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NFkappaB activation is essential for miR-21 induction by TGFβ1 in high glucose conditions

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ABSTRACT

Transforming growth factor beta1 (TGFβ1) is a pleiotropic growth factor with a very broad spectrum of effects on wound healing. Chronic non-healing wounds such as diabetic foot ulcers express reduced levels of TGFβ1. On the other hand, our previous studies have shown that the microRNA miR-21 is differentially regulated in diabetic wounds and that it promotes migration of fibroblast cells. Although interplay between TGFβ1 and miR-21 are studied in relation to cancer, their interaction in the context of chronic wounds has not yet been investigated. In this study, we examined if TGFβ1 could stimulate miR-21 in fibroblasts that are subjected to high glucose environment. MiR-21 was, in fact, induced by TGFβ1 in high glucose conditions. The induction by TGFβ1 was dependent on NFκB activation and subsequent ROS generation. TGFβ1 was instrumental in degrading the NFκB inhibitor IκBα and facilitating the nuclear translocation of NFκB p65 subunit. EMSA studies showed enhanced DNA binding activity of NFκB in the presence of TGFβ1. ChIP assay revealed binding of p65 to miR-21 promoter. NFκB activation was also required for the nuclear translocation of Smad 4 protein and subsequent direct interaction of Smad proteins with primary miR-21 as revealed by RNA-IP studies. Our results show that manipulation of TGFβ1–NFκB–miR-21 pathway could serve as an innovative approach towards therapeutics to heal diabetic ulcers.

1. Introduction

Wound healing is a complex, yet normal physiological response to tissue injury and is an outcome of well-synchronized interaction between multiple biological pathways. A typical response to tissue injury occurs in three overlapping but distinct stages: inflammation, proliferation, and remodelling [1]. Dysregulation of timely interactions between these processes can manifest in pathological abnormalities, either as delayed wound healing or excessive healing. Most chronic wounds, fail to progress through the normal phases of wound repair [2], but instead remain in a chronic inflammatory state [3]. Diabetic foot ulcer is a typical example of chronic wound that displays impaired healing pattern. Wound healing in diabetes mellitus is impaired, as a result of poor blood supply, impaired leucocyte function, infection, and excessive callus formation [4]. At the cellular level, diabetic wounds are characterized by increased number of acute inflammatory cells and reduced levels of growth factors [5,6]. Growth factors play prominent roles as mediators of cellular interactions that occur during wound healing. Transforming growth factor β1 (TGFβ1) is a growth factor intimately involved in wound healing through its action on trans-differentiation of epithelial cells to mesenchymal cells besides regulation of extracellular matrix [7]. In addition, it also works as a potent chemo-attractant for cells such as monocytes, macrophages, lymphocytes, neutrophils, and fibroblasts, the migration of which is necessary for proper healing of the wounds [4]. TGFβ1 treated wounds are characterized by a more organised and denser collagen matrix [8]. Remarkably, TGFβ1 is reduced in diabetic chronic foot ulcers and chronic venous ulcers [9,10].

MicroRNAs (miRNAs) are a group of small non-coding single stranded RNA molecules that negatively modulate target gene expression through interaction with the 3′UTR of the target mRNA. MiRNAs are first transcribed by RNA polymerase II enzyme as long primary transcripts (Pri-miRNA). These are processed in the nucleus by RNAse III enzyme Drosha, to form precursor miRNA (Pre-miRNA). Pre-miRNAs are transported to cytoplasm and further processed by RNAse III enzyme Dicer to produce about 22 nucleotide long mature miRNA. Mature miRNA gets incorporated into RNA-induced silencing complex (RISC) and mediates the

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silencing of target genes [11]. The last decade has witnessed the emergence of microRNAs as strategic players in diverse cellular processes, including development, differentiation, proliferation, migration, stress response, angiogenesis, cell death, and carcinogenesis [12–17]. While miRNAs are linked to a variety of pathological conditions such as neurological disorders, cancer, and metabolic diseases, research studies on the role of miRNAs in wound healing is relatively new and few [18–20]. Our recent research findings revealed that miRNAs may be indispensable for wound healing, and that miR-21 is necessary for fibroblast migration, a process that is retarded in diabetic wounds [20]. Understanding the mechanisms that control miRNA biogenesis in diabetic wounds could open new doors for treatment of chronic wounds. In the current study, we show that miR-21 can be induced by TGFβ1 in high glucose conditions and that NFκappaB (NFκB) activation is required for the induction.

