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Nitrosylation of Tissue Transglutaminase enhances fibroblast migration and regulates MMP activation

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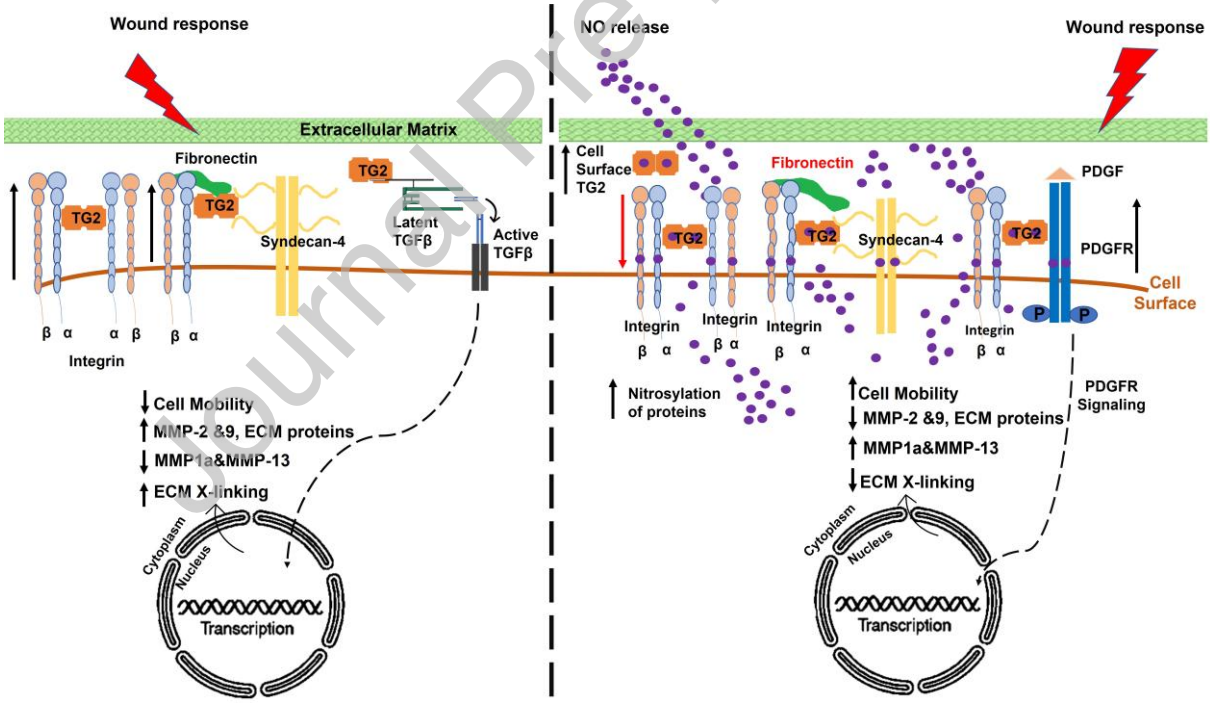
Highlights

- Overexpression of TG2 in Swiss 3T3 fibroblasts delayed cell migration in a TGF-β dependent manner.
- Attenuation in cell migration was rescued by TG2 active site inhibitor and nitric oxide donor.
- Induction of TG2 expression increased the active MMP-2 and MMP-9 levels and decreased active MMP-1a and MMP-13 a TGF-β dependent manner.
- Increasing dose of nitric oxide donor led to the loss of TG2/ITGβ-1 and TG2/SDC-4 complex formation, while an increase TG2 and PDGFRβ protein interaction was detected.

Graphical Abstract



Abstract

In wound healing, the TG2 enzyme plays a dual functional role. TG2 has been shown to regulate extracellular matrix (ECM) stabilization by its transamidase activity while increasing cell migration by acting as a cell adhesion molecule. In this process, nitric oxide (NO) plays a particularly important role by nitrosylation of free cysteine residues on TG2, leading to the irreversible inactivation of the catalytic activity.

In this study, transfected fibroblasts expressing TG2 under the control of the tetracycline-off promoter were treated with NO donor s-nitroso-n-acetyl penicillamine (SNAP) to analyze the interplay between NO and TG2 in the regulation of cell migration/invasion as well as TGF-β1-dependent MMP activation. Our results demonstrated that inhibition of TG2 cross-linking activity by SNAP promoted the migration and invasion capacity of fibroblasts by hindering TG2-mediated TGF-β1 activation. While the inhibition of TG2 activity by NO downregulated the biosynthesis and activity of MMP-2 and MMP-9, that of MMP-1a and MMP-13 shown to be upregulated in a TGF-β1-dependent manner under the same conditions. In the presence of SNAP, interaction of TG2 with its cell surface binding partners Integrin-β1 and Syndecan-4 was reduced, which was paralleled by an increase in TG2 and PDGF association. These findings suggests that migratory phenotype of fibroblasts can be regulated by the interplay between nitric oxide and TG2 activity.

Keywords: Tissue transglutaminase (TG2), nitric oxide (NO), cell migration, extracellular matrix (ECM) remodeling, wound healing, matrix metalloproteinases (MMPs)

Abbreviations: Tissue transglutaminase (TG2), nitric oxide (NO), extracellular matrix (ECM), matrix metalloproteinases (MMPs), guanosine diphosphate (GDP), guanosine triphosphate (GTP), fibronectin (FN), integrinβ-1 (ITGβ-1), syndecan-4 (SDC-4), platelet-derived growth factor receptor (PDGFR), ransforming growth factor β-1 (TGFβ-1), latent TGFβ-binding protein-1 (LTBP1), latency-associated peptide (LAP), nuclear factor kappa B (NFκB), inducible nitric oxide synthase (iNOS), S-Nitroso-N-acetyl penicillamine (SNAP).

Introduction

Wound healing is characterized by three interconnected and overlapping phases; inflammation, tissue formation/fixation, and new tissue matrix modelling. These phases take place by the cooperation of different well-characterized cells interacting with each other under the coordination of various factors such as cytokines, growth factors [1,2]. Proteases are suggested to play an essential role in different wound healing stages whereby the existing extracellular matrix (ECM) is restructured to maintain tissue integrity [3]. Tissue transglutaminase (TG2) is known to be one of the active mediators in different phases of the wound-healing process [1,4,5].

TG2 is a member of the transglutaminase enzyme family, which mediates various biological processes in different cell and tissue types [6]. TG2, in the calcium-bound reduced state, cross-links proteins by forming ε(γ-glutamine)-lysin peptide bonds that are resistant to proteolytic and mechanical degradation [7].

Although it was initially thought that the enzyme functioned as a cytosolic Ghα protein upon binding of GDP/GTP, studies have pointed out that TG2 is released from the cells by through atypical mechanism that may involve recycling endosomes [8], a direct molecular trap mechanism [9] or recently reported exovesicles [10,11]. Once externalized, TG2 has a role in increasing the rigidity of the ECM by cross-linking its proteins [1,12,13]. Accumulating evidence suggested that TG2 loses its cross-linking enzyme activity upon binding to the extracellular fibronectin (FN) and becomes a novel surface adhesion molecule that acts as a co-receptor for integrin and syndecan-4 (SDC-4) [14–16]. Moreover, in fibroblasts, cell surface TG2 has been shown to interact with platelet-derived growth factor receptor (PDGFR), leading not only to receptor aggregation but also to the regulation of PDGFR-integrin interactions resulting in PDGFR-mediated cell migration and proliferation [17–19].

TG2 can also indirectly modulate wound healing through activation of the transforming growth factor β-1 (TGFβ-1). TG2 activates TGFβ-1 by cross-linking the latent TGFβ-binding protein-1 (LTBP1) in the ECM, which favors the dissociation of latency-associated peptide (LAP) and the release of active TGFβ-1 factor [20–22]. TGFβ-1 activates the signaling mechanisms necessary for the activation of genes that regulate many different cellular functions such as cell proliferation, viability, and differentiation, as well as ECM homeostasis. In proliferative and maturation phases of wound healing, TGFβ-mediated collagen and FN synthesis and matrix metalloproteinases activation play an important role in the remodeling of

ECM [23]. TG2 is also transcriptionally activated in the downstream of the TGFβ-SMAD signal pathway by the TGFβ response element in the *TGM2* promoter region [4,24,25]. Following matrix remodeling, connective tissue generation culminates in scar tissue formation and small-scale fibrosis to reach the end point [3]. In fibrotic tissue formation, as a result of TGFβ-1 mediated activation of SMAD signaling, gene expression levels of ECM proteins are stimulated, while gene expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are differentially regulated, leading to increased matrix deposition [26–28]. We have previously suggested that TG2 enzyme activity was responsible for the increased TGFβ-1 cytokine levels in fibroblasts and hence ECM synthesis by activation of the nuclear factor kappa B (NFκB) signaling pathway [29]. In the same study, we showed that nitric oxide (NO), an extracellular second messenger, which is responsible from the manipulation of protein function through the nitrosylation of cysteine and tyrosine residues, nitrosylates TG2 resulting in the inhibition of the enzyme's cross-linking activity and TG2-mediated TGFβ-1 activation and ECM synthesis.

