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IHG-1 must be localised to mitochondria to decrease Smad7 expression and amplify TGF-β1-induced fibrotic responses

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ABSTRACT

TGF-β1 is a prototypic profibrotic cytokine and major driver of fibrosis in the kidney and other organs. Induced in high glucose-1 (IHG-1) is a mitochondrial protein which we have recently reported to be associated with renal disease. IHG-1 amplifies responses to TGF-β1 and regulates mitochondrial biogenesis by stabilising the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator-1-alpha. Here we report that the mitochondrial localisation of IHG-1 is pivotal in the amplification of TGF-β1 signalling. We demonstrate that IHG-1 expression is associated with repression of the endogenous TGF-β1 inhibitor Smad7. Intriguingly, expression of a non-mitochondrial deletion mutant of IHG-1 (Δmts-IHG-1) repressed TGF-β1 fibrotic signalling in renal epithelial cells. In cells expressing Δmts-IHG-1 fibrotic responses including CCN2/connective tissue growth factor, fibronectin and jagged-1 expression were reduced following stimulation with TGF-β1. Δmts-IHG-1 modulation of TGF-β1 signalling was associated with increased Smad7 protein expression. Δmts-IHG-1 modulated TGF-β1 activity by increasing Smad7 protein expression as it failed to inhibit TGF-β1 transcriptional responses when endogenous Smad7 expression was knocked down. These data indicate that mitochondria modulate TGF-β1 signal transduction and that IHG-1 is a key player in this modulation.

1. Introduction

Induced in high glucose-1 (IHG-1) also known as tRNA-histidine guanylyltransferase 1-like (THG1L) is a mitochondrial associated protein first identified in a screen for genes associated with diabetic nephropathy [1,2]. IHG-1 amplifies responses to the major fibrotic mediator transforming growth factor (TGF)-β1 and regulates mitochondrial biogenesis by stabilising the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α [3,4]. Increased activation of TGF-β1 is believed to be a pivotal mediator of disease development in the diabetic kidney and in other fibrotic disorders [5,6].

TGF-β1 initiates signalling through ligand dependent activation of a complex of heteromeric transmembrane serine/threonine kinases, consisting of type I and type II receptors. Upon phosphorylation by the type II receptor, the type I receptor phosphorylates receptor-regulated Smads (R-Smads) 2 and 3, the R-Smad complex with the common-mediator Smad (Co-Smad) Smad4 and translocates to the nucleus where they regulate gene transcription. Inhibitor Smads (I-Smads) 6 and 7 inhibit TGF-β signalling. Smad7 typically resides in the nucleus in unstimulated cells and upon receptor activation translocates to the plasma membrane [7]. At the plasma membrane Smad7 may inhibit TGF-β1 signal transduction by complexing with the TGF-β type I receptor (TβR1) and preventing phosphorylation of R-Smads [8–10], by recruiting WW-HECT-type E3 ubiquitin ligases (e.g. Smurf proteins) to induce degradation of TGF-β type I receptor (TβR1) [11–14] and by facilitating dephosphorylation of TβR1 by phosphatases [15]. In the nucleus Smad7 prevents the binding of functional R-Smad/Smad4 complexes to target gene promoters [16]. Smad7 also associates with histone deacetylases such as HDAC and SIRT1, and the acetyltransferase p300, suggesting that Smad7 may be involved in regulating the epigenetic status of chromatin and therefore TGF-β1 target gene transcription [17–19]. Here we report that a non-mitochondrial deletion mutant of IHG-1 increases Smad7 levels and inhibits TGFβ1 responses. Conversely expression of mitochondrial IHG-1 is associated with decreased Smad7 levels. These data indicate that mitochondrial localisation of IHG-1 is pivotal to its role in amplifying TGF-β1 signalling.

2. Materials and methods

2.1. Cell culture

HEK293T (ATCC, Middlesex, UK) was cultured in DMEM and MEM respectively, containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin

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and 1 mg/ml streptomycin — all from Invitrogen (Paisley, UK). Human proximal tubular cells (HK-2) (ATCC, Middlesex, UK) were maintained in low glucose DMEM (1 g/L glucose) (Invitrogen, Paisley, UK), 2 mM glutamax (Invitrogen), 10 mM HEPES (Sigma-Aldrich, Dublin, Ireland), 10 ng/ml EGF (Sigma-Aldrich), 36 ng/ml hydrocortisone (Sigma-Aldrich), ITS (10 μg/ml insulin; 5.5 μg/ml transferrin; 5 ng/ml sodium selenite) (Sigma-Aldrich), 3 pg/ml triiodothyronine (Sigma-Aldrich),, 25 ng/ml prostaglandin E1 (Sigma-Aldrich), 100 U/ml penicillin and 1 mg/ml streptomycin (Invitrogen). Stably transduced cell lines have been described previously [4].

2.2. Lentivirus production, transfections and reporter gene assays

Δmts-IHG-1 (deletion of amino acids 9–22) was generated using the QuickChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, Cork, Ireland). Plenti6/TGFβR1 was generated from pRK5 TGFβR1 (T202D) Flag (Addgene (Cambridge MA) plasmid 1483)[20]. Recombinant lentivirus was produced by the transfection of HEK293T cells with pCMV_R8.9, pMD.2G, and Plenti6/TGFβR1, pGIPZ/Scr, pGipZ/Smad7 shRNAi (Thermo Fisher Scientific, Surrey, UK), or empty vector control (EV) using a calcium phosphate transfection kit (Invitrogen). Transient transfection of cells with plasmids was performed using either the Fugene 6 or Fugene HD reagent (Roche, Dublin, Ireland) according to the manufacturer's instructions. The cells were stimulated with TGF-β1 following 24 h in serum and supplement free conditions, times and concentrations indicated in the figure legends. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany).

2.3. RNA isolation and RT-PCR

Total RNA was extracted by using Trizol reagent (Invitrogen). 1 μg of total RNA was reverse-transcribed using random primers and SuperScript II (Invitrogen). RT-PCR was performed on an Applied Biosystems 7900HT fast real-time PCR system using Taqman gene specific assays (Applied Biosystems, Warrington, UK). Results were normalised to 18 s rRNA expression.