2. Materials and methods

2.1. Cell culture and treatment

NIH-3T3 cells purchased from American Type Culture Collection (ATCC, USA) were grown in Alpha Modified Eagle's Medium (αMEM, Sigma, Japan) supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin under standard culture conditions of 37 °C, 5% CO_2 and 95% humidity. Cells were cultured for 24 h in αMEM containing 25 mM D-glucose (high glucose – HG) or 25 mM L-glucose (Control-NG). Cells were treated with 5 ng/ml TGFβ1 1 h prior to HG treatment. When required, 10 mM N-acetylcysteine (NAC) or 10 nM InSolution NFκB activation inhibitor (NFI, Millipore, Japan) were added 2 h prior to TGFβ1 treatment.

2.2. Transfection of NFkappaB construct

NFκB reporter construct (Qiagen, Japan) was transfected to semi-confluent cells using lipofectamine (Invitrogen, Japan), one day prior to TGFβ1 treatment in HG conditions. Twenty-four hours later, miRNA enriched total RNA was isolated using miRNeasy kit (Qiagen, Japan).

2.3. Real-time RT-PCR

MiRNA enriched total RNA was isolated using miRNeasy kit (Qiagen, Japan). One μg miRNA enriched total RNA was used to synthesize first strand cDNA by using miScript II RT kit (Qiagen). The first strand cDNA was further used in conjunction with miScript Primer assays and SYBR Green PCR kit (Qiagen) for analyses of primary, precursor and mature forms of miR-21 by Real-time RT-PCR, using ABI Prism 7500 HT sequence detection system (Applied Biosystems, CA, USA).

2.4. Immunoblot assay

Whole cell lysates from cells were obtained by lysing cells in M-PER reagent (Pierce, USA) containing protease inhibitors. Ten microgram proteins were resolved over 10% SDS–PAGE gels, electroblottted onto nitrocellulose membrane and incubated with primary antibodies specific for the proteins to be assessed. Antibodies anti SMAD2/3, anti SMAD4, NFκB p65 and NFκB–IκBα were obtained from Cell Signaling Technology Inc., USA). β-Actin and PCNA (Cell Signaling Technology Inc.) were used as house-keeping proteins for cell lysates and nuclear extracts, respectively.

2.5. Immunofluorescence staining

Cells cultured on coverslips were subjected to TGFβ1 treatment in HG conditions, with or without NFI, and fixed for immunofluorescence using Image-iT® Fix-Perm kit (Molecular Probes, USA). Following fixing and blocking, the cells were incubated with primary antibodies rabbit anti-Smad2/3, anti-Smad4, or anti-p65 at 4 °C overnight. Cells were washed in PBS and incubated with goat anti-rabbit AlexaFluor 488 secondary antibody. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma, USA). Images were obtained with a confocal microscope (Nikon Eclipse TiE, Japan).

2.6. Reactive oxygen species (ROS) generation assay

Following 24 h treatment of cells with TGFβ1 in HG conditions, 10 μM dichlorofluorescein diacetate (DCF-DA) was added to the cells. Nonfluorescent DCF-DA is converted to fluorescent DCF in proportion to the amount of ROS generated in the cells. The fluorescent signal was measured at excitation 485 nm and emission 530 nm, using FP-6200 spectrofluorometer (Jasco, Japan).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from TGFβ1 treated cells were obtained using NE-PER extract (Thermoscientific, USA). NFκB oligonucleotides (Forward: AGTTGAGGGGACTTTCCCAGGC; Reverse: GCCTGGGAAAGTCCCC) were annealed, and biotin labelled using Biotin 3′ end DNA labelling kit (Thermoscientific). Nuclear extracts were incubated with the labelled probes in the presence of binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), glycerol and NP-40 for 20 min at room temperature. The reaction mixtures were electrophoresed on 6% native acrylamide gel and transblotted to Hybond N filters (Amersham Pharmacia Biotech, England). The nuclear protein/oligonucleotide complex bands were detected by an immunoassay following the protocol of LightShift Chemiluminescent EMSA kit (Thermo Scientific). The signal obtained were visualized with a Luminescent Image Analyser LAS-3000 (Fuji, Japan).