In the light of our previous findings, in this study, we aimed to elucidate the role of TG2 nitrosylation in cell migration using an *in vitro* model mimicking the tissue/matrix remodeling phase of wound healing. Using transfected fibroblasts expressing TG2 under the control of the tetracycline-off promoter, we investigated the effect of TG2 upregulation on the activation of gelatinases, MMP-2 and MMP-9, and collagenases, MMP-1a and MMP-13. The effects of TG2 nitrosylation on cell migration and MMP patterns were elucidated by the treatment of TG2-overexpressing fibroblasts with the NO donor S-Nitroso-N-acetyl penicillamine (SNAP) treatment. In the second part of this study, the effects of TG2-nitrosylation on the enzyme's interaction with cell surface binding partners integrinβ-1 (ITGβ-1), SDC-4 and PDGFR-β were investigated.

Results

TG2 transamidation activity hinders fibroblast migration by modulating MMP biosynthesis and activity

Given that TG2 cross-linking activity is paramount in the regulation of matrix stiffness [30,31] and hence may affect the migration of cells, we investigated the effect of TG2 activity on the migration of non-induced and induced fibroblast cells using wound scratch assays in the presence and absence of 50 μM TG2 inhibitor Z-DON. Our previous results showed that 72 hours after tetracycline withdrawal from the culture medium, TG2 expression was induced

on both the cell membrane and ECM [29]. The NI and IND fibroblasts, allowed to attach on the tissue culture plastic and reach 100% confluency, were scratched using a pipette tip to form a wound bed. TG2 inhibitor was added onto samples every 24 hours. Evaluation of NI and IND cells migration over 24 and 48 hours revealed that NI fibroblast cells migrated more rapidly into the wound bed than the IND cells (Fig. 1A). Z-DON-treated IND fibroblasts exhibited a migration pattern similar to NI cells. At the 24-hour time point, while 70% of NI fibroblasts migrated into the wound bed, only 43% of IND fibroblast cells showed migration into the scratch (Fig. 1B, $p<0.01$). Following 48 hours, 72% of IND fibroblasts migrated into the wound area while NI fibroblast completely closed the wound bed. The Z-DON-treated fibroblast cell migration potential of IND fibroblasts increased by 30% at the end of 24 hours and another 33% at 48 hours, reaching comparable levels with NI. Taken together, our data suggested that TG2 cross-linking activity hinders fibroblast migration potential.

To investigate whether the TG2-impeded cell migration was acted on by the modulation of biosynthesis and activity of gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1a and MMP-13), expression and activity profiles of the MMP genes were evaluated by quantitative real-time PCR and zymography, respectively. At 24 hours, IND cells expressed 2.1 times more MMP-2 levels when compared to NI, while treatment of IND fibroblasts with Z-DON led to a decrease in the MMP-2 expression levels comparable to NI (Fig. 2A). Similarly, at 48 hours, a 1.3-fold increase was detected in levels of MMP-2 in IND cells (Figure 2A, $p<0.01$), which was brought down to NI levels when these cells were treated with Z-DON. Consistently, TG2 induction led to a 2.7-fold increase in the activation of gelatinase ($p<0.05$). As a result of the inhibition of TG2 activity by Z-DON, it was determined that Z-DON treated IND cells had similar MMP-2 activity with NI fibroblast at 24 hours (Supplemental Fig. 1A, p=ns) At the end of the 48th hour, MMP-2 activity increased by 1.44-fold in IND compared to NI cells (Supplemental Fig. 1A, $p < 0.0001$), while Z-DON treatment resulted in a 1.40-fold reduction in MMP-2 activation (Fig. 2A and Supplemental Fig. 1A,). This result indicates that Z-DON-treated fibroblasts and NI at 48 hours exhibit similar MMP-2 activity, just as after 24 hours (p=ns).

TG2 induction led to a respective 25.8-fold and 3.2-fold increase in MMP-9 expression levels at 24 and 48 hours (Fig. 2B, $p<0.05$). Inhibition of TG2 activity by Z-DON led to a decrease in MMP-9 expression levels like NI at both time points (p=ns). In Fig. 2B, the comparison of the NI and IND cells at 24th hour indicated that the activity of MMP-9 in the IND cells was increased by 2.6-fold. In the IND cells treated with Z-DON, no significant change in MMP-9

activity levels was observed after 24 hours compared to NI (Supplemental Fig. 1B). When a similar comparison was performed for the 48 hours of MMP-9 activity, 1.92-fold increased activity was detected for IND (Fig. 2B and Supplemental Fig. 1B, $p < 0.0001$), and inhibition of TG2 with Z-DON allowed these cells to exhibit MMP-9 activity similar to NI cells (p=ns).

As shown in Fig. 2C and 2D, gene expression profiles and activation of MMP-1a and MMP-13 (collagenases) in NI and IND fibroblasts in the presence and absence of Z-DON were investigated by quantitative real-time PCR and zymography to understand the effect of TG2 induction on the biosynthesis and activation of collagenases. TG2 induction decreased the levels of both collagenases at 24 and 48 hours in a transamidase activity-dependent manner. In NI fibroblasts, MMP-1a expression levels were 2-fold higher than that of IND at 24 hours (Fig. 2C). When IND cells were treated with Z-DON, the MMP-1a expression levels were increased to the levels of NI at 24 hours (p=ns). After 48 hours, there still was a significant 1.7-fold increased MMP-1a expression in NI cells compared to IND (Fig. 2C, $p<0.05$). No significant difference in MMP-1a levels was detected between NI and Z-DON-treated IND fibroblasts. Investigation of MMP-1a enzyme activity showed that at 24 hours, there was a 1.97-fold decrease in MMP-1a enzyme activity of IND compared to NI (Fig. 2C and Supplemental Fig. 1C). After the Z-DON treatment, IND fibroblasts were found to demonstrate a similar MMP-1a activity to NI (Supplemental Fig. 1C, p=ns). At 48 hours, MMP-1a enzyme activity of IND was 2.08-folds lower compared to NI (Supplemental Fig. 1C, $p<0.01$), and that of NI was similar to Z-DON treated fibroblasts (Supplemental Fig. 1C, p=ns). Fig. 2D demonstrated that the induction of TG2 activity caused a 2.8-fold deduction in the MMP-13 expression levels compared to that of the NI at 24 hours, and the Z-DON treatment brought up MMP-13 levels ($p<0.05$). Similar results were obtained for 48 hours by a 1.7-fold reduction in MMP-13 levels in IND ($p<0.01$), while no significant difference was recorded between NI and Z-DON samples. At 24 hours, MMP-13 activity decreased by 62% after TG2 induction, whereas at the end of 48 hours, the activity of MMP-13 was decreased by 43% in IND (Supplemental Fig. 1D, $p<0.001$), and Z-DON-treated IND fibroblasts showed similar active MMP-13 levels with NI at both time points (Supplemental Fig. 1D, p=ns).

NO rescues TG2-impeded fibroblast migration by modulating MMP biosynthesis and activity

Previously we have shown that NO donor SNAP not only led to the nitrosylation of TG2, which decreased the transamidation activity but also retention of the protein on the cell

surface, enabling TG2 to act as a cell adhesion molecule [29]. In order to investigate how TG2 can affect cell migration in the presence of SNAP, wound scratch experiments were performed on NI and IND fibroblasts in the presence of non-toxic concentrations of 50, 150, and 300 µM SNAP.

Increasing concentrations of SNAP triggered migration of IND cells at both 24 and 48 hours (Fig. 3A). While the migration potential of IND fibroblasts treated with 50 µM SNAP increased to 23% (24 hours) and 33% (48 hours), this rate was respectively increased by 23% and 39% in cells treated with 150 µM SNAP. There was a respective 14% and 22% increase in cell migration potential of 300 µM SNAP-treated IND cells at 24 hours and 48 hours. NI fibroblasts displayed a 27% and 28% increase in the rate of wound closure when compared to IND at 24 and 48 hours, respectively (Fig. 3B, $p<0.05$).

In order to understand whether NO rescues TG2-impeded fibroblast migration by modulating MMP biosynthesis and activity, the expression levels and activities of gelatinases and collagenases in IND fibroblasts were investigated using real-time PCR and zymography (Fig. 4). Although treatment of IND cells with 50 µM SNAP did not result in a decrease in MMP-2 expression levels at 24 h, a 1.2-fold reduction was detected at 48 h. An increase in SNAP concentration (150 µM and 300 µM) led to a significant reduction in the MMP-2 mRNA levels down to that of NI at both time points. Similarly, when MMP-2 activity of SNAP-treated IND cells was compared to their non-treated counterpart, 1.5-fold (50 µM), 2.1-fold (150 µM), and 3.18-fold (300 µM) reductions in MMP-2 activity were detected at 24 hours. At 48 hours, treatment of IND cells with 50, 150, and 300 µM SNAP resulted in a 1.3-, 2.2- and 2.4-fold reduction in MMP-2 activity (Fig. 4A and Supplemental Fig. 2A). SNAP treatment also led to a decrease in MMP-9 expression and activity in IND cells (Fig. 4B). 50 µM SNAP led to 11-fold and 1.8-fold decrease in the MMP-9 expression levels and a 1.8-fold and 1-fold reduction in the MMP-9 activity at 24 and 48 hours, respectively (Supplemental Fig. 2B). In the presence of 150 µM SNAP, 14.1-fold and 1.9-fold reductions in the MMP-9 expression levels (Fig. 4B) and 2.6-fold and 1.04-fold decreases in the MMP-9 activity were observed compared to the IND fibroblasts at 24 and 48 hours, respectively (Fig. 4B and Supplemental Fig. 2B). Incubation with 300 µM SNAP for 24 and 48 hours caused a ~21-fold and a 3.14-fold decrease in the MMP-9 expression levels of IND fibroblasts (Fig. 4B), while a respective 4.1-fold and 1.5-fold decrease in the MMP-9 activity was evident at the time points (Fig. 4B and Supplemental Fig. 2B).

