



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

James B. Corcoran^a, Sarah McCarthy^a, Brenda Griffin^a, Andrew Gaffney^a, Una Bhreathnach^a, Emma Börgeson^a, Fionnuala B. Hickey^a, Neil G. Docherty^b, Debra F. Higgins^a, Fiona Furlong^{a,c}, Finian Martin^a, Catherine Godson^a, Madeline Murphy^{a,*}

^a UCD Diabetes Complications Research Centre, UCD Conway Institute, School of Medicine and Medical Sciences, University College Dublin, Belfield, Dublin 4, Ireland

^b Department of Physiology, School of Medicine, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland

^c School of Pharmacy, QUB, Lisburn Road, Belfast BT9 7BL, Northern Ireland, U.K.

ARTICLE INFO

Article history:

Received 7 August 2012

Received in revised form 28 February 2013

Accepted 26 March 2013

Available online 6 April 2013

Keywords:

IHG-1

TGF- β 1

Mitochondria

Smad7

ABSTRACT

TGF- β 1 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- β 1 and regulates mitochondrial biogenesis by stabilising the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator-1- α . Here we report that the mitochondrial localisation of IHG-1 is pivotal in the amplification of TGF- β 1 signalling. We demonstrate that IHG-1 expression is associated with repression of the endogenous TGF- β 1 inhibitor Smad7. Intriguingly, expression of a non-mitochondrial deletion mutant of IHG-1 (Δ mts-IHG-1) repressed TGF- β 1 fibrotic signalling in renal epithelial cells. In cells expressing Δ mts-IHG-1 fibrotic responses including CCN2/connective tissue growth factor, fibronectin and jagged-1 expression were reduced following stimulation with TGF- β 1. Δ mts-IHG-1 modulation of TGF- β 1 signalling was associated with increased Smad7 protein expression. Δ mts-IHG-1 modulated TGF- β 1 activity by increasing Smad7 protein expression as it failed to inhibit TGF- β 1 transcriptional responses when endogenous Smad7 expression was knocked down. These data indicate that mitochondria modulate TGF- β 1 signal transduction and that IHG-1 is a key player in this modulation.

© 2013 Elsevier B.V. All rights reserved.

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)- β 1 and regulates mitochondrial biogenesis by stabilising the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 α [3,4]. Increased activation of TGF- β 1 is believed to be a pivotal mediator of disease development in the diabetic kidney and in other fibrotic disorders [5,6].

TGF- β 1 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- β 1 signal transduction by complexing with the TGF- β type I receptor (T β R1) 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 (T β R1) [11–14] and by facilitating dephosphorylation of T β R1 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- β 1 target gene transcription [17–19]. Here we report that a non-mitochondrial deletion mutant of IHG-1 increases Smad7 levels and inhibits TGF β 1 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- β 1 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

* Corresponding author. Tel.: +353 1 7166818.

E-mail address: madeline.murphy@ucd.ie (M. Murphy).

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 Arbor, 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

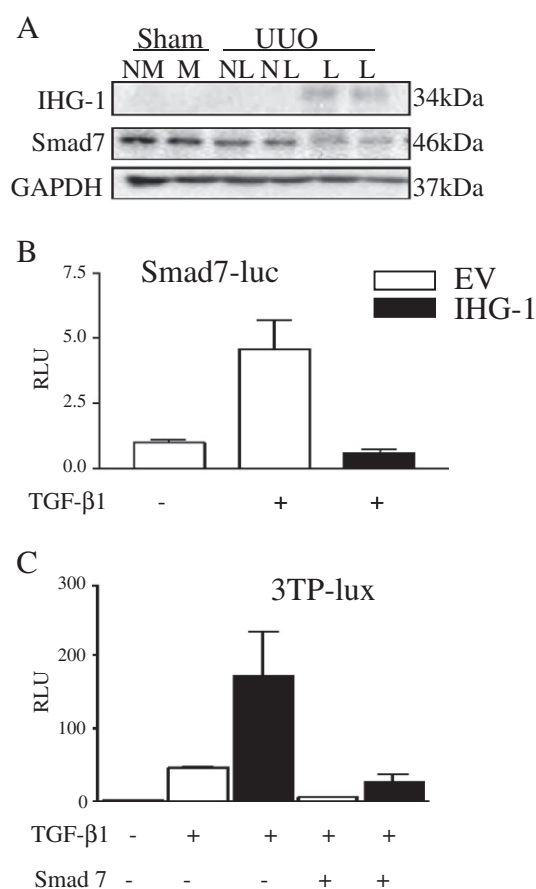


Fig. 1. IHG-1 decreases Smad7 expression. **A.** Altered expression in a 3-d UUO model. Total protein extracts were prepared from non manipulated (NM) sham, M (manipulated) (sham), L (ligated) NL (non-ligated) rat kidneys. Shown are representative western analyses of these extracts probed for IHG-1, Smad7 and GAPDH. **B.** HK2 cells were co-transfected with Smad 7-luciferase reporter and pRL-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-Luc reporter and pRL-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 licensee or by licenced technicians in accordance with institutional, ethical, and legal guidelines.

