THE ROLE OF THE INTERACTION BETWEEN TISSUE TRANSGLUTAMINASE AND NITRIC OXIDE IN WOUND HEALING

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ABSTRACT

THE ROLE OF THE INTERACTION BETWEEN TISSUE TRANSGLUTAMINASE AND NITRIC OXIDE IN WOUND HEALING

Tissue transglutaminase (TG2) plays a bifunctional role in wound healing by catalyzing crosslinking reactions to mediate the stabilization of the extracellular matrix (ECM) and by acting as a cell adhesion protein to induce cell migration. In this process, nitric oxide (NO) plays a pivotal role by altering the biological activity of TG2 through nitrosylation of free cysteine residues. The neutralization of TG2 activity via NO blocks the transcriptional activity of TGF-β1, which has an essential role in the synthesis of the ECM and the modulation of matrix metalloproteinases (MMP), and regulates cell adhesion and migration possibly through controlled ECM degradation. In this study, to further elucidate the effects of TG2 mediated TGF-β1 activation and the role of NO in the control of TG2 cross-linking activity on cell migration, tet-off Swiss 3T3 fibroblasts stably transfected with TG2 cDNA were used as the cell model. Wound scratch and transwell migration assays performed in the presence of TG2 inhibitor, TGF-β1 function blocking antibody, and NO donor S-Nitroso-N-acetylpenicillamine (SNAP) indicated that the migration of fibroblasts was significantly promoted by the inhibition of TG2 cross-linking activity. The nitrosylation of TG2 by NO possibly enforces a conformational change in TG2 causing the enzyme to function as a cell adhesion protein. Analysis of the gene expression levels of MMP-2, MMP-9, MMP-1a, and MMP-13 showed that the inhibition of TG2 activity by NO not only downregulates MMP-2 and MMP-9 but also upregulates MMP-1a and MMP-13 expression levels by the reduction of TGF-β1 activity.

ÖZET

DOKU TRANSGLUTAMİNAZI VE NİTRİK OKSİT ARASINDAKİ İLİŞKİNİN YARA İYİLEŞMESİNDEKİ ROLÜ

Yara iyileşmesinde TG2 enzimi iki fonksiyonlu bir rol oynar. Bir yandan transamidaz aktivitesiyle hücreler arası matrisin (HAM) sabitlenmesini düzenlerken, diğer yandan hücre adezyon molekülü olarak görev yaparak hücre göçünü arttırdığı görülmüştür. Bu süreçte nitrik oksit, serbest sistein rezidülerinin nitrosilasyonu yoluyla TG2'nin biyolojik aktivitesini değiştirerek oldukça önemli bir rol oynar. TG2 aktivitesinin NO tarafından engellenmesi, HAM'ın sentezlenmesinde ve aynı zamanda HAM'ı keserek hücre göçü ve adezyonunu düzenleyen MMP'lerin düzenlenmesinde önemli bir rol oynayan TGF-β1'in transkripsiyonel aktivitesinin bloke olmasına yol açtığı gözlemlenmiştir. Bu çalışmada, TG2 enzimine bağlı olarak çalışan TGF-β1 aktivasyonun ve NO'in bir çapraz bağlama enzimi olarak görev yapan TG2'yi etkileyerek hücre göçü üzerindeki etkisi incelenmiştir. Model hücre hattı olarak TG2 genini indüklenebilir bir şekilde tetrasiklin promoter kontrolü altında ifade eden Swiss 3T3 fibroblast hücre hattı kullanılmıştır. TG2 enziminin çapraz bağlama aktivitesinin NO donörü SNAP ve TG2 enzim inhibitörü tarafından inhibe edilerek fibroblast hücre göçünü arttırdığı yara yatağı ve transwell hücre göçü yöntemleriyle gözlemlenmiştir. Bunlara ek olarak, TGF-β1 fonksiyon bağlayıcı antikor kullanılarak TGF-β1 enzim aktivitesinin engellenmesinin hücre göçünü arttırdığı yine bu yöntemler ışığında açıklığa kavuşturulmuştur. NO tarafından TG2'nin çapraz bağlama aktivitesinin durdurulması ve muhtemelen TG2'nin üç boyutlu yapısının değiştirilerek bir hücre adhezyon molekülü halini almasının fibroblast göçünü arttırdığı işaret edilmektedir. Bunlara ek olarak, MMP-2, MMP-9, MMP-1a, ve MMP-13 gen ifadelerinin analizleri ışığında NO'in TG2 çapraz bağlama enzimin aktivitesini engelleyerek TGF-β1 yolağından sadece MMP-2 ve MMP-9 gen ifadelerinin azalmasına değil aynı zamanda MMP-1a ve MMP-13 gen ifadelerinin artmasına yol açtığı bu çalışmamız doğrultusunda gözlemlenmiştir.

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bFF	Basic Fibroblast Factor
BTC	Biotin cadaverine, trifluoroacetate salt (N-(5-aminopentyl) biotinamide, trifluoroacetic acid salt)
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
FN	Fibronectin
IL-1	Interleukin-1
Ind	Induced
LAP	Latency Associated Peptide
LTBP	Latent Transforming Growth Factor-Beta 1 Binding Protein
LTGF-β	Latent Transforming Growth Factor-Beta
M	Molar
McolA	Murine Collagenase-like A
ml	Milliliters
MMPs	Matrix Metalloproteinases
MT-MMPs	Membrane-type Matrix Metalloproteinases
µg	Microgram
µl	Microliter
µM	Micromolar
NI	Non-Induced
nm	Nanometer
NO	Nitric Oxide
PDGF	Platelet-Derived Growth Factor
RGD	Arginine-Glycine-Aspartate
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
q-PCR	Quantitative Polymerase Chain Reaction
S.D.	Standard Deviation
S.E.M.	Standard Error of the Means
SNAP	(±)-S-Nitroso-N-acetylpenicillamine
Swiss 3T3	Mouse Fibroblast Cell Line

TG2	Tissue Transglutaminase-2 protein
TGF-β	Transforming Growth Factor-Beta
TIMP	Transcription of Tissue Inhibitor of Metalloproteinases
TMB	3,3’,5,5’-Tetramethyl benzidine
TNF-α	Tumor Necrosis Factor-Alpha
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1. WOUND HEALING

Wound healing is composed of three interrelated, tightly controlled and overlapping phases of haemostasis and inflammation, new tissue formation followed by tissue remodeling as shown in Figure 1.1. These phases are complex but well-characterized and require the interactions between different cell types and tissues. It is suggested that various factors such as cytokines, growth factors and proteases have significant roles at the different phases of the wound healing process [1, 2].

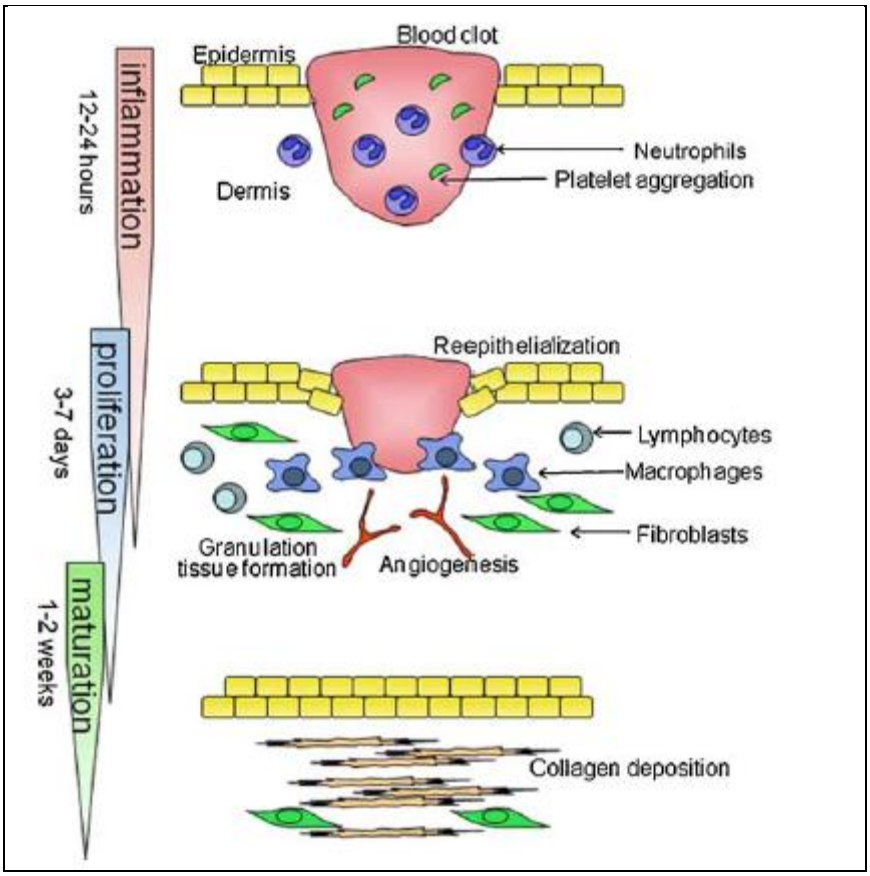

Figure 1.1. Phases of the wound healing [2]

1.1.1. Haemostasis and Inflammation

The inflammatory phase begins instantaneously after wounding as an aim to providing hemostasis [3, 4]. Blood loss is reduced by the vasoconstriction of smooth muscle cells and haemostasis is initiated by the platelet activation and aggregation resulting in the release of clotting factors at the site of vascular injury [5-8]. These factors activate the intrinsic and extrinsic coagulation pathways which lead to the formation of fibrin clots preventing further blood loss through the obstruction of the disrupted blood vessels [9]. Due to the activation of intrinsic and extrinsic coagulation pathways provisional matrix is formed from cross-linked fibrin and fibronectin (FN) that is deposited into the wound site in response to tissue damage [1, 10, 11]. Platelets initiate the healing process by producing growth factors such as platelet-derived growth factor (PDGF) which not only induces the recruitment of neutrophils and macrophages, fibroblasts and smooth muscle cells but also stimulates proliferation [12]. Attraction of neutrophils and macrophages into the wound area commences the inflammatory phase and is necessary for the removal of bacteria and foreign debris from the wound bed [3, 6, 7]. Macrophages are attracted into the site of injury by transforming growth factor-beta (TGF-β) which stimulates them to produce additional cytokines such as PDGF, tumor necrosis factor alpha (TNFα), fibroblast growth factor (FGF), interleukin-1 (IL-1), and several other chemokines. Secretion of these factors by macrophages enhances the inflammatory response and ameliorates the chemotaxis of smooth muscle cells and fibroblasts, thereby regulating the expression of collagenase and the synthesis of collagen. Subsequently, macrophages initiate the formation of new tissue and the proliferative phase begins toward the end of the inflammatory phase [12].

1.1.2. Granulation Tissue Formation

Due to tissue damage there is a loss of vascularity, which is restored through the formation of vascular connective tissue, commonly referred to as ‘granulation tissue’. In granulation tissue formation, macrophages and fibroblasts also migrate to the wound site besides the blood vessels and play a role in the release of growth factors including the vascular endothelial growth factor (VEGF), basic fibroblast factor (bFF), and TGF-β to the wound area, which activates neovascularization and angiogenesis. The release of TGF-β and PDGF from macrophages stimulates fibroblast gene expression and proliferation. Upon

stimulation fibroblasts start to express and deposit the structural protein FN as well as TGF-β and PDGF in an autocrine manner. The provisional matrix formed as a result of the coagulation process is important as it provides the initial scaffold for cell migration but is later on degraded by proteases and replaced in the newly synthesized matrix by wound fibroblasts [13-15]. In other words, while this provisional matrix gives structural support for infiltrating fibroblasts, leukocytes and endothelial cells, it hinders cell migration and acts as a physical barrier due to its highly cross-linked nature, hence needs to be replaced [10, 14, 16-19]. Cell migration through cross-linked fibrin is tightly regulated by the cleavage of the ECM, leading to a path for cell movement [20, 21]. Fibroblasts initiate ECM synthesis subsequent to migration and provisional matrix is progressively replaced with a collagenous matrix due to the action of TGF-β1 on fibroblasts [22, 23].

1.1.3. Tissue Remodeling

The final phase of the wound healing process is tissue remodeling. During the remodeling step, there is an increase in the synthesis of the collagen content, although the number of fibroblasts and macrophages are reduced. Collagen maintains its maturation with the additional crosslink formation between the lysyl and hydroxylysyl residues which increase the tensile strength of the wound [24].

The major components of the extracellular matrix formed after tissue granulation are FN; hyaluronic acid; and fibrils of type I, III, and V collagen. The levels of cross-linked type I collagen and proteoglycans are increased and the amounts of type III collagen, FN, and hyaluronic acid levels are decreased in the remodeling step. In remodeling and matrix formation, growth factors such as TGF-β, PDGF, and fibroblast growth factor (FGF) entail discrete roles. For example, TGF-β augments the transcription of the tissue inhibitor of metalloproteinases (TIMP), a specific collagenase inhibitor and reduces matrix metalloproteinases (MMP) gene transcription *in vitro*, while PDGF and FGF induce the collagenase gene expression [25]. ECM remodeling leads to scar tissue formation through the conversion of granulation tissue into collagen rich scar tissue, in which is there is a continual synthesis and degradation of collagen fibrils [1, 26].

1.2. TISSUE TRANSGLUTAMINASE

Tissue transglutaminase (TG2) is a member of the transglutaminase enzyme family which mediates several distinct biochemical functions in different cell and tissue types [27]. TG2 resides in different compartments of cells including the nucleus, nuclear membrane, mitochondria, cytosol, cell surface plasma membrane and the extracellular matrix. TG2 is encoded by the 32.5 kilobase TGM2 gene and has been located to chromosome 20 [28, 29].

1.2.1. Structure of Tissue Transglutaminase

TG2 is a 76-85kDa monomeric protein of 685-691 amino acids [30]. In human TG2, amino acid sequence of the protein has revealed an active site with a cysteine residue located at position 277 [31]. TG2 protein that is isolated from guinea pig liver, has a total 17 cysteine residues and six potential glycosylation sites [32]. It is implicated that the transamidating activity of TG2 is regulated by the calcium-binding site of the enzyme [33].

The three dimensional structure of TG2 consists of an N-terminal β-sandwich domain, followed by a large α/β catalytic core, and two C-terminal barrels [34, 35]. The C277 residue of TG2 is essential for the enzyme's Є-(γ-glutamyl)-lysine crosslinking activity [36]. Studies also indicated that the transamidating activity of TG2 is not only mediated by the catalytic core domain, which is required for the hydrolysis of GTP and ATP, but also by the flanking domains that play a significant role in TG2 activity [37].

1.2.2. TG2 Activity

TG2 mediates the formation of Є-(γ-glutamyl)-lysine cross-links in a Ca^{+2}-dependent manner and the post-translational modification of proteins by amine incorporation [38]. In physiological conditions, concentrations of Ca^{2+} and nucleotides (GTP/GDP and ATP/ADP) are important factors for the regulation of TG2 enzymatic activity. In cellular conditions the low levels of intracellular Ca^{2+} and high concentrations of GTP/ATP lead to the inhibition of TG2 cross-linking activity, and the hydrolytic GTPase and ATPase activity of the enzyme is activated. On the other hand, in the extracellular milieu the high concentrations of Ca^{2+} and low concentrations of GTP/GDP and ATP/ADP results in a

conformational change in the enzyme leading to the induction of the cross-linking activity of TG2 [39]. Upon deposition into the ECM, TG2 cross-links the ECM proteins and increases the stability of the ECM [40]. Externalization of TG2 is dramatically augmented in response to tissue damage and cellular stress situations and it accumulates in the ECM [41-45]. On the cell surface, TG2 was shown to regulate the interaction of integrins with FN serving as a cell adhesion protein independent from its transamidating activity. Here, TG2 act as a signaling molecule in cell adhesion via interacting with cell surface β1 and β3 integrins and syndecan-4 [46, 47]. Cell adhesion is mediated via TG2 by binding to its extracellular substrate FN [48].

FN is a glycoprotein and one of the main components of the ECM. It functions in wound healing, angiogenesis and embryogenesis [49]. FN binds to α5β1 and αIIBβ3 integrins through its Arg-Gly-Asp (RGD) region and mediates cell adhesion [50].

1.2.3. TG2 in Wound Healing

TG2 expression was observed in all phases of rat skin wound repair. It was observed in the provisional matrix, re-epithelization, new tissue formation and tissue remodeling [51]. TG2 mediates the reconstitution of tissue integrity by its transamidating activity subsequent to wound healing. The wound area is stabilized by increased TG2 activity which occurs both in the intracellular and extracellular compartments and increased protein polymerization enhances cell-matrix interactions in the wound site [52]. Previous studies implicated that the stability of the sheet of TG2-/- MEF cells was improved by the exogenous addition of purified guinea pig liver TG2. The treatment of TG2-/- MEF cells with exogenous TG2 led to alterations in the cell migration pattern comparable to the wild-type phenotype [53]. TG2 contributes to the wound healing process not only by its transamidating activity, but also by its function as a cell adhesion protein. Neutralization of TG2 by iodoacetamide inhibited the activity of the enzyme without any inhibitory effect on cell adhesion [54]. TG2 can also indirectly modulate the wound healing through activation of the growth factor TGF-β1. This illustrated that TG2 activates TGF-β1 which plays a significant role in the synthesis and proliferation of the ECM in fibroblasts [55].

1.3. TRANSFORMING GROWTH FACTOR-β

1.3.1. Structure and Function of Transforming Growth Factor-β1

TGF-β is a 25 kD homodimeric peptide and there are five known isoforms of TGF-β, three of which are expressed in mammalian cells (TGF-β 1, 2, 3) [56, 57]. While all cell types express TGF-β receptors, TGF-β is expressed and synthesized only by several cell types such as macrophages and fibroblasts. TGF-β in a latent form in complex with a propeptide called latency associated peptide (LAP), and a latent TGF-β binding protein (LTBP). LAP consists of a pro-domain, called Latency Associated Peptide (LAP), an amino terminal (5') signaling sequence and a carboxy (3') mature protein domain [58]. The large latent TGF-β complex is activated by the incorporation of LTBP into the matrix by TG2 protein where the latent TGF-β complex can be degraded and active TGF-β can be released as shown in Figure 1.2 [60-62].

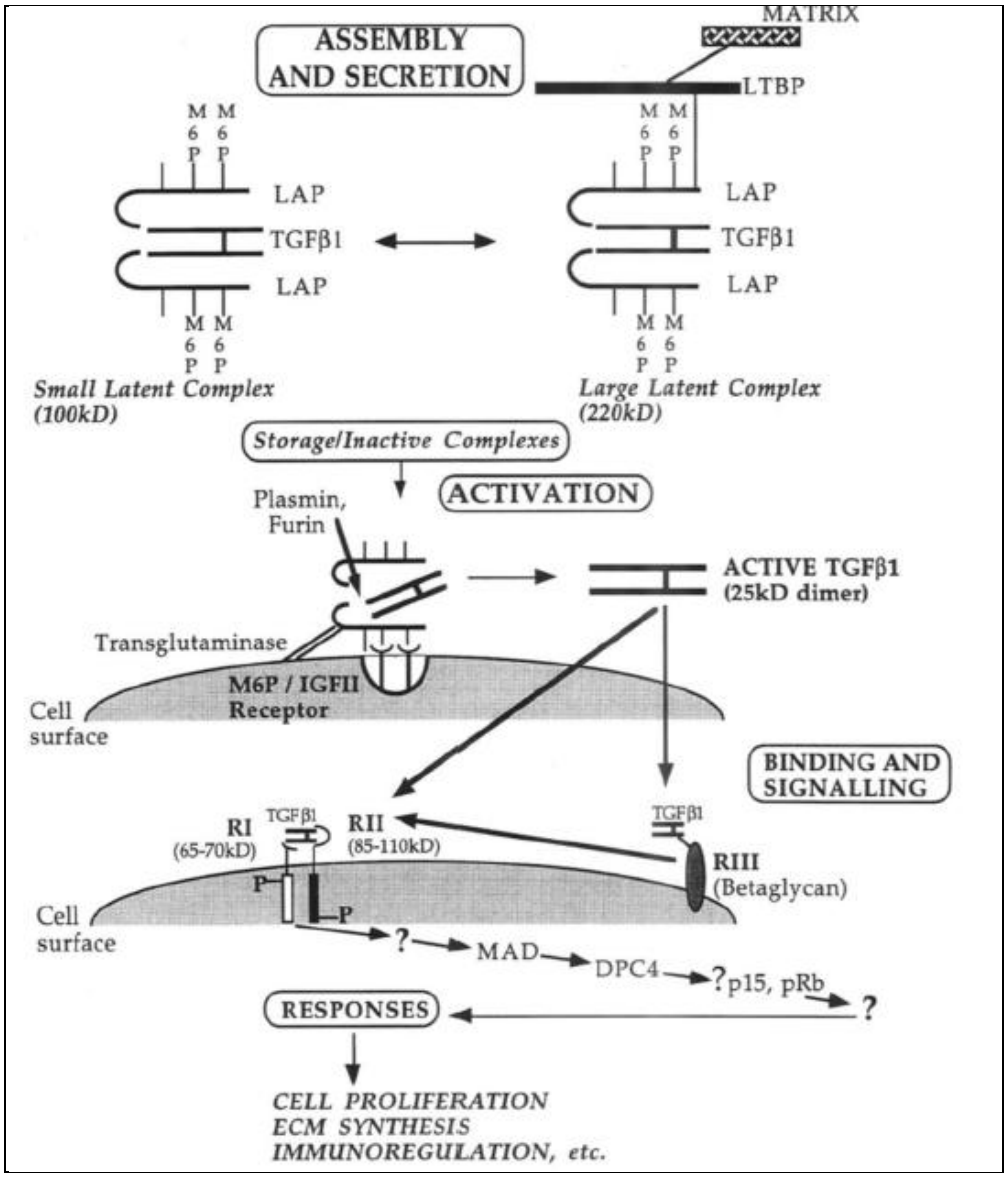

Figure 1.2. Activation of TGFβ-1 by TG2 [63]

1.3.2. TGF-β1 in Wound Healing

TGF-β1 also has a crucial role in wound healing, not only by attracting cells to the wound site, but also by promoting the deposition of collagen and matrix [63]. While, low concentrations of TGF-β leads to fibroblast division, high concentrations of TGF-β stimulates the differentiation of fibroblasts. Differentiated fibroblasts cause increased collagen, FN and ECM production [64, 65]. *In vitro* models suggest the role of TGF-β1 as the modulator of the provisional matrix composition is to induce the migration of epithelial

cells [66]. TGF-β1 is also important in the regulation of wound healing since it modulates the synthesis of certain matrix metalloproteinases [67-69]. In wound healing models, the treatment of TGF-β resulted in a decrease in the synthesis of collagenases and enhanced the production of the collagenase inhibitor TIMP-1 leading to an inhibition in the degradation of newly synthesized collagen matrix in fibroblasts [70, 71]. It is well documented that TG2 is important for the activation of TGF-β1 which plays a pivotal role in the synthesis of ECM and proliferation in fibroblasts [72]. However, it is also known that LTGF-β could be also activated by proteolysis or by the acidic environment in the wound site [73].

