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ERK and p38 MAPK inhibition controls NF-E2 degradation and profibrotic signaling in renal proximal tubule cells

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Abstract

Aims: Transforming growth factor-β (TGF-β) mediates fibrotic manifestations of diabetic nephropathy. We demonstrated proteasomal degradation of anti-fibrotic protein, nuclear factor-erythroid derived 2 (NF-E2), in TGF-β treated human renal proximal tubule (HK-11) cells and in diabetic mouse kidneys. The current study examined the role of mitogen-activated protein kinase (MAPK) pathways in mediating NF-E2 proteasomal degradation and stimulating profibrotic signaling in HK-11 cells.

Main methods: HK-11 cells were pretreated with vehicle or appropriate proteasome and MAPK inhibitors, MG132 (0.5 μM), SB203580 (1 μM), PD98059 (25 μM) and SP600125 (10 μM), respectively, followed by treatment with/without TGF-β (10 ng/ml, 24 h). Cell lysates and kidney homogenates from FVB and OVE26 mice treated with/without MG132 were immunoblotted with appropriate antibodies. pUse vector and pUse-NF-E2 cDNA were transfected in HK-11 cells and effects of TGF-β on JNK MAPK phosphorylation (pJNK) was examined.

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CRediT authorship contribution statement

Jia Li, Shunyin Jin and Sanjana Rane performed the research. Madhavi J. Rane designed the study. Madhavi Rane and Jia Li analyzed the experimental data. Michelle Barati, Lu Cai provided critical advice during the study. Qian Lin and Yi Tan provided the animal kidney tissues. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Key findings: We demonstrated activation of p38, ERK, and JNK MAPK pathways in TGF-β treated HK-11 cells. Dual p38 and ERK MAPK blockade prevented TGF-β-induced pSer^{82}Hsp27, fibronectin and connective tissue growth factor (CTGF) expression while preserving NF-E2 expression. Blockade of JNK MAPK inhibited TGF-β-induced CTGF expression without preserving NF-E2 expression. MG132 treatment prevented TGF-β-induced pJNK in HK-11 cells and in type 1 diabetic OVE26 mouse kidneys, demonstrating that TGF-β- and diabetes-induced pJNK occurs downstream of proteasome activation. A direct role for NF-E2 in modulating pJNK activation was demonstrated by NF-E2 over-expression.

Significance: ERK and p38 MAPK promotes NF-E2 proteasomal degradation while proteasome activation promotes pJNK and profibrotic signaling in renal proximal tubule cells.

Keywords

Transforming growth factor-β; Diabetic nephropathy; Renal fibrosis; Nuclear factor-erythroid derived 2; Type 1 diabetes

1. Introduction

Diabetic Nephropathy (DN) is a leading cause of end-stage renal disease (ESRD) and has created a great burden on people's health and economy worldwide [1,2]. DN is associated with glomerulosclerosis, tubular atrophy and interstitial fibrosis. While the research is focused on determining mechanisms underlying glomerular pathology associated with DN, the tubular consequences of DN are underappreciated. Given that renal fibrosis is a biological process involving various cells in the kidney, efforts must be made to examine roles of various cell types, profibrotic factors and multiple signaling pathways contributing to renal fibrosis. One such profibrotic factor, transforming growth factor-β (TGF-β) is a critical mediator of the hypertrophic and fibrotic manifestations of DN [3]. Efforts to block profibrotic responses of TGF-β were not successful as TGF-β also possesses anti-inflammatory properties. Moreover, targeting another profibrotic protein connective tissue growth factor (CTGF) was initially successful as the anti-CTGF antibody phase I clinical trial had promising results [4]. However, the Phase II clinical trial was terminated (ClinicalTrials.gov identifier NCT00913393). Given that fibrosis is the final common pathway in all kidney diseases, if left untreated, can progress to chronic kidney disease and finally to ESRD. Therefore, finding therapeutic targets between activation of TGF-β and CTGF signaling may lead to identification of therapies to prevent/slow progression of fibrosis to ESRD. We recently identified nuclear factor-erythroid derived 2 (NF-E2) as an anti-fibrotic protein in renal proximal tubule cells [9]. Thus, making it a potential therapeutic target to combat kidney diseases.

NF-E2 is a transcription factor composed of two basic leucine zipper (bZIP) proteins (45 kDa and 18 kDa respectively). NF-E2 is mainly expressed in hematopoietic cells and regulates hematological diseases [5]. However, a growing number of evidence demonstrates that NF-E2 is also expressed in and plays a role in non-hematopoietic cells [5–7]. Tang et al. showed NF-E2 mRNA was identified in renal tubules and glomerulus of diabetic patients [8]. Recently, we have identified NF-E2 protein as an anti-fibrotic protein whose expression is significantly decreased in TGF-β treated human proximal renal tubule cells

(HK-11) and in type 1 and type 2 diabetic (T1D or T2D) mouse kidneys with a concurrent increase in expression of profibrotic proteins [9]. Heat shock protein 27 (Hsp27) is a member of small heat shock proteins' family which are molecular chaperones [10]. Hsp27 has been shown to modulate protein quality control in cells exposed to stress. Hsp27 is also identified as an actin-binding protein and is known as an anti-apoptotic protein [11–14]. Hsp27 phosphorylation is associated with increased expression of CTGF in TGF-β treated human keloid fibroblasts [15]. We previously demonstrated a novel association between NF-E2 and phosphorylated Ser^{82}Hsp27 ($pSer^{82}$Hsp27) in HK-11 cells, which was enhanced further in the presence of TGF-β, concurrent with NF-E2 degradation at the proteasome, suggesting a role for $pSer^{82}$Hsp27 in targeting NF-E2 for degradation and thus modulating profibrotic responses [9]. However, signaling pathways contributing to TGF-β-induced NF-E2 proteasomal degradation and induction of profibrotic pathways were not examined. Identification of mechanisms that regulate NF-E2 degradation may lead to the generation of therapies to slow progression of fibrotic kidney disease.