2.4. Immunofluorescence analysis

Cells were plated onto 2 well chamber glass slides and allowed to attach in serum containing medium. Cells were transfected with tagged IHG-1 encoding plasmids (IHG-1-V5, Δmts-IHG-1-V5) using Fugene 6 (Roche) and cultured in serum containing medium for 48 h. Cells were rinsed with PBS and fixed in 3.7% paraformaldehyde (Sigma) in PBS for 18 min. The cells were permeabilised in 0.1% Triton in PBS on an orbital shaker for 10 min and then washed twice for 10 min in PBS. Slides were blocked in 5% normal goat serum (NGS) (Sigma) in PBS for 1 h at room temperature and incubated with primary antibodies, anti-V5 (1/400 Invitrogen) and anti-MnSOD (Stressgen Ann Arbour, Michigan, USA), (1/300), diluted in 5% NGS in PBS for 1 h at room temperature. After rinsing with PBS, slides were incubated with rabbit anti-mouse (for anti-V5) or goat anti-rabbit antibody (for anti-MnSOD) conjugated to FITC (1/400, Invitrogen) for 20 min at room temperature. Blocking and antibody incubations were performed on an orbital shaker. Cells were mounted in an antifade solution (Invitrogen). Secondary antibody only controls were performed. The cells were fixed and visualised with an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and Axio Vision 3.0.6 software (Carl Zeiss, Inc., Thornwood, NY) or in some cases by laser confocal microscopy (Zeiss SM 510) and Zeiss software using an ×63 oil immersion objective lens.

2.5. SDS PAGE and immunoblotting

Whole-cell extracts were prepared in Cell Lytic Extraction buffer (Sigma) with protease inhibitor mixture (Sigma) and phosphatase inhibitor cocktail (Roche). Protein content was quantified and normalised by using the Bradford method (Bio-Rad, Hemel Hempstead, UK). Proteins were separated by electrophoresis on SDS/PAGE gels. Subcellular fractionation was performed using Qproteome Nuclear Protein Kit (Qiagen, Crawley, UK). Antibodies: β-Actin (Sigma), Fibronectin (BD Biosciences), Flag (Invitrogen), CTGF, GAPDH, Jagged-1, Jun-C, Smad2, (Cell Signaling, Hertfordshire, UK), MnSOD (Stressgen), pSmad2, Smad3 pSmad3 (Abcam, Cambridge, UK), Smad7 (Tebu Biosciences, Peterborough, UK), and V5 (Invitrogen and Universal Biologicals, Cambridge, UK). Densitometry was performed using ImageJ software.

2.6. Unilateral ureteral obstruction

The UUO model used was carried on male Wistar rats weighing 250–350 g (2–3 months of age) out under licence as described previously

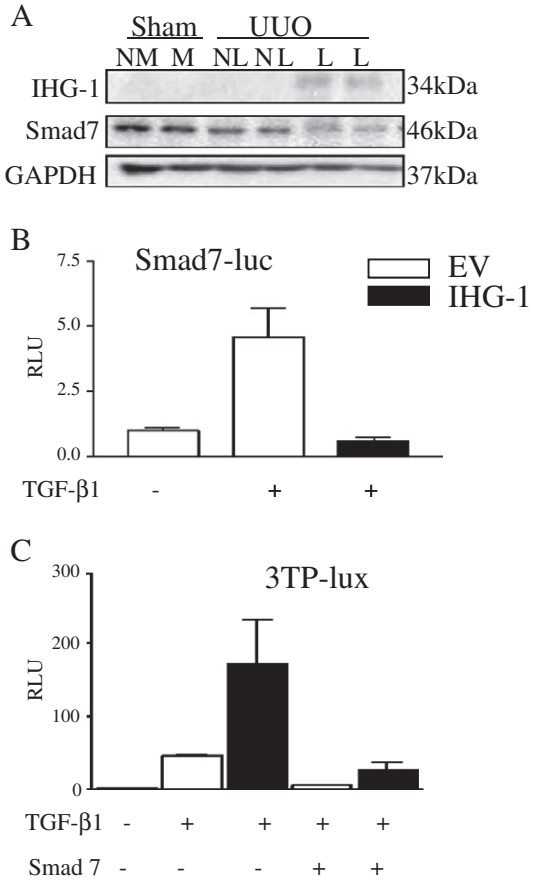

Fig. 1. IHG-1 decreases Smad7 expression. A. Altered expression in a 3-d UUO model. Total protein extracts were prepared from non manipulated (NM) sham, M (manipulated) (sham), L (ligated) NL (non-ligated) rat kidneys. Shown are representative western analyses of these extracts probed for IHG-1, Smad7 and GAPDH. B. HK2 cells were co-transfected with Smad 7-luc reporter and phRL-CMV plus or minus pcDNA6-IHG-1-V5, as indicated. Firefly luciferase activity normalised to Renilla luciferase activity was determined as directed by manufacturer (Promega). Where indicated, cells were stimulated with 5.0 ng/ml TGF-β1 24 h after transfection. Luciferase activity was measured 24 h later. The results shown are means ± s.e.m. of at least three independent experiments. Differences in mean are significant ($P < 0.05$). C. Cells were co-transfected with the 3TP-Lux reporter and phRL-CMV plus or minus combinations of IHG-1-V5, Smad7 expression plasmids, as indicated. Smad 7 significantly inhibits IHG-1 enhancement of TGF-β1 induced luciferase activity ($P < 0.05$).

[21] and were handled either by the licencee or by licenced technicians in accordance with institutional, ethical, and legal guidelines.

2.7. Statistical analysis

Results are expressed as mean ± s.e.m. and were analysed for significance by ANOVA. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. IHG-1 suppresses Smad7 expression

We have previously shown that IHG-1 mRNA expression is increased by high extracellular glucose [1] and in the tubulointerstitium of diabetic nephropathy patients and in the UUO model of tubulointerstitial fibrosis [3]. We have proposed that IHG-1 contributes to fibrotic renal disease as it potentiates TGF-β1 signalling in HK-2 cells (renal proximal tubule cell line [22]) leading to an increase in the expression of proteins associated with fibrosis i.e. profibrotic mediator CCN2/connective tissue growth factor (CTGF) and extracellular matrix protein fibronectin [3]. Increased IHG-1 mRNA levels were detected following early injury in UUO and when TIF was evident [3], IHG-1 protein expression was also increased at 3 days post obstruction in the UUO model of kidney fibrosis concomitant with decreased Smad7 protein expression (Fig. 1A), indicating that IHG-1 may enhance TGF-β profibrotic responses by modulating Smad7 activity. TGF-β1 increases the expression of its own inhibitor Smad7 in a well described negative feedback loop [23]. Recent research has demonstrated a role for Smad7 in renal protection in vivo using models of both