1.4. MATRIX METALLOPROTEINASES

1.4.1. Metalloproteinases

Matrix metalloproteinases (MMPs) constitute a large family of calcium-dependent zinc-containing endopeptidases that include gelatinases, collagenases, matrilysins, stromelysins and membrane-type metalloproteinases which have a role in tissue remodeling and the degradation of most structural components of the ECM. Varieties of cells such as fibroblasts, endothelial cells, osteoblasts, neutrophils, lymphocytes, and macrophages are responsible for secreting MMPs as zymogens which are subsequently activated by other proteolytic enzymes. MMPs are usually expressed minimally in normal physiological conditions and over-expression of MMPs abrogates the balance between the activity of MMPs and tissue inhibitors of MMPs (TIMPs) which could result in a variety of pathological conditions such as ulcers, cancer and cardiovascular diseases [26, 74-76].

The MMP family includes 26 types of human MMPs which are classified into collagenases, gelatinases, stromelysins, and matrilysins according to their specificity. MMPs also contain another subclass of MMPs which are represented by the membrane-type MMPs (MT-MMPs) that additionally contain a transmembrane domain, a membrane linker domain, and an intracellular domain [77, 78]. Besides the confined role of MMPs in the degradation of the ECM, MMPs have a role in the cleavage of growth factor receptors from the cell surface, release of growth factors from the cell membrane, shedding of cell adhesion molecules and activation of other MMPs [79-81].

1.4.2. MMPs in Wound Healing

MMPs have significant roles in all phases of wound healing by performing many functions [82, 83]. MMPs could contribute to inflammation by cleaving cytokines and chemokines from the cell surface to activate these mediators, or processing these mediators to enhance their activity. MMPs were also shown to inhibit inflammatory signals by degrading the cytokines and chemokines as shown in Figure 1.3. MMPs also play a crucial role in re-epithelization by cleaving the components of cell-cell and cell-matrix interactions within the epithelium. MMPs also involved in the remodeling of the ECM directly by degrading the proteins such as collagens as shown in Figure 1.3. *In vitro* analysis demonstrated that MMPs which are produced by fibroblasts in collagen lattices cause the contraction and remodeling of the collagen that is similar to the tissue remodeling in wound repair [84, 85]. Many MMPs have overlapping functions in these processes and human MMPs 1, 2, 3, 7, 8, 9, 10, 12, and 13 have a major role in wound remodeling [82, 83, 86-88].

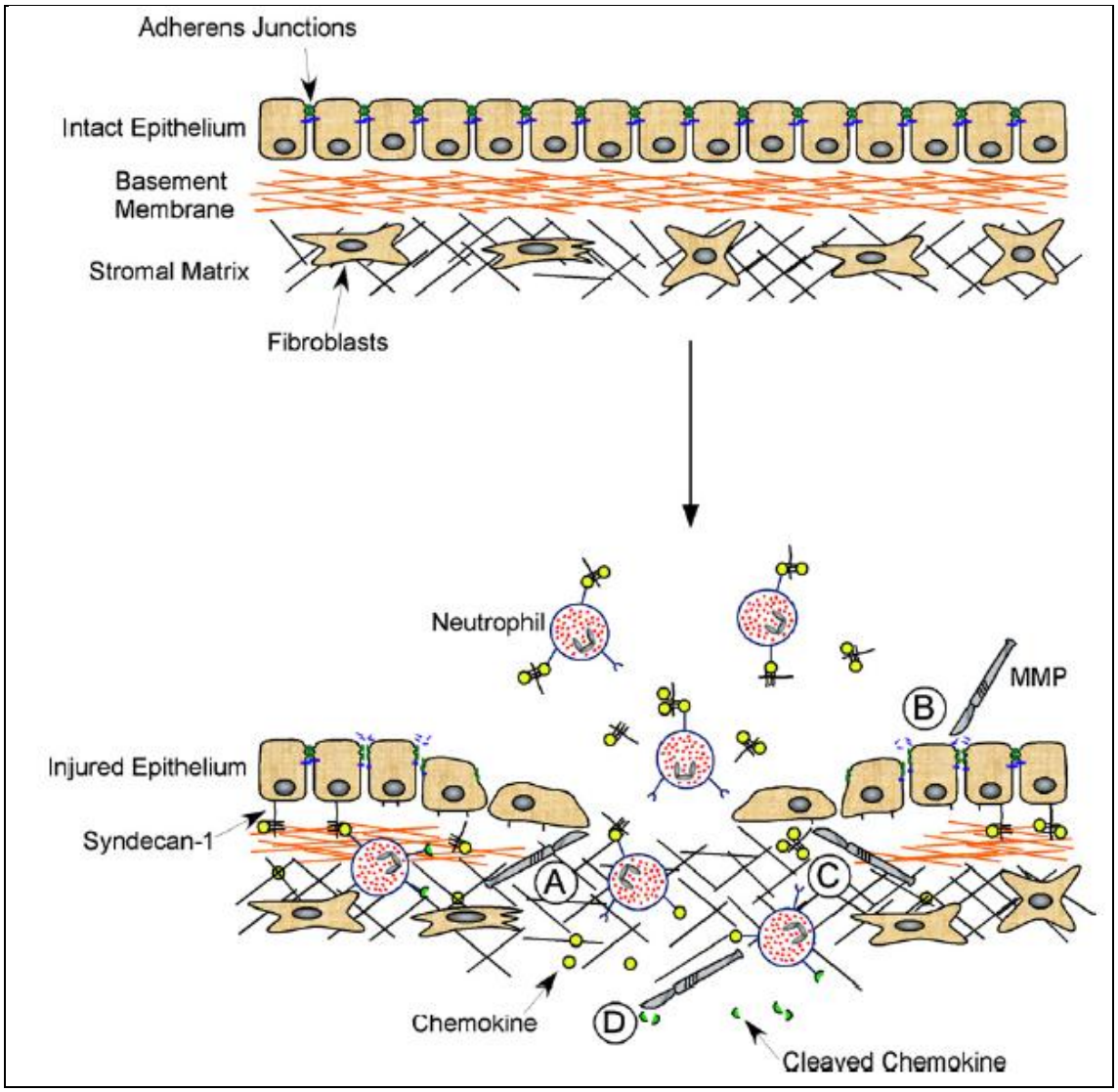

Figure 1.3. Various functions of MMPs during wound healing. (A) Cell migration: MMPs facilitate epithelial cell migration by cleaving cell-ECM and cell-cell adhesion proteins on the cell surface. (B) Re-epithelialization: MMPs cleave junctional proteins, and degrade cell–cell adhesions. (C) Leukocyte influx: MMPs shed the proteoglycan/chemokine complexes from the basolateral cell surface and lead to the transepithelial migration of neutrophils. (D) Inflammation: MMPs cleaves multiple cytokines and chemokines [83]

The wound healing process requires ECM synthesis, deposition and degradation. Several cell types such as fibroblasts, keratinocytes, macrophages and neutrophils in the wound site are responsible for ECM degradation and consequently modification of ECM composition and organization is performed by recruiting MMPs into the wound site [89]. The action of MMPs is tightly regulated in the tissue repair process at transcriptional, translational, and

post-transcriptional levels, because of their high potency [90]. Extracellular activation of MMPs from latent form finally through the action of TIMPs is also stringently controlled in the wound repair process [91-93].

In vivo analyses implicated that the expression of collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), matrilysin (MMP-7), stromelysins (MMP-3, -10), macrophage metalloelastase (MMP-12), membrane-type MMPs (MMP-14) and TIMP-1, -2, -3 are distributed among cells in skin repair [26, 94-96].

MMP-2 and MMP-9 (gelatinases) which are responsible from the cleavage of type IV, V, VII, and X collagen, and other basement-membrane proteins have crucial role in the remodeling phase [82, 83, 97, 98]. It was also suggested that MMP-2 and MMP-9 function in the proteolytic cleavage of the provisional matrix [16, 99, 100]. MMP-2 promotes cell migration by clearing the ECM and facilitates wound healing [101]. Studies also implicated that MMP-9 has a role in fibrinolytic events in an *in vivo* nephritis model and studies on MMP-9 null mice show a dramatic increase in fibrin deposition [102-104].

During the dermal wound healing process, MMP-9 shows sudden upregulation in the early phase of healing which suggests that it could have a role in re-epithelization, on the other hand, MMP-2 has sustained and prolonged expression which could indicate that it could also play a significant role in angiogenesis and matrix remodeling [74, 95]. It was also implicated that MMP-2 and MMP-9 expression levels dramatically increased by the addition of TGF-β1 in an *in vitro* model of airway epithelial cells [105].

Human MMP-1, MMP-8, and MMP-13 (collagenases) mainly cleave type I, II, and III fibrillar collagen in the ECM [82, 83, 97]. MMP-1 (Interstitial collagenase) is induced in the later stages of wound healing and lasts longer [106] and MMP-13 which is synthesized from the wound fibroblasts is a significant factor in tissue remodeling [107]. In mice, the original murine interstitial collagenase (MMP-13) is the homologue to human MMP-13. It has also been suggested that mouse MMP-13 is a functional homologue of human MMP-1, because they both are expressed in similar situations [108]. It was also indicated that the new enzyme murine collagenase-like A (McolA) or MMP-1a has significant sequence identity with the human MMP-1. MMP1a degrades collagen *in vitro* and it is located in the

mouse chromosome 9, MMP gene cluster. It was proposed that MMP-1a could represent a true orthologue and homologue of human MMP-1 which has a role as the interstitial collegenase in murine tissues [109]. Balance between ECM synthesis which consists of granulation tissue formation and collagen deposition, and degradation of matrix should be controlled in the normal wound healing process. Synthesis of matrix is important to allow cell migration [110, 111]. In cultured fibroblasts, the treatment of TGF-β leads to a decrease in the synthesis of collagenases and induces the production of collagenase inhibitor TIMP-1 leading to an inhibition in the degradation of newly synthesized collagen [70, 71].

1.5. NITRIC OXIDE

1.5.1. Structure and Function of Nitric Oxide

Nitric oxide (NO) is a short-lived free radical involved in many diverse biological processes such as neurotransmission, inflammatory and immune response and vasodilatation [112-113]. NO is synthesized from the conversion of L-arginine into L-citrulline by three different isoforms of nitric oxide synthase (NOS) such as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed and regulated by intracellular Ca^{2+} levels, whereas, iNOS is expressed independent from the Ca^{2+} levels and induced in response to the inflammatory signals [114]. NO mediates its biological effects by targeting and modifying iron and zinc clusters, cysteine residues and haem groups.

NO modifies cysteine thiols by the S-nitrosylation reaction which results in the regulation of proteins function. S-nitrosylation could be mediated directly in the presence of electron acceptors via the interaction with NO or indirectly by NO carriers and NO derivatives [115].

1.5.2. NO in Wound Healing

NO may play a pivotal role in all phases of wound healing by expression of three isoforms of NOS [116]. It has been demonstrated that while, nNOS expression was observed in

melonocyres and kerotinocytes, eNOS is expressed in dermal fibroblasts and endothelial cells and the upregulation of iNOS was observed in fibroblasts, kerotinocytes and endothelial cells [117].

It was documented that NO inhibits the cross-linking activity of TG2 via the nitrosylation of cysteine residues located in the active site of the protein. It was also implicated that TGF-β1 activity diminished through the inhibition of TG2 activity by NO which causes a decline in the ECM production and stability. TG2 modification by nitrosylation leads to the localization of TG2 on the cell surface instead of ECM and enhances the TG2 function as a cell adhesion protein [118].

1.6. AIM OF THE STUDY

The aim of the study is to investigate the role of TG2 and NO interaction in cell migration in order to acquire an understanding of the initial phases of wound healing. In the previous study performed on non-induced and induced Swiss 3T3 fibroblasts under the control of the tetracycline-regulated inducible promoter [120], it was clarified that inhibition of the cross-linking activity of TG2 by NO leads to the inhibition of transcriptional activation of TGF-β1 through NFκB pathway. TG2 functions as an RGD-independent cell adhesion protein, independent to its transamidating activity [118].

In accordance with the previous data, the role of transamidating activity of TG2 and the TG2 dependent activity of TGF-β1 in cell migration were analyzed via wound scratch and transwell migration assays by using the same cell model. In these experimental models, the effect of NO regulated TG2 and TGF-β1 activity in the cell migration was examined. In conjunction to this study, the expression profiles of the major ECM proteases MMP-2, MMP-9, MMP-1a, and MMP-13 in wound healing was evaluated in order to explain the molecular downstream effectors assigned in the NO regulated cell migration of TG2 induced fibroblasts.

2. MATERIALS

2.1. INSTRUMENTS

The instruments used in this study are as follows:

- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A, Singapore)
- CO2 incubator (Nuaire NU5510/E/G, USA)
- Inverted phase contrast microscope (Nikon Eclipse TS-100, USA)
- Fluorescence microscope (Nikon Eclipse TE-200, USA)
- Centrifuge (Hettich mikro 22R, Germany and SIGMA 2-5 centrifuge, Germany)
- Vortex (Stuart SA8, UK)
- pH meter (Hanna instruments PH211, Germany)
- Spectrophotometer (Implen Nanophotometer, USA)
- PCR Thermal Cycler (Biorad MyCycler, USA)
- Real-time PCR Thermal Cycler (Biorad iCycler iQ multicolor detection system, USA)
- -80 °C freezer (Thermo Forma -86C ULT Freezer, USA)
- ELISA plate reader (Bio-Tek Elx800, USA)
- Liquid nitrogen (Arpece 110, France)
- Vacuum-dryer (Eppendorf Concentrator 5301, Germany)
- Water bath (Grant OLS 200, UK)

2.2. EQUIPMENT

The laboratory equipment used in this study are as follows:

- Cell culture flasks, T-25, T-75, T-150 and cell culture plates, 6-well, 12-well, 96-well, (TPP, Switzerland or Grenier-Bio, Germany)
- Serological pipettes 25, 10, 5, 2 ml (Lp Italiana Spa, Italy or Axygen, USA)
- Polypropylene centrifuge tubes, 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany)

- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Micro pipette tips 1000, 200, 10 µl (Lp Italiana Spa, Italy or Capp, USA)
- Cryovials (TPP, Switzerland)
- Transwells (Costar, USA)

2.3. CHEMICALS

The chemicals used in this study are as follows:

- Cell culture media:
  - Dulbecco's Modified Eagle's Medium – high glucose (Sigma D6429 or Gibco 41966, USA)
  - Serum-free AIM-V (Gibco, USA)
- Growth supplements:
  - Fetal Bovine Serum (FBS) – Cell culture tested (Sigma F9665)
  - Non-essential amino acid (Sigma M7145)
  - G-418 sulphate (Gibco 11877-023, UK)
  - Xanthine sodium salt (Sigma X2502, Germany)
  - HAT media supplement Hybri-Max (Sigma H0262, USA)
  - Mycophenolic acid (Santa Cruz, sc-200110, USA)
- 2-propanol (AppliChem A3928, Germany)
- 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma T2885,China)
- Absolute Ethanol (AppliChem A3678, Germany)
- Biotin cadaverine, trifluoroacetate salt (N-(5-aminopentyl) biotinamide, trifluoroacetic acid salt) (Biotium 90063, USA)
- Bovine serum albumin (Sigma A7030, USA)
- CaCl2 (Riedel-de Haen 12022, Germany)
- Chloroform (Aldrich 528730, USA)
- Dimethyl sulfoxide (Santa Cruz sc-202581, USA)
- Dulbecco's Phosphate Buffered Saline (DPBS) (PAN Biotech P04-53500, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Merck 108418, Germany)
- Fibronectin from human plasma (Sigma F0895, USA)

- H2SO4 (Riedel-de Haen 07208, Germany)
- L-Glutamine (Invitrogen 25030, USA)
- Penicillin-streptomycin (Thermo Scientific SV30010, USA or Biochrom A2213, Germany)
- Phosphate-Citrate Buffer with Urea Hydrogen Peroxide (Sigma P4560, USA)
- Sodium deoxycholate (Sigma D6750, New Zeland)
- Tris-base (Merck 108387, Germany)
- Tris-HCl (Merck 108219, Germany)
- TriPure Isolation Reagent (Roche 11667157001, USA)
- Trypsin-EDTA (Biochrom L2153, Germany)

2.4. KITS AND SOLUTIONS

The commercially available kits that were used in this study were as follows:

- Cell Proliferation Reagent WST-1 (Roche 05015944001, Germany)
- (±)-S-Nitroso-N-acetylpenicillamine (Calbiochem 487910, USA)
- Tetracycline hydrochloride (Sigma T7670, China)
- Extravidin peroxidase (Sigma E2886, Israel)
- Zedira Z-DON-VPL-Ome (Zedira Z006, Germany)
- Taq DNA Polymerase (Fermentas EP0402, USA)
- Oligo dT primer (Qiagen 79237, Germany)
- QuantiTect Primer Assay MMP1a (Qiagen QT00138894, Germany)
- QuantiTect Primer Assay MMP13 (Qiagen QT00111104, Germany)
- QuantiTect SYBR Green PCR Kit (Qiagen 204145, Germany)
- Sensiscript RT Kit (Qiagen 205213, Germany)

2.5. ANTIBODIES

- TGF-β1, 2, 3 Antibody Monoclonal Mouse IgG1 Clone # 1D11 (R&D Systems MAB1835, USA)

2.6. CELL LINES

- Swiss Albino mouse embryo 3T3 fibroblast (ATCC CCL-92, USA)
    - TG3 Clone Swiss 3T3 fibroblasts stably transfected with tetracycline-controlled transactivator [119] and transactivator-controlled tissue transglutaminase cDNA [120].

3. METHODS

3.1. CELL CULTURE METHODS

3.1.1. Cells and Culture Conditions

Cell lines used in the project included the wild type and tissue transglutaminase (TG2) transfected Swiss Albino mouse embryo 3T3 fibroblast obtained from American Type Culture Collection (ATCC).

Swiss 3T3 fibroblasts stably transfected with tetracycline-controlled transactivator [119] and transactivator-controlled tissue transglutaminase cDNA [120] constructs (clone TG3) were grown in DMEM containing 10%(v/v) heat inactivated FBS, 200mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 400μg/ml active G418, 250μg/ml xanthine, HAT supplement, 10μg/ml mycophenolic acid, and 2μg/ml tetracyline. Swiss 3T3 TG3 clone was cultured in the tetracycline containing medium to express low endogenous levels of TG2, and referred as the non-induced cells throughout the study. The medium was replaced every 2 days with fresh medium containing tetracycline and to keep the cells in the non-induced state. Cells were grown in the absence of tetracycline for at least 72 hours to induce the TG2 expression, as described previously [120]. All cells were cultured in a humidified atmosphere at 37^{o}C, 5% (v/v) CO_2, 95% (v/v) air.

3.1.2. Cell Passaging

The passage of cells was undertaken when cells have reached an approximate 70% confluency. After the removal of media, the cell monolayer was rinsed once with DPBS solution (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, and 2 mM potassium phosphate. Cells were then detached with 0.25% (w/v) trypsin-5mM EDTA solution in PBS, pH 7.4 at 37^{o}C in 5% CO2 humidified incubator. The detached cells were collected in the 10% (v/v) FBS containing media to inactivate the trypsin and centrifuged at 300xg for 5 minutes. Subsequent to centrifugation, the

supernatant was discarded and cells were resuspended in the growth media and seeded to the tissue culture flasks.

3.1.3. Cell Counting

Cell counting was performed using the haemocytometer. 10µl aliquots of the cell suspension were applied to the haemocytometer and the number of cells was calculated by counting the 5 squares in the large middle square of the haemocytometer using inverted phase contrast microscope (Nikon, USA). The cells were counted three times and an average of cell number per ml was calculated using the "counted number of cells x dilution factor / mm2 of counted area x chamber depth" formula.

3.1.4. Cryopreservation of the Cell Lines

Cells were trypsinised and centrifuged as described above (Method Section 3.3.2.). After discarding the supernatant, cell pellet was resuspended in the freezing media containing 90% (v/v) heat inactivated FBS and 10% (v/v) dimethylsulfoxide (DMSO). Cells were then pipetted in 1 ml aliquots into cryovials and slowly frozen at -80°C and then transferred to the liquid nitrogen for long term storage.

3.1.5. Cell Thawing

Cryovials were removed from the liquid nitrogen storage and rapidly thawed at 37°C. The cell suspension was then transferred into a sterile centrifuge tube and 5ml of serum supplemented growth media was added onto the cells suspension in a drop wise manner with gentle shaking. Centrifugation at 300xg for 5 minutes was performed and supernatant was removed. Cells were then resuspended in the growth media and transferred into a T-25 tissue culture flask. Following a12-24 hour incubation, the media was removed and cells were rinsed with PBS, pH 7.4 before the addition of fresh media onto the cell monolayer.

3.1.6. Cell Proliferation Assay

To determine the optimum TG2 inhibitor (Zedira, Germany) and TGF-β neutralizing

antibody (R&D Systems, USA) concentration, 30µM, 40µM, 50µM, 75µM TG2 inhibitor and 5µg/ml and 10µg/ml TGF-β neutralizing antibody (R&D Systems, USA) concentrations were respectively studied. Cell Proliferation Reagent WST-1 (Roche, Germany) was used for the determination of cell proliferation rate. Cells were seeded in quadruplicate on 96-well plates at a density of 10.000 cells/well in 100 µl culture media supplemented with 10% (v/v) FBS concentration and incubated for 16 hours. Cells were then incubated with the growth media supplemented with 4% (v/v) FBS containing various concentrations of the TG2 inhibitor and TGF-β antibody for 24 hours and 48 hours. At the end of each time point, the media was discarded and cells were rinsed with PBS, pH 7.4 and 50 µl of fresh media containing 5 µl WST-1 reagent were added to each well. Following a1 hour incubation at 37°C, the absorbance of each sample was measured at 450 nm with 630 nm reference wavelength in an ELISA plate reader (Bio-Tek, USA).

WST-1 working principle depends on the reduction of the tetrazolium salt WST-1 to the colored formazan compounds by the succinate-tetrazolium reductase, which exists in the mitochondria of the viable cells. As the number of cells increases, the formation of highly water soluble formazan residues also increases due to the metabolic activity of the cells. Therefore, this assay gives the colorimetric measurement of cell proliferation making the quantification of cell number possible when performed in parallel with a standard plate containing known number of cells.

3.2. MEASUREMENT OF CELL SURFACE TG2 ACTIVITY VIA BIOTIN CADAVERIN INCORPORATION

96-well plates were coated with 10 µg/ml FN from human plasma (Sigma, USA) in 50 mM Tris-HCl, pH 7.4 for 16 hours at 4°C or 1 hour at 37°C and blocked with 3% (w/v) BSA in PBS, pH 7.4 for 30 minutes at 37°C. The wells were then gently washed three times with 50 mM Tris-HCl, pH 7.4 for 5 minutes on a shaker. Swiss 3T3 TG3 clone cells were trypsinized and resuspended in serum free media. The cells were counted and seeded on FN coated plates at 20.000 cells/well density in serum free media containing the TG2 inhibitor (Zedira, Germany) at concentrations of 5µM, 10µM, 20µM, 30µM, 40µM, 50µM, and 75µM in the presence of 0.132 mM Biotin cadaverine, trifluoroacetate salt (N-(5-aminopentyl) biotinamide, trifluoroacetic acid salt) (BTC) (Biotium, USA). FN coated

wells were incubated only with serum free media containing 0.132 mM BTC for the negative background control. Following a three-hour incubation at 37°C, wells were rinsed twice with PBS, pH 7.4 and 100 µl of 0.1 % (w/v) sodium deoxycholate and 2mM EDTA solution was added and incubated at room temperature for 10 minutes by gentle shaking. Following the incubation, wells were washed three times for 5 minutes with 50 mM Tris-HCl, pH 7.4 and 100 µl of extravidin peroxidase (dissolved 1:1000 in 3% (w/v) BSA) was pipetted to each well to reveal incorporated BTC. After 90 minute incubation at 37°C, washing steps were performed with 50 mM Tris-HCl, pH 7.4 three times for 5 minutes by gentle shaking. Wells were then equilibrated with the developing buffer containing 0.1 M NaOAc, pH 6, for 5 minutes by gentle shaking. Following the equilibration, the reaction was then developed by addition of 100 µl of developing buffer containing 3,3',5,5'-Tetramethyl benzidine (TMB) (dissolved in DMSO), which is the substrate for extravidine peroxidise, to each well. Plate is then incubated at room temperature or 37°C for 15-30 minutes until blue color formation occurred. The reaction was subsequently terminated by the addition of 50 µl of 2.5 M H_2SO_4 to each well, and the absorbance was measured at 450 nm using an ELISA plate reader (Bio-Tek, USA).

