protein inhibits TGF-β1-induced migration in scleroderma fibroblasts. Scleroderma fibroblasts were scratched with a pipette tip and left untreated or treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 48 hours. At 0 hours, the wounds were equal in width in all three groups. 48 hours after wound creation the TGF-β group showed faster cell migration (wound closure). nAG treatment at a concentration of 10 nM decreased TGF-β1-induced migration (wound closure) to a level similar to the control group. Quantitation of results is presented in Figure 8.

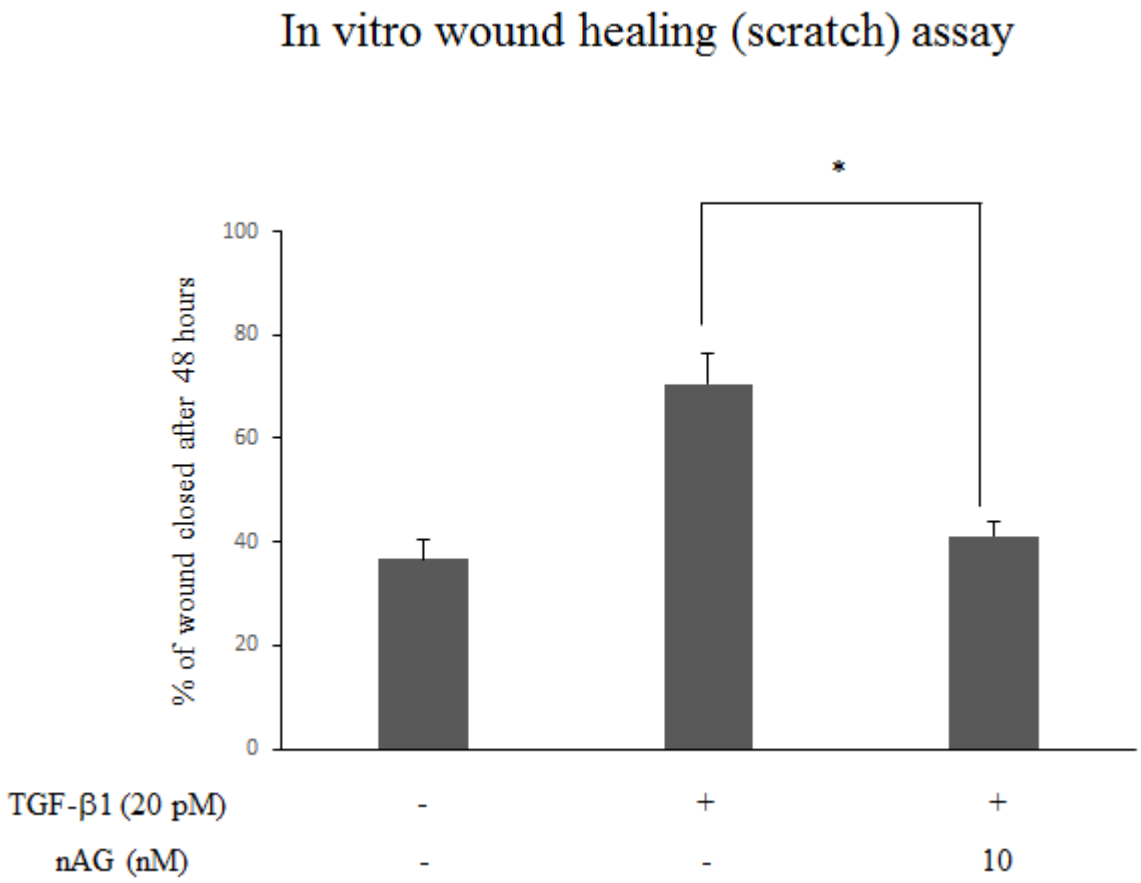


Figure 8. nAG protein inhibits TGF-β1-induced migration in scleroderma fibroblasts. Scleroderma fibroblasts were scratched with a pipette tip and left untreated or treated with 20 pM TGF-β1 with or without 10 nM nAG protein for 48 hours. Experiments were performed on fibroblasts from 3 different scleroderma patients. Densitometry was performed on western blot band intensities using Image J software. The nAG treatment group at a 10 nM concertation showed a significant inhibition of TGF-β1-induced cell migration ($P < 0.05$).