2.8. Chromatin immunoprecipitation (CHIP) assay

Cells were cross-linked for 10 min with 1% formaldehyde, and chromatin was isolated using Pierce™ Chromatin Prep module (Thermo Scientific). The chromatin was subjected to immunoprecipitation with anti-NFκB p65 antibody or IgG as a negative control, using Pierce™ agarose ChIP kit (Thermo Scientific). After stringent washing, DNA was eluted and subjected to PCR using pri-miR-21 primers encompassing NFκB binding sites (set A: Forward 5′-GGAGTGGATGGGTTCTGCCTTA-3′ and Reverse 5′-CAAGGTGGATTGCATCGAGG-3′; set B: Forward 5′-TGCAACAGACTGGCCTTC-3′ and Reverse 5′-CATGCAAGACTGTTATCCAATCT-3′).

2.9. RNA-immunoprecipitation (RNA-IP) assay

RNA-IP assay was performed using Ribocluster profiler™ RIP-assay kit for microRNA (MBL, Japan). In brief, cell lysates were obtained from TGFβ1 treated cells and immunoprecipitated with anti Smad 2/3 or anti Smad 4, followed by stringent washing and elution. RNA obtained was used as a template for cDNA synthesis, followed by RT-PCR using primary miR-21 primers (Forward: ATATGCCTTCCTTTCCCACC; Reverse: GAAAACGGACACAATTTCGG). Cell lysates immunoprecipitated with anti IgG were used as control.

2.10. Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was done with two-tailed Student's *t*-test. Differences between groups were considered to be significant at *P* values < 0.05.

3. Results

Fibroblast cells were treated with TGFβ1 in normal or high glucose conditions, and analysed for expression of primary, precursor, and mature forms of miR-21. High glucose did not elicit any change in miR-21 expression. TGFβ1 upregulated all the three forms of miR-21 in both normal and high glucose conditions (Fig. 1A). The influence of TGFβ1 on the primary and mature forms of miR-21 was significantly higher in high glucose conditions (Fig. 1A), and was curtailed in the presence of NFI (inhibitor of NFκB activation) or the antioxidant, NAC (Fig. 1B). In order to investigate the involvement of NFκB, cells were transfected with NFκB plasmid and subjected to TGFβ1 treatment. Transfection with NFκB plasmid by itself increased miR-21 expression (Fig. 1C). Addition of TGFβ1 further elevated the expression; this was subdued in the presence of NAC, implying that miR-21 upregulation could be via generation of ROS. Intracellular ROS assay showed that TGFβ1 significantly increased intracellular ROS in the fibroblasts (Fig. 1D).