In Fig. 4C, no significant change in MMP-1a mRNA levels was detected following 50 µM SNAP treat of IND at both time points (p=ns). The treatment of IND fibroblasts with 150 µM SNAP led to a respective 1.9-fold and 1.4-fold increase in MMP-1a expression at 24 and 48 hours, while in the presence of 300 µM SNAP an ~2-fold and 1.71-fold induction in the MMP-1a expression levels were observed, at indicated time intervals. Comparison of MMP-1a activity indicated that IND cells treated with 50 µM, 150 µM, and 300 µM SNAPs showed a 1.3-fold (50 µM), 2.5-fold (150 µM) and 2.1-fold (300 µM) increase of MMP-1a activity at the 24th hours. There was a 1.2-fold increase in 50 µM and a 3-fold increase in 150 µM and 300 µM SNAP-treated IND cells for MMP-1a activity at 48 hours (Supplemental Fig. 2C). Fig. 4D demonstrated that MMP-13 mRNA expression in IND cells was not significantly increased in response to 50 µM SNAP treatment (p>0.05), while a significant increase with 2.9- and 1.5-fold and 4.4 and 1.5-fold was evident for IND cells treated with 150 and 300 µM SNAP at 24 and 48 hours, respectively. When MMP-13 activities of IND cells in response to increasing concentrations of SNAP was compared a 1.8- (24 hours) and 1.1-fold (48 hours) increase for 50 µM, a 3.1- (24 hours) and 2.2-fold (48 hours) increase for 150 µM and finally, a 4.6- (24 hours) and 1.8-fold (48 hours) increase for 300 µM SNAP in MMP-13 activity was recorded (Fig. 4D and Supplemental Fig. 2D).

TG2 controls fibroblast migration by TGF-β dependent MMP regulation

Given that TG2 induction in fibroblasts led to TGF-β-mediated biosynthesis of ECM proteins [29], the role of TGF-β-dependent MMP regulation in TG2-impeded fibroblast migration was investigated next. As shown in Fig. 5A, the use of TGFβ neutralizing antibody (MAB1835) enhanced the migration of IND fibroblasts compared to untreated or control mIgG-treated IND fibroblasts at both time points. The presence of 10 µg/mL MAB1835 led to a respective 33% and 22% escalation in the cell migration of IND fibroblasts at 24 and 48 hours (Fig. 5B). When the migration rate of IND cells was compared to that of IND cells treated with 10 µg/mL of control mIgG, no significant difference was found (p=ns).

The effect of TGF-β activity on the expression and activity of gelatinases (MMP-2 and MMP-9) in IND fibroblasts was assessed. As shown in Fig. 6, 10 µg/mL MAB1835 antibody treatment led to an approximate 1.5-fold and 1.4-fold decrease in the MMP-2 expression levels of IND fibroblasts compared to their non-treated counterparts at 24 and 48 hours, respectively. On the other hand, the comparison of MMP-2 expression levels of non-treated and mIgG-treated fibroblasts showed no statistical difference at 24 hours, while a slight decrease in MMP-2 expression levels was evident at 48 hours. Consistently, our zymography

results showed that NI and IND cells treated with MAB1835 antibody showed similar MMP-2 activity at 24 and 48 hours, while MMP-2 activity was increased by 2.4-fold in IND and 2.5-fold in mIgG-treated IND cells (Fig. 6A and Supplemental Fig. 3A). In the presence of MAB1835, MMP-9 expression in IND fibroblasts was reduced 5.6 times at 24 hours and 1.8 times at 48 hours, while no significant change was detected in the MMP-9 expression levels of IND cells treated with control mIgG (Fig. 6B). When the non-treated IND was compared with the MAB1835-treated fibroblasts, the activity of MMP-9 was found to be decreased by 2.8-fold and 1.6-fold, respectively, at 24 and 48 hours (Supplemental Fig. 3B). In that, NI- and MAB1835-treated IND fibroblasts exhibited similar MMP-9 activity levels (Supplemental Fig. 3B, p=ns). When the mIgG-treated IND cells were compared with IND at 24 and 48 hours, there was no significant statistical difference in terms of the MMP-9 enzyme activity (Supplemental Fig. 3B, p=ns). As indicated in Fig. 6C, inhibition of TGFβ function by MAB1835 antibody resulted in a 2.2-fold and 1.7-fold increase in MMP-1a expression levels in IND cells at 24 and 48 hours, respectively. When the media of cells were examined for MMP-1a activity at 24 and 48 hours, it was seen that MMP-1a activity of MAB1835-treated IND cells mimicked that of NI cells, while mIgG treatment caused no change in MMP-1 activity of IND fibroblasts (Fig. 6C and Supplemental Fig. 3C). Treatment of IND fibroblasts with MAB1835 antibody, as shown in Fig. 6D, significantly increased MMP-13 expression levels by 2.2 times at 24 hours and by 1.7 times at 48 hours. Interestingly, incubation of the IND cells with control mIgG also resulted in a marked incline in MMP-13 expression levels at 24 hours, which was brought back down to the levels of IND at 48 hours. Determination of MMP-13 activity indicated similar levels both for NI and MAB1835-treated IND cells. No change in MMP-13 activity was evident in IND fibroblasts in the presence of the mIgG control antibody ((Fig. 6D and Supplemental Fig. 3D).

NO regulates TG2 interaction with ITGβ-1, SDC-4 and PDGFR-β

Given that our previous study showed that nitrosylation of TG2 increased its retention on the cell surface, we have next investigated the interaction of cell surface TG2 with its binding partners ITGβ-1, SDC-4, and PDGFR-β in the presence and absence of SNAP. The treatment of IND cells with increasing concentrations of SNAP resulted in a decrease in the association of TG2 with ITGβ-1 on the cell surface. The treatment of IND cells with 50 μM and 150 μM NO donor SNAP led to a respective 45% and 55% decrease in the TG2/ITGβ-1 complex formation on the cell surface. An extreme 78% reduction was measured in the TG2/ ITGβ-1 cell surface interaction in 300 μM SNAP-treated IND cells (Fig. 7B). A similar trend was

observed for the TG2/SDC-4 complex in that TG2 association with SDC-4 was decreased with increasing concentrations of SNAP in IND fibroblasts. The treatment of IND cells with 50 μM, 150 μM, and 300 μM NO donor SNAP led to a respective 52% and 80% and 67% decrease in the TG2/SDC-4 interaction (Fig. 7C).

In order to determine how the interaction of TG2 changes with its cell surface binding partner PDGFR [32], membrane fractions isolated from IND fibroblasts treated with increasing doses of SNAP were probed for TG2 following PDGFR co-immunoprecipitation (Fig. 7D). SNAP treatment led to an increase in the TG2 and PDGFR-β association in a concentration-dependent manner (bottom blot). Treatment of IND cells with 50 μM and 150 μM SNAP resulted in a 40% and 58% increase in TG2/PDGFR complex formation, while 300 μM of SNAP led to a 96% increase in PDGFR-β/TG2 interaction when compared to NI and non-treated IND fibroblasts (Fig. 7D). In order to understand whether the interaction of TG2 with PDGFR-β leads to the phosphorylation of PDGFR-β on tyrosine 751 (pY751PDGFR-β), Western blot analysis was performed on NI and IND cells treated with SNAP. Fig. 7E shows that levels of pY751PDGFR-β were respectively increased by 40%, 60%, and 96% in IND fibroblasts treated with 50 μM, 150 μM, and 300 μM of SNAP, suggesting that interaction of TG2 with PDGFR may lead to phosphorylation and activation of PDGFR signaling.

When the SNAP concentration was increased, the phosphorylation of PDGFR-β on tyrosine 751 (pY751PDGFR-β) was respectively increased by 39.86, 59.71, and 95.64-fold.