3.4 nAG decreases SMAD3-driven transcriptional activity

To assess the specificity of nAG's inhibitory potential on different TGF-β isomers, a luciferase assay was done using a TGF-β responsive, Smad3-specific $CAGA_{12}$-lux reporter in 293T cells. The results indicate that all three TGF-β isoforms increase $CAGA_{12}$-lux activity in 293 cells as expected. Importantly, when nAG was added in a concentration of 10 nM, luciferase activity was decreased by 30% in TGF-β2 stimulated cells, 50% in TGF-β3 stimulated cells and most potently with an 83% reduction in TGF-β1-stimulated cells (Fig 9). This indicates that the nAG protein was most specific in inhibiting the isomer TGF-β1 which is the most important isomer involved in fibrosis.

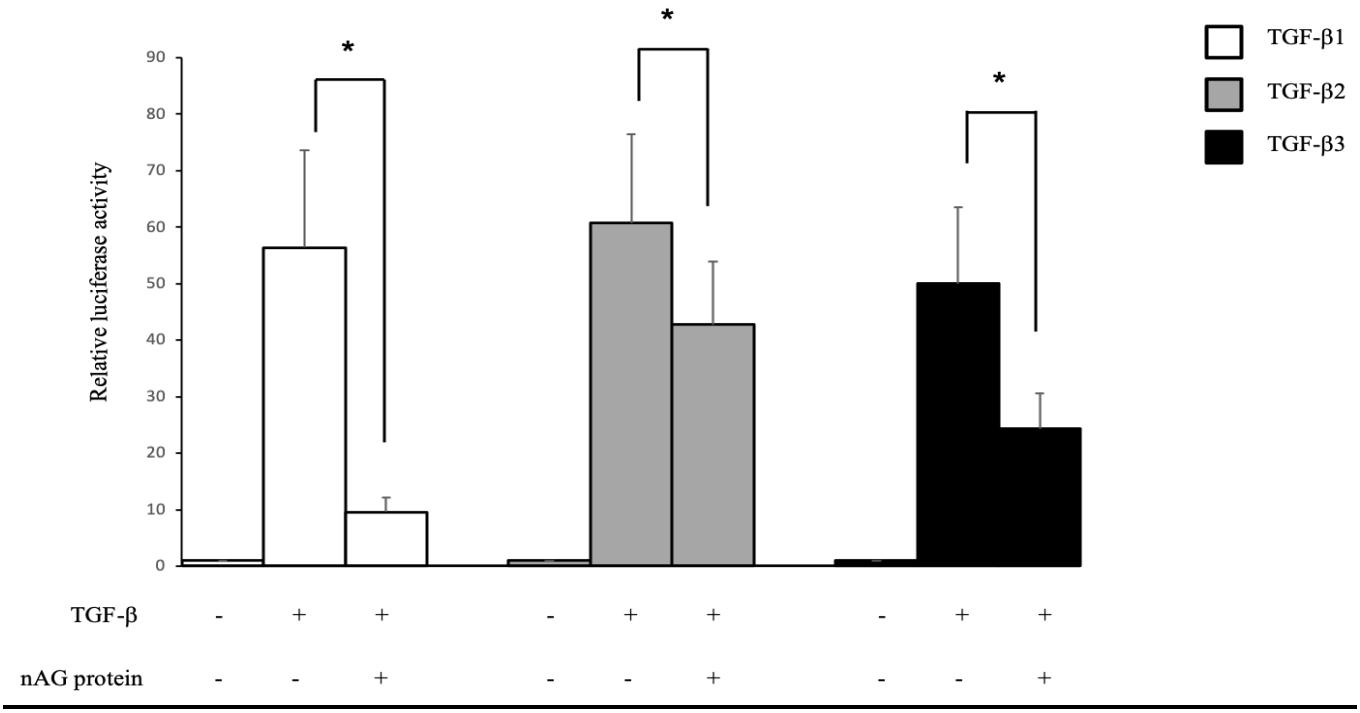


Figure 9. nAG protein inhibits TGF-β-induced Smad3-driven ($CAGA_{12}$-lux) transcriptional activity in 293T cells. Using a luciferase assay with CAGA reporter cells with different isomers of TGF-β revealed nAG protein to be effective in inhibiting TGF-β1 signaling 83%. TGF-β2 and TGF-β3 isomer activity were also reduced by 30% and 50% respectively. All three isomer inhibitions were statistically significant ($P < 0.05$).