3.3. RNA ISOLATION

3.3.1. RNA Isolation from Cell Lines

Swiss 3T3 TG3 clone cells grown in special-conditioned medium for with or without tetracycline for 24 hours were seeded at 200.000 cells/well density on 6 well plates and allowed to attach for 12-16 hours. Following the incubation, cells were washed with PBS, pH 7.4. and the special-conditioned medium supplemented with 4% (v/v) FBS was added to all wells with tetracycline for non-induced cells and without tetracycline for induced cell lines. Non-induced cells were used as negative control. In the experiments performed with induced cells, cells were exposed to 7 different treatment conditions. Induced cells were incubated with (±)-S-Nitroso-N-acetylpenicillamine (SNAP) (Calbiochem, USA) at concentrations of 50µM, 150µM, and 300µM as determined in the previous study [118]. For inhibition of TG2 activity, TG2 inhibitor (Zedira, Germany) was used to treat the induced cells at 50µM concentration for 24, and 48 hours. In order to block TGF-β activity, 10µM TGF-β neutralizing antibody (R&D, USA) and 10µM mouse serum IgG as

negative control was used to treat the induced cells as for 24, and 48 hours, as described [121, 122]. Time points were optimised according to the previous study [118]. Subsequent to the end of each time point, medium was removed, cells were rinsed with PBS, pH 7.4 and 500 µl TriPure reagent was applied onto the cell monolayer. After the lysis of cells by scrapping in TriPure reagent, cells were collected into the sterile micro centrifuge tubes and 100 µl chloroform was added into the tubes. The tubes were then shaken vigorously, incubated for 10 minutes at room temperature, and then centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase, which contains the RNA was carefully transferred into a new tube without disturbing any other phases and mixed with 250 µl isopropyl alcohol. The samples were then incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4°C. Afterwards, supernatant was removed and RNA pellet was rinsed with 500 µl 75% (v/v) ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. RNA pellet was dried via vacuum-dry prior to the removal of supernatant and dissolved in 50 µl RNase-free water. RNA samples were stored at -80°C.

3.3.2. Quantification of RNA

The concentrations of isolated total RNA samples were measured and quantified spectrophotometrically at 260 nm using RNase-free water as blank. Total RNA concentration was calculated from the absorbance at 260 nm using the equation below:

‘‘Concentration of RNA sample (ng/µl) = 40 x A260 x dilution factor’’ (Equation 3.1)

The protein contamination in total RNA samples was measured by calculating the A260/A280 ratio.

3.4. POLYMERASE CHAIN REACTION

3.4.1. cDNA Synthesis

Target RNAs were reverse transcribed using the Qiagen Sensiscript RT Kit. In the first step, 1000 ng total RNA was transferred into a new tube in 13 µl of RNase-free water, onto which 7 µl reaction mixture was added in the presence of 2 µl 10x Buffer RT, 2 µl dNTP

Mix (5 mM each dNTP), 2 µl Oligo-dT primer (10 µM), 1 µl Sensiscript Reverse Transcriptase to make up a total volume of 20 µl. The samples were then incubated at 37°C for 60 minutes. All samples from a single experiment were reverse transcribed simultaneously, to minimize the possible variations in reverse transcriptase efficiency. Samples were then stored at -20°C.

3.4.2. Polymerase Chain Reaction

Amplification reactions were performed using the Thermus aquaticus (Taq) polymerase to optimize the PCR conditions for the designed primers. Primer sequences that were used are presented in the Table 3.1. Reaction mixtures containing 2.5 µl of 10X Taq Buffer, 25mM MgCl2, 0.5 µl of dNTP mix, 0.75 µl of forward primer (0.3 µM), 0.75 µl of reverse primer (0.3 µM), 0.3 µl of Taq Polymerase, 16.2 µl of dH2O were prepared as the master mix solutions for 3 different gene amplifications and added onto 1 µl of cDNA containing PCR tubes. Conditions for the PCR reaction were as shown in the Table 3.2. Annealing temperature of 18SrRNA was chosen as 60.9 ºC. The PCR products were visualized after electrophoresis on a 1.5% agarose gel containing ethidium bromide.

Table 3.1. PCR primer sequences

	Primer sequences	**Product size (bp)**	**Annealing temp. (°C)**
MMP-2	5’- ATTCTGTCCCGAGACCGCTATG-3’ (sense)	134	60.9
	5’-ACCACACCTTGCCATCGTTG-3’ (antisense)		
MMP-9	5’-CGTGTCTGGAGATTCGACTTG-3’ (sense)	131	60.9
	5’-CAGAAATAGGCTTTGTCTTGGT-3’ (antisense)		
18SrRNA	5’-AACTGAGGCCATGATTAAGAGG-3’ (sense)	189	60.9

Table 3.1. PCR primer sequences (continue)

	5’-GGCATCGTTTATGGTTGGAAC-3’ (antisense)		

Table 3.2. PCR conditions

Cycle	**Temperature**	**Time**	**Phase**
1	95 ºC	3 minutes	Initial denaturation
2 (30 repeats)	95 ºC	30 seconds	Denaturation
	60.9ºC	30 seconds	Annealing
	72ºC	2 minutes	Extension
3	72ºC	5 minutes	Final extension

3.4.3. Quantitative Polymerase Chain Reaction

The mRNA expression levels of MMP1a, MMP13, MMP2, and MMP9 genes in the cell lines were determined using QuantiTect SYBR Green PCR Kit in Bio-Rad iCycler iQ5 instrument. PCR reaction for the MMP expression was performed in conditions given in the Table 3.3 with the real-time PCR master mix for MMP1a and MMP13 prepared as follows: 6.25 µl of SYBR Green PCR master mix, 1.25 µl of primer mix (QuantiTect Primer Assays, MMP1a, MMP13) and 50 ng of cDNA (1 µl) in a total volume of 12.5 µl. Real-time PCR master mix for the MMP2, MMP9 and the 18SrRNA as an internal control was prepared as follows: 6 µl of SYBR Green PCR master mix, 0.75 µl of forward primer (0.3 µM) and 0.75 µl of reverse primer (0.3 µM) and 50 ng of cDNA (1 µl) in a total volume of 12.5 µl. The PCR reaction was performed in conditions given in the Table 3.4.

Table 3.3. Real-time PCR conditions for MMP1a, MMP13

Cycle	**Temperature**	**Time**	**Phase**
1	94°C	15 min	
2	95°C	5 min	Initial denaturation

Table 3.3. Real-time PCR conditions for MMP1a, MMP13 (continue)

3 (40 repeat)	95°C	60 sec	Denaturation
	55°C	60 sec	Annealing
	72°C	60 sec	Extension
4	72°C	10 min	Final extension
5 (90 repeat)	50-95°C 0.5 °C increase /12 sec		Melt curve
6	4°C	∞	Cooling

Table 3.4. Real-time PCR conditions for MMP2, MMP9, and 18SrRNA

Cycle	**Temperature**	**Time**	**Phase**
1	94°C	15 min	
2	95°C	5 min	Initial denaturation
3 (40 repeat)	95°C	60 sec	Denaturation
	60.9°C	60 sec	Annealing
	72°C	60 sec	Extension
4	72°C	10 min	Final extension
5 (90 repeat)	50-95°C 0.5 °C increase /12 sec		Melt curve
6	4°C	∞	Cooling

3.5. WOUND SCRATCH ASSAY

The scratch assay was performed on the 24 hour induced or non-induced Swiss 3T3 TG3 clone cells that were seeded on 12 well plates at 600.000 cells/well in special-conditioned medium as mentioned above (Method Section 3.1.1). Cell monolayer was scratched using a p1000 pipette tip in a straight line to create a wound bed. Medium was then immediately discarded to remove the debris and cells were washed with PBS, pH 7.4 to smooth the edge

of the scratch. The special-conditioned medium supplemented with 4% (v/v) FBS was added to all wells with tetracycline for the non-induced cells and without tetracycline for the induced cell lines. Non-induced cells were used as negative control. In the experiments performed with induced cells, cells were incubated in 7 different conditions for 24, and 48 hours as described above in the Method Section 3.3.1. At the end of each time point, the cells were photographed alive to obtain the same field image and to avoid the possible variation caused by the difference in the width of the scratches. Markings were created as the reference points close to the scratch by a marker pen and images were captured by leaving the reference mark outside of the image field but within the eye-piece field of view under the phase-contrast microscope (Nikon Eclipse TS-100, USA). 10 images were acquired at 4X magnification from one well for the each time point. Images were quantified by ImageJ software and the distances for each scratch closure were measured taking the images from time point 0 as the base point.

3.6. TRANSWELL MIGRATION ASSAY

Non-induced, induced, and TGF-β neutralizing antibody treated cells were seeded at 200.000 cells/well density onto the 6-well plates and incubated at 37 °C for 24h. In another experimental set-up, non-induced, induced, and induced cells treated with 50 μM SNAP, 150 μM SNAP, 300 μM SNAP and 50μM TG2 inhibitor treated cells were incubated at 37 °C for 48h. Boyden chamber type Transwells (Costar, USA) were used to examine the ability of cells to migrate through FN-coated 8 μm pore size membrane. The undersurfaces of the 6 well Transwell membranes were coated with FN in 50mM Tris-HCl, pH 7.4 and incubated at 37°C for 1 h at the day of the experiment. Meanwhile, treated cells were trypsinized and washed once with serum-free AIM-V (Gibco, NY) medium and counted. After 1 h incubation of inserts with FN, transwells were rinsed with 50mM Tris-HCl, pH 7.4 and then submerged in the wells containing 2 ml of serum-free AIM-V media. In one ml serum-free AIM-V medium, 170.260 cells/well were seeded into the inner chamber of inserts and cells were allowed to migrate through the FN coated undersurface at 37°C in a CO2 incubator. Early time point of cell migration was chosen as 4 h and the late time point was determined as 8 h through the optimization experiments. At their determined incubation time points, cells from the inner surface of the insert were wiped clean with a cotton swab from the surface and the inserts were washed once with PBS, pH 7.4. Cells

were then fixed and stained with 0.5% Crystal Violet in 75% ethanol solution for 15 minutes at room temperature. Next, the inserts were washed two times with PBS, pH 7.4., and once with dH_2O of and air-dried. The images of cells migrated through the FN were captured with Nikon X Camera attached to an inverted phase contrast microscope (Nikon Eclipse TS-100, USA) at 40X magnification. At least 5 images/inserts were acquired for each time point and quantified as described before [47].

3.7. 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 $P<0.05$.

4. RESULTS

4.1. MMP-2, MMP-9, MMP-1a, MMP-13, 18SrRNA PRIMER OPTIMIZATION

In order to determine the specific PCR conditions for the designed MMP-2, MMP-9, and 18SrRNA primers, RNA was isolated from non-induced (NI) and induced (Ind) Swiss 3T3 fibroblasts as described in the Method Section 3.3.1., and reverse transcriptase polymerase chain reaction (RT-PCR) was performed as explained previously in the Method Section 3.4.1.

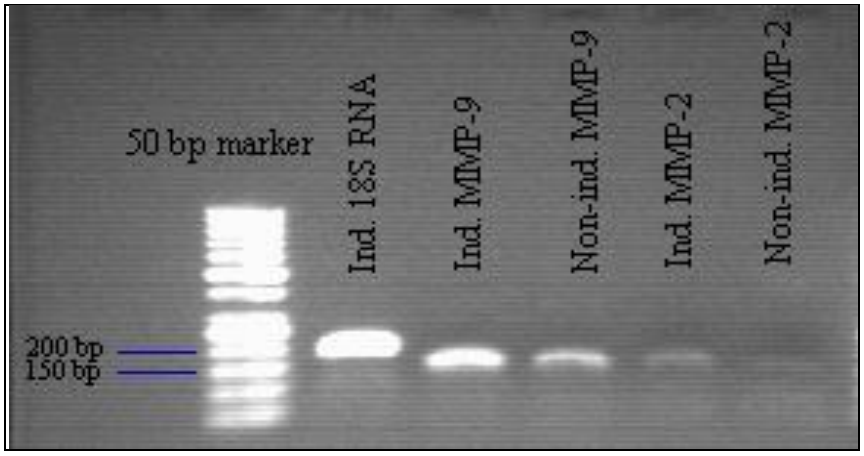

Figure 4.1. Representative figure of MMP-2, MMP-9 and 18SrRNA PCR products on 1.5% agarose gel using the PCR conditions described in Table 3.2

Figure 4.1 showed that at 60.9°C annealing temperature, a single band at the expected product size is given for the MMP9 and MMP2, while a predominant ~200bp product was observed when using an annealing temperature of either 55°C or 60.9°C for the 18sRNA.

After determination of the PCR conditions, the compatibility of these conditions to the real-time PCR for the amplification of MMP-2, MMP-9, MMP-1a, MMP-13 and 18SrRNA were confirmed and the optimum concentrations and the annealing temperatures of primers were determined for each gene product as described in the Method Section 3.4.3.

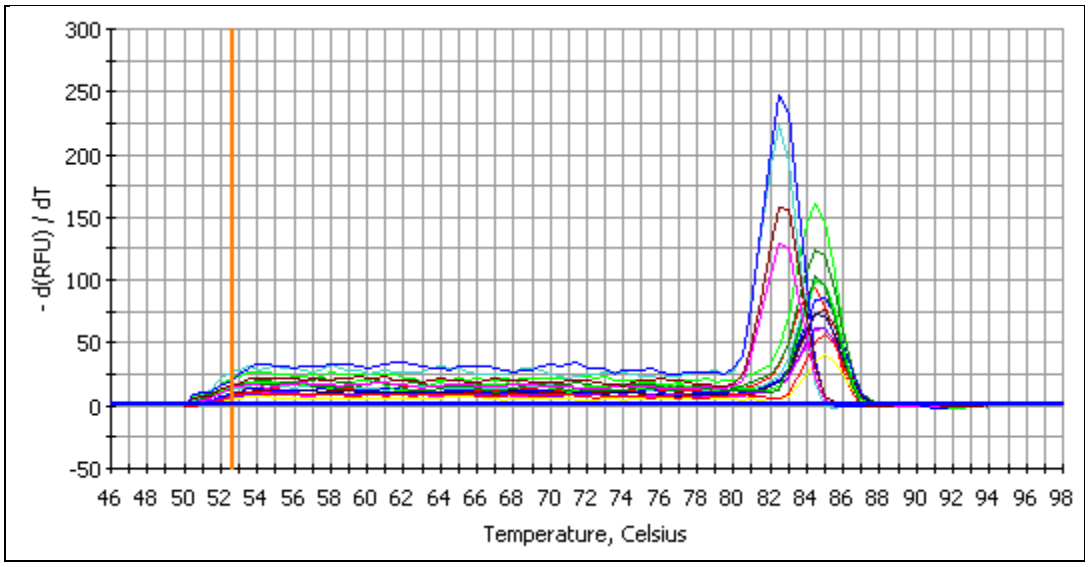

Figure 4.2. Melt curve analysis showing the amplicons for MMP-2, MMP-9, and 18SrRNA genes using 60.9°C as annealing temperature shown as opd file from iCycler software

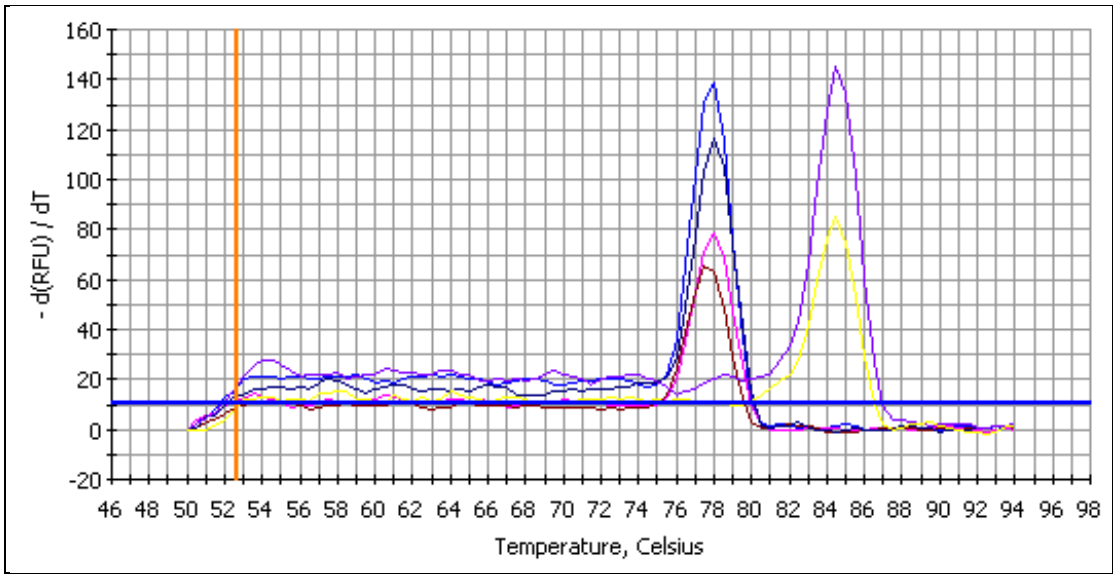

Figure 4.3. Melt curve analysis of MMP-1a and MMP-13 genes using 55°C as annealing temperature shown as opd file from iCycler software

Figure 4.2 and Figure 4.3 demonstrated the melt curve analysis of MMP-2, MMP-9, MMP-1a, MMP-13 and 18SrRNA. As can be seen from the representative figures, the primers are specific and amplify a single product at their optimized concentrations and temperatures.

4.2. EFFECT OF TG2 INHIBITOR ON THE MIGRATION OF FIBROBLASTS

4.2.1. TG2 Inhibitor Concentration Analysis

In order to decide the adequate concentration of TG2 inhibitor that fully block the TG2 enzymatic activity, the cells were treated with different concentrations of TG2 inhibitor and then seeded on FN coated microtiter plates in the presence of BTC. For this purpose, Swiss TG3 clone fibroblasts which were induced for 72 hours were seeded on FN in the presence of BTC and 7 different concentrations of TG2 inhibitor (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, and 75 μM) for 2 hours and TG2 activity on the cell surface was measured through the detection of FN incorporated BTC by extravidin peroxidase. Following the addition of extravidin peroxidase which has high affinity to BTC, the reaction was visualized by the addition of TMB, which is the colorimetric substrate of peroxidase and data was quantified at 450 nm.

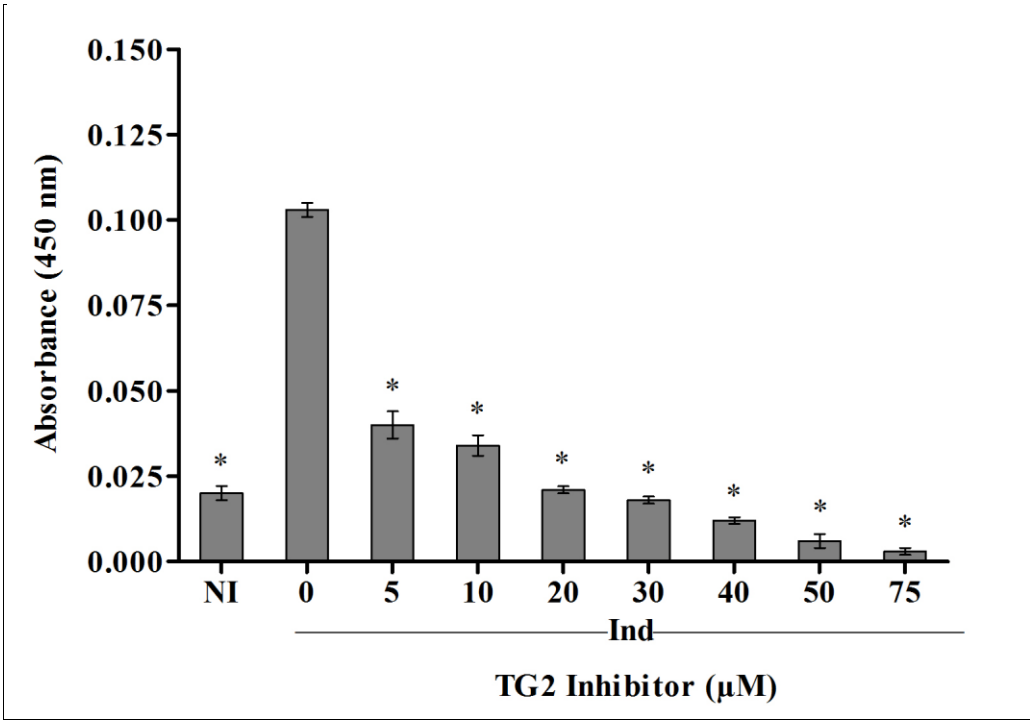

Figure 4.4. Inhibition of FN-bound TG2 activity by TG2 Inhibitor. The inhibition of the cross-linking activity of cell surface TG2 at increasing concentrations of active site TG2 inhibitor was performed as described previously in the Method Section 3.2.1. Values represent the mean ± S.D. absorbance at 450nm from one typical experiment which was performed in quadruplicates. The final values were obtained by subtracting the background values of the only BTC containing wells from the absorbance values obtained for samples. The * represents statistical significance ($p<0.05$) for the absorbance values when compared with the absorbance values obtained for the control induced cells to which no inhibitor was added

The data shows that at all concentrations, TG2 inhibitor caused an average of a 4-fold decrease on the cell surface associated TG2 activity when compared to the control induced fibroblasts which was not treated with the TG2 inhibitor as shown in Figure 4.4. At 50 µM the inhibitor led to a significant 17.1-fold decrease in TG2 activity which was not significantly dropped further down by the higher 75 µM inhibitor concentration.

In order to choose a non-toxic TG2 inhibitor concentration, a cell proliferation assay was performed to observe the cytotoxic effect of TG2 inhibitor at different concentrations. Inducible Swiss 3T3 fibroblasts (Ind) were incubated with 30 µM, 40 µM, 50 µM, and 75

μM for 24 and 48 hours as described in the Method Section 3.1.6. TG2 inhibitor was added every 24 hours into the media at defined concentrations. Cell proliferation rate at different concentrations of TG2 inhibitor were determined by cell proliferation reagent WST-1 as described in the Method Section 3.1.6.