Non-canonical TGF-β pathways include activation of mitogen-activated protein kinases (MAPKs). MAPKs are a group of protein kinases containing three subfamilies which are c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 [16]. Both p38 and ERK MAPK pathways have been shown to promote Hsp27 phosphorylation [14,17]. MAPKs regulate many cellular functions including proteasomal degradation [18–20]. ERK, p38 and JNK MAPK pathways are involved in kidney injury and fibrosis [21–23]. However, whether MAPK pathways mediate NF-E2 degradation in DN is still unknown. Therefore, in the current study, we examined the role of MAPK pathways in modulating Hsp27 phosphorylation, NF-E2, CTGF and fibronectin (FN) expression in TGF-β treated HK-11 cells. We also examined the role of MAPKs in mediating proteasomal degradation of NF-E2 and stimulating profibrotic signaling in HK-11 cells. These key results were also confirmed in T1D kidney tissues.

2. Materials and methods

2.1. Mouse model

3-month-old female transgenic OVE26 mice on the FVB background were used as the T1D mouse model, which has been characterized in our previous studies [24,25], age- and sex-matched FVB mice as controls. The mice were divided into four groups: diabetic group (OVE26), MG132 treated OVE26 group (OVE26/MG132), control group (FVB) and MG132 treated FVB group (FVB/MG132), minimum $n = 4$ for each group. In MG132 treated groups, four 3-month-old OVE26 or FVB mice were injected intraperitoneally with MG132 (0.0025 μg/ml dissolved in dimethyl sulfoxide (DMSO) solution, then 10 μg/kg diluted with saline, Cat # M8699, Sigma-Aldrich, St. Louis, MO, USA) daily for 3 months as described previously [26]. In sham groups, four 3-month-old OVE26 mice or FVB mice were given same volume of physiological saline with DMSO (Cat # D8418, Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 0.0025 μg/ml for 3 months. All the mice were euthanized at 6-month of age and renal cortex lysates were generated for western blot. All animal procedures were approved by the University of Louisville Animal Care and Use Committee, which conformed to NIH guidelines and were certified by the American

Association for Accreditation of Laboratory Animal Care. All mice housed in the University of Louisville Research Resources Center were housed with a constant temperature at 22 °C, a 12-hour light/dark cycle, and free access to standard chow and tap water.

2.2. HK-11 cell culture

Human renal proximal tubule epithelial cells (HK-11) immortalized by transduction with adenovirus 12-SV40 were obtained from Dr. Racusen [27]. HK-11 cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) (1:1) (Cat # 11966025, Cat # 11765047, Thermo Fisher Scientific, Waltham, MA, USA) containing 5% fetal bovine serum (Cat # 10082-147, Thermo Fisher Scientific, Waltham, MA, USA) and maintained in a cell culture incubator at 37 °C in a humidified atmosphere with 5% CO_2 as previously described [14]. HK-11 cells were plated on 6-well plates and were pretreated with/without JNK inhibitor SP600125 (10 μM) (Cat # S5567, Sigma-Aldrich, St. Louis, MO, USA), p38 inhibitor SB203580 (1 μM) (Cat # 559389, Calbiochem, Burlington, MA, USA), ERK inhibitor PD98059 (25 μM) (Cat # 513000, Calbiochem, Burlington, MA, USA) and proteasome inhibitor MG132 (0.5 μM) (Cat # M8699, Sigma-Aldrich, St. Louis, MO, USA), for an hour prior to treatment with TGF-β (10 ng/ml) (Cat # cyt-716, Prospec Protein Specialists, East Brunswick, NJ, USA) for 24 h. Cell lysates were immunoblotted with appropriate antibodies. The HK-11 cell supernatants were also collected, and equal volume of cell supernatants were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blotting with anti-FN antibody (1:1000, Cat # F3648, Sigma-Aldrich, St. Louis, MO, USA).

2.3. Transfection of HK-11 cells

HK-11 cells were transfected with the indicated plasmid cDNAs using lipofectamine™ 2000 (Cat # 11668019, Thermo Fisher Scientific, Waltham, MA, USA) as previously described [9].

2.4. Western blot

Mouse renal cortex and HK-11 cells were lysed by radio immunoprecipitation assay (RIPA) lysis buffer (Cat # sc-364162A, Santa Cruz Biotechnology, sc-364162A, Dallas, TX, USA) with protease inhibitors, supplement with 200 mM phenylmethylsulfonyl fluoride (PMSF) (Cat # 78830-1G, Sigma-Aldrich, St. Louis, MO, USA) on ice. The lysates were collected after centrifugation at 12,000 rpm at 4 °C for 20 min. The protein concentration was measured using a Bradford protein–binding assay (Cat # 5000006, BIO RAD, Hercules, CA, USA) and the lysates were heated with a 4× laemmli sample buffer (Cat # 1610747, BIO RAD, Hercules, CA, USA) at 95 °C for 5 min. Total protein was separated by SDS–PAGE on gradient gels and transferred to a 0.2 μM nitrocellulose membrane (Cat # 1620112, BIO RAD, Hercules, CA, USA). The membranes were blocked with 5% milk/0.5% bovine serum albumin (Cat # A7906-50G, Sigma-Aldrich, St. Louis, MO, USA) in Tris buffered saline with Tween-20 (Cat # P7949, Sigma-Aldrich, St. Louis, MO, USA) (TBST) for 1 h at room temperature, then incubated with primary antibody at 4 °C overnight. Next day, the membranes were washed three times with TBST for 10 min and incubated with secondary antibody for 1 h at room temperature, then washed three times with TBST for 10 min to be ready for exposure. All films were optically scanned and quantified by measuring

the density of each band using ImageJ software [28] (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/). Each band's density was normalized by its respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control density. The primary antibodies used in the current study included anti-NF-E2 (1:500, Cat # 11089-1-AP, Proteintech, Chicago, IL, USA), anti-FN (1:1000, Cat # F3648, Sigma-Aldrich, St. Louis, MO, USA), anti-CTGF (1:1000, Cat # sc14939, Santa Cruz Biotechnology, Dallas, TX, USA), anti-GAPDH (1:3000, Cat # ab37168, Abcam, Cambridge, MA, USA) and anti-pJNK (1:1000, Cat # 9251), anti-pERK (1:1000, Cat # 9106), anti-pP38 (1:1000, Cat # 9211), anti-pSer^{82}Hsp27 (1:1000, Cat # 2401) were all obtained from Cell Signaling Technology, Danvers, MA, USA.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

HK-11 cells were transfected with pUse vector or pUse-NF-E2 cDNA for 24 h followed by treatment with 10 ng/ml TGF-β for additional 24 h and then total cell RNA was extracted by TRIzol reagent (Cat # cs502, Amsbio, Cambridge, MA, USA) according to the manufacturer's instructions. Then the concentration and purification of RNA was measured by Nano-Drop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA). Reverse transcription and qPCR were performed as previously described [29]. The primers utilized in the study include CTGF (Hs00170014_m1), FN (Hs01549976_m1), plasminogen activator inhibitor-1 (PAI-1) (Hs00167155_m1), and GAPDH (Hs02786624_g1) which were all purchased from Thermo Fisher Scientific, Waltham, MA, USA.