3.5 nAG colocalizes withALK5 receptor

To investigate whether the nAG protein may be interacting with TGF-βR1 (ALK5), immunofluorescence confocal microscopy was used. Using a concentration of 10 nM of nAG showed colocalization of nAG protein with the ALK5 receptor mainly within the cytoplasm of the fibroblast cell 24 hours after nAG treatment (Fig 10). To ensure there was no autofluorescence, a group with only a secondary antibody was analyzed and no staining was noted.

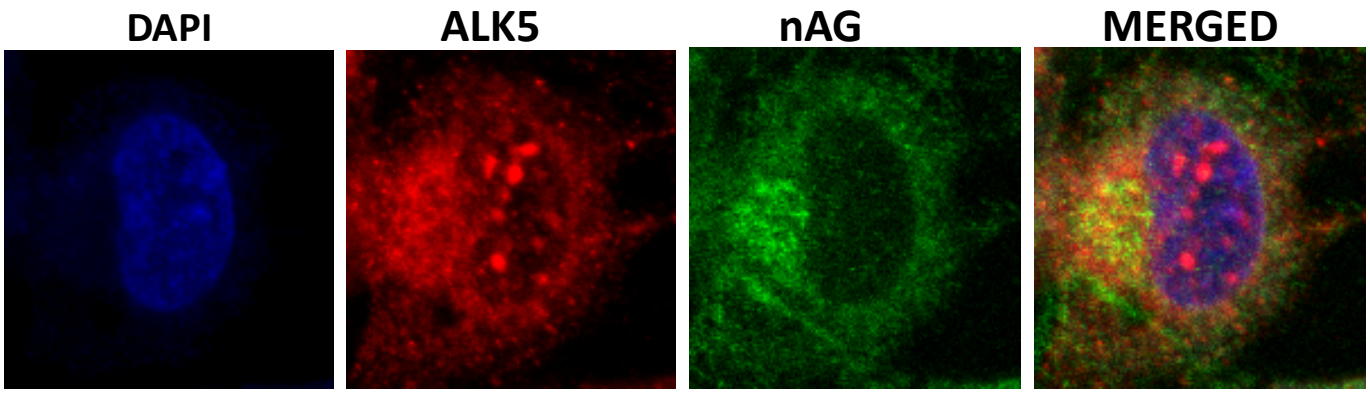


Figure 10. nAG protein colocalizes with ALK5 in scleroderma fibroblasts. Scleroderma fibroblasts were treated with 10 nM of nAG protein for 24 hours. Cells were fixed and immunostained for nAG protein (green), ALK5 (red) and counterstained with DAPI (nuclear stain). Immunofluorescence confocal microscopy reveals colocalization of ALK5 (red) and nAG protein (green) shown by the yellow color in the merged image mostly within the cytoplasm.

CHAPTER 4:
DISCUSSION

DISCUSSION

nAG protein was first identified as a critical mediator of nerve-dependent limb regeneration in salamanders (1). Studies using nAG protein in mammalian systems have revealed a potential anti-fibrotic effect *in vitro* and *in vivo* (6, 7). Here I investigate the potential role of nAG protein in modulating TGF-β signaling and fibrotic responses in scleroderma patient skin fibroblasts. My results indicate that nAG protein inhibits TGF-β-induced production of ECM-related proteins including fibronectin, collagen III, CTGF and a-SMA in scleroderma fibroblasts. In addition, my results indicate that nAG protein inhibits TGF-β-induced Smad2/3 signaling, decreases ALK5 protein levels and reduces TGF-β-induced migration in scleroderma fibroblasts. Interestingly, nAG protein inhibits TGF-β1 to a greater extent than TGF-β2 or -β3, in a Smad3-driven luciferase assay, suggesting that nAG may have different affinities for binding and neutralizing TGF-β isoforms. On the other hand, nAG protein may also inhibit TGF-β signaling by binding to TGF-β receptors (i.e. ALK5) and promoting their degradation. Taken together, these findings indicate that nAG protein is a potent inhibitor of TGF-β signaling and fibrotic responses in scleroderma fibroblasts and that it may serve as a candidate for further development as an antifibrotic agent for the treatment of scleroderma.