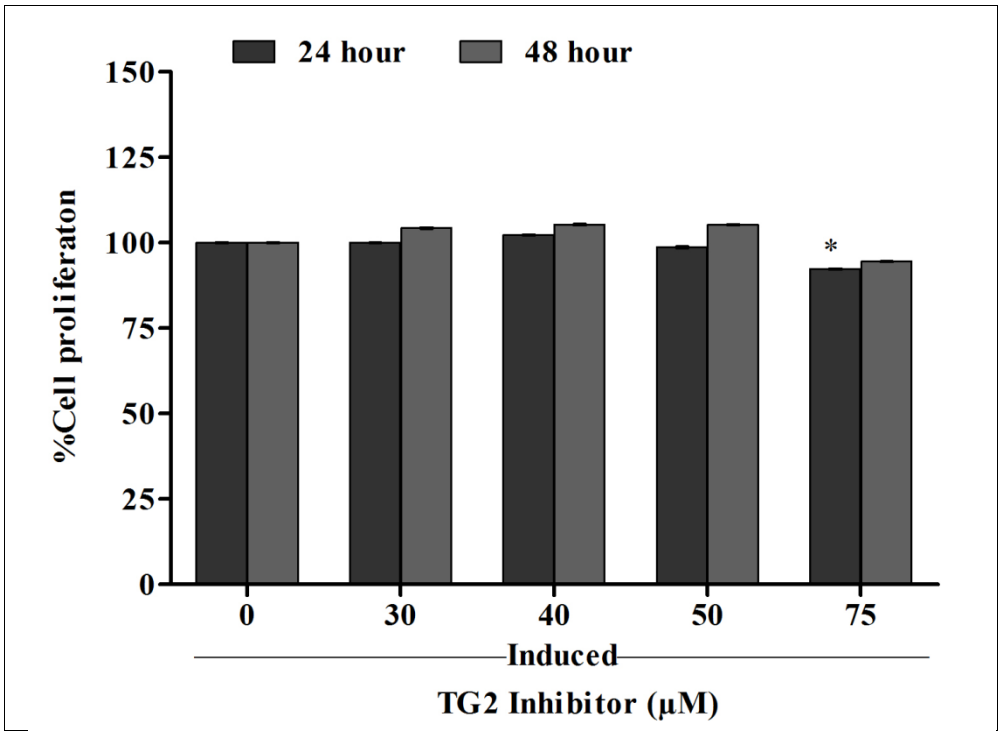

Figure 4.5. Effect of TG2 inhibitor on the inducible Swiss 3T3 fibroblast cell proliferation. The measurement of cell proliferation levels for transfected inducible (Ind) Swiss 3T3 fibroblasts was performed after 24 and 48 hours incubation with different TG2 inhibitor concentrations. Each data point corresponds to the mean percentage of cell proliferation ± S.D. of one typical experiment performed in 4 replicates, and * symbol indicates the statistically different values of untreated induced fibroblasts when compared to the TG2 inhibitor treated induced fibroblasts ($P<0.05$)

Results indicated that 75 μM TG2 inhibitor concentration leads to a significant 8% decrease in cell proliferation at 24 hours as shown in Figure 4.5. Taken together, as the 50 μM TG2 inhibitor concentration is as effective as 75 μM TG2 inhibitor in blocking the cross-linking activity cell surface bound TG2 as shown in Figure 3.4 and possess no toxic effect on the induced fibroblasts viability as shown in Figure 4.5, it was chosen as the inhibitor concentration to be used for the rest of the study.

4.2.2. Effect of TG2 Inhibitor on the Migration of Fibroblasts

The effect of TG2 activity on the migration of non-induced and induced fibroblast cells was investigated using wound scratch assays in the presence and absence of 50 μM TG2 inhibitor. The non-induced and induced Swiss 3T3 fibroblasts were allowed to attach on the tissue culture plastic for 16 hours and after cells reached 100% confluency, a wound bed was formed using a pipette tip. TG2 inhibitor was added onto samples every 24 hours as described in the Method Section 3.5.

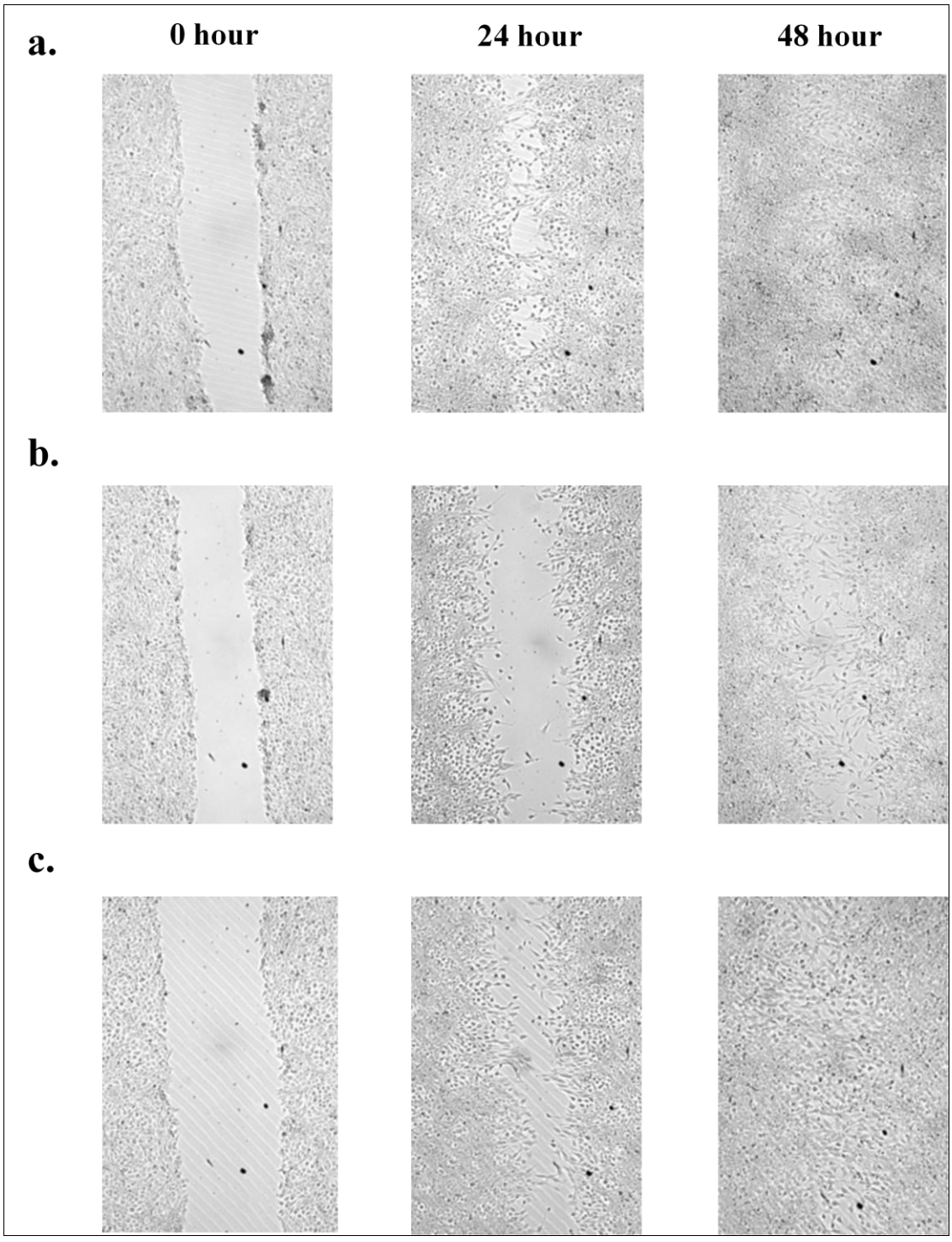

Figure 4.6. The role of TG2 inhibitor on the migration of induced fibroblasts. Representative photographs of wound scratch assay in the absence and presence of TG2 inhibitor. a. Non-induced Swiss 3T3 fibroblasts b. Induced Swiss 3T3 fibroblasts c. TG2 inhibitor treated induced Swiss 3T3 fibroblasts

As can be seen from the Figure 4.6, the induced cells migrated more slowly into the wound bed area compared to the non-induced cells, however the rate of cell migration of induced cells in the presence of TG2 inhibitor was similar to that of non-induced.

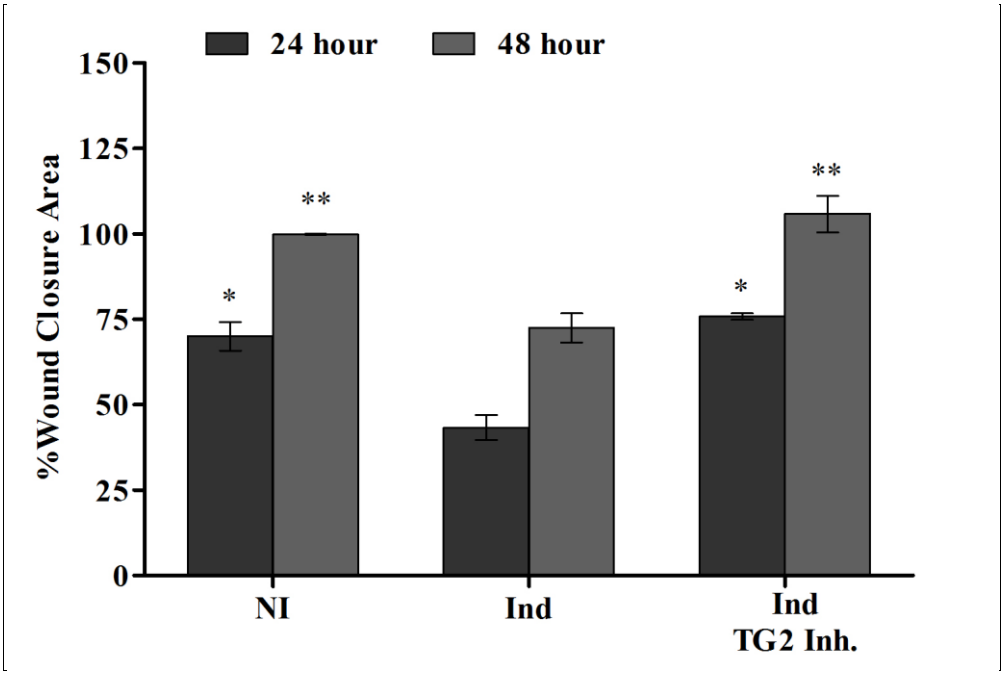

Figure 4.7. The role of TG2 inhibitor on the wound closure of induced fibroblasts. Each data point represents the mean percentage for closure of the wound area ± S.E.M. of three independent experiments performed in duplicate. The wound closure rate was expressed as the mean percentage of migration of non-induced (NI) fibroblast cells at 48 hour (control) ± S.E.M., which represents 100%. 48 hour of NI fibroblast cells were taken as the control due to their full closure of the denuded area. The migration rate of induced (Ind) fibroblasts in the absence and presence of the TG2 inhibitor (TG2 Inh) in 24 hours were calculated and expressed as the mean percentage of control. * and ** symbols represent the significant difference between NI and Ind and the comparison of untreated induced and inhibitor treated induced fibroblasts ($P<0.05$)

When the effect of TG2 activity on the cell migration of Swiss 3T3 TG3 clone fibroblasts was quantified using the Image J program, results demonstrated that the treatment of induced Swiss 3T3 with 50 µM TG2 inhibitor led to a 30% and 33% increase in cell migration compared to the untreated induced Swiss 3T3 fibroblasts at 24 and 48 hours, respectively as shown in Figure 4.7. These data suggest that the cross-linking activity of

TG2 inhibits the cell migration of fibroblasts and the inhibition of TG2 activity by TG2 inhibitor increases fibroblast migration.

4.2.3. Effect of TG2 Inhibitor on the Expression of MMP-2 and MMP-9 in Fibroblasts

In order to investigate the effect of TG2 activity on the biosynthesis of gelatinases (MMP-2 and MMP-9), the expression profiles of these genes were assessed using quantitative real-time PCR. For this purpose, RNA was isolated from non-induced fibroblasts, untreated induced, and TG2 inhibitor treated induced Swiss 3T3 fibroblasts after 24 and 48 hours incubation as described in the Method Section 3.5. Each individual data point in the following graphs corresponds to each conditions average expression value of the interested gene normalized against the 18SrRNA.

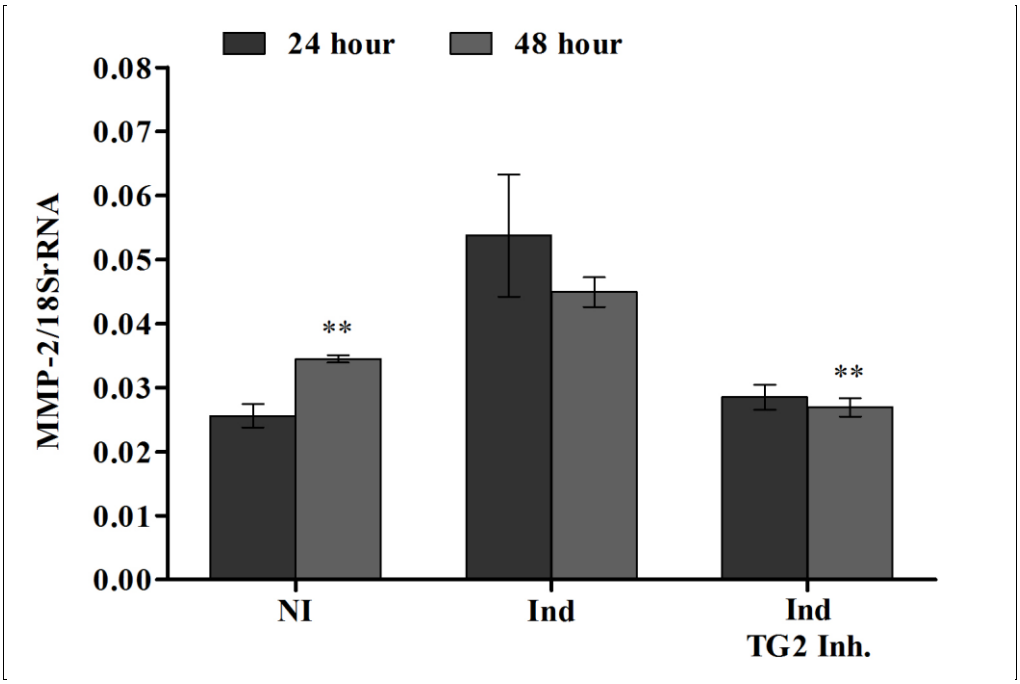

Figure 4.8. Effect of TG2 inhibitor on the MMP-2 expression levels. Each point represents the mean MMP-2 expression value ± S.E.M. of non-induced fibroblasts, untreated Ind and TG2 Inh. treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. ** symbol indicates statistical difference ($P<0.05$)

Results showed a significant 1.3-fold difference in the expression levels of MMP-2 between the non-induced fibroblasts and untreated induced fibroblasts at 48 hours. In the MMP-2 expression levels ~2-fold and ~1.8-fold difference was observed in non-induced fibroblasts and TG2 inhibitor treated induced fibroblasts when compared to untreated induced fibroblasts at 24 hours, respectively ($p>0.05$). Inhibition of TG2 enzymatic activity with the TG2 inhibitor led to a 1.7-fold decrease in MMP-2 expression at 48 hours compared to untreated induced Swiss 3T3 fibroblast cell lines.

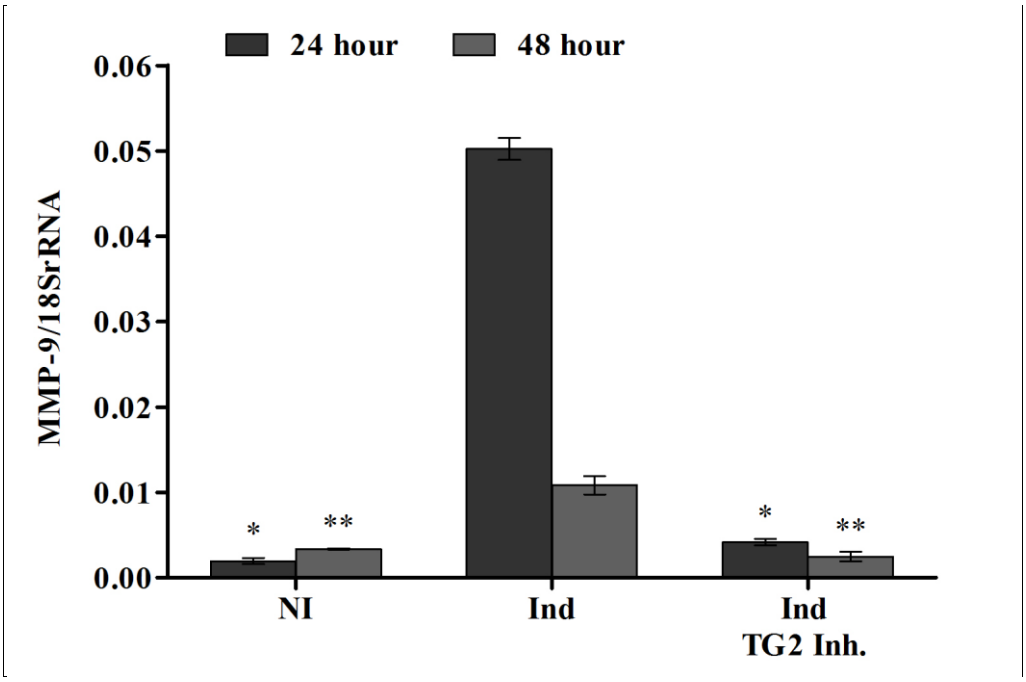

Figure 4.9. Effect of TG2 inhibitor on MMP-9 expression levels. Each point represents the mean MMP-9 expression value ± S.E.M. of NI, untreated Ind and TG2 Inh. treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. The significant difference was observed between non-induced fibroblasts and untreated * and ** symbols indicate statistical difference ($P<0.05$)

Figure 4.9 demonstrates that induction in TG2 led to ~26-fold increase in the MMP-9 expression levels compared to non-induced Swiss 3T3 fibroblasts at 24 hours. The treatment of induced fibroblasts with the TG2 inhibitor resulted in a 12.1-fold decrease in the MMP-9 expression levels at 24 hours. After 48 hours incubation, 3.3-fold and 4.5-fold

reduction in MMP levels were observed in non-induced and inhibitor treated induced Swiss 3T3 fibroblasts, respectively, when compared to the induced fibroblasts.

4.2.4. Effect of TG2 Inhibitor on the Expression of MMP-1a and MMP-13 in Fibroblasts

In order to investigate the role of TG2 activity on the biosynthesis of collagenases (MMP-1a and MMP-13) in transfected Swiss 3T3 fibroblast cell lines, expression profiles of these genes were assessed by quantitative real-time PCR. For this purpose, RNA was isolated from non-induced fibroblasts, untreated and TG2 inhibitor treated induced Swiss 3T3 fibroblasts after 24 and 48 hours incubation as described in the Method Section 3.5. Each Individual data point in the following graphs corresponds to average expression value of the interested gene normalized against the 18SrRNA expression value.

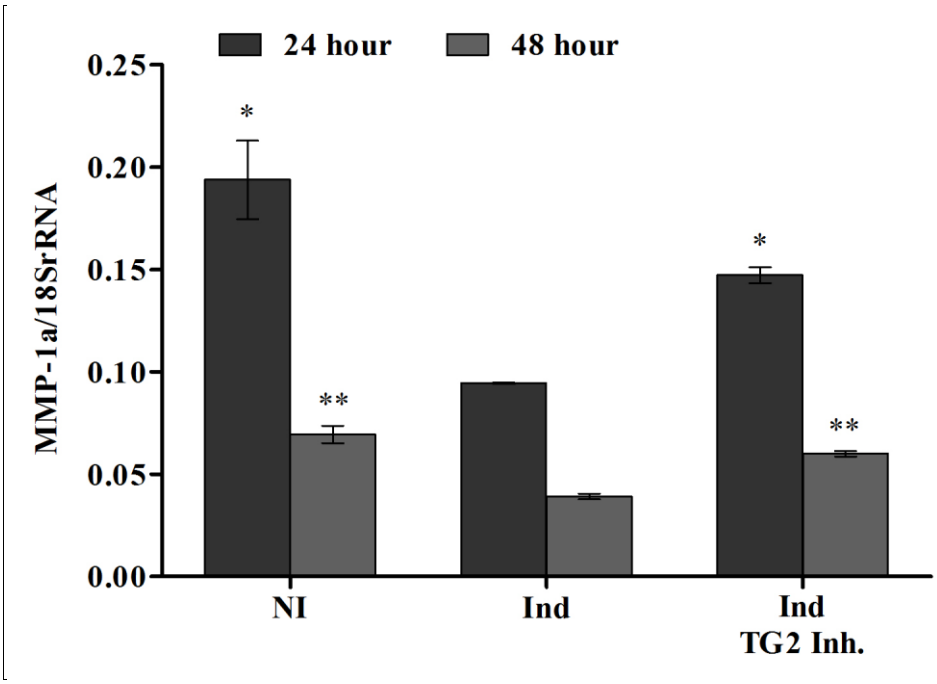

Figure 4.10. Effect of TG2 inhibitor on the MMP-1a expression levels. Each point represents the mean MMP-1a expression value ± S.E.M. of NI, untreated Ind and TG2 Inh. treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

Non-induced fibroblasts Swiss 3T3 fibroblasts which have low TG2 expression and enzymatic activity displayed a significant 2.1-fold induction in MMP-1a expression levels compared to the untreated induced Swiss 3T3 fibroblasts at 24 hours as shown in Figure 4.10. The inhibition of cross-linking activity of TG2 by the inhibitor treatment resulted in a significant 1.5-fold increase in the MMP-1a expression levels of induced cells at 24 hours. After 48 hours incubation, significant 1.7-fold and 1.5-fold inductions were observed in the MMP-1a expression levels of non-induced and inhibitor treated induced fibroblasts, respectively, when compared with the induced fibroblasts.

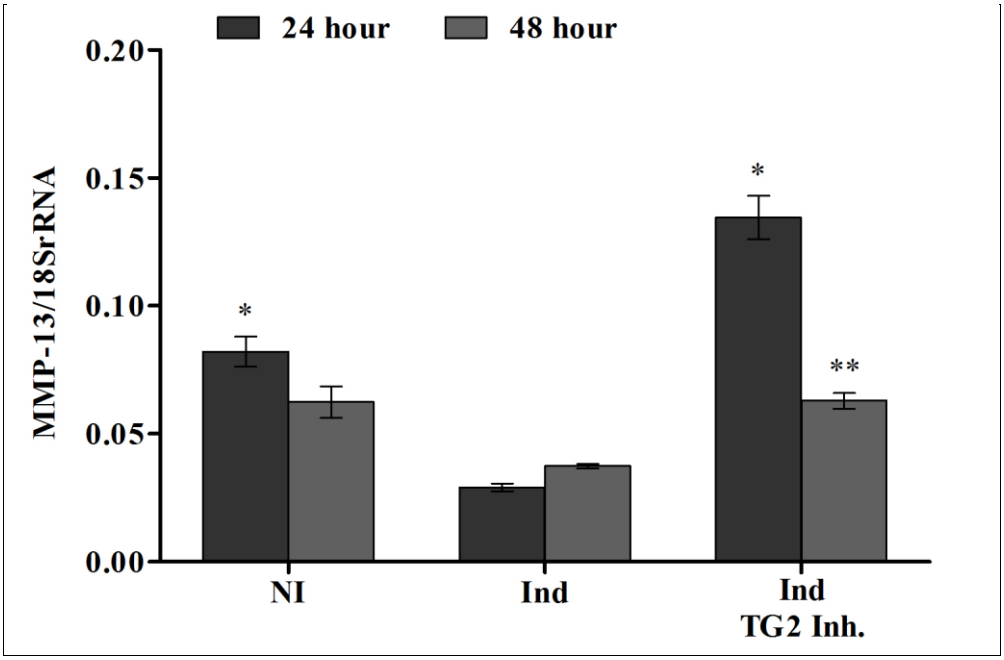

Figure 4.11. Effect of TG2 inhibitor on MMP-13 expression levels. Each point represents the mean MMP-13 expression value ± S.E.M. of non-induced fibroblasts, untreated Ind and TG2 Inh. treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

Figure 4.11 demonstrates that the induction of TG2 activity caused a 2.8-fold deduction in MMP-13 expression levels compared to that of the non-induced at 24 hours ($p<0.05$). Non-induced fibroblasts displayed a ~1.6-fold induction in MMP-13 expression levels when compared to induced fibroblasts at 48 hours ($p>0.05$). The abrogation of TG2 activity with the TG2 inhibitor led to a significant 4.6-fold and 1.7-fold increase in the MMP-13 expression levels compared to the induced fibroblasts at 24 and 48 hours, respectively.