2.6. Statistical analysis

At least three independent experiments were conducted for *in vitro* studies and four mice were included in each group, to analyze the significance of the data. All statistical analyses were performed using GraphPad Prism version 8.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The results were shown as means ± standard deviations. Comparison between 2 groups were analyzed by two tailed, unpaired *t*-tests. Significance in more than two groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test between two interested groups. P values ≤0.05 were considered statistically significant.

3. Results

3.1. TGF-β stimulates p38 and ERK MAPK activation in HK-11 cells

In our previous study, we demonstrated TGF-β induced proteasomal degradation of NF-E2 with concurrent induction of profibrotic signaling in HK-11 cells. In addition, we identified a novel interaction between NF-E2 and pSer^{82}Hsp27 in HK-11 cells, which was enhanced further in the presence of TGF-β, possibly identifying NF-E2 as a Hsp27 cargo protein targeting NF-E2 at the proteasome [9]. To further explore the signaling pathways contributing to these effects, here we first examined activation (phosphorylation) of MAPKs namely, p38 and ERK MAPK in HK-11 cells. TGF-β-induced pP38 (Fig. 1A and B) and pERK (Fig. 1C and D) in HK-11 cells.

3.2. Simultaneous blockade of ERK and p38 MAPK inhibited pSer^{82}Hsp27 and preserved NF-E2 expression in TGF-β treated HK-11 cells

As p38 MAPK has been implicated in profibrotic signaling in the kidney [30] and TGF-β-induced pP38 in HK-11 cells (Fig. 1A and B), we next examined its contribution to NF-E2 degradation. Blockade of p38 MAPK pathway only partially preserved NF-E2 expression in the presence of TGF-β, without any statistical significance (Fig. 2A and B). p38 MAPK blockade on the other hand significantly enhanced TGF-β-induced CTGF expression (Fig. 2C and D). As SB203580 has been shown to non-specifically induce pERK [31], we examined effects of SB203580 on TGF-β-induced pERK in HK-11 cells. TGF-β alone and SB203580 alone significantly induced pERK which was further exacerbated by co-treatment of SB203580 and TGF-β (Fig. 2E and F). Based on these findings and given that TGF-β-induced ERK MAPK contributes to profibrotic signaling in renal tubule cells [32], we further examined the effects of simultaneous blockade of ERK and p38 MAPK pathways on TGF-β mediated decrease in NF-E2 expression and an increase in pSer^{82}Hsp27. Simultaneous blockade of both ERK and p38 MAPK in HK-11 cells significantly preserved NF-E2 expression (Fig. 2G and H) and inhibited pSer^{82}Hsp27 (Fig. 2I and J) in the presence of TGF-β.

3.3. Simultaneous blockade of ERK and p38 MAPK prevented CTGF and FN expression in TGF-β treated HK-11 cells

We next examined effects of simultaneous blockade of ERK and p38 MAPK, which preserved NF-E2 expression, on modulating expression of profibrotic proteins CTGF and FN in TGF-β treated HK-11 cells. Immunoblotting the aforementioned HK-11 cell lysates demonstrated that blockade of ERK and p38 MAPK inhibited TGF-β-induced CTGF (Fig. 3A and B) and FN expression, as well as FN release in HK-11 cell supernatant (Fig. 3C and D).

3.4. Pharmacological blockade of JNK MAPK inhibits TGF-β-induced CTGF expression but fails to preserve NF-E2 expression in HK-11 cells

We have previously demonstrated TGF-β induced NF-E2 degradation at the proteasome [9] while Lee et al. demonstrated that JNK MAPK activation promoted NF-E2 degradation in murine erythroleukemia cells [33]. Therefore, we examined the role of JNK MAPK phosphorylation (pJNK) in modulating NF-E2 and CTGF expression in TGF-β treated HK-11 cells. Incubation of HK-11 cells with JNK inhibitor (SP600125) completely blocked TGF-β stimulated pJNK (Fig. 4A and B). Blockade of JNK MAPK also completely inhibited TGF-β-induced CTGF expression (Fig. 4C and D). It should be noticed that inhibition of pJNK alone significantly reduced NF-E2 expression and treatment of cells with JNK inhibitor plus TGF-β synergistically decreased NF-E2 expression (Fig. 4E and F).

3.5. Blockade of proteasome inhibited TGF-β-induced JNK phosphorylation in renal tubule cells and in OVE26 diabetic mice

To examine the role of proteasome activation on TGF-β- and diabetes-induced JNK activation, HK-11 cells, T1D OVE26 mice and their control FVB mice were treated with proteasome inhibitor MG132, as previously described [9,26]. MG132 (0.5 μM) alone

significantly inhibited pJNK in HK-11 cells and pretreatment with MG132, significantly inhibited TGF-β-induced pJNK in TGF-β treated HK-11 cells (Fig. 5A and B). Similarly, diabetes-induced pJNK was also prevented by MG132 in OVE26 mouse kidneys (Fig. 5C and D).

3.6. Over-expression of NF-E2 prevented TGF-β-induced JNK MAPK activation in HK-11 cells

We have previously demonstrated that over-expression of NF-E2 inhibited CTGF expression [9]. Moreover, JNK activation induced CTGF expression. Therefore, we next examined the direct effects of NF-E2 over-expression on TGF-β-induced pJNK, as a measure of JNK activation in HK-11 cells. HK-11 cells were transfected with pUSE vector alone or pUSE-NF-E2 cDNA constructs following which the cells were stimulated with/without TGF-β strongly increasing NF-E2 expression under both conditions (Fig. 6A and B), as shown in our recent study [9]. As seen in Fig. 6A and B, TGF-β induced pJNK in vector transfected cells. However, over-expression of NF-E2 significantly inhibited TGF-β-induced pJNK.