My results show that the nAG protein significantly inhibits TGF-β1-induced fibrotic responses and Smad2/3 phosphorylation in human scleroderma fibroblasts. A similar effect of

nAG protein might be occurring during limb regeneration in salamanders. For example, TGF-β and PSMAD2/3 are maximally expressed between 6 and 48 hours post amputation, which constitutes the preparation phase of limb regeneration, and was almost not detectable at day 5-7 post amputation which corresponds to the same time nAG protein is secreted from the amputated nerve Schwann cells (1, 76). The disappearance of TGF-β and PSMAD 2/3 signaling at the late stage of the preparation phase until the end of the re-development phase is possibly correlated with nAG protein's ability to inhibit TGF-β signaling. This concept is further strengthened by the fact that in the absence of nAG, limb regeneration does not occur and instead heals by fibrosis which might be due to uninhibited TGF-β signaling. During wound healing of salamanders, it was found that TGF-β1 is only transiently elevated for the first 4 days with very few myofibroblasts and immune cells. Whether nAG plays a role in salamander wound healing is yet to be determined. Immune cells have been known to be responsible for activating TGF-β and the absence of neutrophils or macrophages at the site of the wound in salamanders could explain why they heal without a scar. Similarly in humans it is believed that up until the 2nd trimester the fetus can undergo scarless healing due to the lack of development of the immune system (5).

The mechanism(s) by which nAG protein inhibits TGF-β signaling and responses is not clear. The inhibitory effect that nAG protein has on TGF-β-induced signaling and fibrotic responses could be explained by its' possible binding with ALK5 receptors, as shown by the colocalization of nAG protein and ALK5 by confocal microscopy, increasing the internalization and degradation of TGF-β receptors and thus preventing TGF-β1 from inducing fibrosis. The 50% reduction of ALK5 receptor protein by nAG protein treatment as determined by western blot further supports the notion of increased TGF-β receptor degradation. Further experiments are required however to confirm this.

nAG protein decreased TGF-β-induced collagen III and fibronectin possibly not only due to the reduced TGF-β Smad2/3 signaling but also due to an increase in MMPs since in previous studies nAG transfection in human fibroblasts was found to increase MMPs significantly, which degrade the ECM (7). nAG recombinant protein was more effective in inhibiting collagen III and although reduced collagen I the inhibition did not reach significance. This is in agreement with previous research that found nAG transfection with normal human fibroblasts to be more pronounced with collagen III (7).

TGF-β in salamander limb regeneration was found to be important for the migration of stem cells to form a blastemal. However, during the re-development phase cell migration is no long required and the appearance of nAG protein might be responsible for the decreased cell migration. In the present study, similar results were found when adding nAG recombinant protein to scleroderma fibroblast cells and performing an in vitro wound healing (scratch) assay. TGF-β1 significantly enhanced cell migration and the addition of nAG protein completely blocked the effect of TGF-β on cell migration.

The most important TGF-β isomer in fibrosis is TGF-β1. A luciferase assay measuring activity of the three different TGF-β isoforms revealed that nAG protein was most effective in inhibiting the activity of TGF-β1, reducing its Smad3-driven ($CAGA_{12}$-lux) transcriptional activity by 83%.

Scleroderma fibroblasts have been shown to be more sensitive to TGF-β signaling than normal skin fibroblasts with an increase in collagen production and deposition. Treatment of this disease is challenging and so far, remains limited. nAG protein was able to inhibit the fibrotic responses induced by TGF-β despite sclerodermal cells increased responsiveness to TGF-β and

has shown promise as a potential antifibrotic agent. Further studies of this protein on human cells and in vivo models might prove to be promising and learning more on how nAG works in salamanders might provide us with insight on how these animals remain scarless through the test of time.

In summary, my results indicate that nAG protein acts as a potent inhibitor of TGF-β-induced production of ECM-related proteins, Smad2/3 signaling and migration in scleroderma fibroblasts. This inhibition appears to be most specific for TGF-β1 as compared to the other isoforms but may also involve interaction of nAG protein with ALK5 receptor to promote internalization and receptor degradation.

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