Discussion

Wound healing has a complex and highly developed chain of events after injury, and it is completed as a result of serial phases. These phases are hemostasis, inflammation, reepithelization, neovascularization, and remodeling by stabilization of ECM, respectively [33,34]. TG2 participates in several aspects of the wound healing process including the stabilization of fibrin clot, cross-linking of ECM proteins to enhance the stability, association with FN to promote cell adhesion, regulating the activation of TGF-β1, and hence MMP activity during granulation, tissue formation, and matrix remodeling [1,4,5]. Another important mediator in different phases of wound healing is NO, a small radical, derived from the amino acid L-arginine by three different isoforms of nitric oxide synthase. Starting from the early phase of wound healing, NO is mainly synthesized by inducible isoform (iNOS) expressed in fibroblasts, keratinocytes, macrophages and other inflammatory cells in nM-μM

concentrations to regulate collagen synthesis [35]. Furthermore, NO participates in wound contraction and cell proliferation in wound healing. Experiments on purified recombinant TG2 showed that up to 15 cysteine residues on TG2 can be nitrosylated by NO, which results in the irreversible inactivation of the transamidation activity of the enzyme [36]. In addition, our previous study revealed that NO modulates not only TG2 activity but also TG2-mediated TGF-β regulation and matrix deposition/turnover [29], suggesting that, as the fibrosis goes in parallel with a decrease in NO levels [29,37,38] the release of TGF-β and TG2 by myofibroblast was controlled in the presence of NO to prevent excessive fibrotic scar tissue formation in a normal wound healing setting. In order to test this hypothesis, here, we aimed to investigate the effect of NO released by SNAP in μM concentrations on TG2-regulated fibroblast migration and TGF-β dependent MMP activation by focusing on changes the interactions of cell-surface TG2 with ITGβ-1, SDC-4, and PDGFR. For this purpose, a previously established cell model that is fibroblasts expressing TGM2 under the control of tetracycline-off promoter was used in the current study [39].

Induction of TG2 expression in fibroblasts delayed the wound closure in an activity-dependent manner as treatment of IND fibroblasts with TG2 inhibitor Z-DON restored the TG2 impaired-cell migration. Similarly, NO donor SNAP known to apprehend TG2 inactive on the cell surface rescued TG2-impeded fibroblast migration in a dose-dependent manner. Knowing that TG2 induction in tubular epithelial cells and fibroblasts leads to increased deposition and cross-linking of ECM as well as a reduction in matrix turnover [29,40], the observed attenuation in IND fibroblast migration could be ascribed to the transamidase-dependent modeling of ECM plasticity. Our data was also consistent with the previous reports, which suggest that the upregulation and activation of TG2 enzyme were associated with the high levels of ε-(γ-glutamyl) lysine cross-linked ECM proteins leading to fibrotic conditions interfering with the cell proliferation and migration [4,41,42]. It is known that TG2 transamidase action is involved in the activation of TGF-β, which is a key cytokine responsible for MMP activation and ECM dynamic/modeling/homeostasis directing cell migration. Previously we showed that elevated ECM deposition in IND fibroblasts was mediated by TGF-β activation through NF- κB pathway in a transamidase activity-dependent manner [29]. We therefore reason that TG2 transamidase-dependent TGF-β activation may be responsible for the attenuation in cell migration. Consistent with this hypothesis, treatment of IND fibroblasts with TGFβ neutralizing antibody (MAB1835) rescued the decreased

migration to the levels comparable to NI as well as that of SNAP- or Z-DON-treated IND fibroblasts.

TGF-β plays an important role in regulating cell migration during wound closure by modulating the activities of MMPs [43,44]. In the ECM, proteolytic cleavage of LAP by MMP-9, MMP-2, or MMP-9 bound to CD44 in the cell surface results in the activation of TGF-β1 [45]. Furthermore, Hsieh et al. have shown that TGFβ-1 directly upregulates MMP-9 expression levels via TGF-β receptor, ROS-dependent activation of JNK1/2 and ERK1/2, and transcription factor NF-κB pathway, which promotes cell migration in RBA-1 cells [46]. Though unlike MMP-9, MMP-2 was shown to mediate degradation of fibrillar collagen [47], increased MMP-2 activity was documented in myofibroblastic hepatic stellate cells [48] and in myofibroblasts in various tumor stroma [49–51]. Similarly, MMP-9 expression correlates with myofibroblast differentiation [52].As our initial findings that suggests a myofibroblastic phenotype for IND fibroblasts [29], results showing higher MMP-2 and MMP-9 expression and activity levels for IND fibroblasts than in NI was in line with the given literature. Increased MMP-2 and MMP-9 activity was reduced to basal levels not only by the presence of Z-DON and SNAP but also by the inhibition of TGF-β activity by MAB1835 suggested that TG2 transamidase activity-mediated upregulation of gelatinases was TGF-β dependent.

On the other hand, studies with fibroblasts showed that TGF-β leads to a decrease in the synthesis of collagenases and enhances the production of collagenase inhibitor TIMP-1, inhibiting the degradation of newly synthesized collagen [53,54]. Collagenases MMP-1a and MMP-13 contain TGF-β inhibitory element (TIE) in their promoters, suggesting that TGF-β can potentially negatively regulate the expression of these MMPs via TIE sites. It has been demonstrated that TGF-β downregulates the transcription of MMP-1 and MMP-7 [55]. Furthermore, TGF-β modulates MMP-13 gene expression partly via the AP1 site [56]. Our findings also coincide as far as the correlation of MMP-1a/MMP-13 and TGF-β1 is considered. The induction of TG2 in fibroblasts lowered active MMP-1a and MMP-13 levels in a transamidase-dependent manner through TGF-β1 activation. Consistently, SNAP treatment of IND fibroblasts reverted the collagenase activity profile to resemble NI fibroblasts in a dose-dependent manner.

Previously, we showed that SNAP treatment not only led to the loss of TG2 transamidation activity in IND fibroblasts but also apprehension of TG2 on the cell surface where it potentially acted as a cell adhesion protein. It is possible that nitrosylation of TG2 leads to a conformational change in the enzyme structure leading to the loss of enzyme activity but

facilitating the interaction of the TG2 protein with the cell surface proteins such as β1, β3 integrins, SDC-4, or PDGFR [4,15,17,57,58]. On the other hand, nitrosylation of these cell surface partners can also bring about changes in their affinity towards TG2, which should be further investigated with proteomic analysis. In support of our hypothesis, we showed for the first time that the association of TG2 with ITGβ-1 and SDC-4 on the cell surface was decreased in the presence of SNAP while increasing TG2/PDGFR interaction triggering PDGFR Y751 phosphorylation (Fig. 7), which was reported to be important for the activation of downstream phosphatidylinositol 3-kinase signaling [59]. When taken together with earlier studies demonstrating that TG2 facilitates focal complex maturation to initiate cell migration through protein-protein interactions [60] and TG2-mediated activation of PDGFR-PI3K-AKT signaling induces cell migration and neointima formation [18,19], our findings suggest that NO-rescued cell migration in IND fibroblasts may be not only due to the inhibition of TG2 transamidation activity but also the promotion of TG2-PDGFR interaction.

In conclusion, our results shown in this paper support the idea that in the physiological settings of wound healing, the temporary matrix containing cross-linked fibrin and fibronectin in the wound area was degraded by TG2-mediated TGFβ-1 activation of MMP-2 and MMP-9 until NO release hinders the TG2 enzyme activity, promoting MMP-1 and MMP-13 activation and ECM remodeling to facilitate cell migration by reinforcing TG2/PDGFR association. Given that, constitutive activation of TG2 and subsequent induction of TGF β-1 leads to generation of an ECM with increased matrix strength, potential translational relevance of our findings can be the use of NO donor drugs as anti-fibrotic agents to finely tune the fibrotic response in the late stages of wound healing. In support, previous studies showed that application of exogenous NO ameliorates wound healing response [61] and prevents accumulation of excessive connective tissue by activating NO-guanylate cyclase pathway [62].

Experimental procedures

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM) (Cat No: D6429), HAT media supplement Hybri-Max (Cat No: H0262), G-418 (Cat No: A1720), Xanthine sodium salt (Cat No: X2502), Fetal Bovine Serum (FBS) (Cat No: F9665), Tetracycline hydrochloride (Cat No: T7670), monoclonal anti-β-actin (Cat No: A5441) were purchased from Sigma-Aldrich®.

TGM2 monoclonal antibody (CUB 7402) (Cat No: MA5-12739) was purchased from Thermo Fischer (USA). Anti-mouse IgG-HRP (Cat No: sc-2031), integrin beta 1 antibody (M-106) (Cat No:sc-8978) and PDGFR-β Antibody (958) (Cat No: sc-432) were purchased from Santa-Cruz. TGF-beta (MAB 1835) (Cat No: MAB1835) and mouse IgG1 isotype control (Cat No: MAB002) antibodies were from R&D Systems. Anti-syndecan 4 antibody (Cat No: ab24511) was purchased from Abcam. Phospho-PDGF Receptor β (Tyr751) (Cat No: 4549S) was purchased from Cell Signaling Technology. QuantiTect Primer MMP-2 (Cat No: 249900, QT00116116), MMP-9 (Cat No: 249900, QT00108815), MMP-1a (Cat No: 249900, QT00138894) and MMP-13 (Cat No: 249900, QT00111104) were purchased from Qiagen. MMP-2 (Cat No: PF037), MMP-9 (Cat No: PF038), MMP-1a (Cat No: PF063) and MMP-1a (Cat No: 444248) proenzyme standards were purchased from Merck.