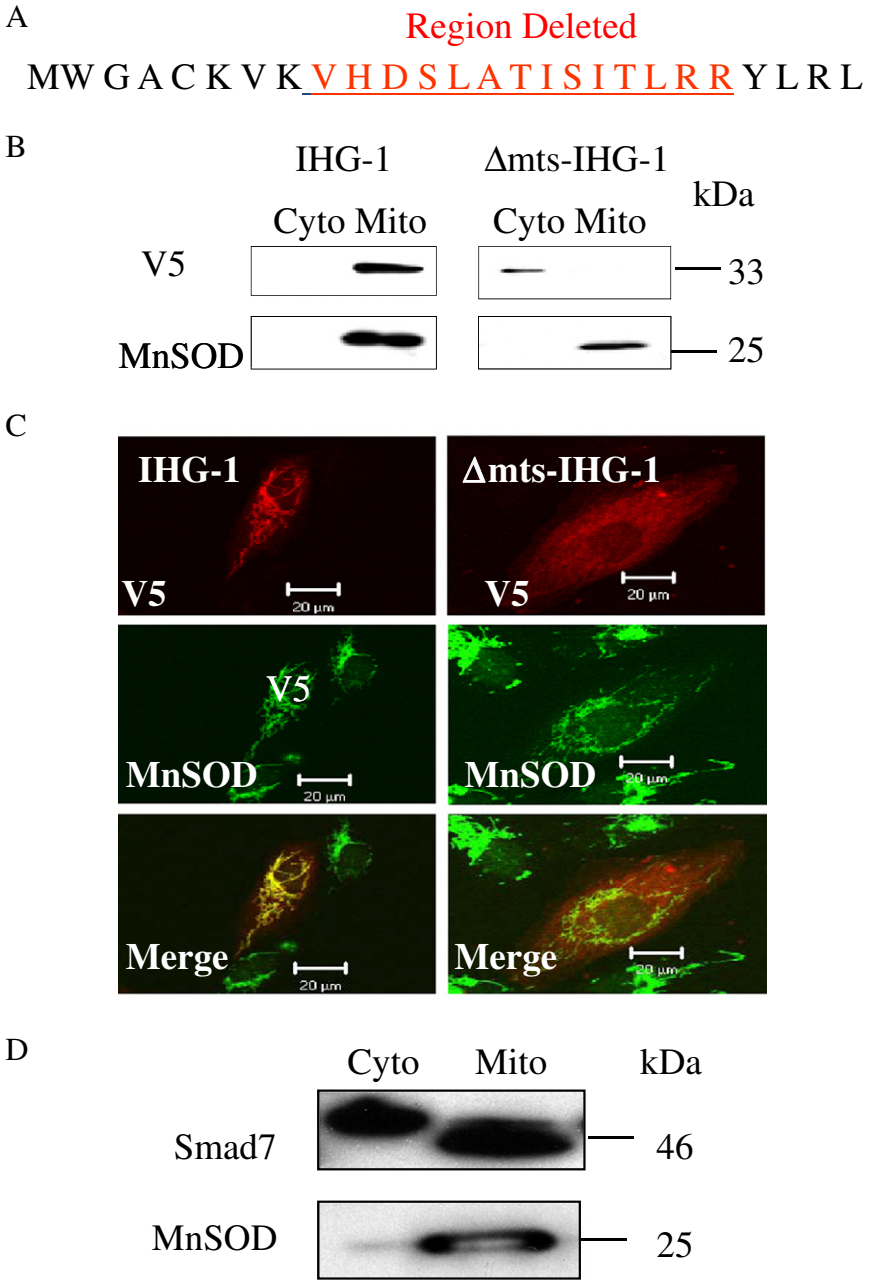

Fig. 2. Mitochondrial targeting sequence (mts) is required to localise IHG-1 to mitochondria. A. Schematic representation of region deleted from IHG-1. Sequence encoding amino acids coloured red (9–22) were deleted from IHG-1 to generate Δmts-IHG-1 B. Cytoplasmic (C) and mitochondrial (M) protein extracts were prepared from HK-2 cells that had been transfected with IHG-1-V5 or Δmts-IHG-1-V5. Shown is representative western analysis of these extracts probed for V5 and MnSOD. C. Cells from (B) were fixed and probed with anti-V5 and anti-MnSOD antibodies and analysed by immunofluorescence microscopy. D. Cytoplasmic (C) and mitochondrial (M) protein extracts were prepared from HK-2 cells. Shown is western analysis of these extracts probed for Smad7 and MnSOD.

fibrosis [24] and DN [25]. Smad7 expression is reduced in renal epithelial cells cultured in high extracellular glucose [26]. We investigated if IHG-1 amplified TGF-β1 responses by altering Smad7 expression. We report that IHG-1 inhibited TGF-β1 mediated transcription from Smad7-luc (Fig. 1B). TGF-β1 stimulation of the PAI promoter was inhibited when Smad7 was overexpressed (Fig. 1C), as has previously been reported [8]. Smad7 also inhibited the ability of IHG-1 to enhance TGF-β1 induced reporter gene expression (Fig. 1C). These data indicate that IHG-1 modulates TGF-β1 signalling responses by regulating Smad7 expression levels.

3.2. Predicted N-terminal mitochondrial targeting sequence (mts) is functional

We have previously shown that IHG-1 is localised to mitochondria in mammalian cells and in rat kidneys [4] as determined by a combination of subcellular fractionation and immunocytochemistry. At the amino terminal of human IHG-1 there is a predicted mitochondrial targeting sequence (mts) [3]. To investigate the functionality of the predicted mts amino acids 9–22 were deleted (Fig. 2A) and the resulting mutant V5 tagged IHG-1 protein (Δmts-IHG-1-V5) was expressed in HK-2 cells. In contrast to IHG-1-V5, Δmts-IHG-1-V5 did not co-localise with the mitochondrial marker MnSOD as judged by western analysis (Fig. 2B). This observation was confirmed by immunofluorescence microscopy (Fig. 2C) indicating that the mts was essential for mitochondrial localisation. These data indicate that the mts is necessary for the localisation of IHG-1 to mitochondria. Smad7 was found to be expressed in both cytoplasm and mitochondrial fractions prepared from HK-2 cells (Fig. 2D).

3.3. Δmts-IHG-1 increases Smad7 expression

To determine if IHG-1 mitochondrial localisation was important for its ability to regulate Smad7 expression, we investigated the expression of Smad7 in HK-2 cells expressing Δmts-IHG-1. Δmts-IHG-1 increased the expression of Smad7 mRNA (Fig. 3A). Stimulation with TGF-β1 did not alter Smad7 protein expression in HK-2 cells (Fig. 3B) similar to the findings in primary human renal cells (HKCs) [27]. Δmts-IHG-1 resulted in an increased Smad7 protein expression (Fig. 3B). Δmts-IHG-1 expression significantly enhanced transcription from a transfected Smad7 promoter reporter construct (Fig. 3C). In the absence of TGF-β1 stimulation levels of reporter gene expression were on average six-fold greater in HK-2 cells expressing Δmts-IHG-1 (Fig. 2C). Following TGF-β1 stimulation Δmts-IHG-1 doubled the levels of reporter gene expression (20-fold vs 10-fold). These data indicate that Δmts-IHG-1 inhibits TGF-β1 signalling by increasing Smad7 expression.