4.3. EFFECT OF NO ON TG2-INDUCED CELL MIGRATION IMPAIRMENT

4.3.1. Effect of NO Donors on the Migration of Non-induced and Induced Fibroblasts

The role of NO on the TG2-induced abrogation of cell migration of the TG2 induced fibroblasts through the inhibition of the enzymatic activity by nitrosylation of the enzyme active site residues was investigated. In order to examine the effect of NO donor SNAP on non-induced and induced fibroblasts, wound scratch assay was performed in the presence of non-toxic 50 μM, 150 μM, 300 μM SNAP concentrations which is determined in the previous study [118] at 24h and 48h time points as mentioned in the Method Section 3.5.

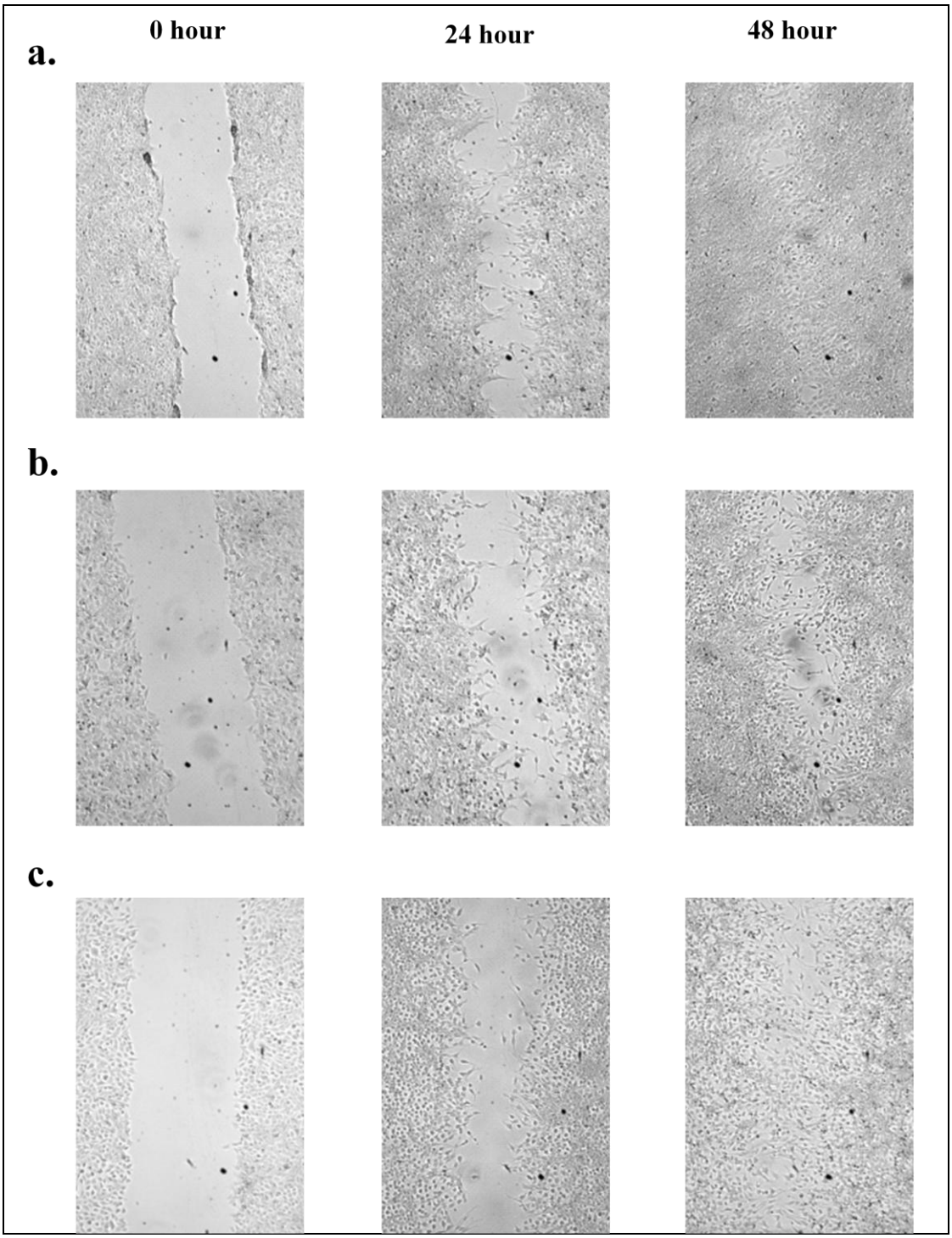

Figure 4.12. The role of NO on the migration of induced fibroblasts. Representative photographs of wound scratch assay in the absence and presence of NO donor. a. NI b. Ind fibroblasts c. 50 µM SNAP treated Ind fibroblasts d. 150 µM SNAP treated Ind fibroblasts e. 300 µM SNAP treated Ind fibroblasts

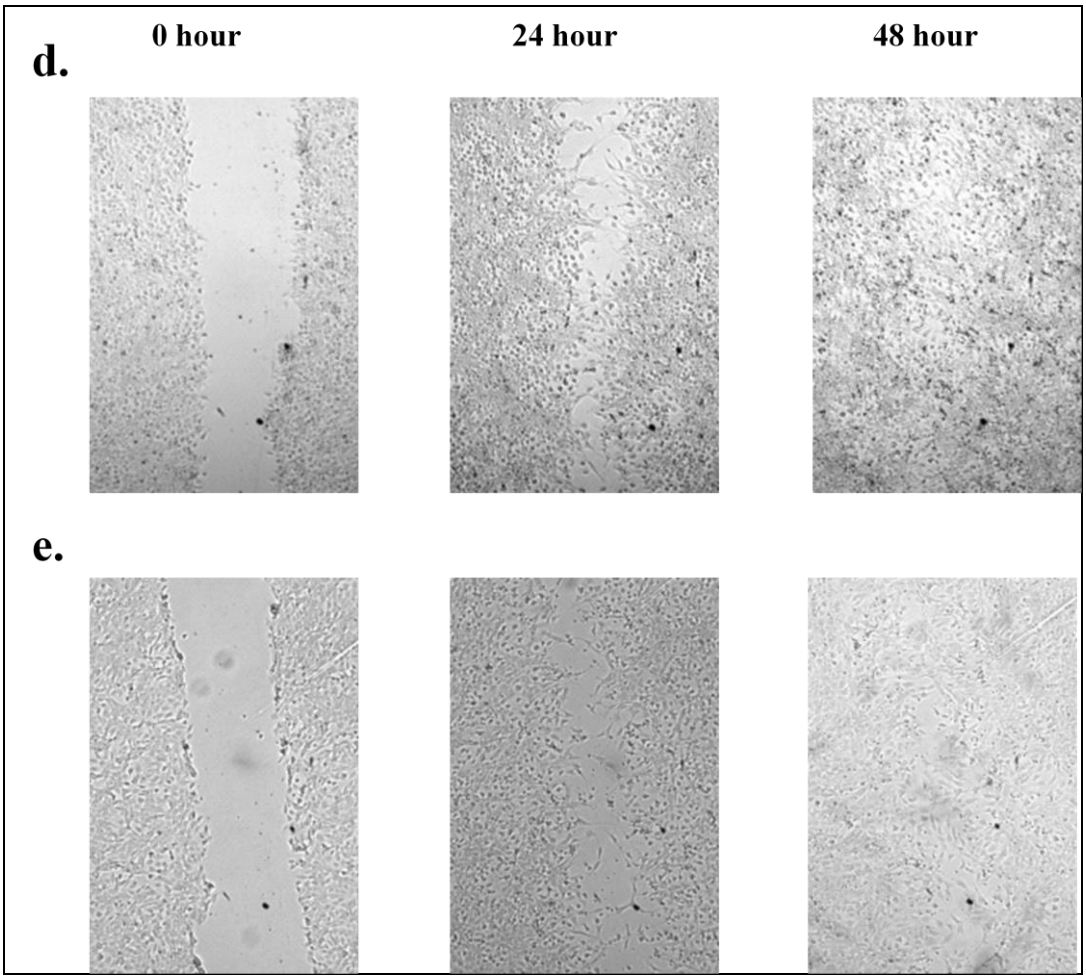

Figure 4.12. The role of NO on the migration of induced fibroblasts. Representative photographs of wound scratch assay in the absence and presence of NO donor. a. NI b. Ind fibroblasts c. 50 μM SNAP treated Ind fibroblasts d. 150 μM SNAP treated Ind fibroblasts e. 300 μM SNAP treated Ind fibroblasts (continue)

Wound bed closure was higher in the non-induced fibroblasts and in the 50, 150 and 300 μM concentrations of NO donor when compared to wound closure in the induced Swiss 3T3 fibroblasts as shown in Figure 4.12.

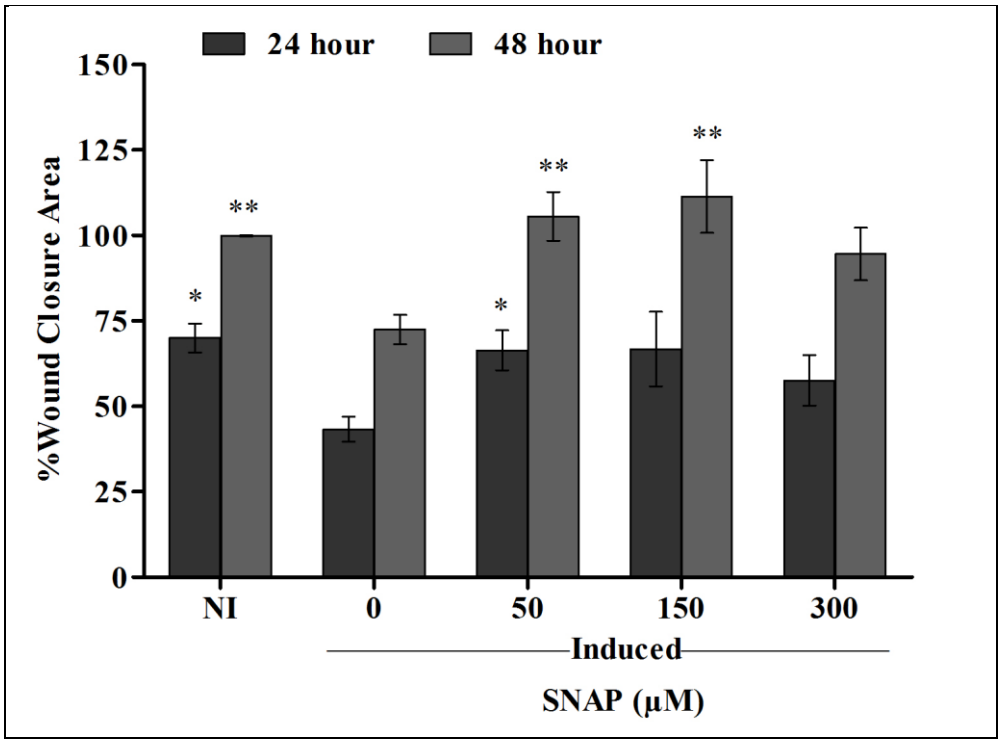

Figure 4.13. The role of nitric oxide on the wound closure of induced fibroblasts. Each data point represents the mean percentage of closure of wound area ± S.E.M. of three independent experiments performed in duplicate. The wound closure rate was expressed as the mean percentage of migration of NI Swiss 3T3 fibroblast cells at 48 hours (control) ± S.E.M., which represents 100%. 48 hours of NI Swiss 3T3 fibroblast cells were taken as the control due to their full closure of the denuded area. * and ** symbols represent significant difference between NI and Ind in comparison to the untreated Ind and SNAP treated Ind Swiss 3T3 fibroblasts ($P<0.05$)

Results for the role of SNAP on the cell migration of induced Swiss 3T3 fibroblasts demonstrated that non-induced fibroblasts which possess low levels of TG2 activity displayed 27% and 28% increase rate of wound closure when compared to induced Swiss 3T3 fibroblasts at 24 and 48 hours, respectively. It can be seen in Figure 4.13 that the treatment of induced fibroblasts with 50 µM SNAP resulted in a 23% and 33% increase in cell migration comparable to the rate of wound closure displayed by the non-induced fibroblasts at 24 and 48 hours, respectively. There was a 23% and ~39% augmentation in cell migration of induced cells due to 150 µM SNAP treatment for 24 and 48 hours, respectively, compared to the non-treated negative induced control. The treatment of

induced fibroblasts with 300 μM SNAP led to 14% and 22% induction in cell migration compared to the rate of wound closure displayed by the non-induced fibroblasts at 24 and 48 hours, respectively. There was no significant difference in the rate of scratch closure between the induced cells and induced cells treated with 150 μM SNAP at 24 hours. In addition, the treatment of induced cells with 300 μM SNAP did not cause any significant effect in the rate of wound closure when compared to that of induced fibroblasts. Data suggests that NO could induce the migration of induced fibroblasts by inhibiting the cross-linking activity of TG2 and possibly enforcing a change in protein conformation causing TG2 to function as a cell adhesion protein.

4.3.2. Effect of NO on the Expression of MMP-2 and MMP-9 in Fibroblasts

TG2 activity dependent effect of nitric oxide on the expression levels of gelatinases (MMP-2 and MMP-9) in transfected Swiss 3T3 fibroblast cell lines was examined. Total RNA isolated from non-induced, induced, and 50 μM, 150 μM, and 300 μM SNAP treated induced Swiss 3T3 fibroblasts at 24h and 48h time points were subjected to quantitative real-time PCR as described in the Method Section 3.4.3. Each individual data point in the following graphs corresponds to the average expression value of the interested gene normalized against the 18SrRNA for each sample.

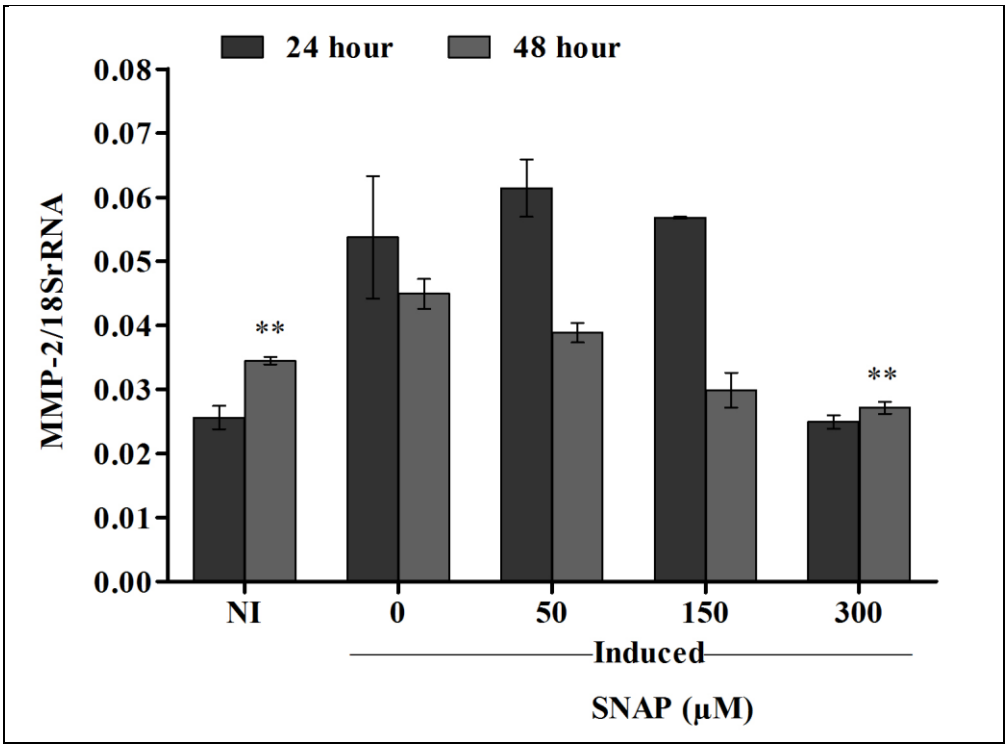

Figure 4.14. Effect of SNAP on the MMP-2 expression levels. Each point represents the mean MMP-2 expression value ± S.E.M. of NI, untreated and 50 µM, 150 µM, 300 µM SNAP treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. ** symbol indicates statistical difference ($P<0.05$)

At 24 and 48 hours incubation the induction of TG2 in fibroblasts led to a insignificant 2-fold and significant 1.3-fold increase in MMP-2 levels compared to their non-induced counterparts, respectively, as shown in Figure 4.14. The treatment of induced fibroblasts with 50 µM SNAP led to an insignificant 1.1-fold increase and 1.1-fold decrease at 24 and 48 hours, respectively. The treatment of induced fibroblasts with 150 µM SNAP led to an insignificant 1-fold increase and 1.5-fold decrease at 24 and 48 hours, respectively ($p>0.05$). Inhibition of TG2 enzymatic activity by 300 µM SNAP resulted in a insignificant 2.1-fold and significant 1.6-fold decrease in MMP-2 expression at 24 and 48 hours, respectively, compared to the untreated induced Swiss 3T3 fibroblast cell lines.

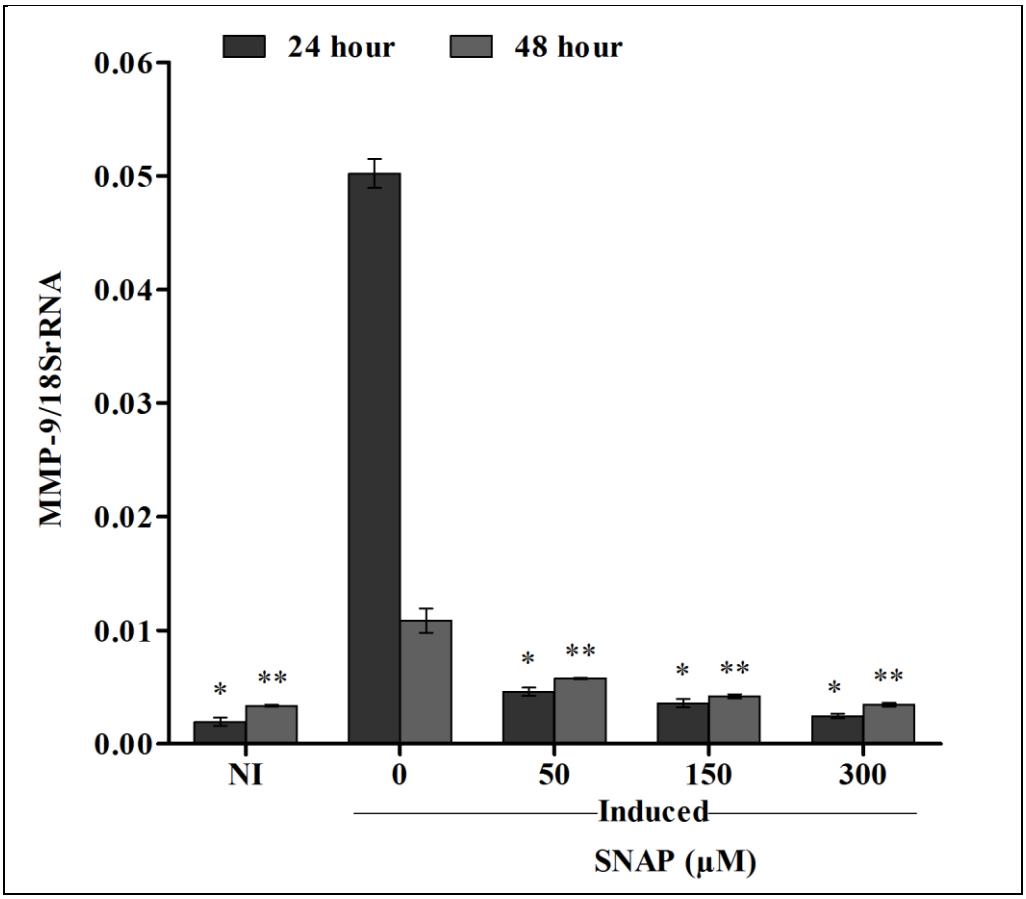

Figure 4.15. Effect of SNAP on the MMP-9 expression levels. Each point represents the mean MMP-9 expression value ± S.E.M. of NI, untreated and 50 µM, 150 µM, 300 µM SNAP treated induced Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

Non-induced fibroblasts demonstrated a ~26-fold and 3.3-fold reduction in MMP-9 expression levels compared to the untreated induced Swiss 3T3 fibroblasts at 24 and 48 hours, respectively. While, the inhibition of cross-linking activity of TG2 with 50 µM SNAP resulted in a 11-fold and 1.8-fold decrease in the MMP-9 expression levels at 24 and 48 hours, respectively. After 24 and 48 hours incubation in the presence of 150 µM SNAP, 14.3-fold and 2.6-fold reductions in the MMP-9 expression levels were observed compared to the untreated induced fibroblasts, respectively. Incubation with 300 µM SNAP for 24 and 48 hours caused a ~21-fold and a 14.7-fold decrease in the MMP-9 expression levels compared to the untreated induced Swiss 3T3 fibroblasts, respectively. Taken together, data suggests that the inhibition of TG2 activity by the increasing concentrations of SNAP resulted in a dramatic decrease in the MMP-9 expression levels. It

can also be concluded that that 24 hours was an important time point to see the dramatic change in the MMP-9 expression levels.

4.3.3. Effect of NO on the Expression of MMP-1a and MMP-13 in Fibroblasts

TG2 activity dependent effect of nitric oxide on the expression levels of collagenases (MMP-1a and MMP-13) in the transfected Swiss 3T3 fibroblast cell lines was examined. Total RNA isolated from non-induced, induced, and 50 µM, 150 µM, 300 µM SNAP treated induced Swiss 3T3 fibroblasts at 24h and 48h time points were subjected to quantitative real-time PCR as described in the Method Section 3.4.3. Each Individual data points in the following graphs correspond to the average expression value of the interested gene normalized against the 18SrRNA for each sample.