Cell culture and wound scratch assay

Swiss 3T3 fibroblasts stably transfected with tetracycline (tet)-controlled transactivator [63] and transactivator-controlled tissue transglutaminase cDNA [39] constructs (clone TG3) were cultured as described before in the special-conditioned medium containing 10% (v/v) heat-inactivated FBS, 200 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 400 μg/mL active G418, 250 μg/ml xanthine, HAT supplement, 10 μg/mL mycophenolic acid, and 2 μg/mL tetracycline [15,39]. TG2 expression was induced (IND) by the withdrawal of tetracycline from the medium for 72 hours. For the scratch assay, cells seeded on 12-well plates (600.000 cells/well) for 24 hours were scratched and incubated in 4% FBS containing special-conditioned medium with (Non-induced; NI) or without tet following a PBS-wash. While NI cells were used as the negative control, IND cells were treated either with TG2 inhibitor Z-DON (50 μM) or previously optimized low (50 μM), medium (150 μM), and high (300 μM) concentrations of NO donor S-nitroso-N-acetylpenicillamine (SNAP). TGF-β neutralizing and control mouse serum IgG was used at 10 μM of concentration to determine the role of TGF-β in TG2-mediated suppression of cell migration. Images of the whole wound bed were captured at 24 and 48 hours using the phase-contrast microscope (Nikon Eclipse TS-100, USA) at 4x magnification. At least 10 images/well were analyzed using the image analysis program Image J (National Institute of Health), and the percentage wound closure area was calculated as the mean percentage of NI cell-wound closure rate (which represented 100%) recorded at 48 hours.

Analysis of MMP expression and activity

The effect of TG2 inhibitor, SNAP and TGF-β neutralizing antibody and the effect of TGF-β on the expression levels of gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1a and MMP-13) in IND Swiss 3T3 fibroblast cell lines were by quantitative real-time reverse transcription-PCR as described [29]. Briefly, RNA transcribed to cDNA using Sensiscript RT kit (Qiagen) was subjected to real-time PCR analysis performed with pre-validated QuantiTect MMP-2, -9, -1a, -13 primer sets in SYBR Green PCR master mix (Qiagen) using iCycler iQ Real-Time PCR detection system (Bio-Rad). 18sRNA was used as the house-keeping reference gene in PCR reactions for each sample. For the detection of MMP activity, gelatin and collagen zymography was performed as described in Chau et al. [64]. Band intensities in zymography gels were quantified using the image analysis program Image J (National Institute of Health) and band intensities for NI was defined as 1-fold.

Immunoblotting and Co-immunoprecipitation

Swiss 3T3 cells transfected with TG2 under the control of a tetracycline (tet)-off promoter were cultured as described previously [15,29]. Cells were treated with 50, 100, and 300 μM of SNAP and cell lysates were used incubated with anti-ITGβ-1 or SDC-4 or PDGFR-β1 antibody to immunoprecipitate immune complexes which were then resolved in SDS-PAGE and analyzed for TG2 association via Western blot. The membrane was labeled and visualized with an anti-TG2 Cub7402 antibody to determine the TG2 protein interacting with the ITGβ-1 or SDC-4 or PDGFR-β1 [15]. In-put samples and β-actin antibody were used for normalization to show that the protein was loaded in equal amounts in the SDS-PAGE wells.

Statistical analysis

The statistical differences between the gene expression results were analyzed using Student's t-test. The migration assay results were also examined using Student's t-test. Student's t-test analyses were calculated in GraphPad Prism (version 5.03) program. All results were considered statistically significant when $p<0.05$.

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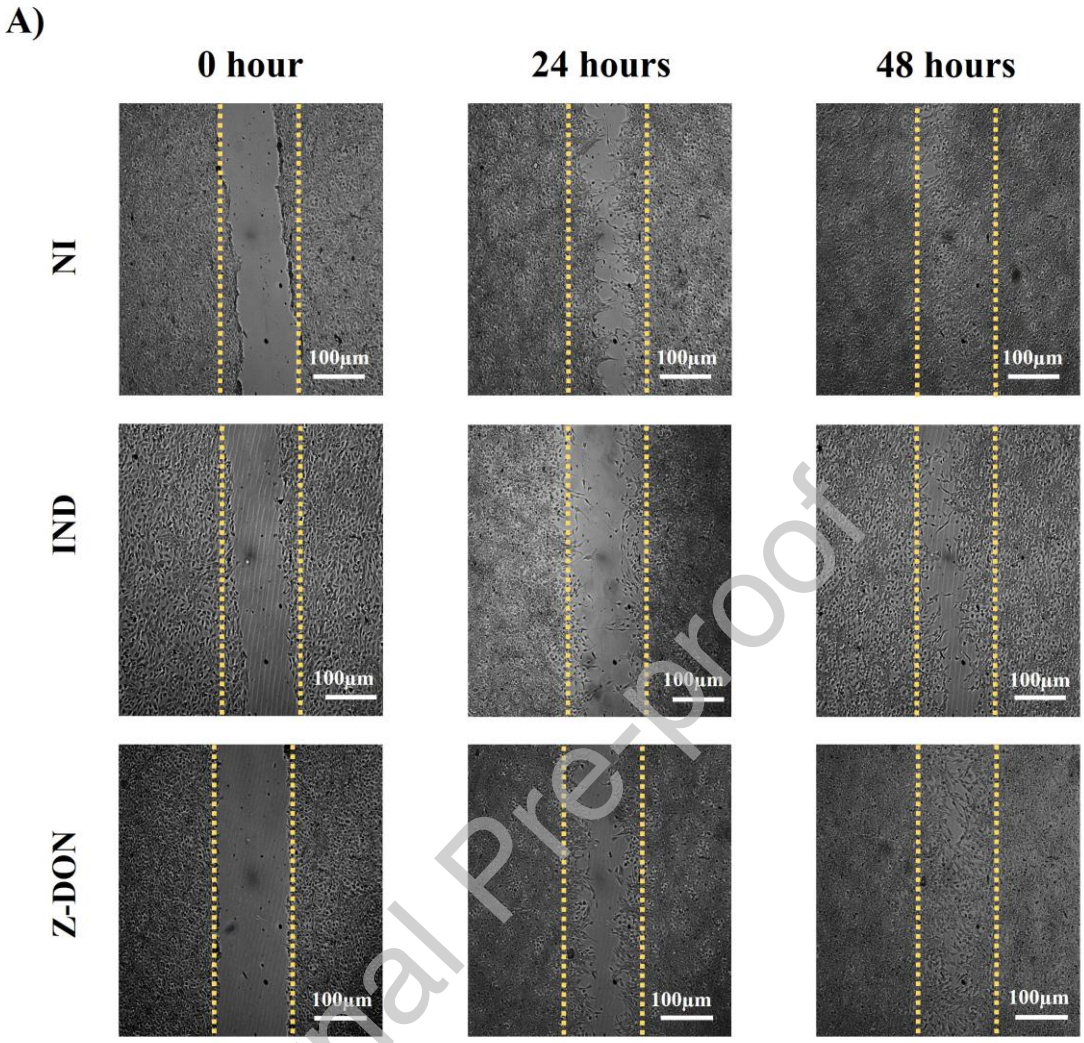
Figure Captions



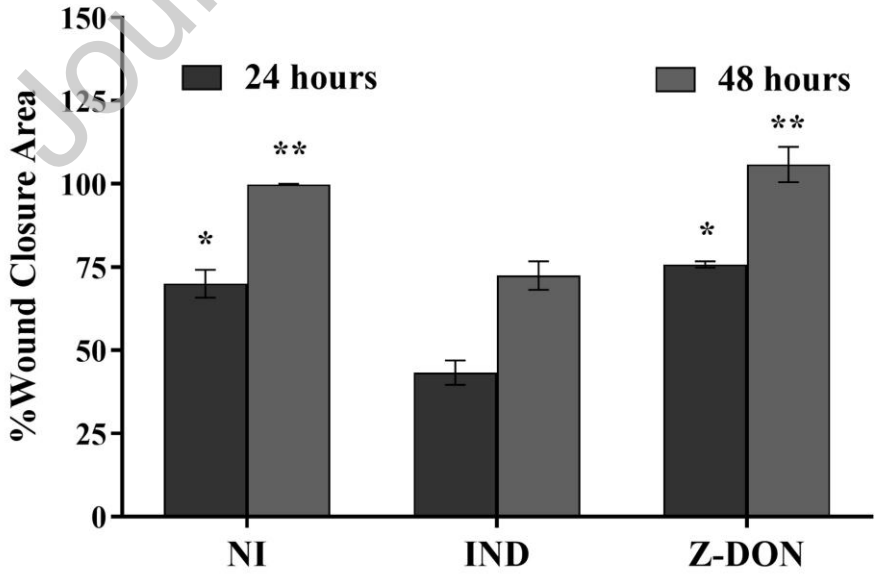


Figure 1. Effect of TG2 Activity on Cell Migration. a) Migration potential of non-induced (NI), induced fibroblasts to overexpress TG2 (IND) was investigated using wound scratch assays in the presence and absence of 50 µM TG2 inhibitor (Z-DON). Cell migration to wound bed was visualised at 24 and 48 h with a phase-contrast microscope (at 4x magnification) **b)** Each data point represents the mean percentage for closure of the wound area ± S.E.M. of three independent experiments performed in duplicate. The wound closure rate was expressed as the mean percentage of migration of NI fibroblasts at 48 hours (control) ± S.E.M., which represents 100%. The migration rate of IND fibroblasts in the presence and absence of Z-DON in 24 hours were calculated and expressed as the mean percentage of control ± S.D. * and ** represent the significant difference $p<0.05$ and $p<0.01$, respectively, by paired sample t-test.