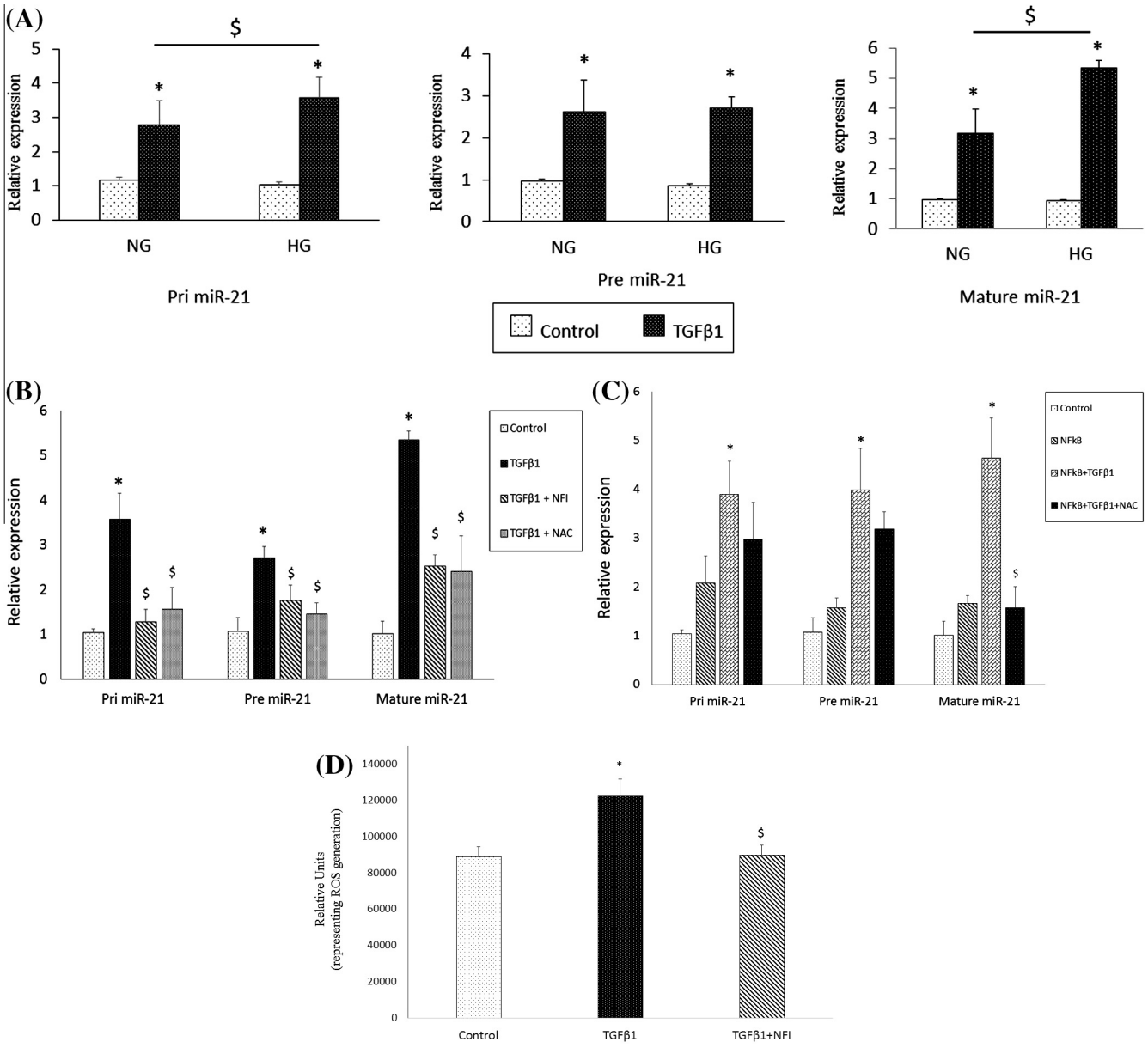

Fig. 1. TGFβ1 increases miR-21 expression in fibroblasts via NFκB activation and ROS generation. (A) Fibroblasts were treated with 5 ng/ml TGFβ1 in normal glucose (NG; 25 mM L-glucose) or high glucose (HG; 25 mM D-glucose) conditions for 24 h. Expression levels of primary (Pri miR-21), precursor (Pre miR-21) and mature (Mature miR-21) forms of miR-21 were determined by qRT-PCR. The amount of miR-21 was obtained by normalizing to the level of SnRNA U6 in the samples. (B) Fibroblasts cultured in HG conditions were treated with TGFβ1 in the presence of 10 nM NFI (Insolution NFκB activation inhibitor) or 10 mM of antioxidant NAC (N-acetyl cysteine). Expression levels of miR-21 were determined by qRT-PCR. (C) Fibroblasts transfected with NFκB reporter construct were treated with TGFβ1 in HG conditions with or without NAC. Expression levels of miR-21 were determined by qRT-PCR. (D) Fibroblasts in HG condition were treated with TGFβ1 for 24 h and intracellular ROS was determined in a fluorescence assay. Data represent results of three independent experiments. $^{*}P < 0.05$ *t*-test versus control; $^{\$}P < 0.05$ *t*-test versus TGFβ1 group. Data are presented as mean ± S.D. of three independent experiments.

TGFβ1 induced ROS generation was annulled in the presence of NFI. Results obtained reveal that TGFβ1 stimulates the activation of NFκB, which in turn induces ROS generation and miR-21 upregulation.