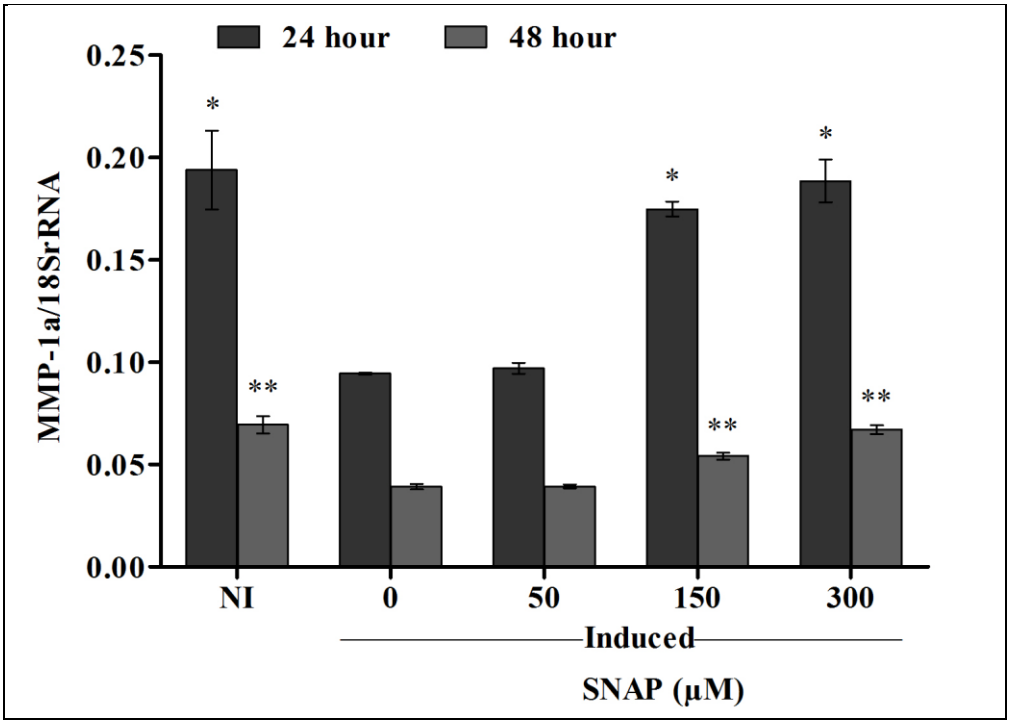

Figure 4.16. Effect of SNAP on the MMP-1a expression levels. Each point represents the mean MMP-1a expression value ± S.E.M. of NI, untreated and 50 µM, 150 µM, 300 µM SNAP treated induced fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

MMP-1a expression levels was 2-fold and 1.7-fold higher in the non-induced fibroblasts compared to the untreated induced Swiss 3T3 fibroblasts at 24 and 48 hours, respectively, as shown in Figure 4.16 ($p<0.05$). There was 1-fold induction observed in 50 µM SNAP treated induced fibroblasts compared to untreated induced fibroblasts at both 24 and 48 hours ($p>0.05$). The treatment of induced fibroblasts with SNAP concentrations higher than 50 µM demonstrated a significant increase in the MMP-1a expression levels. For example, treatment of induced fibroblasts with 150 µM SNAP led to a 1.8-fold and 1.3-fold increase in, MMP-1a mRNA levels at 24 and 48 hours, respectively. After 24 and 48 hours incubation of induced fibroblasts in the presence of 300 µM SNAP, a ~2-fold and 1.7-fold induction in the MMP-1a expression levels were respectively observed, compared to their untreated counterpart. Data suggests that the inhibition of enzymatic activity of TG2 by increasing concentration of SNAP led to a significant increase in the MMP-1a expression levels.

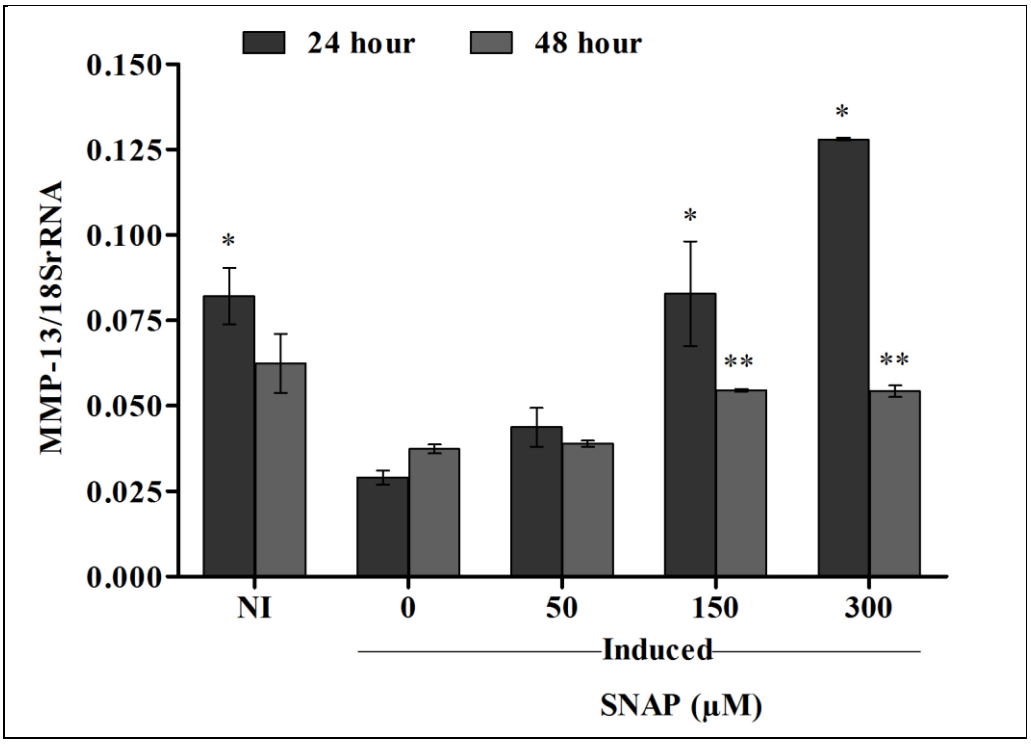

Figure 4.17. Effect of SNAP on the MMP-13 expression levels. Each point represents the mean MMP-13 expression value ± S.E.M. of NI, untreated and 50 µM, 150 µM, 300 µM SNAP treated induced fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

As can be seen from the Figure 4.17, MMP-13 mRNA expression was significant 2.8-fold and insignificant ~1.7-fold more in non-induced fibroblasts than the untreated induced fibroblasts at 24 and 48 hours, respectively. Induced fibroblasts treated with 50 μM SNAP led to a 1.5-fold and 1-fold induction at 24 and 48 hours, respectively ($p>0.05$). SNAP treatment led to a significant increase in MMP-13 expression only at 150 and 300 μM. Treatment of induced fibroblast with 150 μM SNAP resulted in a 2.8-fold and 1.5-fold increase in the MMP-13 expression levels at 24 and 48 hours, respectively. After 24 and 48 hours incubation in the presence of 300 μM SNAP, a respective 4.3-fold and a 1.4-fold induction in the MMP-13 expression levels were observed compared to the untreated induced fibroblasts. These results indicate that the inhibition of enzymatic activity of TG2 with increasing concentrations of SNAP can result in a significant increase in the MMP-13 expression levels.

4.4. ROLE OF TGF-β IN TG2-INDUCED CELL MIGRATION IMPAIRMENT

4.4.1. Determination of Non-toxic TGF-β Neutralizing Antibody Concentrations

The role of TGF-β on TG2 mediated impairment of migration in fibroblasts was examined by the utilization of TGF-β neutralizing antibody (clone MAB1835). In order to decide the optimum non-toxic concentration of TGF-β neutralizing antibody, a cell proliferation assay was performed and the cytotoxic effect of different concentrations of TGF-β neutralizing antibody was analyzed. Inducible fibroblasts were incubated with 5 μg/ml and 10 μg/ml TGF-β neutralizing antibody for 24 and 48 hours as described in the Method Section 3.1.6.

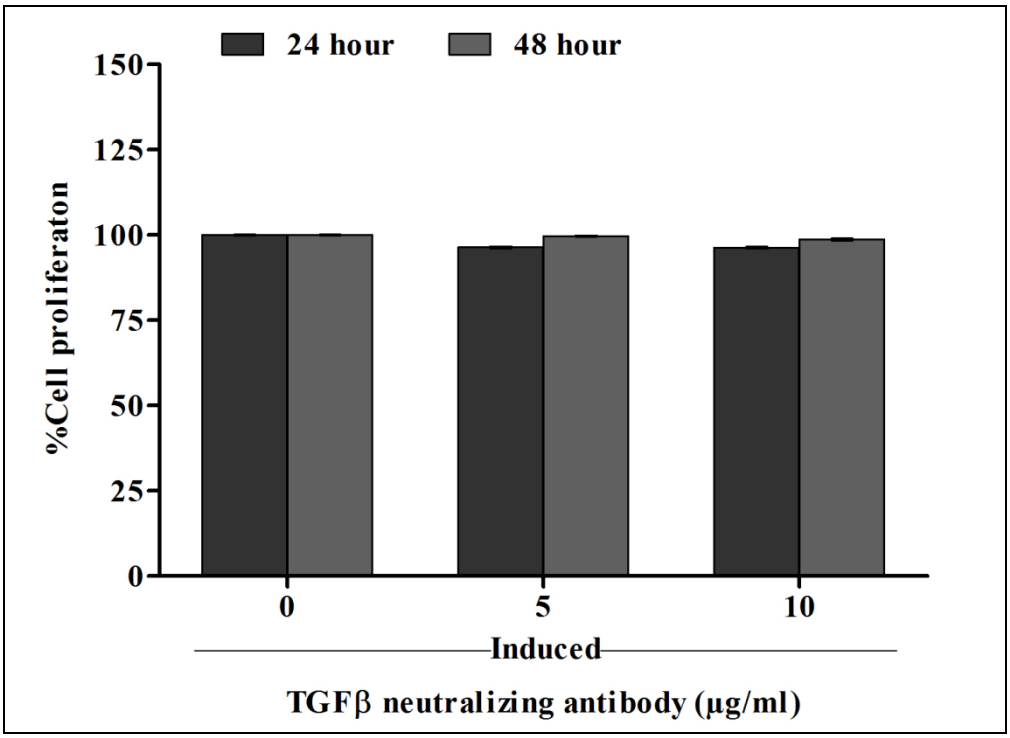

Figure 4.18. Effect of TGF-β neutralizing antibody on the inducible fibroblast cell viability. The measurement of cell proliferation levels of transfected induced Swiss 3T3 fibroblasts was performed after 24 and 48 hours incubation with 5 µg/ml and 10 µg/ml TGF-β neutralizing antibody concentrations. Each data point corresponds to the mean percentage of cell proliferation ± S.D. of one typical experiment performed in 4 replicates

The concentrations of 5 µg/ml and 10 µg/ml TGF-β neutralizing antibody (clone MAB1835) concentrations did not show any significant cytotoxic effect on the cell proliferation at both 24 and 48 hours. Since, it was reported that at 10 µg/ml MAB1835 blocks the TGF-β function, induced cells were treated with 10 µg/ml TGF-β neutralizing antibody was decided to be used in blocking of the TGF-β activity [121, 122].

4.4.2. Effect of TGF-β Neutralizing Antibody on Migration

The role of TGF-β on the TG2 activity mediated perturbation of migration in the induced fibroblasts was examined by the utilization of TGF-β neutralizing antibody (clone MAB1835). Inducible Swiss 3T3 fibroblasts were incubated in the presence or absence of 10 µg/ml TGF-β neutralizing antibody for 24 and 48 hours as described in Section 3.5.

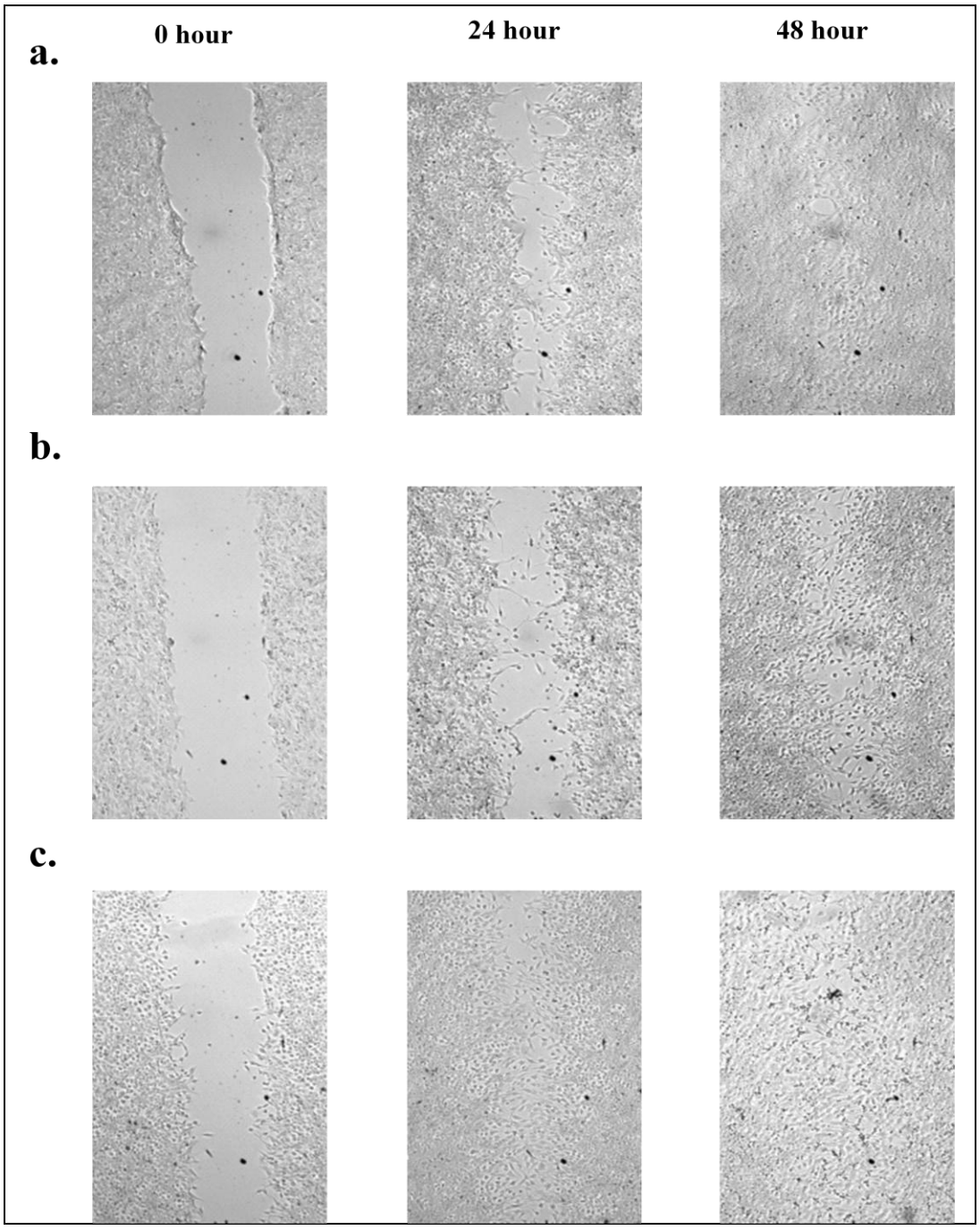

Figure 4.19. The role of TGF-β on the migration of induced fibroblasts. Representative photographs of wound scratch assay in the absence and presence of TGF-β neutralizing antibody. a. Non-induced (non-induced fibroblasts) Swiss 3T3 fibroblasts b. Induced (Ind) Swiss 3T3 fibroblasts c. TGF-β neutralizing antibody (MAB1835) treated induced Swiss 3T3 fibroblasts

Complete wound bed closure occurred only in non-induced fibroblasts and TGF-β neutralizing antibody treated induced Swiss 3T3 fibroblast as shown in Figure 4.19 at 48 hours.

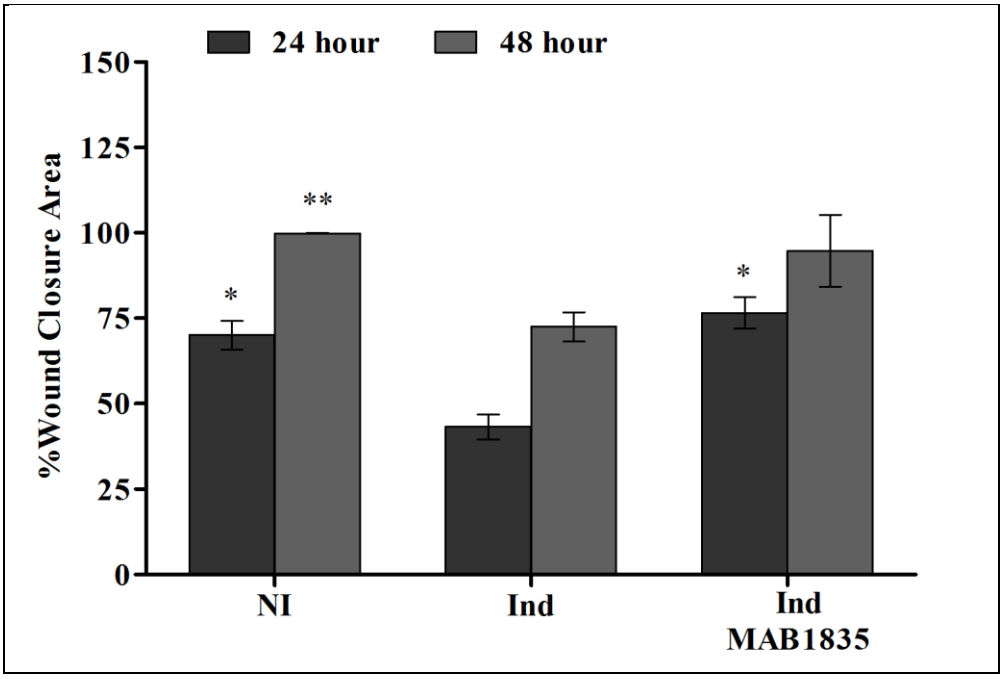

Figure 4.20. The effect of TGF-β on the migration of transfected induced fibroblasts. Each data point represents the mean percentage of closure of wound area ± S.E.M. of three independent experiments performed in duplicate. The wound closure rate was expressed as the mean percentage of migration of NI Swiss 3T3 fibroblast cells at 48 hours (control) ± S.E.M., which represents 100%. The area of wound closure for the control NI fibroblasts at 48 hours was accepted as 100%. * and ** symbols represent significant difference between NI and Ind and comparison of untreated Ind and MAB1835 treated Ind Swiss 3T3 fibroblasts ($P<0.05$)

Non-induced fibroblast displayed significant 27% and 28% increase in the rate of cell migration resulting in scratch closure when compared to the induced fibroblasts at 24 and 48 hours, respectively, as shown in Figure 4.20. The presence of 10 μg/ml TGF-β neutralizing antibody led to a significant 33% and insignificant 22% increase in the cell migration of induced fibroblasts at 24 and 48 hours, respectively. These data suggest that TG2 enzyme works in concert with TGF-β to inhibit the migration of fibroblasts.

4.4.3. Effect of TGF-β on the Expression of MMP-2 and MMP-9 in Fibroblasts

The effect of TGF-β on the expression levels of gelatinases (MMP-2 and MMP-9) in the transfected Swiss 3T3 fibroblast cell lines was assessed. Total RNA isolated from non-induced, induced, and 10 µg/ml TGF-β neutralizing antibody treated induced Swiss 3T3 fibroblasts at 24h and 48h time points were subjected to the quantitative real-time PCR as described in the Method Section 3.4.3. Each Individual data points in the following graphs correspond to the average expression value of the interested gene, normalized against the 18SrRNA.

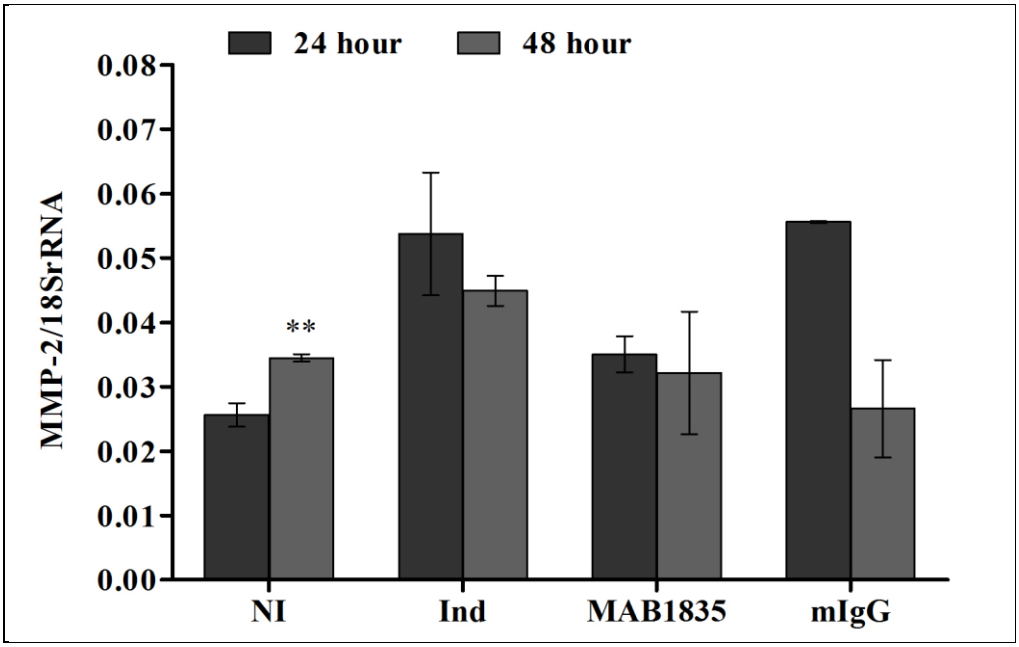

Figure 4.21. Role of TGF-β on the MMP-2 expression levels. Each point represents the mean MMP-2 expression value ± S.E.M. of NI, untreated Ind and 10 µg/ml TGF-β neutralizing antibody (MAB1835) treated Ind Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. mIgG was used as a negative control of the TGF-β neutralizing antibody. ** symbol indicates statistical difference ($P<0.05$)

Figure 4.21 demonstrated that 10 µg/ml TGF-β neutralizing antibody treatment was led to an approximate 1.5-fold and ~1.3-fold decrease in the MMP-2 expression levels, albeit insignificant, compared to the untreated induced cells at 24 and 48 hours, respectively. Non-induced fibroblasts displayed insignificant ~2.1-fold and significant 1.3-fold decrease

in the MMP-2 expression levels when compared to the induced fibroblasts at 24 and 48 hours, respectively.