3.7. Over-expression of NF-E2 inhibited TGF-β-induced CTGF, FN, and PAI-1 mRNA expression in HK-11 cells

NF-E2 over-expression was shown to significantly inhibit TGF-β-induced CTGF, FN, and PAI-1 protein expression [9]. In the current study, we examined role of NF-E2 over-expression on TGF-β-induced CTGF, FN and PAI-1 mRNA expression. As shown in Fig. 7A–C, TGF-β induced CTGF, FN, and PAI-1 mRNA expression in pUse vector transfected cells, which was significantly inhibited in the presence of NF-E2 over-expression.

4. Discussion

We recently demonstrated a critical role for NF-E2 in modulating TGF-β-induced renal fibrosis in HK-11 cells. We demonstrated degradation of NF-E2 protein expression in TGF-β treated HK-11 cells and in the kidney homogenates of both T1D and T2D mice [9]. However, the signaling pathways underlying NF-E2 degradation at the proteasome were not identified. Here, we identified a role for ERK and p38 MAPK in inducing expression of profibrotic genes including CTGF and FN while downregulating expression of anti-fibrotic protein NF-E2. While JNK MAPK also contributes to TGF-β-induced CTGF expression, blockade of JNK MAPK does not preserve NF-E2 expression.

ERK, p38 and JNK MAPK pathways modulate diabetic kidney disease [21–23]. Moreover, TGF-β is a mediator of diabetes, and a role for TGF-β-induced ERK1/2 and p38 MAPK activation in renal interstitial fibrosis in the rat proximal tubule cells is documented [34]. Therefore, we examined the role of MAPKs in modulating NF-E2 expression. We demonstrated a critical role for both ERK and p38 MAPK in promoting TGF-β-induced NF-E2 degradation at the proteasome, as illustrated in the proposed mechanisms (Fig. 8). While MAPKs regulate many cellular functions including proteasomal degradation [18–20], this is a first report demonstrating their role in modulating NF-E2 expression. Furthermore, we demonstrated a role for pERK and pP38 MAPK in mediating TGF-β-induced Hsp27 phosphorylation, CTGF and FN expression in HK-11 cells. Our findings

are in agreement with previous studies, showing that both p38 and ERK MAPK pathways promote Hsp27 phosphorylation [14,17] and CTGF expression [35]. In our recent study, we demonstrated enhanced association of pSer^{82}Hsp27 and NF-E2 concurrent with enhanced NF-E2 degradation in HK-11 cells [9]. Loss of NF-E2 coincided with increased expression of profibrotic proteins CTGF, FN and PAI-1. A direct role for NF-E2 in inhibiting the aforementioned profibrotic proteins expression was documented by NF-E2 over-expression studies in TGF-β treated HK-11 cells [9]. Moreover, in the current study we demonstrated over-expression of NF-E2 significantly decreased TGF-β-induced FN, CTGF, and PAI-1 mRNA expression in HK-11 cells. TRANSFAC analysis identified NF-E2 binding site on the promoter region of CTGF [36] suggesting a role for NF-E2 in modulating CTGF expression.

JNK MAPK signaling was shown to mediate cell death in tubulointerstitial injury of kidney [21] and was reported to play a role in proteasomal degradation of p45/NF-E2 in murine erythroleukemia cells by phosphorylating NF-E2 on Ser157 [33]. Moreover, JNK MAPK pathway modulates CTGF expression [21]. Similar to previous reports, we also demonstrated dependence on JNK activation for induction of CTGF expression. However, blockade of JNK MAPK further exacerbated NF-E2 degradation and failed to preserve NF-E2 expression in TGF-β treated HK-11 cells suggesting that JNK activation occurred downstream of proteasome activation, as shown in Fig. 8. JNK inhibition may lead to activation of negative feedback loop to exacerbate proteasome activation to possibly restore JNK phosphorylation. JNK activation upstream of proteasome activation [20] or induction of JNK activation as a consequence of inhibiting the proteasome have been documented [37]. Our study suggests JNK activation occurs downstream of proteasome activation, as blockade of the proteasome in diabetic OVE26 mice and in TGF-β treated HK-11 cells prevented JNK activation. Interestingly, blockade of the proteasome with MG132 in diabetic OVE26 mice, preserved NF-E2 expression [9], decreased CTGF in mouse kidneys and prevented renal functional decline and renal pathological changes associated with type 1 diabetes [26]. Other studies also have documented a protective role for MG132 in diabetic nephropathy by preserving SnoN protein expression [38]. Our studies demonstrate JNK activation occurs downstream of the proteasome and that blockade of JNK activation by inhibiting the proteasome preserved NF-E2 expression (Fig. 8) and prevented renal damage in diabetic mice [9,26]. Moreover, a direct role for NF-E2 in modulating JNK activation was demonstrated in control and TGF-β treated HK-11 cells such that NF-E2 overexpression inhibited TGF-β-induced pJNK. NF-E2 over-expression may lead to activation of dual-specificity phosphatases, known to dephosphorylate and inactivate JNK [39] or may inhibit expression of upstream activators of JNK MAPK pathway [40,41]. NF-E2 is known to heterodimerize with small musculoaponeurotic fibrosarcoma (Maf) proteins such as BZIP transcription factors K, F, G (MafK, MafF, MafG) [42]. While, ubiquitously expressed small Maf proteins are known to homodimerize, or heterodimerize with other transcription factors such as c-Jun/Fos and inhibit NF-E2 mediated gene transcription [43]. Moreover, homodimerized large Maf protein, namely, c-Maf, upon phosphorylation, can associate with its co-activator and induce transcription of collagen 2a1, collagen 27a1, CTGF and matrix metalloproteinase 13 genes [44,45]. In the absence of NF-E2, in TGF-β treated HK-11 cells, large Maf proteins could homodimerize and associate with their co-activators in a

JNK-dependent manner and induce transcription of CTGF. Alternatively, in the absence of NF-E2, small Maf proteins could heterodimerize with other transcription factors to induce transcription of profibrotic genes. Collectively, our data demonstrate that TGF-β-induced p38 and ERK MAPK activation promotes pSer^{82}Hsp27/NF-E2 association and NF-E2 degradation at the proteasome. Activation of the proteasome promotes pJNK leading to induction of CTGF expression (Fig. 8).