TGFβ1 elicits its action through SMAD proteins. TGF signalling through Smad proteins involves the phosphorylation of R-Smads (Smad 2 and Smad 3), complex of R-Smads with Co-Smad (Smad 4), nuclear translocation of the complex and regulation of target genes [21]. We studied the expression and localization of Smad 2/3 and 4. Western blot analysis confirmed the nuclear translocation of these proteins (Fig. 2A). In addition, TGFβ1 was also effective in increasing the Smad 4 expression in the cytoplasm. The increase in cytoplasmic Smad 4 and its nuclear translocation was curtailed in the presence of NFI. Immunofluorescence studies using specific antibodies for Smad 2/3 and 4 confirmed nuclear translocation of Smad proteins (Fig. 2B). The presence of Smad binding element like sequence (R-SBE) has been reported in the stem region of primary miR-21 transcript [22]. We used RNA-IP assay to test if the Smad proteins interacted directly with miR-21 via the R-SBE. TGFβ1 induced the recruitment of Smad 2/3 and Smad 4 to the pri-miR-21, in NFκB dependent manner (Fig. 2C). Our results, thus far, showed that NFκB activation is necessary for ROS generation, translocation of Smad 4 and binding of miR-21 by Smad 2/3 and Smad 4.

The NFκB activating pathway that leads to phosphorylation of inhibitory protein IκBα and nuclear translocation of mostly p65-containing heterodimers is activated by proinflammatory stimulus [23]. To assess the role of NFκB activation, we analysed the expression and localization of p65 subunit and the inhibitor IκBα. TGFβ1 treatment resulted in reduced expression of IκBα in the cytoplasm, implying degradation of IκBα (Fig. 3A). In addition, p65 expression was also decreased in the cytoplasm with concomitant increase in the nuclear fraction. These data revealed that TGFβ1 caused degradation of the IκBα, and thus mediated the release and nuclear translocation of p65 subunit (Fig. 3A). Nuclear translocation of p65 subunit was confirmed by immunofluorescence studies (Fig. 3B). In the nucleus, NFκB binds to specific consensus sequence(s) present on the promoter regions of target genes, resulting in gene transcription. TGFβ1 treatment resulted in increased DNA binding activity of NFκB in the nuclear fraction (Fig. 4A). Two potential NFκB binding sites have been reported in the promoter region of miR-21 gene [24]. In order to investigate direct interaction between miR-21 and NFκB, CHIP assay was performed using NFκB p65 antibody and specific miR-21 primers that encompass potential NFκB binding sites. TGFβ1 treatment caused a significant increase in the binding of p65 to both the binding sites in miR-21 promoter; this was attenuated by NFI (Fig. 4B). These results showed that TGFβ1 was instrumental in activating NFκB pathway and mediating miR-21 upregulation in fibroblast cells in high glucose conditions.

4. Discussion

Chronic diabetic wounds pose a clinical challenge and are a major burden to the health care system. At the cellular level, diabetic wounds are characterized by pro-inflammatory status, absence of proliferation and migration of cells, associated with narrowing or occlusion of blood vessels within the wound edge [5]. Previously, using diabetic mouse models, we studied that a set of microRNAs were differentially expressed in diabetic wound healing [20]. Among the miRNAs studied, miR-21 showed a unique signature with increased expression in diabetic skin but decreased expression during diabetic wound healing. Increased expression of miR-21 is associated with pathological conditions [25] including cancer [26], cardiac ischemia [27], tissue fibrosis [28], and skin diseases such as psoriasis and atopic eczema [29]. On the other hand, studies have also highlighted the importance of miR-21 in promoting proliferation and apoptosis of smooth muscle cells [30], angiogenesis [31], cardioprotection following ischaemia and reperfusion injury [32], and fibroblast migration [20], to name a few. It is also upregulated during the proliferative phase of liver regeneration [33]. In this study, we studied the regulation of miR-21 by TGFβ1 in skin fibroblasts that play a vital role in the process of wound healing, through facilitating secretion of growth factors, migration of other cell types, synthesis of extracellular matrix, and production of collagen [2,34]. TGFβ1 had a profound influence on upregulation of miR-21 in fibroblasts cultured in high glucose conditions. Regulation of miR-21 by TGFβ1 required the activation of NFκB signal pathway. NFκB is a transcription factor normally sequestered in the cytoplasm by the inhibitory protein IκBα. Phosphorylation and subsequent degradation of IκBα results in the release, activation and translocation of the subunits of NFκB into the nucleus, where they bind to target sequences on DNA and function as transcription factors. TGFβ1 induced the degradation of IκBα and translocation of NFκB p65 subunit. Our results are concomitant to the findings of Grau et al., wherein TGFβ1 mediated phosphorylation of IκBα and subsequent nuclear translocation, and DNA binding activity of NFκB was demonstrated in colon cancer cells [35]. However, it has also been reported that TGFβ1 decreased NFκB activity through transcriptional activation of IκBα in B cells, salivary gland cells and breast cancer cells [36–38]. In addition, a biphasic response of an initial increase followed by a decrease in NFκB activation by TGFβ1 has also been recorded [39]. It is possible that activation of NFκB by TGFβ1 is not a universal phenomenon but rather context-specific. The cellular stress associated with high glucose conditions could be a stimulating factor for the activation of NFκB by TGFβ1 in our studies.