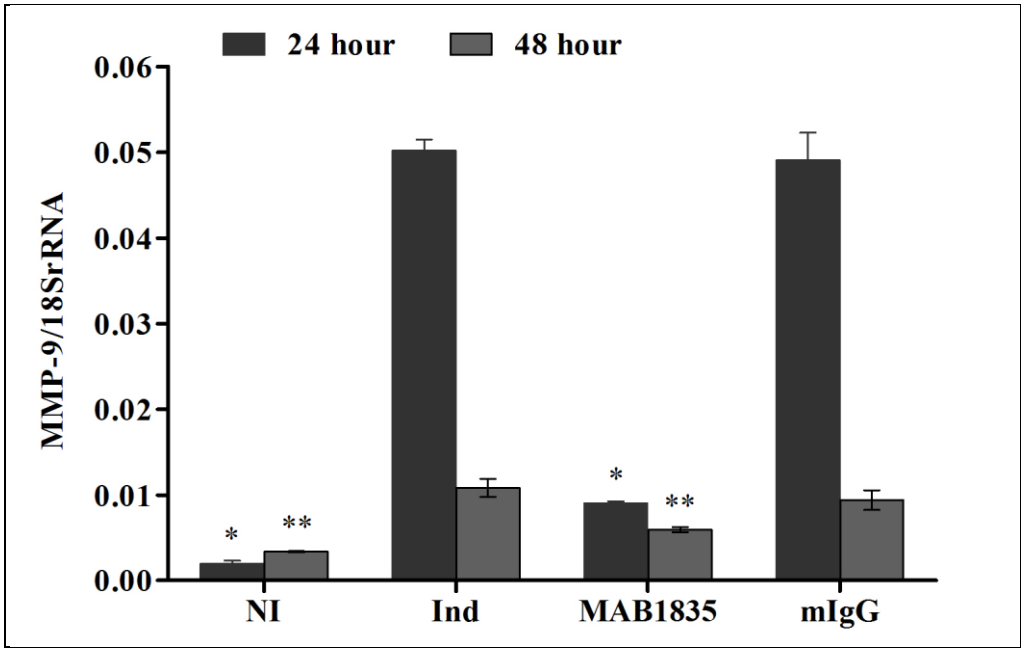

Figure 4.22. Role of TGF-β on the MMP-9 expression levels. Each point represents the mean MMP-9 expression value ± S.E.M. of NI, untreated Ind and 10 µg/ml TGF-β neutralizing antibody (MAB1835) treated Ind Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. mIgG was used as a negative control of the TGF-β neutralizing antibody. * and ** symbols indicate statistical difference ($P<0.05$)

The treatment of induced fibroblast with the TGF-β function blocking antibody resulted in a significant 5.5-fold and 1.8-fold decrease in the MMP-9 expression levels at 24 and 48 hours, respectively, when compared to the expression levels of the non-treated induced fibroblasts as shown in Figure 4.22. Induced fibroblasts displayed significant 26.4-fold and ~3.3-fold increase in the MMP-9 expression levels when compared to the non-induced fibroblasts at 24 and 48 hours, respectively. These experiments indicated that the TGF-β is an important factor in the regulation of gelatinase MMP-2 and MMP-9 expression levels.

4.4.4. Effect of TGF-β on Expression of MMP-1a and MMP-13 in Fibroblasts

Effect of TGF-β on the expression levels of collagenases (MMP-1a and MMP-13) in the

migration of transfected Swiss 3T3 fibroblast cell lines was assessed. Total RNA isolated from non-induced, induced (Ind), and 10 µg/ml TGF-β neutralizing antibody treated induced Swiss 3T3 fibroblasts at 24h and 48h time points were subjected to the quantitative real-time PCR as described in the Method Section 3.4.3. Each individual data points in the following graphs correspond to the average expression value of the interested gene normalized against the 18SrRNA expression.

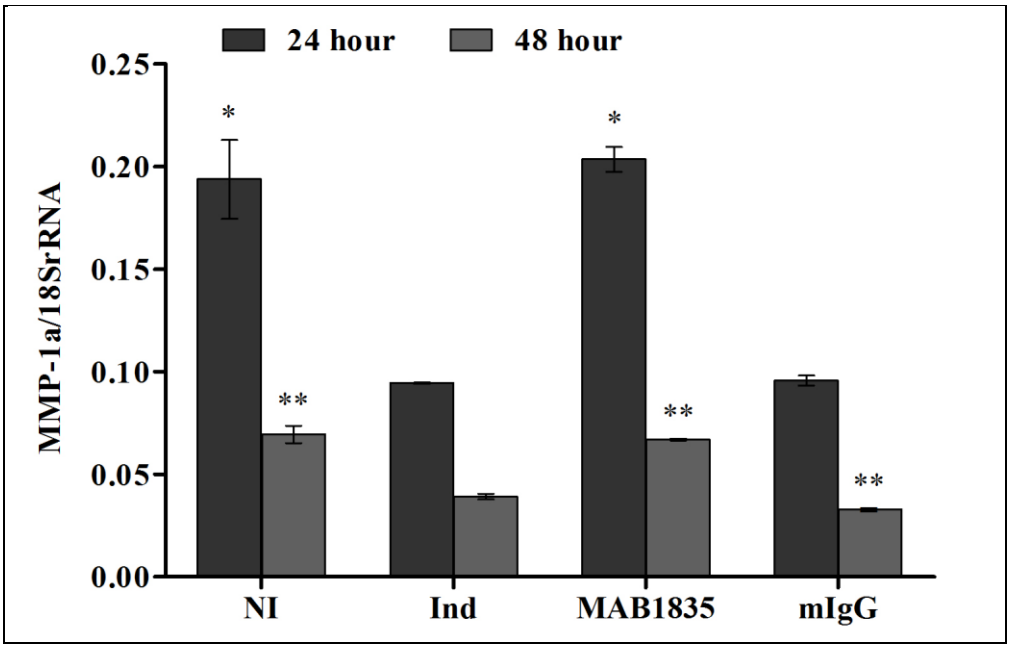

Figure 4.23. Role of TGF-β on MMP-1a expression levels. Each point represents the mean MMP-1a expression value ± S.E.M. of non-induced fibroblasts, untreated Ind and 10 µg/ml TGF-β neutralizing antibody (MAB1835) treated Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. mIgG was used as a negative control of TGF-β neutralizing antibody also significantly changed at 48 hours incubation. * and ** symbols indicate statistical difference ($P<0.05$)

Inhibition of TGF-β function with the neutralizing antibody resulted in a respective 2.1-fold and 1.7-fold increase in MMP-1a expression levels at 24 and 48 hours in the induced fibroblasts. There was observed 2-fold and ~1.8-fold increase in non-induced fibroblasts compared to induced fibroblasts at 24 and 48 hours, respectively ($p<0.05$). Interestingly, at 48 hours incubation with mIgG serum a significant difference was observed in MMP-1a expression levels of induced Swiss 3T3 fibroblasts as shown in Figure 4.23.

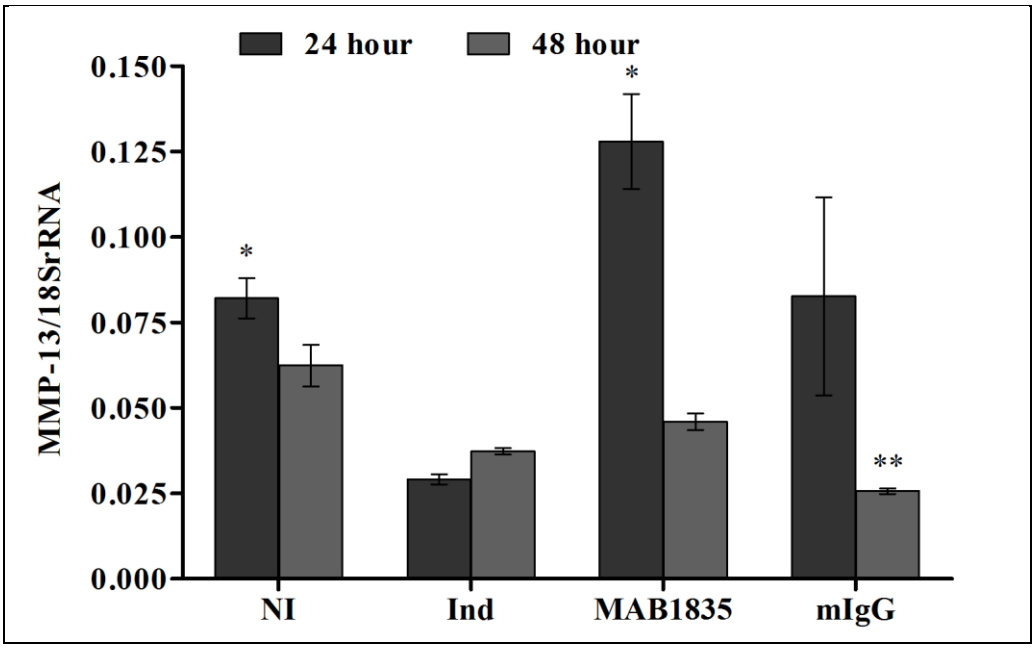

Figure 4.24. Role of TGF-β on the MMP-13 expression levels. Each point represents the mean MMP-13 expression value ± S.E.M. of NI, untreated Ind and 10 µg/ml TGF-β neutralizing antibody (MAB1835) treated Ind Swiss 3T3 fibroblasts from three independent experiments performed in duplicate. * and ** symbols indicate statistical difference ($P<0.05$)

The treatment of induced fibroblasts with the TGF-β neutralizing antibody significantly increased the MMP-13 expression levels by 4.4-fold at 24 hours and by 1.2-fold at 48 hours. There was observed significant 2.8-fold and insignificant ~1.7-fold increase in non-induced fibroblasts compared to induced fibroblasts at 24 and 48 hours, respectively. However, incubation of the induced cells with the control mIgG led to a significant decrease in the MMP-13 expression levels as shown in Figure 4.24.

4.5. EFFECT OF TG2 ON MIGRATION OF FIBROBLASTS ON FN

In order to investigate whether the inhibition of TG2 enzymatic activity affect the migration of fibroblast through FN, transwell assay was performed on non-induced, untreated and TG2 inhibitor treated induced fibroblasts. For this purpose, cells were incubated with 50 µM TG2 inhibitor for 48 hours, seeded on FN coated transwell inserts

and allowed to migrate through FN for at 4 and 8 hours as described in details in Result Section 4.6.

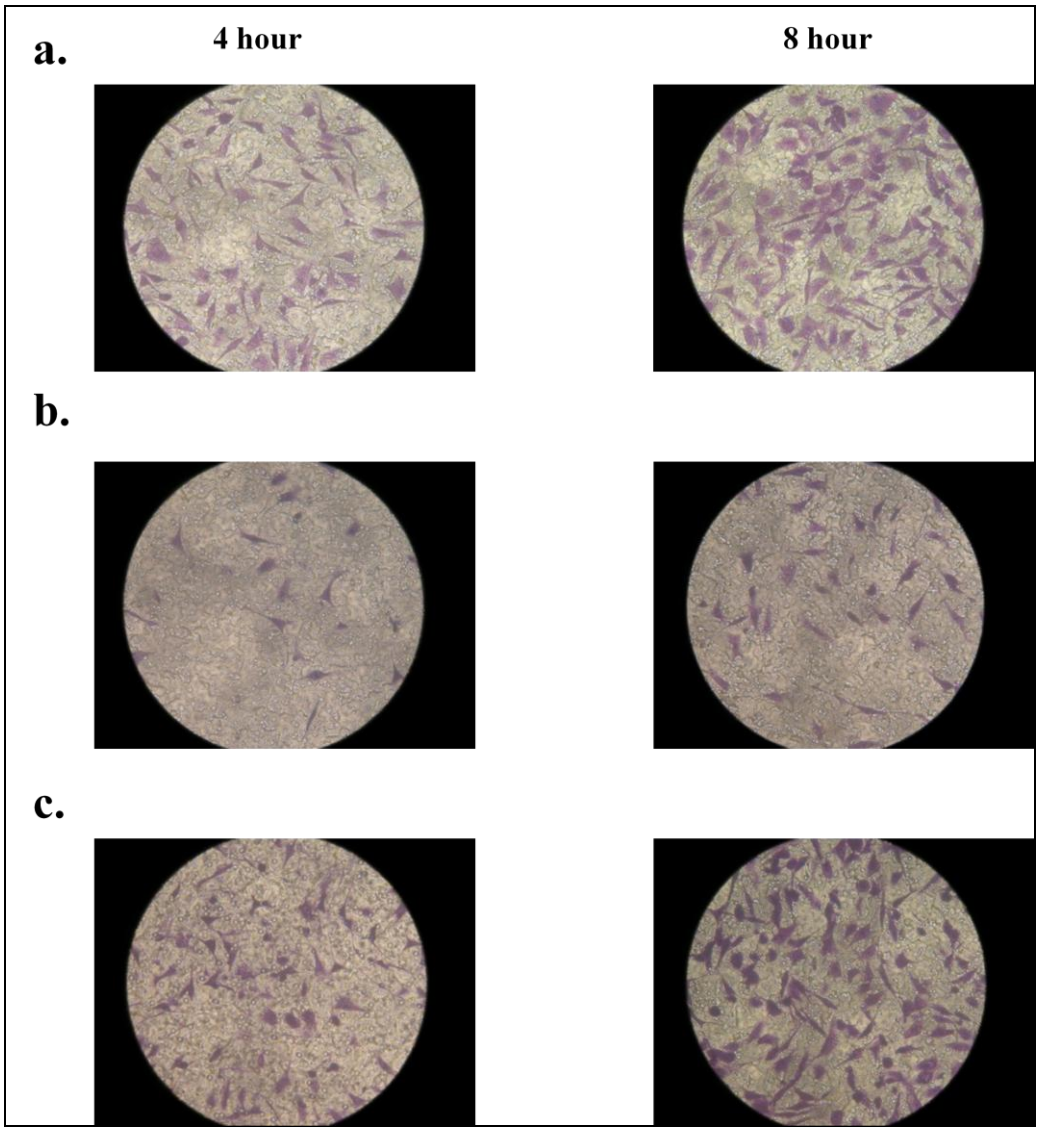

Figure 4.25. Effect of TG2 Inhibitor on the migration of fibroblasts through FN. a. NI, b. Induced, c. TG2 Inh. treated Swiss 3T3 fibroblasts migration through FN at 4 and 8 hours.

Low levels of extracellular TG2 enzyme activity led to an enhanced cell migration potential through FN as shown in Figure 4.25.

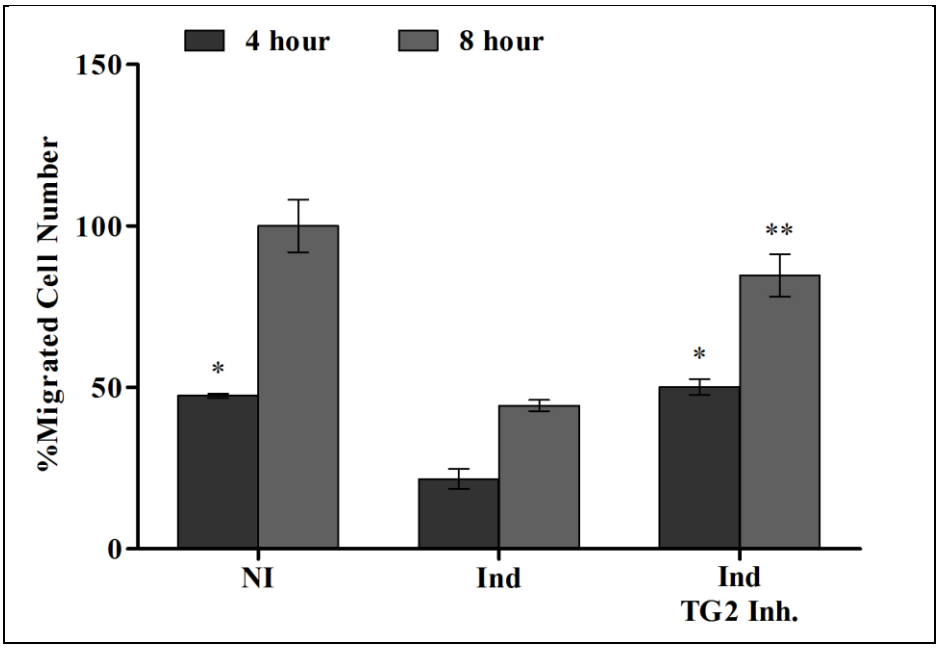

Figure 4.26. Effect of TG2 inhibitor on FN-bound cell migration. Each point represents the mean value of migrated cells±S.E.M. of NI, untreated Ind and 50 μM TG2 Inh. treated Swiss 3T3 fibroblasts from two independent experiments. The migrated cell number was expressed as the mean percentage of migration of NI Swiss 3T3 fibroblast cells at 8 hours (control) ± S.E.M., which represents 100%. * and ** symbols indicate statistical difference ($P<0.05$)

Comparison of the migration potential between induced and non-induced fibroblasts demonstrated a significant ~25.7% and insignificant 55.6% increase in the migration of non-induced cells through FN, at 4 and 8 hours respectively. Similarly, the treatment of induced fibroblasts with TG2 inhibitor led to a significant 28.4% increase in 4 hours and significant 40.2% increase in 8 hours as shown in Figure 4.26.

In the previous study, it was suggested that while nitrosylation of TG2 had an effect on the transamidating activity of the enzyme, it did not affect the binding efficiency of TG2 protein to FN [118]. In order to test the effect of NO donor on the migration of TG2 induced cells through FN matrix, cells were treated with SNAP for 48 hours as before and seeded on FN coated transwell inserts for at 4 and 8 hours as shown in Figure 4.27.

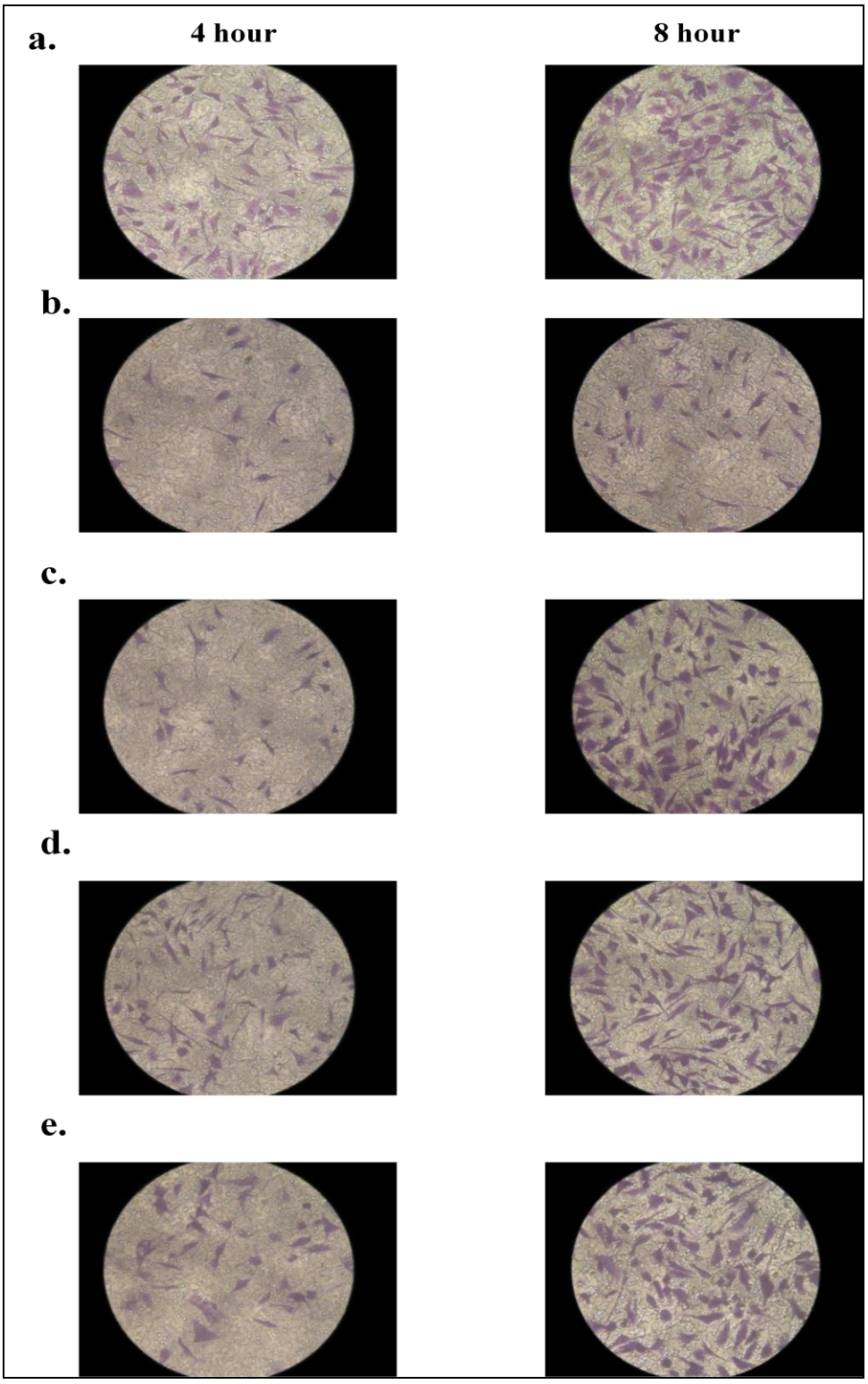

Figure 4.27. Effect of the increasing concentrations of SNAP on the migration through FN. a. NI, b. Ind, c. 50 μM SNAP, d. 150 μM and e. 300 μM SNAP treated Ind Swiss 3T3 fibroblasts migration through FN at 4 and 8 hours.

Nitrosylation of TG2 by NO donor resulted in a significant increase in the migration rate of induced cells through FN in a dose dependent manner as shown in Figure 4.27.

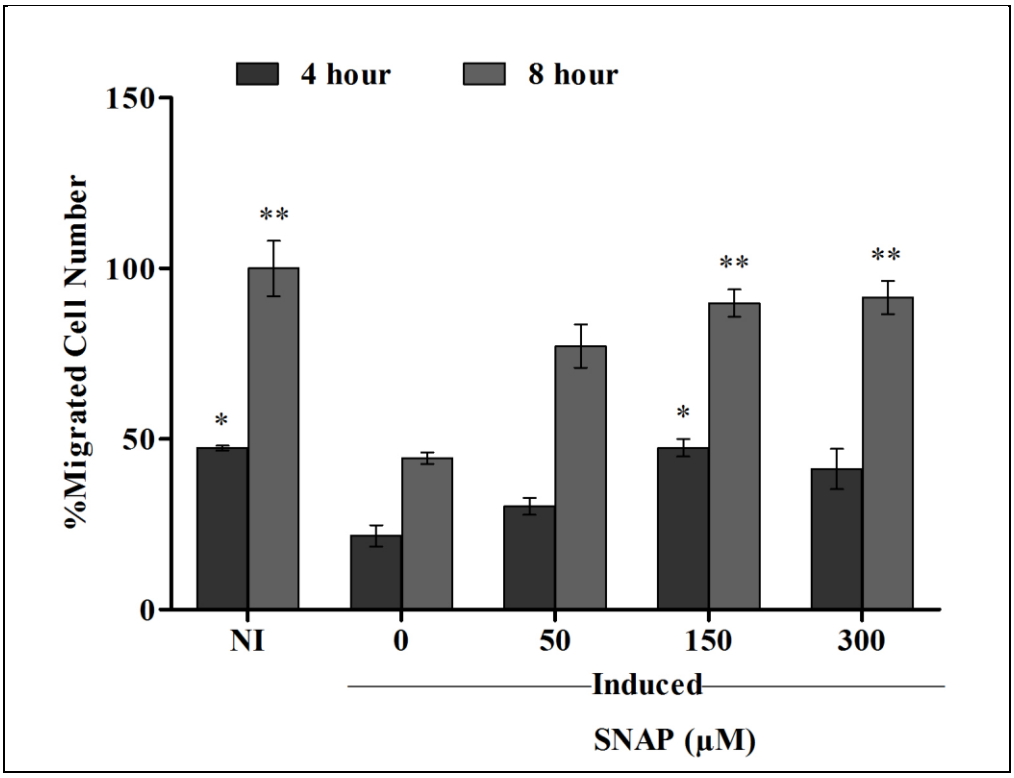

Figure 4.28. Effect of nitrosylated TG2 on FN-bound cell migration. Each point represents the mean value of migrated cells±S.E.M. of NI,, untreated Ind and 50 µM, 150 µM, 300 µM SNAP treated Ind Swiss 3T3 fibroblasts from two independent experiments. The migrated cell number was expressed as the mean percentage of migration of NI Swiss 3T3 fibroblast cells at 8 hours (control) ± S.E.M., which represents 100%. * and ** symbols indicate statistical difference ($P<0.05$)

Figure 4.28. indicates that non-induced fibroblasts demonstrated a significant ~25.7% and 55.6% increase in the migration potential through FN at 4 and 8 hours, respectively, compared to induced fibroblasts. While the treatment of induced cells with 50 µM SNAP led to a ~33% and 11.5% increase in the migration potential of fibroblasts at 4 and 8 hours, respectively ($p>0.05$). 150 µM SNAP resulted in a ~45% and ~25% increase in migration through FN at 4 and 8 hours, respectively. 300 µM SNAP led to a insignificant 19.5% and significant 47.1% augmentation in migration through FN compared to Ind Swiss 3T3 fibroblasts at both 4 and 8 hours incubation, respectively.