In summary, over-expression of NF-E2 in HK-11 cells prevented TGF-β-induced expression of CTGF, FN, PAI-1 [9] and pJNK, a kinase known to induce expression of profibrotic CTGF protein. Our studies have identified NF-E2 as a critical mediator of anti-fibrotic signaling in TGF-β treated HK-11 cells and in diabetic kidneys. Anti-fibrotic roles of NF-E2 could involve modulation of pro or anti-fibrotic proteins and/or miRNAs expression. Future studies will examine direct effects of NF-E2 protein transduction on renal fibrosis in diabetic mouse kidneys.

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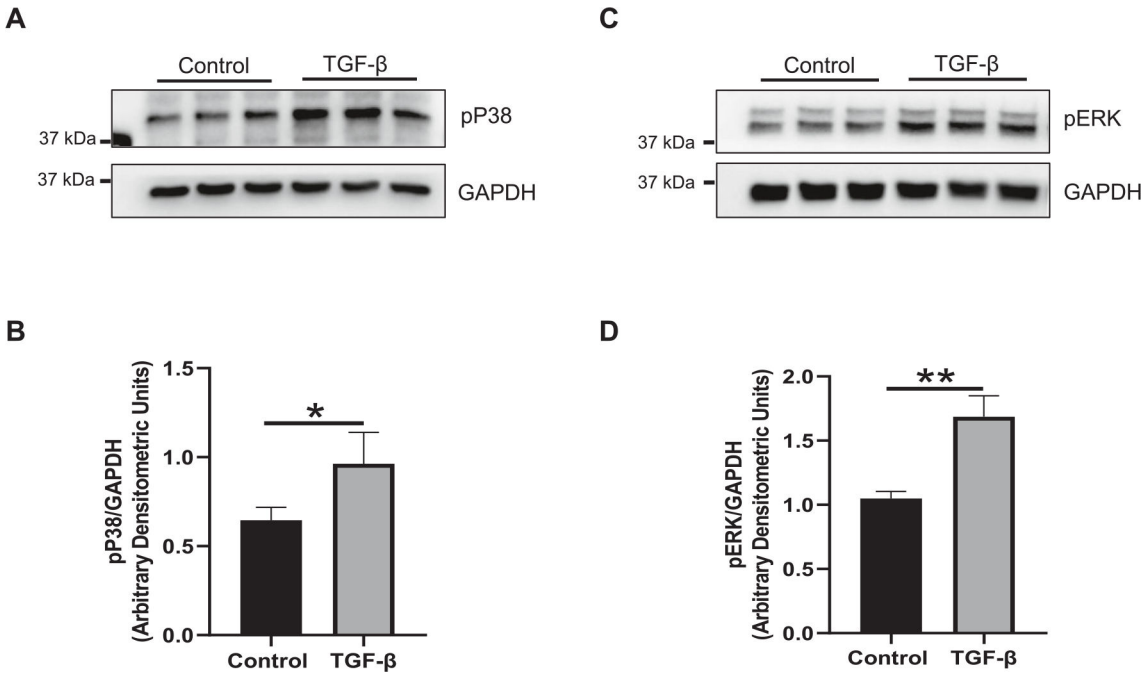

Fig. 1.

TGF-β induced p38 and ERK MAPK activation in HK-11 cells.

HK-11 cells were treated with TGF-β (10 ng/ml) for 24 h and cell lysates were generated. The lysates were immunoblotted with anti-pP38 (A-B), anti-pERK (C-D), and anti-GAPDH antisera (A, C), and the expressions were quantitatively analyzed, respectively (B, D). Data are presented as the mean ± standard deviation (SD), $n = 4$ in each group. Significance was determined by 2 tailed *t*-Test, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control.

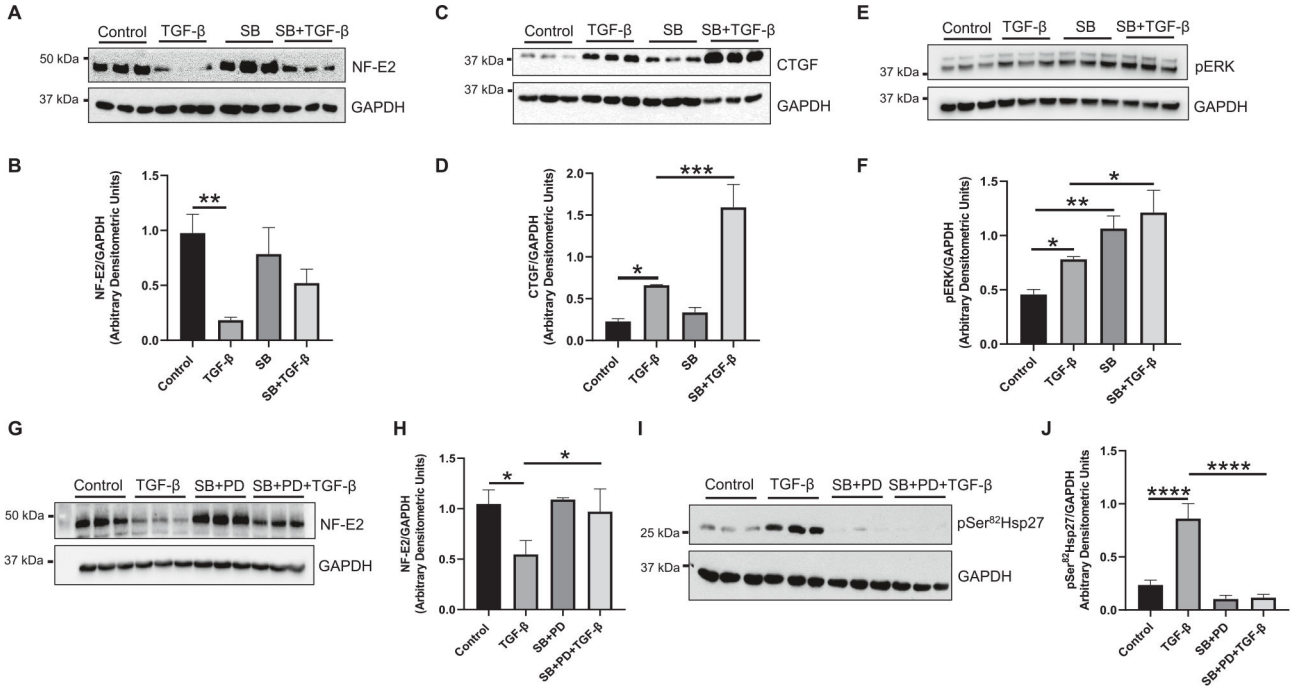

Fig. 2.