The promoter region of miR-21 gene possesses two potential NFκB responsive elements, and direct binding of NFκB p65 subunit to these elements have been reported in human biliary epithelial cells exposed to lipopolysaccharide [24]. In addition, TGFβ1 can directly regulate miR-21 transcription through Smad proteins [40] as well as the processing of primary miR-21 transcript into precursor miR-21 through a post-transcriptional regulation involving the Drosha complex [41]. R-Smad proteins 2/3 and Co-Smad 4 proteins were in fact translocated into the nucleus. Interestingly, the nuclear translocation of Smad 4 was NFκB dependent, in contrast to the R-Smads 2/3. While it is a general concept that the R-Smads and Co-Smads translocate into the nucleus as a complex, recent studies have demonstrated alternative nuclear import machineries that can translocate R-Smads and Co-Smads independent of each other [42,43]. It is hypothesized that the R-Smads not in complex with Smad 4 may participate in miRNA processing through the Drosha complex, while the R-Smad/Smad 4 complex may function in the transcriptional regulation of miR-21 by DNA binding activity [40]. The stem region of primary miR-21 transcript contains a conserved sequence (5′-CAGAC-3′) similar to the Smad-binding element (SBE) found in promoters of TGFβ1 regulated genes [22]. Davis et al. demonstrated a direct association between the Smad proteins and this sequence which they termed as R-SBE; this association is deemed essential for recruitment of Drosha complex to pri-miR-21 [22]. While Davis et al. reported that Smad 3 specifically bound to miR-21 and Smad 4 did not interact directly with miR-21, our findings show that both the R-Smads and co-Smad could interact with miR-21, in NFκB dependent manner. The exact role of Smad proteins in the regulation of miR-21 in high glucose conditions needs to be elucidated.

Thus far, our results support a novel role for TGFβ1 treatment of diabetic wounds. Topical application or injection of TGFβ1 has been reported to restore tensile strength of collagen [44], induce the accumulation of granulation tissue [45], and accelerate wound