3.4. Δmts-IHG-1 inhibits TGF-β1 profibrotic responses

Reduced Smad7 expression in renal fibrosis has been reported to be associated with increased expression of fibrotic markers such as CTGF and fibronectin [24,25]. In an animal model increasing Smad7 expression (by microbubble targeting to the kidney) was sufficient to rescue the fibrotic phenotype [25]. Consistent with its ability to increase expression Smad7 Δmts-IHG-1 was associated with the inhibition of TGF-β1-induced fibrotic responses (CTGF and fibronectin protein expression) (Fig. 4A) in the HK-2 cell line. Δmts-IHG-1 also decreased the expression levels of jagged-1, a Notch receptor ligand [28] (Fig. 4A). Increased expression levels of jagged-1 have been implicated in the pathophysiology of diabetic nephropathy and renal fibrosis [29].

We hypothesised that Δmts-IHG-1 induced Smad7 expression may inhibit TGF-β1 activity by inhibiting TβR1 initiated cellular responses. Increased expression of TβR1 is a feature of tubulointerstitial fibrosis [30]. Overexpression of constitutively active TβR1 in HK-2 cells led to an increased protein expression of fibronectin, CTGF and jagged-1, Δmts-IHG-1 inhibited TβR1 induced expression levels of these proteins (Fig. 4B) further evidence that it could inhibit profibrotic responses initiated from TβR1 in renal epithelial cells.

Furthermore Δmts-IHG-1-V5 reduced TGF-β1 mediated transcription from a transfected TGF-β1-sensitive plasminogen activator inhibitor (PAI) promoter reporter construct (3TP-lux) (Fig. 4C). These

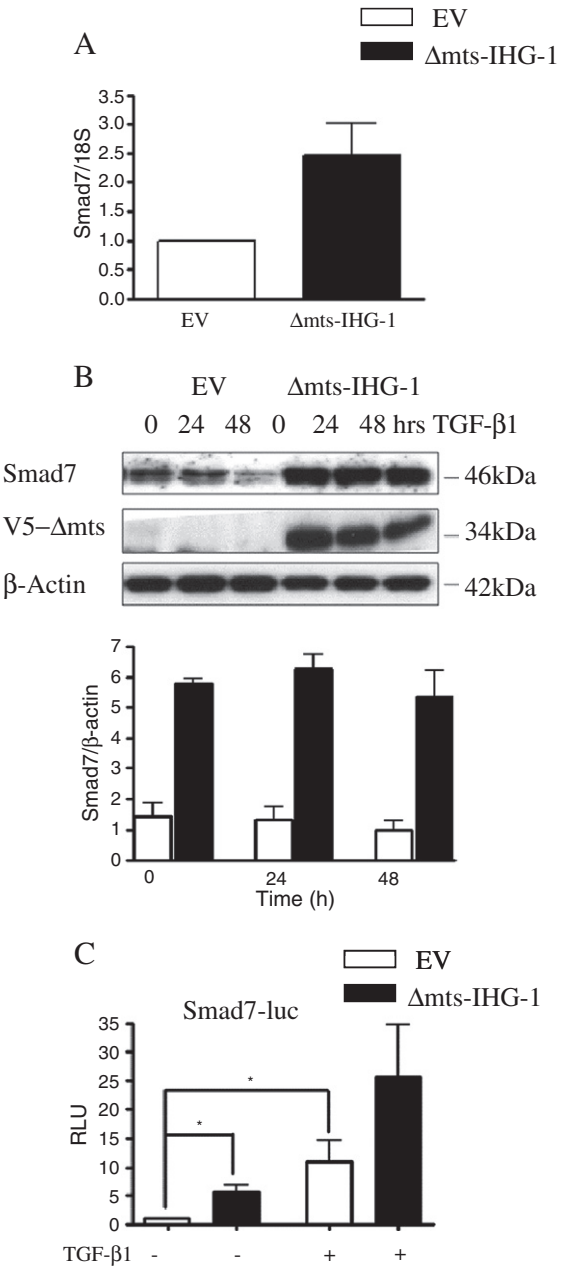

Fig. 3. Δmts-IHG-1 increases Smad7 expression. A. Smad7 mRNA in EV or Δmts-IHG-1 HK-2 cell lines were measured by qRT-PCR. B. Protein extracts were prepared from cells in A ± TGF-β1 (5.0 ng/ml) stimulation for times indicated. Shown are representative western analyses of these extracts probed for V5, Smad7 and β-actin. Alterations in protein expression were measured using densitometric analysis. The results shown are means ± s.e.m. of at least three independent experiments. Differences in mean are significant $P < 0.05$. C. EV or Δmts-IHG-1 HK-2 cell lines were co-transfected with Smad 7-luc reporter and phRL-CMV. Luciferase assay experimental analysis and presentation of results are as in Fig. 1B. Differences in mean are significant ($P < 0.05$).