Simultaneous blockade of p38 and ERK MAPK preserved NF-E2 expression and inhibited pSer^{82}Hsp27 in HK-11 cells.

A-F: HK-11 cells were first pretreated with vehicle or 1 μM SB203580 (p38 inhibitor) followed by treatment with or without 10 ng/ml TGF-β for 24 h. Cell lysates were immunoblotted with anti-NF-E2, anti-CTGF, anti-pERK and anti-GAPDH antisera. Ratios were obtained by normalizing NF-E2, CTGF, and pERK immunoreactive bands to their respective GAPDH controls. G-J: HK-11 cells were simultaneously pretreated with vehicle or 1 μM SB203580 and 25 μM PD98059 (MEK/ERK MAPK inhibitor) for 1 h prior to treatment with 10 ng/ml TGF-β for 24 h. Cell lysates were immunoblotted with anti-NF-E2 and with anti-pSer^{82}Hsp27 antisera. Differences in multiple groups were performed by one-way ANOVA followed by the Tukey test for individual group comparisons. Data is presented as the mean ± SD, $n = 3$ in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

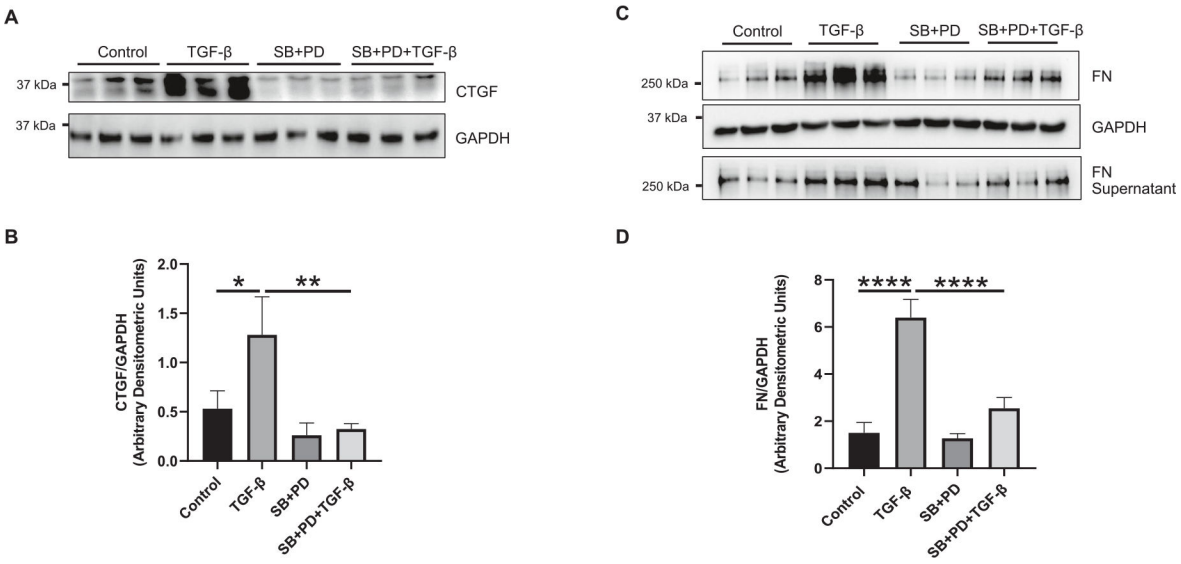

Fig. 3.

Simultaneous blockade of p38 and ERK MAPK pathways inhibited profibrotic signaling in HK-11 cells.

A, C: HK-11 cells were simultaneously pretreated with vehicle or 1 μM SB203580 (p38 inhibitor) and 25 μM PD98059 (ERK inhibitor) for 1 h prior to treatment with 10 ng/ml TGF-β for 24 h and cell lysates were collected. The lysates were immunoblotted with anti-CTGF (A), anti-FN (C), and anti-GAPDH antisera (A, C), followed by quantitative analysis for their expressions, respectively (B, D). HK-11 cell supernatants were immunoblotted with anti-FN (C, D). Data is presented as the mean ± SD, $n \geq 3$ in each group. *P < 0.05, **$P <$ 0.01, ****P < 0.0001.

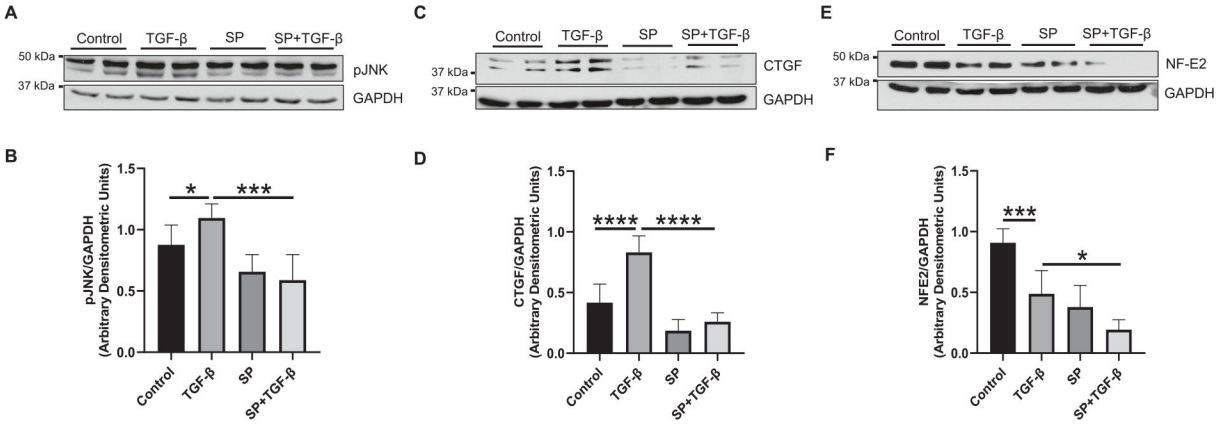

Fig. 4.

Effect of JNK inhibition on NF-E2 & CTGF expression and on JNK phosphorylation in TGF-β treated HK-11 cells.