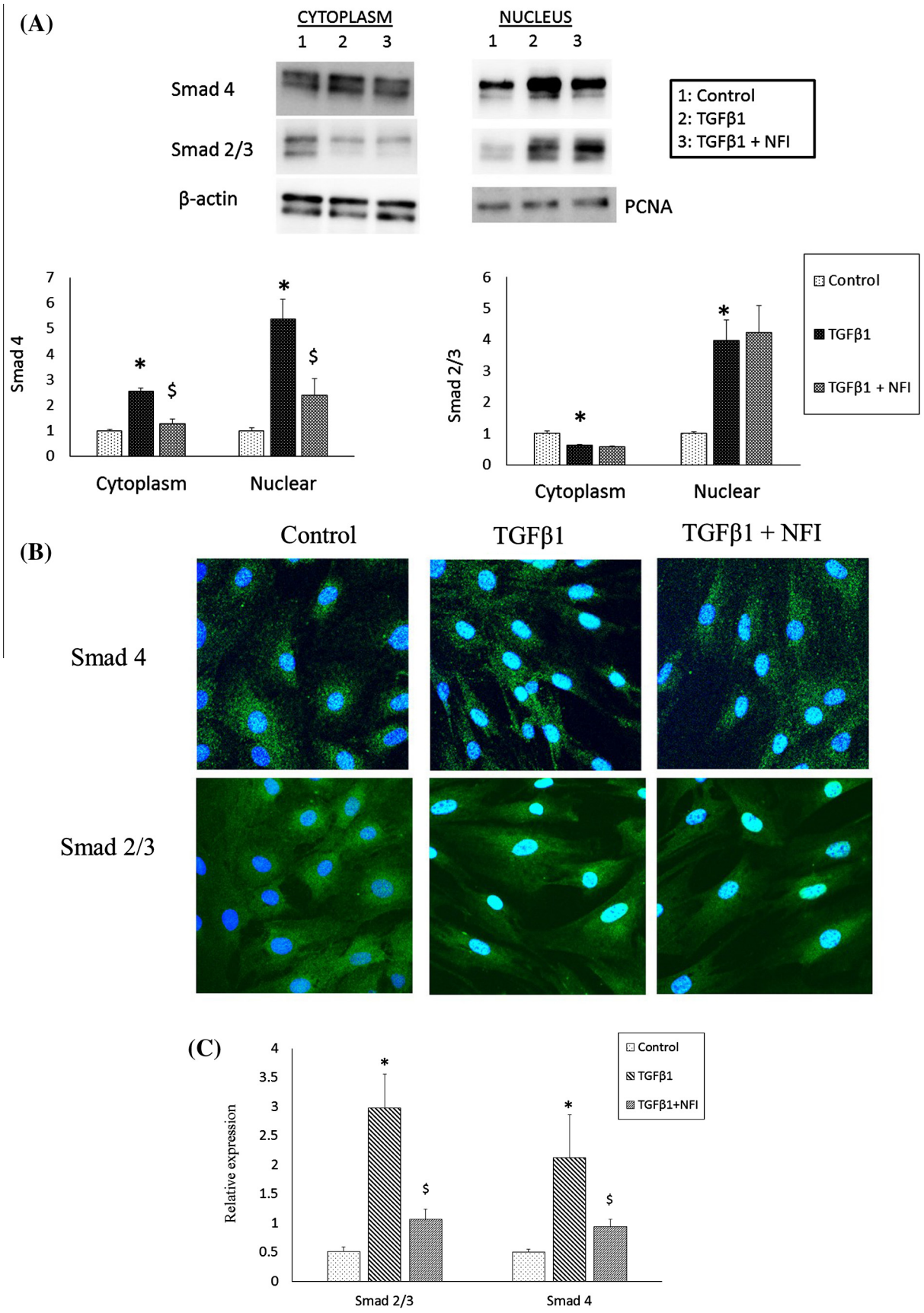

Fig. 2. TGFβ1 activates Smad proteins. (A) Immunoblot of Smad 4 and Smad 2/3 in cytoplasmic and nuclear lysates from fibroblasts treated with TGFβ1 in HG conditions with or without NFI. β-Actin and PCNA were used as respective internal controls. (B) Immunofluorescence of Smad 4 and Smad 2/3 in fibroblasts stimulated with TGFβ1. (C) RNA-IP assay using anti-Smad 2/3 or anti-Smad 4 antibodies, followed by PCR amplification of Pri-miR-21. Relative expression over non-specific control IgG antibody is shown. $^{*}P < 0.05$ t-test versus control; $^{\$}P < 0.05$ t-test versus TGFβ1 group. Representative results of three independent experiments are shown.