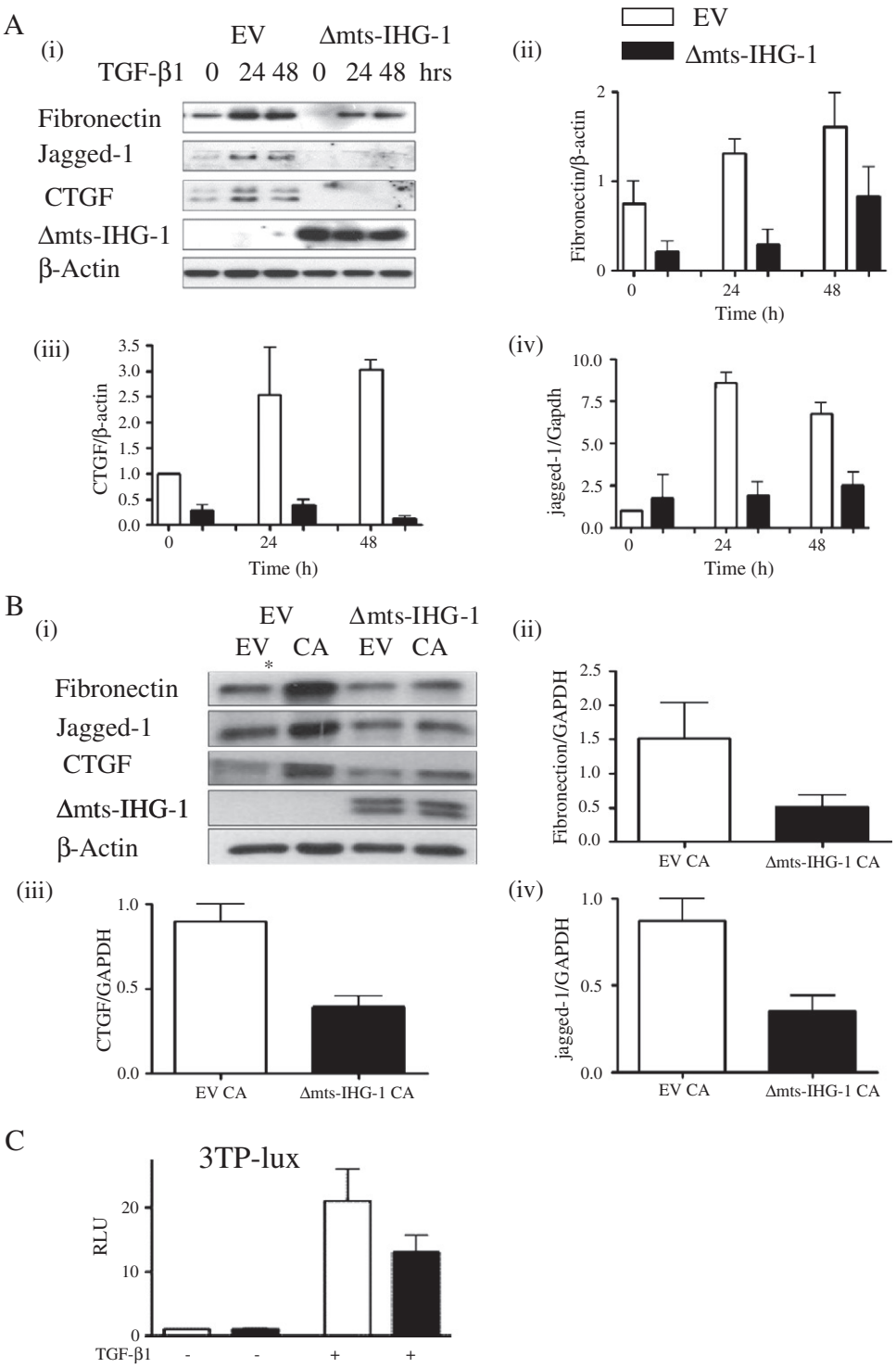

Fig. 4. Δmts-IHG-1 inhibits TGF-β1 profibrotic responses. A. Protein extracts were prepared from stably transfected empty vector (EV) and Δmts-IHG-1 HK-2 cells ± TGF-β1 (5.0 ng/ml) stimulation for times indicated. Shown are (i) representative western analyses of these extracts probed for V5, fibronectin, CTGF, Jagged and β-actin. Alterations in protein expression for (ii) fibronectin, (iii) CTGF and (iv) Jagged were measured using densitometric analysis. The results shown are means ± s.e.m. of at least three independent experiments. Differences in means are significant $P < 0.05$. B. Protein extracts were prepared from EV and Δmts-IHG-1 cell lines that had been transiently transduced with lentivirus overexpressing TβR1 (constitutively active (CA)) or EV control lentivirus. Experimental analysis and presentation of results are as in Fig. 4A. C. EV and Δmts-IHG-1 HK-2 cells ± TGF-β1 (5.0 ng/ml) stimulation for times indicated co-transfected with the 3TP-lux reporter and phRL-CMV. Luciferase assay experimental analysis and presentation of results are as in Fig. 1B. Differences in means are significant ($P < 0.05$).

data indicate that intriguingly IHG-1 must retain the potential to associate with mitochondria in order to amplify TGF-β1-induced transcriptional responses, loss of mitochondrial localisation leads to increases in Smad7 expression and inhibition of TGF-β1 induced profibrotic responses.

3.5. Δmts-IHG-1 inhibits R-Smad mediated transcription but not phosphorylation or translocation

However Δmts-IHG-1 did not alter phosphorylation levels of either Smad2 or Smad3 following stimulation with TGF-β1 (Fig. 5A)

suggesting that Δmts-IHG-1 modulates TGF-β1 responses independently of R-Smad phosphorylation. Δmts-IHG-1 did not alter shuttling of phosphorylated R-Smads to the nucleus following TGF-β1 stimulation (Fig. 5B & C). Although Δmts-IHG-1 inhibited TGF-β1 mediated transcription from a PAI promoter reporter construct (3TP-lux) (Fig. 4C) it did not alter responses to TGF-β1 from a Smad3 responsive