HK-11 cells were simultaneously pretreated with vehicle or 10 μM SP600125 (JNK inhibitor) for 1 h prior to treatment with 10 ng/ml TGF-β for 24 h and cell lysates were collected. Protein expression of pJNK, CTGF and NF-E2 were quantitatively measured by Western blotting. A-F: TGF-β significantly induced expression of pJNK and CTGF while decreased NF-E2 expression. JNK inhibition followed by TGF-β treatment of HK-11 cells, significantly blocked JNK phosphorylation, decreased CTGF expression and decreased NF-E2 expression compared to TGF-β alone condition. Data is presented as the mean ± SD, $n = 6$ in each group. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

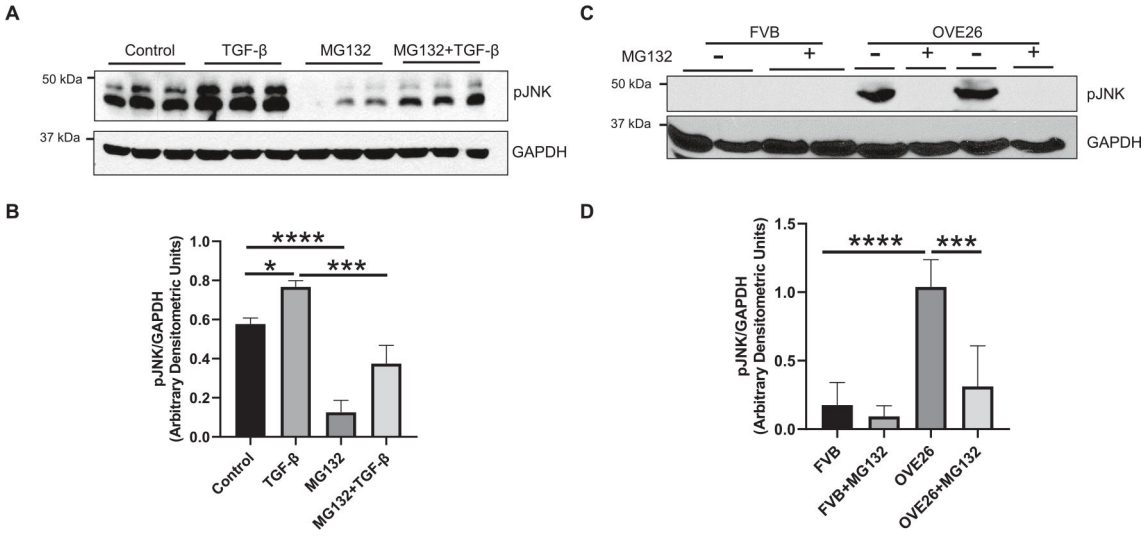

Fig. 5.

Effect of proteasome blockade on TGF-β-induced JNK activation in HK-11 cells and in type 1 diabetic OVE26 mice.

A, B: HK-11 cells were simultaneously pretreated with vehicle (DMSO) or 0.5 μM MG132 (proteasome inhibitor) for 1 h prior to treatment with or without TGF-β (10 ng/ml, 24 h). C, D: Three months control FVB mice and type 1 diabetic OVE26 mice were treated with proteasome inhibitor MG132 at a dose of 10 μg/kg daily for 3 months. Cell and renal cortex lysates were immunoblotted with anti-pJNK and anti-GAPDH antisera (A, C), followed by their expression quantitative analysis (B, D). Differences in multiple groups were performed by one-way ANOVA followed by the Tukey test for individual group comparisons. Data is presented as the mean ± SD, $n = 3$ in each group in cell culture work (A, B) and minimum $n = 4$ for animal studies (C, D). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

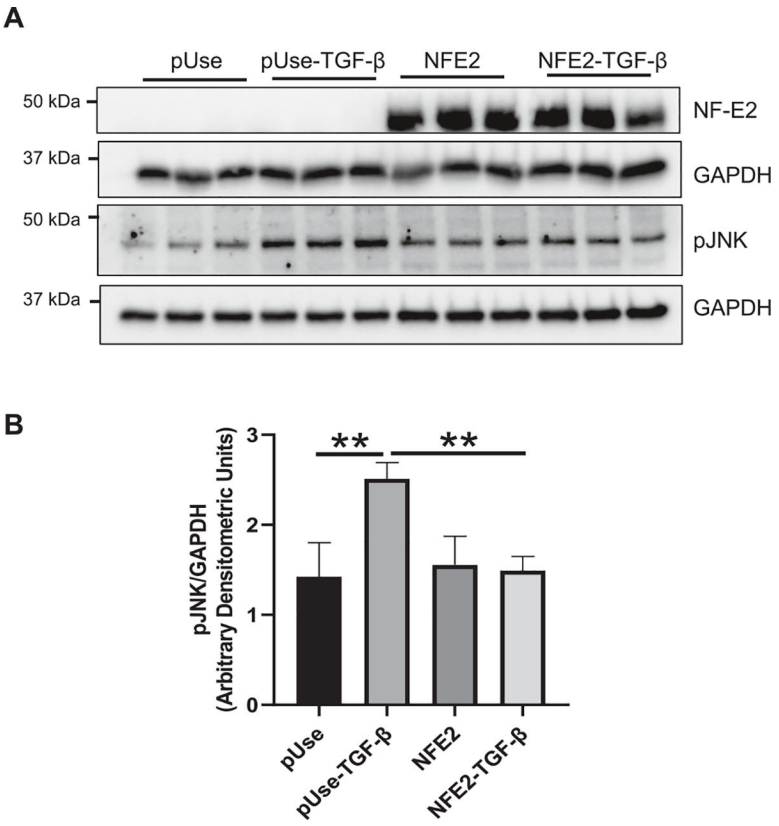

Fig. 6.

Effect of over-expression of NF-E2 on JNK phosphorylation in HK-11 cells.

HK-11 cells were transfected with pUse vector or pUse-NF-E2 cDNA for 24 h followed by treatment with 10 ng/ml TGF-β for additional 24 h and cell lysates were generated. These cell lysates were immunoblotted with anti-NF-E2, anti-pJNK and anti-GAPDH antisera (A), followed by their quantitative analysis (B). Data is presented as the mean ± SD, n = 3 in each group. $^{**}P < 0.01$.