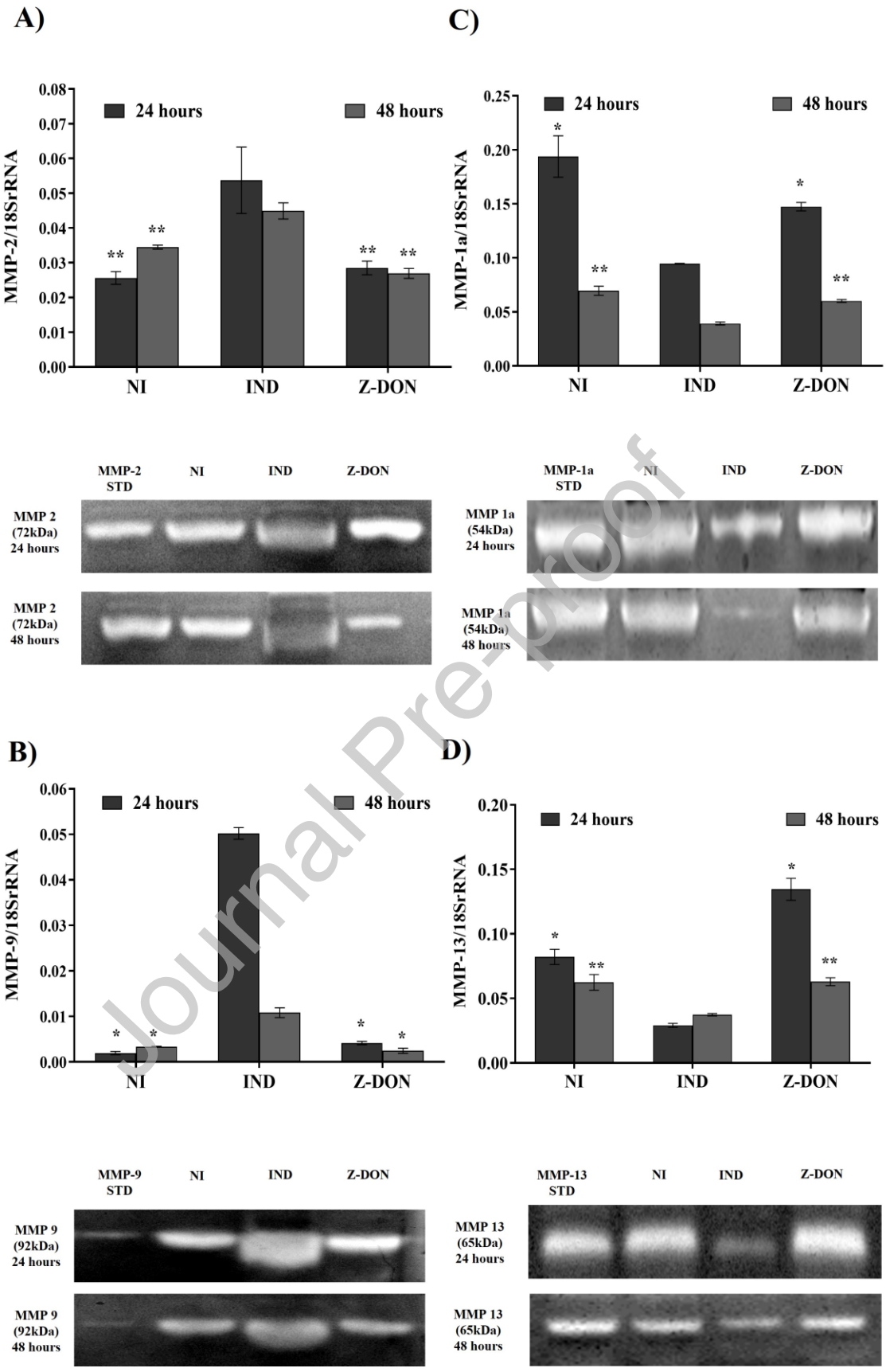


Figure 2. Effect of TG2 expression and activity on MMP levels. The effect of increased TG2 expression (IND) and its inhibition by the TG2 inhibitor (Z-DON) on MMP-2 **(a)**, MMP-9 **(b)**, MMP-1a **(c)**, and MMP-13 **(d)** biosynthesis was examined with RT-PCR, while MMP activity in culture media was determined by gelatine and collagen zymography. RNA samples for RT-PCR experiments were isolated from fibroblasts at 24^{th} and 48^{th} hours as described in "Experimental Procedures." mRNA levels for each gene of interest were normalized to 18 S RNA mRNA (average ± S.D. of the mean for three independent experiments). MMP standards were used as positive loading control. ***$p<0.001$, **$p<0.01$, *$p<0.05$, Student's paired t-test.

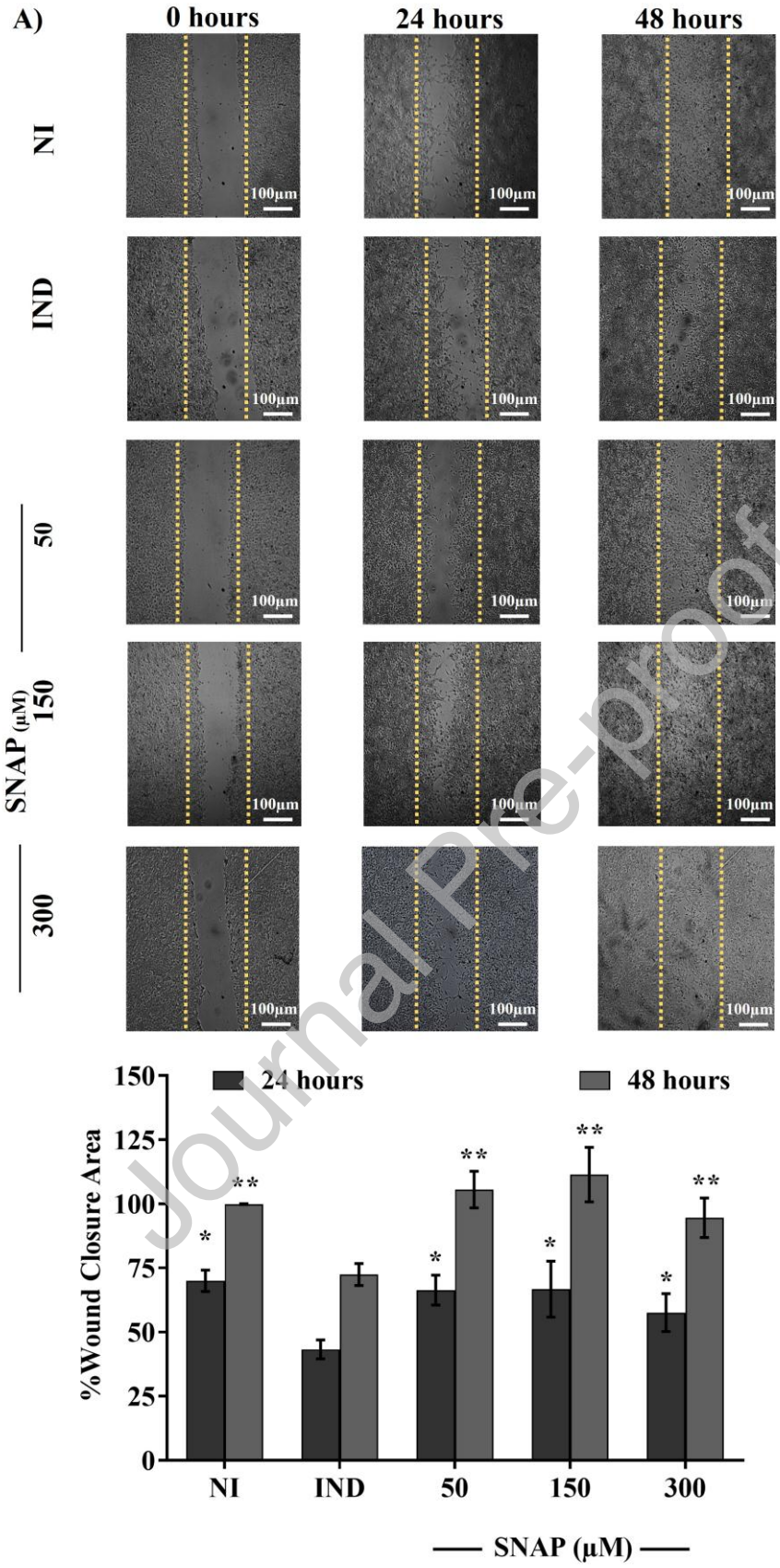


Figure 3. Effect of NO on the migration of TG2 induced fibroblast cells. a) The wound closure rate over 24 and 48h for 50 µM, 150 µM, 300 µM SNAP treated TG2 overexpressing

fibroblasts (IND), was analyzed by wound scratch assay. Representative photographs were taken by 4x objective and bars indicate 100 µm. Non-induced (NI) fibroblast cells was used as positive control. **b)** Mean value of the closed wound area ± S.E.M from three independent experiments was calculated for each data point using Scion Image Analysis Program. Wound closure area for NI fibroblast at 48 hours was used as 100%. Statistical significance between NI and treated and non-treated IND fibroblasts was analyzed by paired sample t-test (**$p<0.01$, *$p<0.05$).