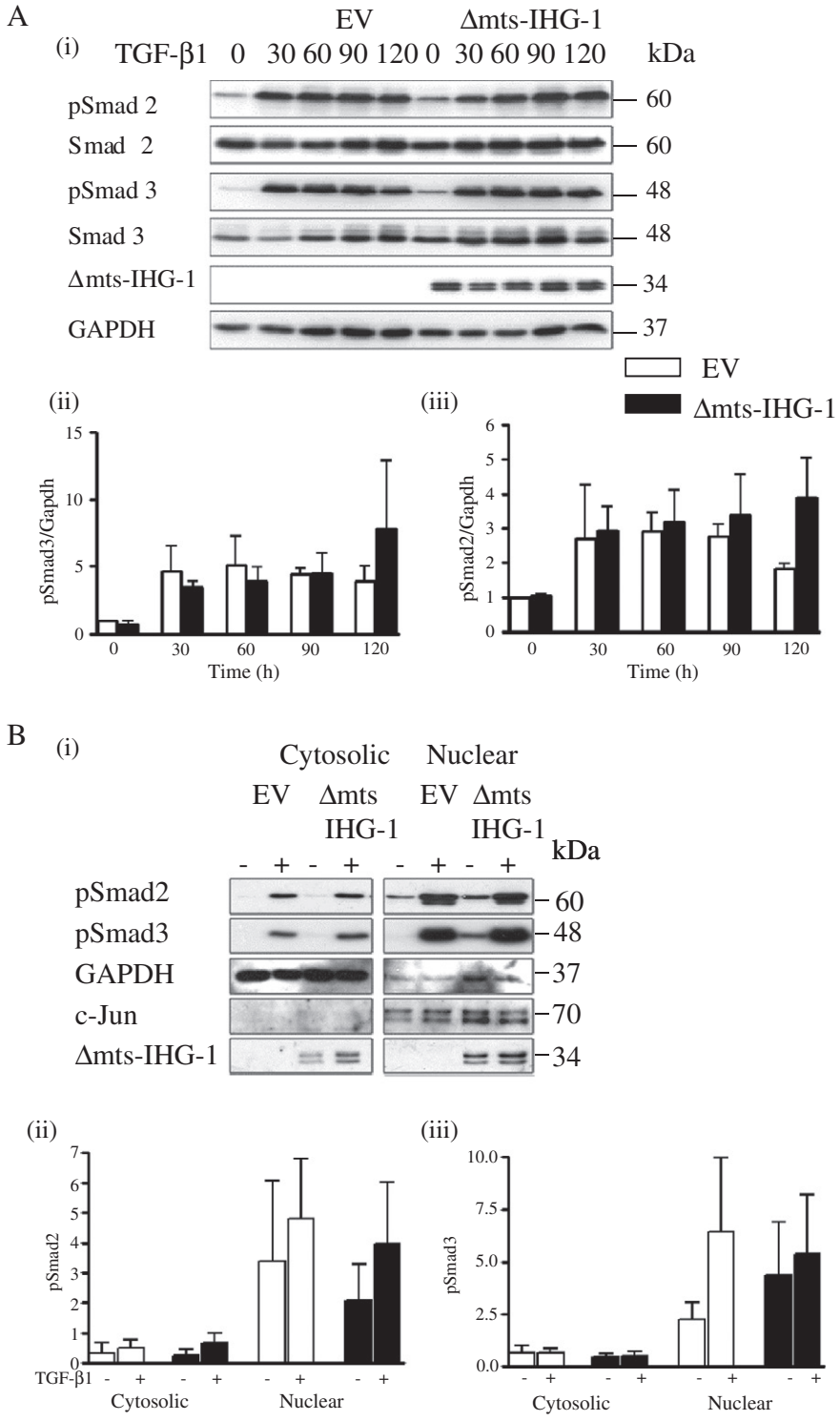

Fig. 5. Δmts-IHG-1 does not alter Smad phosphorylation, translocation or ability to bind SBE. A. Protein extracts were prepared from EV or Δmts-IHG-1 HK-2 cell lines ± 5 ng/ml TGF-β1 stimulation at times indicated. Shown are (i) western analyses of these extracts probed for V5, pSmad2, Smad2, pSmad3, Smad3 and GAPDH. Alterations in protein expression for (ii) pSmad2, (iii) pSmad3 were measured using densitometric analysis. The results shown are means ± s.e.m. of at least three independent experiments. Differences in means are not significant. B. Cytosolic and nuclear extracts were prepared from cells in (B) following stimulation with 5 ng/ml TGF-β1 for 60 min. Shown are western analyses of these extracts probed for pSmad2, pSmad3, and V5, GAPDH and c-Jun. Alterations in protein expression for (ii) pSmad2, (iii) pSmad3 in cytosolic and nuclear compartments were measured using densitometric analysis. The results shown are means ± s.e.m. of at least three independent experiments. Differences in means are not significant. C. EV or Δmts-IHG-1 HK-2 cell lines ± 5 ng/ml TGF-β1 stimulation for 30 min were fixed, probed with Smad2 (stained in red) and analysed by immunofluorescence microscopy. Nuclei were stained with DAPI (blue) and F-actin was visualised with Alexa488 conjugated phalloidin (green). D. EV and Δmts-IHG-1 HK-2 cells ± TGF-β1 (5.0 ng/ml) stimulation for times indicated co-transfected with the (i) SBE-luc and phRL-CMV or (ii) SBE-Smad7 and phRL-CMV. Luciferase assay experimental analysis and presentation of results are as in Fig. 1B. Differences in means for (ii) are significant ($P < 0.05$).

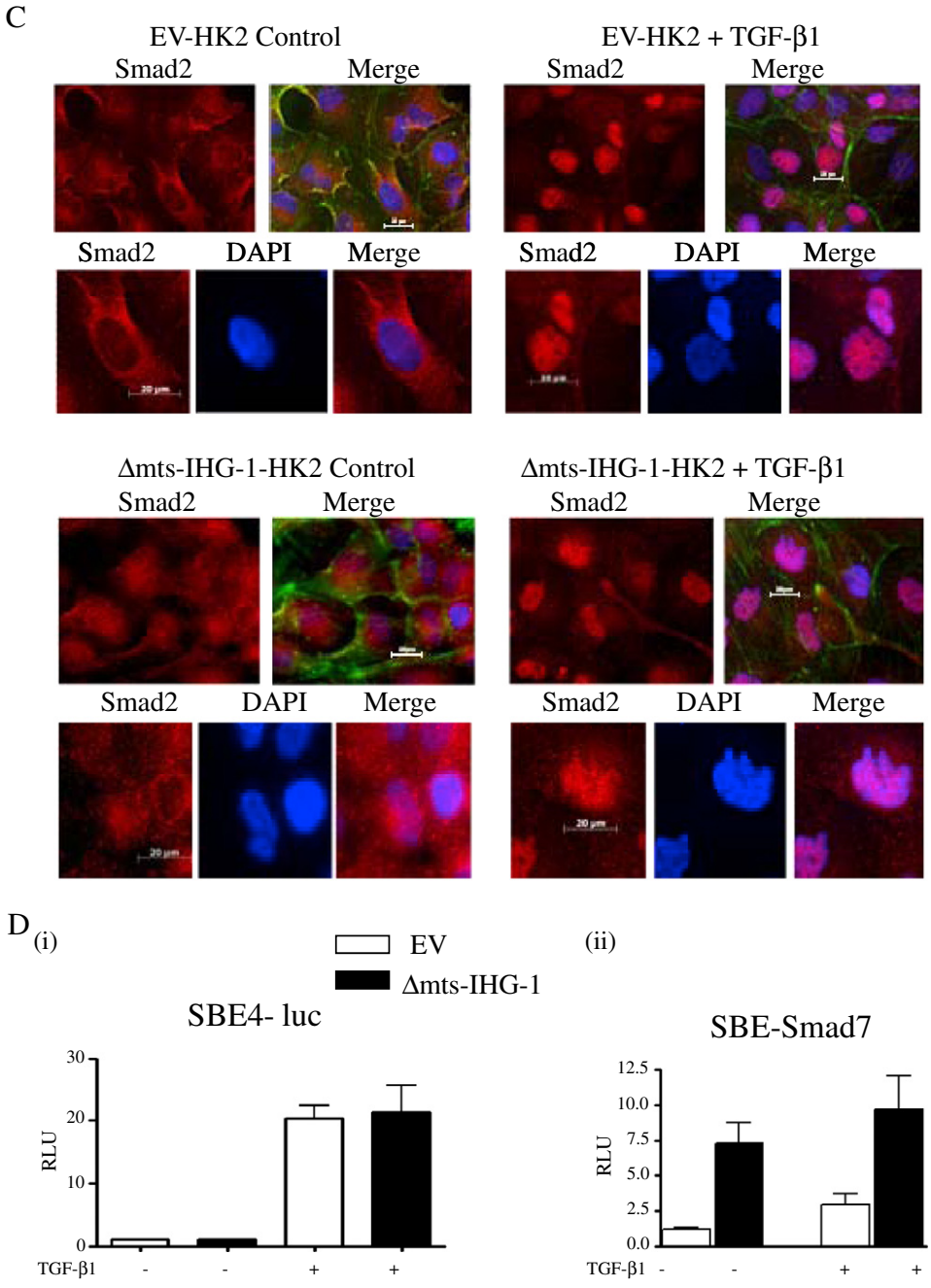

Fig. 5 (*continued*).