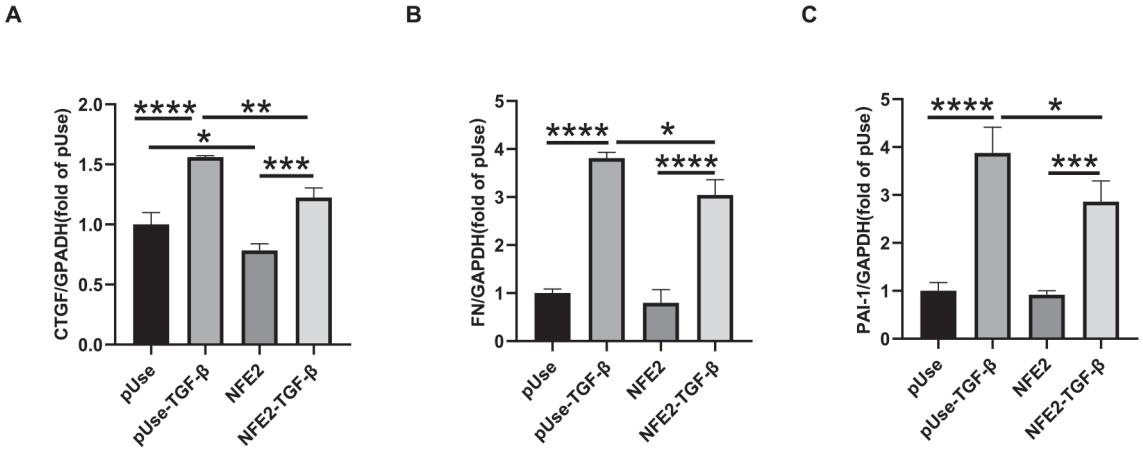

Fig. 7.

Effect of over-expression of NF-E2 on mRNA expression of CTGF, FN, PAI-1 in HK-11 cells.

HK-11 cells were transfected with pUse vector or pUse-NF-E2 cDNA for 24 h followed by treatment with 10 ng/ml TGF-β for additional 24 h and total RNA was isolated. Reverse transcription was performed for the total RNA, and then mRNA expression of CTGF (A), FN (B), PAI-1 (C) were tested by qPCR with CTGF primer, FN primer and PAI-1 primer. Data is presented as the mean ± SD, n = 3 in each group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

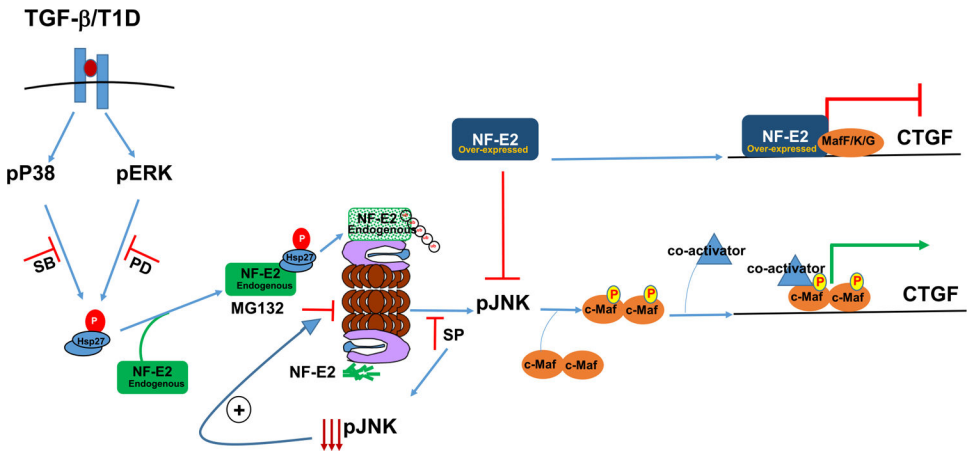

Fig. 8.

Hypothetical model of TGF-β-induced NF-E2 degradation and profibrotic signaling in renal cells.

In our previous study, we demonstrated NF-E2 degradation occurs at the proteasome in TGF-β treated HK-11 cells and in both T1D and T2D mouse kidneys. TGF-β induced pSer^{82}Hsp27 and enhanced pSer^{82}Hsp27/NF-E2 association, with concurrent NF-E2 degradation at the proteasome, possibly identifying NF-E2 as an Hsp27 cargo protein that is targeted to the proteasome. Loss of NF-E2 was associated with increases in profibrotic CTGF expression while over-expression of NF-E2 inhibited CTGF expression. NF-E2 sites are present on the promoter region of CTGF [36] and thus, NF-E2 may have a role in repressing CTGF expression. Results from the present study showed that ERK and p38 MAPK were activated in TGF-β treated HK-11 cells; Simultaneous blockade of p38 and ERK MAPK pathways abrogated pSer^{82}Hsp27 and preserved NF-E2 expression; Preserving NF-E2 expression prevented TGF-β-induced CTGF expression in HK-11 cells. Interestingly, blockade of JNK activation inhibited CTGF expression without preserving NF-E2 expression suggesting JNK activation occurs downstream of proteasome activation. This was confirmed by demonstrating loss of JNK activation and CTGF expression in the presence of MG132 (proteasome inhibitor) plus TGF-β in HK-11 cells and in T1D kidneys [26]. Moreover, MG132 treatment preserved NF-E2 expression in TGF-β treated HK-11 cells and in T1D kidneys [9]. These observations suggested NF-E2 degradation at the proteasome occurs prior to JNK activation. Therefore, blockade of JNK activation did not preserve NF-E2 expression. Thus, in the presence of JNK inhibitor SP600125, pJNK is inhibited which may in turn promote proteasome activation as a feedback mechanism to restore JNK activation. We confirmed a direct role for NF-E2 expression on modulating JNK activation by over-expressing NF-E2 in HK-11 cells and demonstrating blockade of pJNK in presence of TGF-β. Additionally, we demonstrated NF-E2 over-expression inhibited CTGF mRNA and protein expression in HK-11 cells. Moreover, NF-E2 site is present in the promoter region of CTGF [36]. These results collectively suggest that NF-E2 may heterodimerize with small Maf proteins to inhibit CTGF expression while in the absence of NF-E2, small Maf proteins may heterodimerize with other transcription factors to induce CTGF expression or c-Mafs homodimerize and may recruit their co-activator (blue triangle) to induce transcription of profibrotic genes including CTGF [44,45]. Therefore, NF-E2

may be an anti-fibrotic protein in TGF-β treated HK-11 cells and in diabetic kidneys. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)