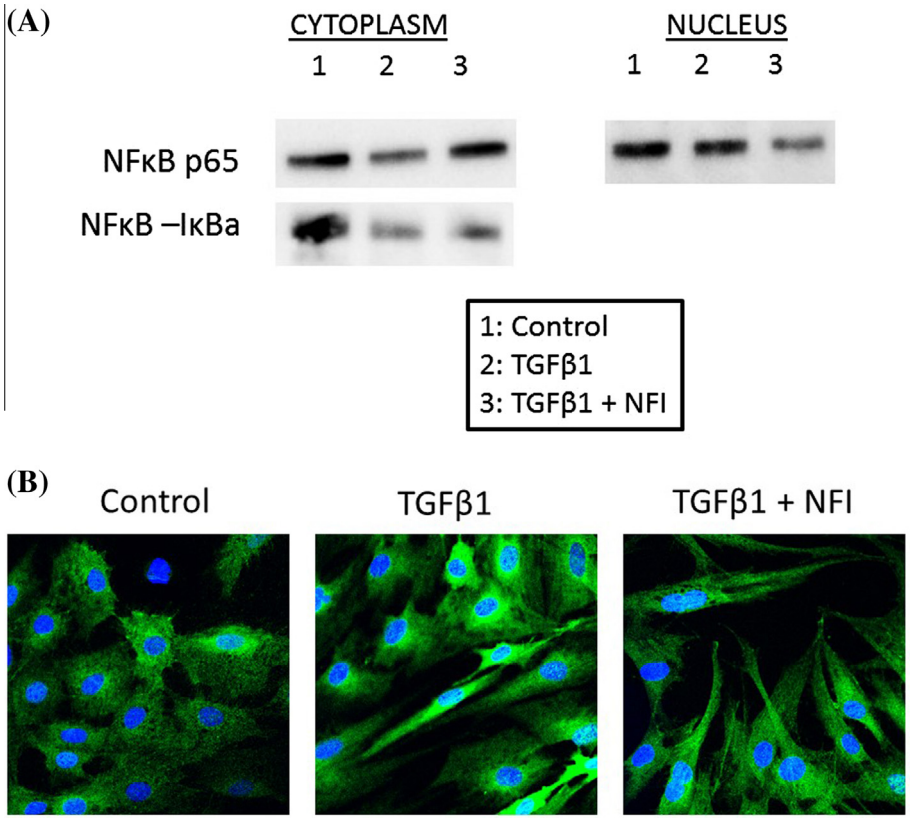

Fig. 3. TGFβ1 activates NFκB. (A) Immunoblot of NFκB p65 and IκBα subunits in cytoplasmic and nuclear lysates from fibroblasts treated with TGFβ1 in HG conditions. β-Actin and PCNA were used as respective internal controls. (B) Immunofluorescence of NFκB p65 subunit in fibroblasts stimulated with TGFβ1. Representative results of three independent experiments are shown.

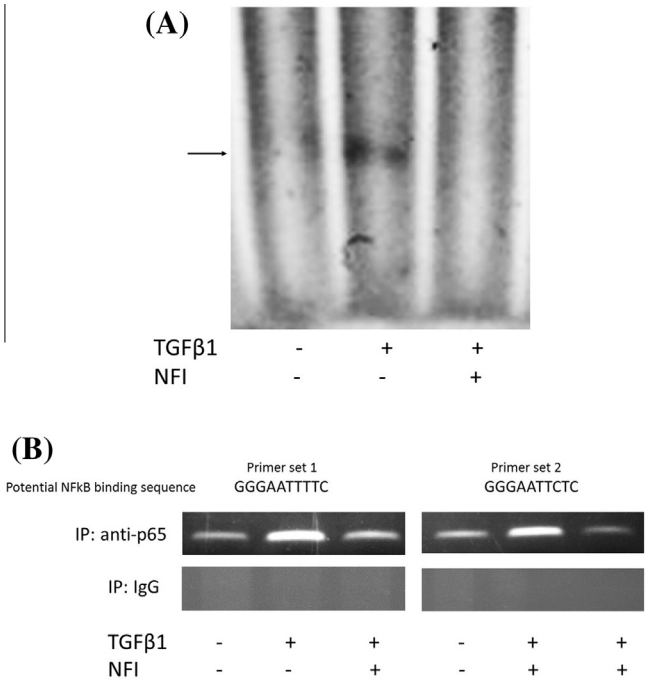

Fig. 4. TGFβ1 induces NFκB binding activity. (A) NFκB EMSA of nuclear lysates from fibroblasts stimulated with TGFβ1 with or without NFI. (B) Fibroblasts were treated with TGFβ1 and ChIP was performed with anti p65 antibody or non-specific IgG (control), followed by PCR amplification with two sets of miR-21 primers that encompass NFκB p65 binding sites. Representative results of three independent experiments are shown.

closure [8] in diabetic wounds. Our studies showed that TGFβ1 treatment can enhance healing of diabetic wounds by its effect on miR-21 that has been shown to be necessary for migration of fibroblasts [20]. We also showed that NFκB activation is necessary for ROS generation, interaction of Smad proteins with miR-21 and it's regulation by TGFβ1.

Conflict of interest

None.

Acknowledgments

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