SBE4-luc [31] minimal promoter reporter construct (Fig. 5D(i)) suggesting that Δmts-IHG-1 does not alter the ability of Smad3 to bind to the Smad binding element (SBE) an 8 bp palindromic sequence (GTCTAGAC). The SBE is found in the human Smad7 promoter and is necessary for the negative regulation of promoter activity by Ski [32]. Mutation of the SBE increases Smad7 promoter basal activity [32] but also results in the loss of response to TGF-β1 in mammalian cell lines (i.e. HeLa, Mv1Lu/L17, and 293 cells). Δmts-IHG-1 significantly increased basal activity from the mutated Smad7 promoter SBE-Smad7 [32] in renal epithelial cells which was unaltered following TGF-β1 stimulation. Surprisingly TGF-β1 stimulation resulted in a trend towards increased activity from the mutated Smad7 promoter reporter in renal epithelial cells but this did not reach significance (Fig. 5D (ii)).

3.6. Δmts-IHG-1 increases nuclear Smad7 expression and requires Smad7 to inhibit TGF-β1 responses

Δmts-IHG-1 was found to localise to both cytosolic and nuclear fractions (Fig. 6A). Δmts-IHG-1 significantly increased Smad 7 levels in the nucleus (Fig. 6A). Nuclear Smad7 has been reported to inhibit R-Smad transcriptional responses by preventing the formation of functional R-Smad/Smad-4-DNA complexes [16]. These data indicate that Δmts-IHG-1 inhibits TGF-β1 signalling by increasing Smad7 expression in the nucleus. To determine whether Smad7 expression was necessary for Δmts-IHG-1 to modulate TGF-β1 mediated transcriptional responses, we investigated the effects of short hairpin microRNA (shRNAmir) induced loss of Smad7 expression on the ability of Δmts-IHG-1 to inhibit TGF-β1 transcriptional responses.

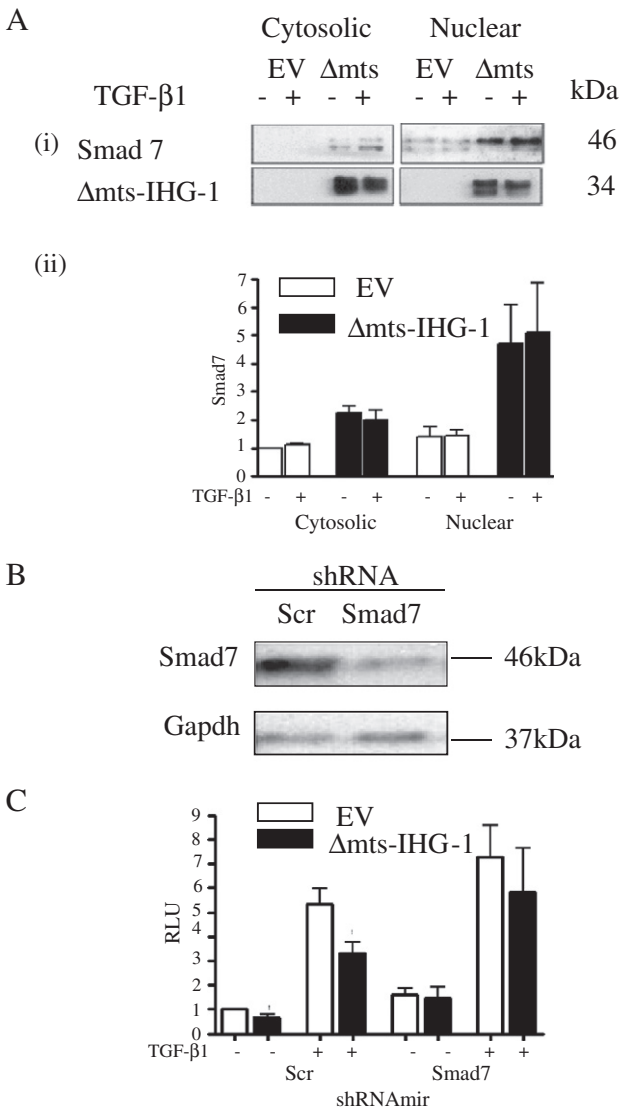

Fig. 6. Δmts-IHG-1 needs Smad7 to inhibit TGF-β responses. A. Cytosolic and nuclear extracts were prepared from from EV and Δmts-IHG-1 cell lines following stimulation with 5 ng/ml TGF-β1 for 60 min. Shown are western analyses of these extracts probed for Smad7 and V5. Alterations in protein expression were measured using densitometric analysis. The results shown are means ± s.e.m. of at least three independent experiments. Differences in means in nuclear fractions are significant (Tukey's Multiple Comparison Test ($P < 0.05$)). B. Whole cell extracts were prepared from HK2 cells expressing short hairpin (sh) RNA interference constructs specific for Smad7 or a scrambled construct (Scr). Shown is western analysis of these extracts probed with Smad7 and GAPDH C. EV and Δmts-IHG-1 cells were cotransfected with 3TP-lux reporter and phRL-CMV plus or minus smad7 shRNA or a scrambled construct (Scr). Luciferase assay experimental analysis and presentation of results are as in Fig. 1C. Differences in mean are significant ($P < 0.05$).

Selective knockdown of Smad7 in HK-2 cells was achieved by transient transfection with a plasmid that expressed Smad7 targeted RNAmir (Fig. 6B). Δmts-IHG-1 over-expression significantly inhibited TGF-β1 mediated expression from 3TP-lux when transfected with a plasmid expressing scrambled shRNAmir (Fig. 6C). In contrast in cells transfected with shRNAmir targeted to Smad7, Δmts-IHG-1 no longer had the ability to inhibit TGF-β1 activation of the 3TP-lux promoter. Loss of Smad7 expression resulted in increased activation of 3TP-Lux transcription as has been previously reported (Fig. 6C).

These data indicate that Δmts-IHG-1 inhibits transcriptional responses to TGF-β1 by enhancing levels of Smad7.

4. Discussion

We have previously described IHG-1 as an amplifier of TGF-β1 in diabetic kidney disease [3]. Our current findings indicate that mitochondrial localisation of IHG-1 is pivotal to its role in TGF-β1 signalling as deletion of the mitochondrial localisation signal of IHG-1(Δmts-IHG-1) not only suppressed its ability to amplify TGF-β1 activity but also inhibited TGF-β1 profibrotic responses. These data indicate a regulatory role for mitochondria in TGF-β1 signal transduction.