In order to investigate the role of TGF-β on the TG2 impaired cell migration of fibroblasts on FN, cells incubated with 10 μg/ml TGF-β neutralizing antibody were seeded on FN coated transwell inserts and allowed to migrate for 4 and 8 hours as shown in Figure 4.28.

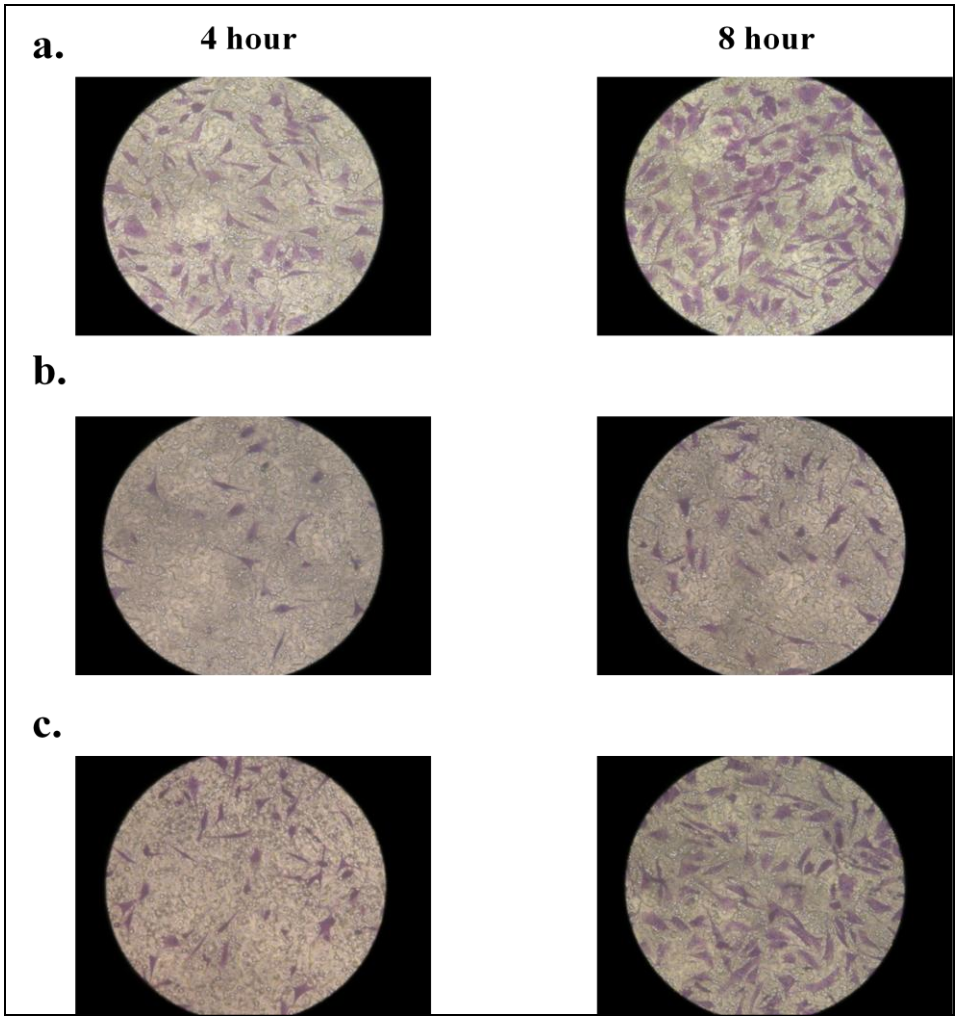

Figure 4.29. Effect of TGF-β on migration through FN. a. NI, b. Ind, and c. TGF-β neutralizing antibody (MAB1835) treated Ind Swiss 3T3 fibroblast migration through FN

When the TGF-β function was blocked by MAB1835 in induced fibroblasts, the rate of cell migration through FN were found to be similar to the levels displayed by the non-induced cells as shown in Figure 4.29.

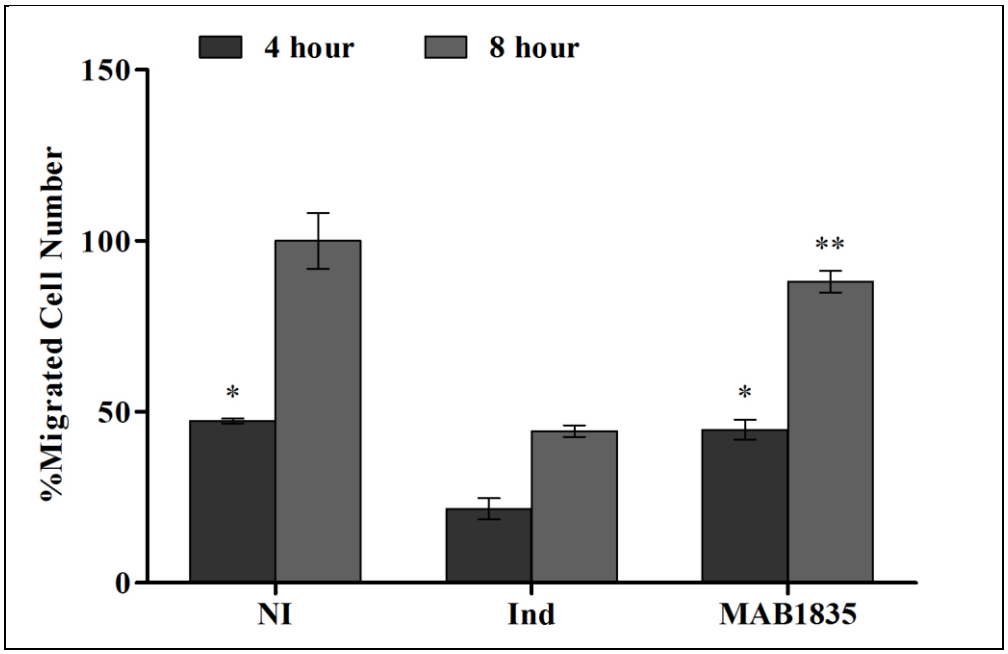

Figure 4.30. Role of TGF-β on FN-bound cell migration. Each point represents the mean value of migrated cells±S.E.M. of non-induced fibroblasts, untreated Ind and 10 µg/ml TGF-β neutralizing antibody (MAB1835) treated Swiss 3T3 fibroblasts from two independent experiments. The migrated cell number was expressed as the mean percentage of migration of NI Swiss 3T3 fibroblast cells at 8 hours (control) ± S.E.M., which represents 100%. * and ** symbols indicate statistical difference ($P<0.05$)

Figure 4.30. indicates that non-induced fibroblasts demonstrated a significant ~25.7% and 55.6 % increase in the migration potential through FN at 4 and 8 hours, respectively, compared to induced fibroblasts. Data showed that the inhibition of TGF-β led to a significant ~23.1% and 43.6% increase in the migration of Swiss 3T3 fibroblasts through FN compared to the untreated induced fibroblasts at both 4 and 8 hours, respectively. Taken together, these data suggest that TGF-β activity is responsible for the delay in the migration of fibroblasts through FN, possibly due to its promoting effects on ECM deposition.

5. DISCUSSION

Wound healing is a dynamic process that correlates with three interrelated phases including haemostasis and inflammation, new tissue formation and tissue remodeling. This study focuses on the initial phases of wound healing.

In the previous study performed on non-induced and induced Swiss 3T3 fibroblasts under the control of the tetracycline-regulated inducible promoter [120], it was elucidated that the neutralization of the enzymatic activity of TG2 by NO leads to the inhibition of NFκB activation which is responsible for the transcriptional activation of TGF-β1 production. However, the nitrosylation of TG2 did not cause the protein to lose its function as a cell adhesion protein since the FN bound-nitrosylated TG2 could still restore RGD-impaired cell adhesion. This study investigates the role of TG2 and NO interplay in cell migration in order to gain on insight into the initial phases of wound healing [118].

Using the same cell model of inducible Swiss 3T3 fibroblasts [118] the role of cross-linking activity of TG2 and the activity of TGF-β1 in the cell migration was further analyzed by performing wound scratch and transwell migration assays. The effect of NO as the modulator of cell migration regulated by the TG2 and TGF-β1 was also investigated in these experimental models. To elucidate the molecular downstream effectors involved in the NO mediated cell migration of TG2 induced fibroblasts, the expression of the wound healing corner stone ECM proteases MMP-2, MMP-9, MMP-1a, and MMP-13 was assessed.

Induced Swiss 3T3 fibroblasts cultured in the absence and presence of TG2 active site inhibitor showed that the rate of migration was increased in the non-induced and TG2 inhibitor treated induced fibroblasts when compared to the untreated induced fibroblasts, suggesting that cell migration is reduced by the transamidating activity of TG2 due to extensive cross-linking of ECM proteins. Data obtained from this study was consistent with previous reports that suggest that the over production and activation of TG2 enzyme were associated with the high levels of ε-(γ-glutamyl) lysine cross-linked ECM proteins leading to fibrotic conditions interferring with cell proliferation and migration [123, 124].

A further objective in undertaking this study was to investigate the role of NO in cell migration through the inhibition of TG2 activity via a nitrosylation reaction which led to the accumulation of the TG2 molecule on cell surfaces thereby resulting in the decreased deposition of TG2 into the ECM [118]. Data from the wound scratch assays demonstrated that SNAP at increasing concentrations ameliorated the cell migration in the induced fibroblasts when compared to untreated induced fibroblasts. It could be suggested that NO could force TG2 to reside on the cell surface which causes the reduction of TG2 in the ECM, which reduces the tensile strength of the ECM making the matrix more suitable for cell migration [24]. It was also demonstrated in the previous study that nitrosylation by increasing concentrations of SNAP caused decreases in the TGF-β1 protein levels via NFκB dependent inhibition of TG2 activity. 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 and β3 integrins and syndecan-4 [46, 47]. Consistent with this hypothesis, recent evidence demonstrated that TG2 on cell surfaces can act as a novel cell adhesion protein independent from its transamidating activity and trigger cell signaling that stimulates cell survival and proliferation at the cell surface [46, 53, 118]. Increasing evidence indicates that continous reduction in NO synthesis and availability is one of the primary causes involved in the initiation and progression of fibrotic response in pathological conditions [125-129]. Hence, it can be concluded that NO can modulate cell migration via the TG2/TGF-β1 dependent pathway and the reduction in the levels of NO could cause delayed wound healing or the development of fibrosis by inhibiting cell migration and proliferation due to the excess deposition and a stiff/non-flexible ECM.

Data from the wound scratch experiments performed to investigate the role of TGF-β1 activity in the migration of fibroblasts over expressing TG2 suggested that the TGF-β1 activation mediated by TG2 stimulated the fibroblasts to assume a secretory profile showing increased collagen, FN, and ECM production [64, 65]. The results here indicated that the blockage of TGF-β1 activity in the induced fibroblasts results in an increase in the rate of cell migration in comparison to the untreated induced Swiss 3T3 fibroblasts. This data could suggest that the inhibition of TGF-β1 in TG2 induced fibroblast increased the cell migration rate due to a reduction in the ECM protein synthesis and deposition which is

the substrate for the TG2 protein. In other words, TG2 will not be effective as a cross linking enzyme due to the low levels of secreted ECM proteins.

To gain deeper insight into the molecular events that control cell migration, the expression of gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1a and MMP-13) which play an important role in ECM turnover were analyzed by using Q-PCR. In the physiological conditions of wound healing a provisional matrix containing cross-linked fibrin and FN is deposited into the wound site as the initial response to tissue damage [1, 10]. Meanwhile, due to the tissue damage and cellular stress TG2 activity in the wound bed shows an increase leading to the activation of TGF-β1 that is responsible from the synthesis of ECM proteins which in turn are cross-linked and stabilized by TG2 [41, 42, 43, 44, 45]. The newly synthesized collagen and FN rich matrix replaces the provisional matrix as the latter is degraded by MMP-2 and MMP-9 [99, 101, 130]. Evidence for MMP-2 and MMP-9 degradation of the components of the provisional matrix are consistent with the *in vivo* events. Results from real time PCR experiments demonstrating that the TG2 mediated activation of TGF-β1 led to an increase in MMP-2 and MMP-9 activity in the induced fibroblasts. Studies suggest that proteinases function in the proteolytic cleavage of the provisional matrix [16, 99, 100]. In this context, MMP-2 initiates cell migration by clearing the ECM and facilitates wound healing [99, 101]. Experiments have shown that MMP-2 and MMP-9 expression levels were lower in induced fibroblasts treated with the TG2 inhibitor and in non-induced fibroblats than in induced fibroblasts. In agreement with our results, recent publications reported that TG2 facilitated ECM stabilization and antisense TG2 cDNA transfection of the fibroblasts caused a decrease in the activation of MMP-2 [131]. In the context of these findings, results from this thesis suggested that MMP-2 and MMP-9 could have an important role in the clearing of the provisional matrix to induce the migration and proliferation of cells in the initial steps of wound healing. Expression analysis showed that the induction of MMP-2 and MMP-9 expression by TG2 is performed through TGF-β1 activation since TGF-β1 neutralizing antibody led to decreased MMP-2 and MMP-9 expression levels. In keeping with this data, a recent study demonstrated that the addition of TGF-β1 into airway epithelial cell cultures dramatically increased the MMP-2 and MMP-9 expression levels resulting in enhanced *in vitro* airway wound repair [105]. In addition, the presence of NO at increasing concentrations caused a

decrease in the expression of MMP-2 and MMP-9 in the induced fibroblasts suggesting that TG2 activity leads to an induction of MMP-2 and MMP-9 expression.

In a normal wound healing process the balance between the ECM synthesis which consists of the granulation tissue formation, collagen deposition, and the degradation of matrix should be preserved. Synthesis of the matrix is important to allow the cell migration. However, excess deposition and stabilization of the ECM by cross-linking was associated with fibrotic conditions. Therefore, the balance between the synthesis and degradation of the ECM must be provided by the mediators of the wound healing [110, 111]**.** In cultured fibroblasts, the treatment of TGF-β leads to the reduction in synthesis of collagenases and enlarges the production of the collagenase inhibitor TIMP-1, inhibiting the degradation of newly synthesized collagen [70, 71]. In consistent with this finding, the results from this thesis showed that expression levels of MMP-1a and MMP-13 (collagenases) were decreased in TG2 induced fibroblasts in TG2 and TGF-β1 activity dependent manner. The treatment of TG2 induced fibroblasts with TGF-β1 activity blocking antibody or TG2 inhibitor caused an up regulation of MMP-1a and MMP-13 levels similar to that of the non-induced fibroblast. Similarly, NO treatment of the induced fibroblasts also showed an increase in MMP-1a and MMP-13 expression levels with respect to the untreated induced fibroblast cell lines. These data suggested that decreased TG2 activity leads to a reduction in TGF-β1 activity that could cause the up regulation of MMP-1a and MMP-13 to facilitate the partial degradation of newly formed collagen and FN matrix that is necessary for cell migration. In physiological conditions of wound healing, the newly synthesized matrix that replaces the provisional matrix is invaded by the fibroblast, endothelial, and epithelial cells. This phase of the wound healing is characterized with the increase in NO synthesis and remodeling of the synthesized matrix with the proteases such as collagenases. In accordance with these events, the addition of NO donor into the induced fibroblasts culture led to an increase in the MMP-1a and MMP-13 as a result of a decrease in TGF-β1 activation that occurred due to the inactivation of TG2 by the NO. As mentioned before under these conditions, TG2 was kept on the cell surface and served as a cell adhesion protein rather than the transamidating enzyme [118]. In order to further confirm this finding, transwell experiments were undertaken on induced cells treated with TGF-β1, TG2 activity blockers and NO donor.

Transwell analysis demonstrated that the migration potential through FN was increased by the inhibition of TG2 transamidating activity of the induced fibroblasts with the treatment of TG2 inhibitor and in the non-induced fibroblast cells compared to untreated induced fibroblasts. The results here indicated that the transamidating activity of TG2 enzyme did not affect the migration potential of the fibroblast cells through FN, this induction could be the result of the TG2 enzyme functioning as cell adhesion protein.

A previous study has shown that the inhibition of TG2 activity through nitrosylation via NO had no effect on the binding efficiency of TG2 to FN [118] and the inhibition of the RGD sequence with RGD-inhibitor peptide fragments did not cause an impairment in cell adhesion, suggesting that TG2 enzyme could mediate its function as the RGD-independent cell adhesion protein on FN [47]. The transwell results showed that the presence of NO at increasing concentrations significantly promoted cell migration potential through FN. This finding fortified the suggestion that TG2 could mediate cell migration by acting on an RGD-independent cell adhesion protein without relying on its transamidating activity.

To further investigate the role of TGF-β1 in cell migration through FN, the transfected fibroblasts induced to express the TG2 enzyme incubated in the presence and absence of the TGF-β1 neutralizing antibody. The findings in these experiments demonstrated that the cell migration potential through FN was stimulated in the absence of TGF-β1 protein. In the context of this data, it could be concluded that TGFβ1 activity is responsible for the delay in the migration potential of fibroblasts through FN, possibly due to its promoting effect on ECM deposition.

The results of this thesis project reveals that under the physiological conditions of wound healing, TG2 is released from the cells into the ECM to stabilize the provisional matrix at the time course of the tissue damage. The elevated levels of TG2 in the ECM causes an induction in the TGF-β1 levels which leads to the up regulation of MMP-2 and MMP-9 expression in order to cleave the provisional matrix. In the physiological conditions of wound healing, the newly synthesized matrix that replaces the provisional matrix is invaded by the fibroblast, endothelial, and epithelial cells. This phase of the wound healing is characterized with the secretion of the NO into the wound site and remodeling of the synthesized matrix with the proteases such as collagenases. As shown in Figure 5.1, the

degradation of the provisional matrix abundant in FN would result in FN fragments containing RGD sequences which could hinder the adhesion and migration of cells onto the newly synthesized. NO secretion inhibits the TG2 activity via nitrosylation to interfere with the extensive stabilization of the newly synthesized matrix by the cross-linking activity of TG2. The reduction in the TG2 activity in ECM via NO leads to a decrease in TGF-β1 activity that could cause the up regulation of MMP-1a and MMP-13 to facilitate the partial degradation of newly formed collagen and FN matrix that is necessary for the cell migration. Previous study showed that the nitrosylation of TG2 by NO resulted in a reduction in the ECM deposition of the enzyme with a subsequent increase in the enzyme levels at the cell surface [118]. On the cell surface nitrosylated TG2 therefore could interact with Syndecan-4 and mediate cell migration and survival signaling, which is important to overcome the inhibitory effect of RGD-containing provisional matrix fragments [120, 47].

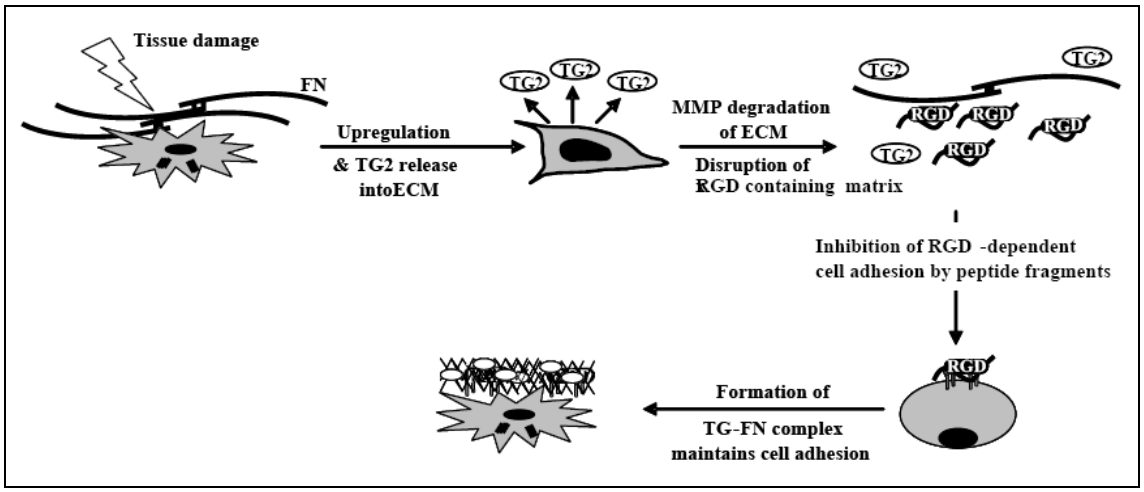

Figure 5.1. Summary of the RGD-independent pathway [47]

6. CONCLUSION

This study investigated the relationship between TG2 and NO through the TGF-β1 pathway at the initial phases of wound healing. The results in this study demonstrated that the inhibition of transamidating activity of TG2 by NO enhanced cell migration through TGF-β1 dependent pathway by enforcing its role as a RGD-independent cell adhesion protein. To gain deeper insight on the downstream effectors of the migration process, this study focused specifically on the gelatinases (MMP-2 and MMP-9) and the collagenases (MMP-1a and MMP-13) which are significant regulators of the wound healing mechanism.

Inhibition of TG2 activity led to the down regulation of MMP-2 and MMP-9 expression levels through TGF-β1 pathway. In other words, increase in TG2 activity caused an induction of the MMP-2 and MMP-9 expression levels. Finally, the role of TG2 in the cell migration through FN was elucidated and findings indicated that inhibition of TG2 activity by its inhibitor and NO led to an increase in the cell migration through FN via a TGF-β1 dependent manner, suggesting that TG2 mediates the cell migration as a RGD-independent cell adhesion molecule. Taken together, all these findings could indicate that an increase in TG2 activity enhances TGF-β1 dependent ECM synthesis and MMP-2 and MMP-9 upregulation which could function in the cleansing of the provisional matrix. As the wound healing progresses increases in NO synthesis interferes with the TG2 cross-linking leading to TGF-β1 dependent upregulation of MMP-1a and MMP-13, which in turn promote cell migration through the remodeling of the newly synthesized matrix. Nitrosylation of TG2, moreover, converts the enzyme into a cell adhesion protein which plays a role in the migration and survival signaling.

7. FUTURE DIRECTIONS

This study revealed the role of NO on the transamidating activity of TG2 and the effect of this interrelation on the cell migration through TGF-β1 mediated role of MMPs in transcriptional level. Transcriptional analysis is not adequate to reveal the exact mechanism underlying wound healing. MMP activity levels, using MMP activity assays and expressional analysis of TIMPs should be investigated to decide exact mechanisms of the MMPs that play a role in wound healing as future prospects. Moreover, the interaction of nitrosylated TG2 with the cell surface integrins and syndecan-4 should be investigated.

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