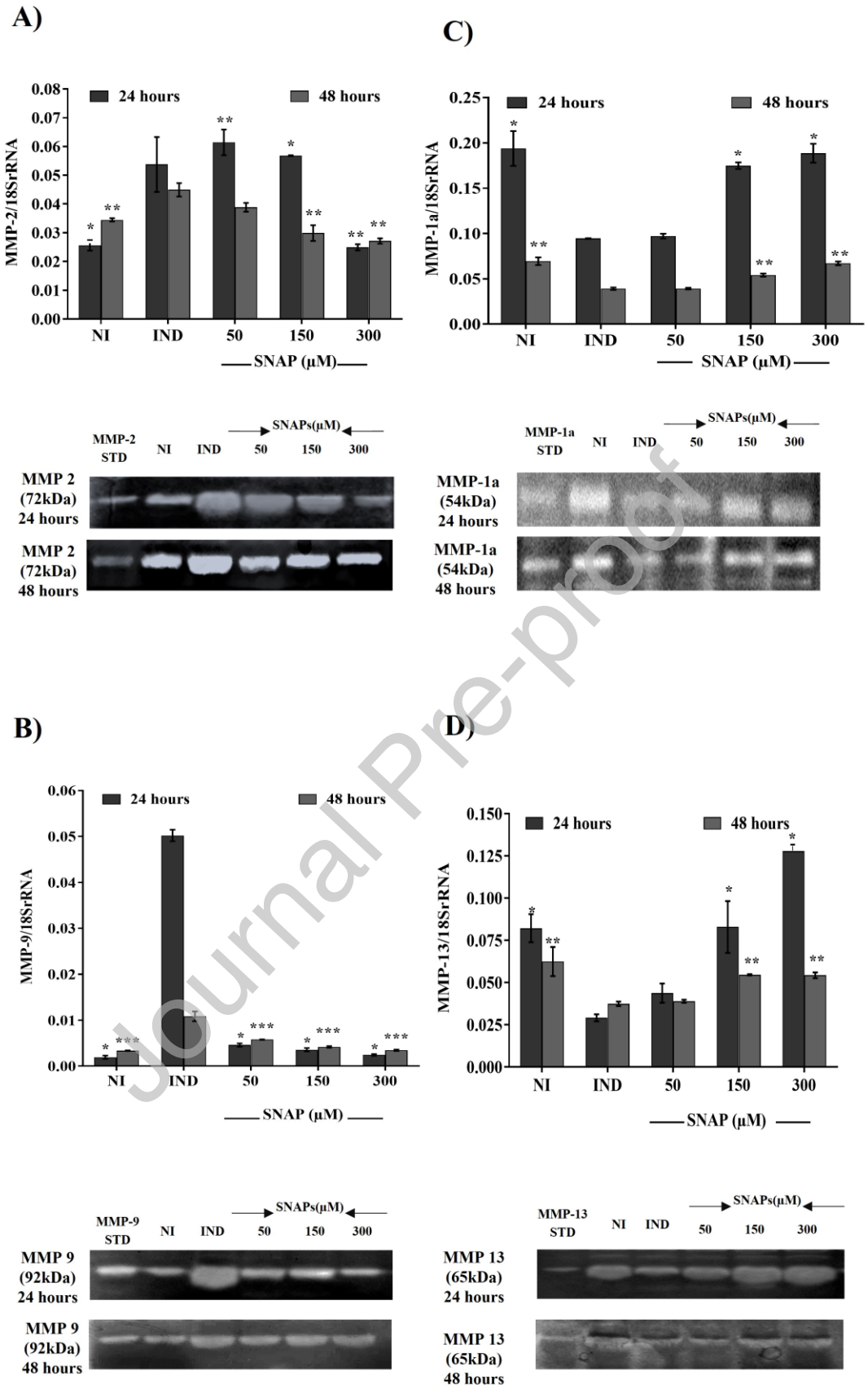


Figure 4. Effect of NO on TG2 regulated MMP expression and activity in fibroblast cells. TG2-induced transcriptional and translational activation of MMP-2 (a), MMP-9 (b),

MMP-1a (c), and MMP-13 (d) was analyzed by RT-PCR and zymography in noninduced (NI) and induced cells (IND) treated with 50, 150, and 300 μm SNAP. Gelatin zymogram was used to analyze MMP-2 and -9 activity, while collagen zymogram was used to analyze MMP-1a and -13 activity in the culture media collected from fibroblasts at 24^{th} and 48^{th} hours as described in "Experimental Procedures." MMP standards were used in zymography to identify the MMP band positions. MMP mRNA levels were normalized to 18sRNA for each MMP was measured in RNA samples isolated from fibroblasts at 24^{th} and 48^{th} hour (means ± S.D. of the mean for three independent experiments). ***$p<0.001$, **$p<0.01$, *$p<0.05$, Student's paired t-test.

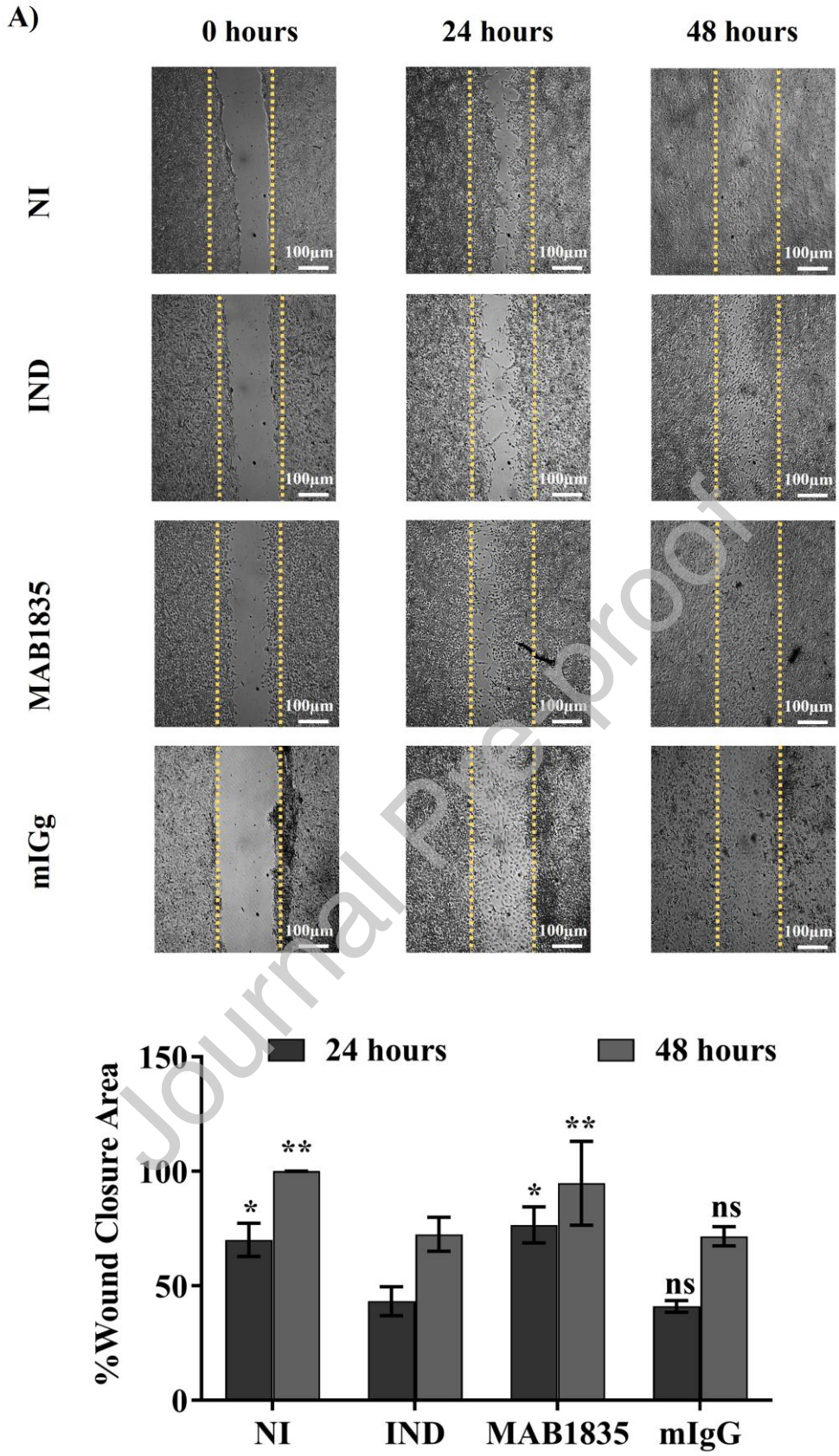


Figure 5. Role of TGFβ in TG2-mediated suppression of cell migration. a) Migration of non-induced (NI), induced cells (IND) incubated in the presence or absence of 10 µg/ml

TGFβ neutralizing antibody (IND MAB1835) for 24 and 48 hours was analyzed by wound-healing assay. 10 µg/ml of mIgG was used as negative control for MAB1835. Images of wound area were taken at 24^{th} and 48^{th} hours by 4x objective. Bars indicate 100 µm. **b)** Each data point represents the mean percentage of closed wound area ± S.D. from three independent experiments performed in duplicate. The mean percentage of wound closure rate recorded for NI fibroblast cells at 48 hours (control) ± S.D. was set at 100%. The significant difference between the NI control and the experimental groups was analyzed by Student's paired t-test (**$p<0.01$, *$p<0.05$, ns was used for non-significant difference).