Mitochondrial dysfunction is a major contributor to hyperglycaemic-induced kidney damage [33,34]. Oxidant stress resulting from increased mitochondrial production of reactive oxygen species (ROS) is believed to play a critical role in disease development [33] and disease associated TGF-β1 activity [34]. Release of mitochondrial ROS is enhanced by the activation of NADPH oxidases [35]. Mitochondrial associated IHG-1 may play a role linking oxidant stress to increased TGF-β1 signalling.

The role of mitochondria in energy metabolism, apoptosis and regulation of calcium is well appreciated in the literature. However there is an increasing awareness in the past decade of the role of mitochondria in the integration and transmission of intracellular signal transduction [36]. Mitochondrial signalling is involved in apoptotic and proliferative pathways, nutrient sensing, inter-organelle communication and in the responses of cells to metabolic transition and physiological stresses [36].

While increased ROS production is associated with the amplification of TGF-β1 responses to date mitochondria have not been described to modulate Smad signalling yet TGF-β1, Smad4 and 5 have been described to localise to mitochondria [37–39] while Smad6 and Smad7 have predicted mitochondrial targeting sequences [37]. Furthermore connections between mitochondria, oxidative stress and TGFβ signalling have been reported [40–42] while recent research on vascular development suggests that TGFβ signalling pathway is dependent on mitochondrial function [43]. Recently mitochondrial ROS generated at complex III has been reported to be required for TGF-β-induced gene expression (i.e. CTGF, Nox4 and α-SMA) in primary normal human lung fibroblasts [44]. Furthermore mitochondrial ROS amplifies TGF-β induced NOX4 (NADPH oxidase 4) expression important in myofibroblast differentiation [45]. Our results substantiate these findings as they indicate a key role for mitochondria in the regulation of Smad7, an important modulator of TGF-β1 activity.

A classical SBE is found in the Smad7 promoter. This element in association with transcription factors AP-1 and Sp1 is important in the TGF-β1 induction of Smad7 [46]. Mutation of the SBE increases Smad7 promoter basal activity [32] but also results in loss of response to TGF-β1 stimulation in HeLa, Mv1Lu/L17, and 293 cells [32]. Maximal induction of the Smad7 promoter requires other transcription factors (e.g. such as CBP/p300, FoxH1, TFE-3 (transcription factor mE3), CBFA (PEBP2/core-binding factor A) and ATF2)[47] and co-factors in addition to Smads [23] suggesting that IHG-1 is important in the regulation of such factors possibly by increasing their stability as we have previously described for the transcriptional co-activator PGC-1α [4].

Both elevated TGF-β signalling and decreased Smad7 protein expression are often present in tissues where an uncontrolled fibrotic response occurs [47]. In kidney disease it has been reported that increased Smad7 mRNA expression is associated with loss of Smad7 protein expression [30,48]. Similarly, TGF-β1 increased Smad7 transcription in renal epithelial cells in this study but did not increase Smad7 protein expression as previously reported for primary human renal cells (HKCs) [27] where TGF-β1 stimulation decreased Smad7 protein due to increased proteasomal degradation. TGF-β1 regulation of Smad7 protein is most likely context dependent as Smad7 protein expression was increased in PC-3U cells by TGF-β1, in these cells increased Smad7 expression resulted in apoptosis [49]. Increased expression of IHG-1 in renal cells most likely contributes to fibrosis by further reducing Smad7 protein expression by inhibiting transcription.

Δmts-IHG-1 increased expression of Smad7 mRNA, protein and promoter activity and inhibited TGF-β1 induced transcription from the PAI promoter and expression of fibrotic proteins yet surprisingly did not alter Smad phosphorylation or translocation. Similarly mitochondrial targeted antioxidants markedly attenuated TGF-β-induced gene expression without affecting Smad phosphorylation or nuclear translocation [44] indicating that Smad activity is regulated in the nucleus. Δmts-IHG-1 was found both in the cytosol and the nucleus and resulted in significantly higher expression of Smad7 in the nucleus but not in the cytosol. Given the increased levels of Smad7 in the nucleus it is possible that Δmts-IHG-1 may retain Smad7 in the nucleus, where it can act as a transcriptional coactivator/corepressor. It has been shown that Smad7 has a nuclear coactivator function that is independent of TGF-β signal transduction [50]. Smad7 interference of R-Smad/Smad4-DNA association could account for the resultant decrease in the transcription of TGF-β1 target genes. When Smad7 expression was knocked down by shRNA, Δmts-IHG-1 no longer altered TGF-β1 transcriptional responses indicating that Δmts-IHG-1 mediates its antifibrotic effects principally by increasing Smad7 expression. Smad 7 has been shown to have anti-fibrotic actions and there is good evidence to support the overexpression of Smad7 as a therapeutic approach to treat fibrosis [25]. In an animal model of diabetes, Smad7 expression (by microbubble targeting to the kidney) was sufficient to rescue the fibrotic phenotype underlying how important Smad7 may be in finding a robust therapeutic agent to DN and to fibrosis in general [25].

5. Conclusions

In summary our findings indicate a key role for mitochondria in the modulation of TGF-β1 signal transduction. Expression of Δmts-IHG-1 similar to inhibition of endogenous IHG-1 expression resulted in an increased expression of Smad7. This increase in Smad7 expression resulted in reduced expression of fibrotic associated proteins CTGF, fibronectin and jagged-1. Δmts-IHG-1 failed to inhibit TGF-β1 transcriptional responses when endogenous Smad7 expression was reduced. Dysregulation of TGF-β signalling contributes to several disease processes (e.g. fibrosis, cancer) making it a popular target for drug development (e.g. small-molecule inhibitors, monoclonal antibodies) [51]. However studies have shown that completely blocking this pathway can lead to cancer, increased inflammation, autoimmunity and cardiovascular disease [51]. Therefore it would be more desirable to decrease excessive levels of TGF-β activity rather than to totally inhibit signal transduction. Smad 7 has been shown to attenuate TGF-β activity and has good potential to treat anti-fibrotic disease [25,51]. Δmts-IHG-1 offers the advantage of stimulating endogenous Smad7 expression and thereby readdressing the imbalance found in kidney fibrosis and perhaps other fibrotic diseases. Although a smaller protein than Smad7, the molecular weight of Δmts-IHG-1 still poses difficulties for delivery. Our future aim is to determine the regions within Δmts-IHG-1 that are necessary to induce the expression of Smad7 and thus develop a more readily deliverable therapeutic. We have renamed Δmts-IHG-1 as ITA1 (inhibitor of TGF-β1 activity 1) and propose ITA1 as a novel biotherapeutic for the treatment of fibrotic renal disease.

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