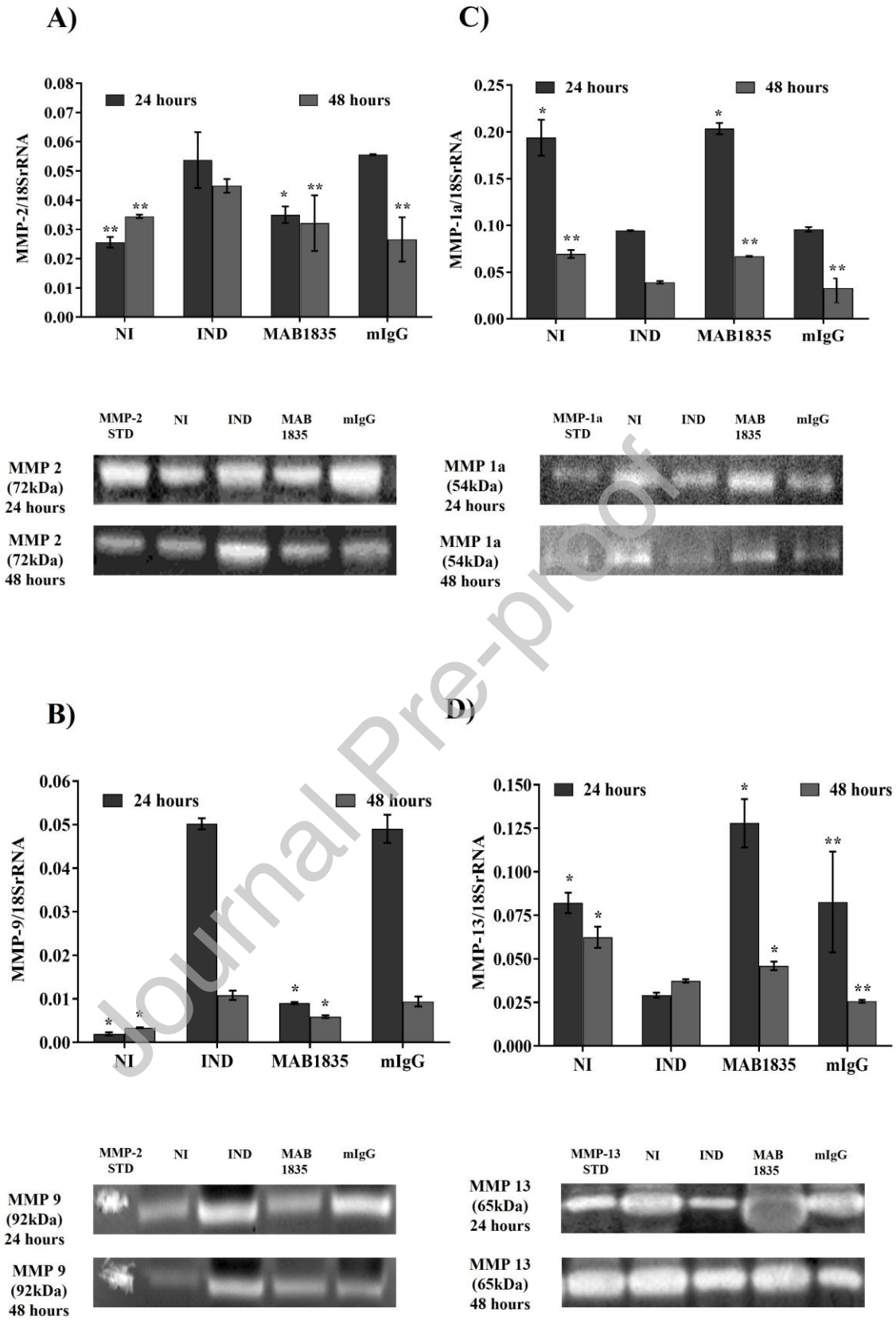


Figure 6. Role of TGFβ on TG2-regulated MMP expression and activity in fibroblast cells. The role of TGFβ on the expression and activity levels of gelatinases MMP-2 **(a)** and MMP-9 **(b)** and collagenase MMP-1a **(c)** and MMP-13 **(d)** was assessed in total RNA and medium samples obtained from non-induced (NI), induced (IND), and 10 µg/ml TGFβ

neutralizing (MAB 1835) and control antibody (mIgG) treated induced fibroblast cells at 24- and 48-hour time points. MMP mRNA levels analyzed by real time-PCR were normalized to 18 S RNA mRNA (means ± S.D. of the three independent experiments). MMP standards were included in the first lanes of zymograms to indicate correct MMP band activity. ***, ** and * indicate the statistical differences $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

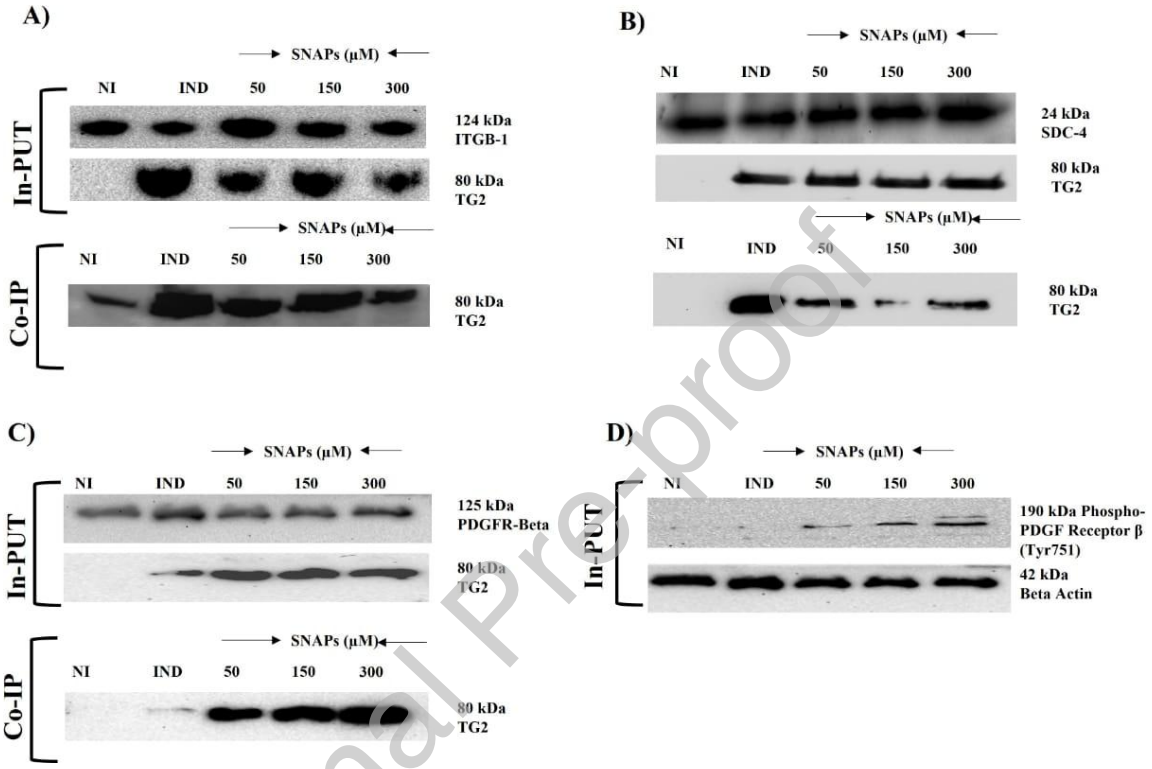


Figure 7. Effect of NO on TG2 association with its cell surface binding partners ITGβ -1, SDC-4 and PDGFR in fibroblast cells. (a) Cell surface TG2 association with integrin β1 (ITGB-1) (**b)**, syndencan-4 (SDC-4) **(c)** and platelet-derived factor receptor β (PDGFRβ) cell surface proteins in in non-induced (NI) and induced (IND) fibroblast treated with 50, 100, and 300 μM of SNAP was determined by co-immunoprecipitation (Co-IP). The cell lysate-in-puts were used to determine the equal loading of proteins (top blots). IP experiments were performed with anti-ITGB-1 or anti-SDC-4 or PDGFRβ and Western blotted for TG2 (bottom blots). **(d)** Western blot analysis of phosphorylated PDGFRβ at Tyrosine 751 residue (PDGFRβ-Y751) and β-actin that is used for equal loading.