2.7. Statistical analysis

Results are expressed as mean $\pm$ 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- β 1 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- β 1 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

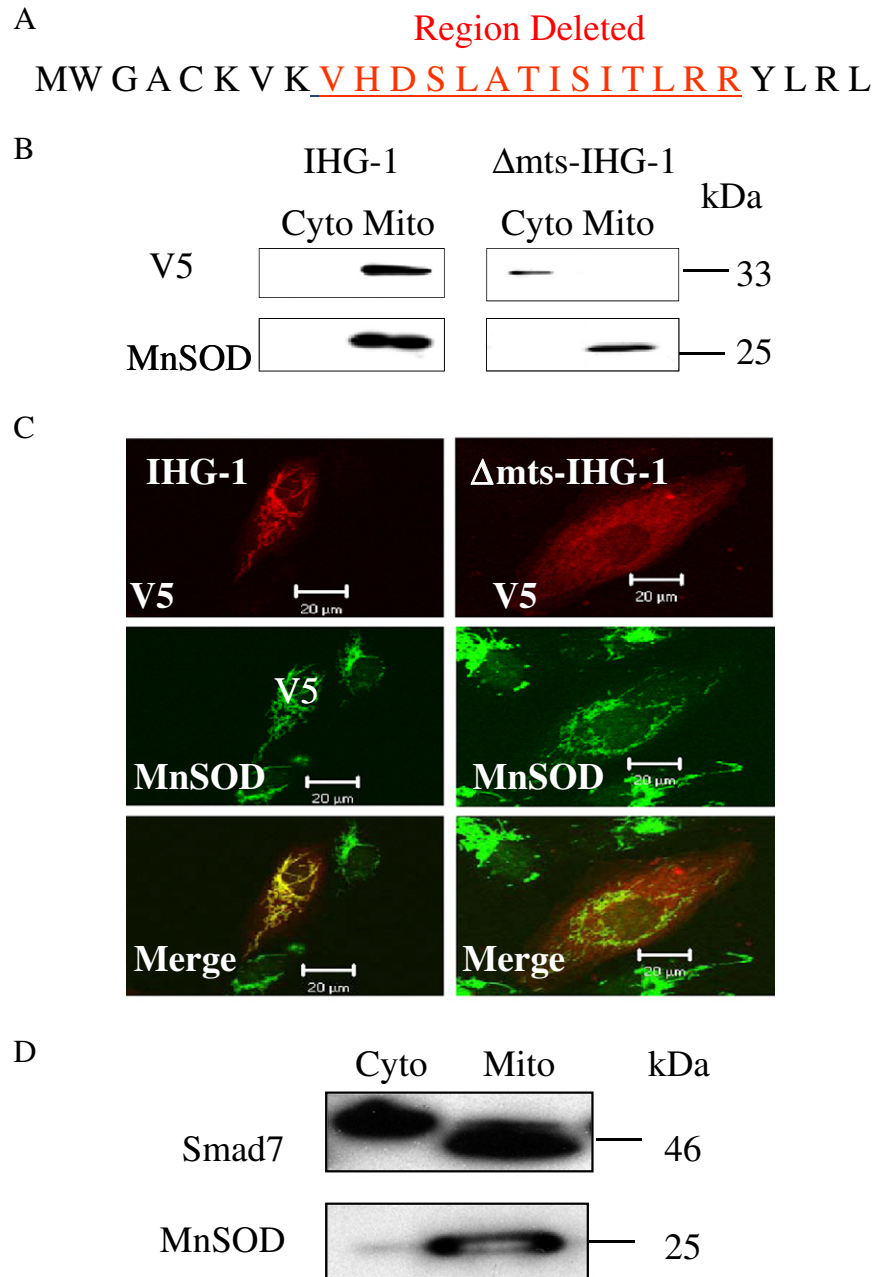


Fig. 2. Mitochondrial targeting sequence (mts) is required to localise IHG-1 to mitochondria. A. Schematic representation of region deleted from IHG-1. Sequence encoding amino acids coloured red (9–22) were deleted from IHG-1 to generate Δ mts-IHG-1. B. Cytoplasmic (C) and mitochondrial (M) protein extracts were prepared from HK-2 cells that had been transfected with IHG-1-V5 or Δ mts-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- β 1 responses by altering Smad7 expression. We report that IHG-1 inhibited TGF- β 1 mediated transcription from Smad7-luc (Fig. 1B). TGF- β 1 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- β 1 induced reporter gene expression (Fig. 1C). These data indicate that IHG-1 modulates TGF- β 1 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 (Δ mts-IHG-1-V5) was expressed in HK-2 cells. In contrast to IHG-1-V5, Δ mts-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. Δ mts-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 Δ mts-IHG-1. Δ mts-IHG-1 increased the expression of Smad7 mRNA (Fig. 3A). Stimulation with TGF- β 1 did not alter Smad7 protein expression in HK-2 cells (Fig. 3B) similar to the findings in primary human renal cells (HKCs) [27]. Δ mts-IHG-1 resulted in an increased Smad7 protein expression (Fig. 3B). Δ mts-IHG-1 expression significantly enhanced transcription from a transfected Smad7 promoter reporter construct (Fig. 3C). In the absence of TGF- β 1 stimulation levels of reporter gene expression were on average six-fold greater in HK-2 cells expressing Δ mts-IHG-1 (Fig. 2C). Following TGF- β 1 stimulation Δ mts-IHG-1 doubled the levels of reporter gene expression (20-fold vs 10-fold). These data indicate that Δ mts-IHG-1 inhibits TGF- β 1 signalling by increasing Smad7 expression.

3.4. Δ mts-IHG-1 inhibits TGF- β 1 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 Δ mts-IHG-1 was associated with the inhibition of TGF- β 1-induced fibrotic responses (CTGF and fibronectin protein expression) (Fig. 4A) in the HK-2 cell line. Δ mts-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 Δ mts-IHG-1 induced Smad7 expression may inhibit TGF- β 1 activity by inhibiting T β R1 initiated cellular responses. Increased expression of T β R1 is a feature of tubulointerstitial fibrosis [30]. Overexpression of constitutively active T β R1 in HK-2

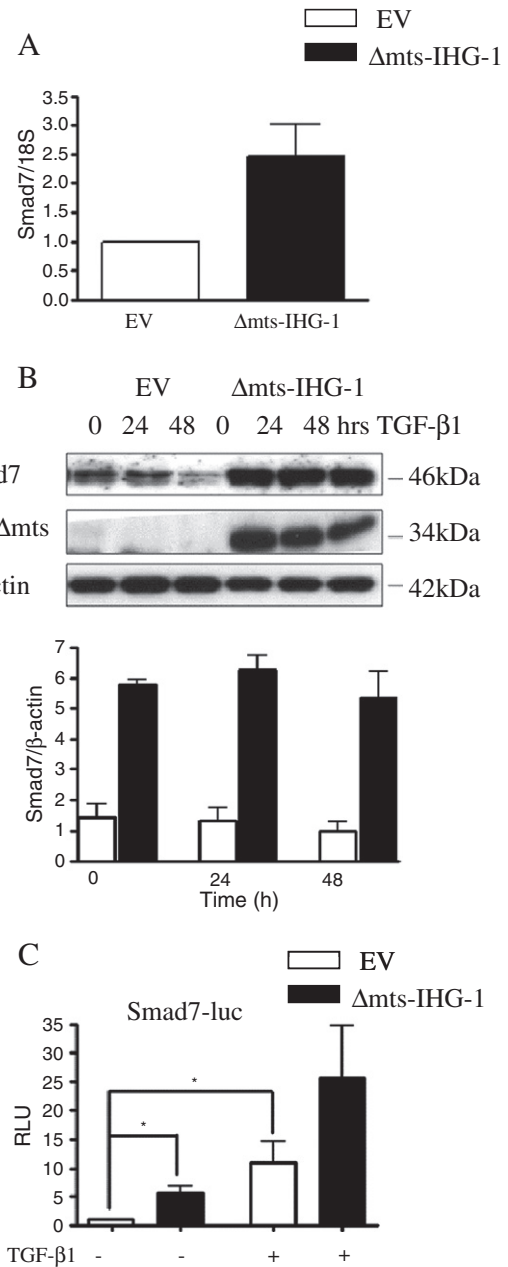


Fig. 3. Δ mts-IHG-1 increases Smad7 expression. A. Smad7 mRNA in EV or Δ mts-IHG-1 HK-2 cell lines were measured by qRT-PCR. B. Protein extracts were prepared from cells in A $\pm$ TGF- β 1 (5.0 ng/ml) stimulation for times indicated. Shown are representative western analyses of these extracts probed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

cells led to an increased protein expression of fibronectin, CTGF and jagged-1, Δ mts-IHG-1 inhibited T β R1 induced expression levels of these proteins (Fig. 4B) further evidence that it could inhibit profibrotic responses initiated from T β R1 in renal epithelial cells.

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

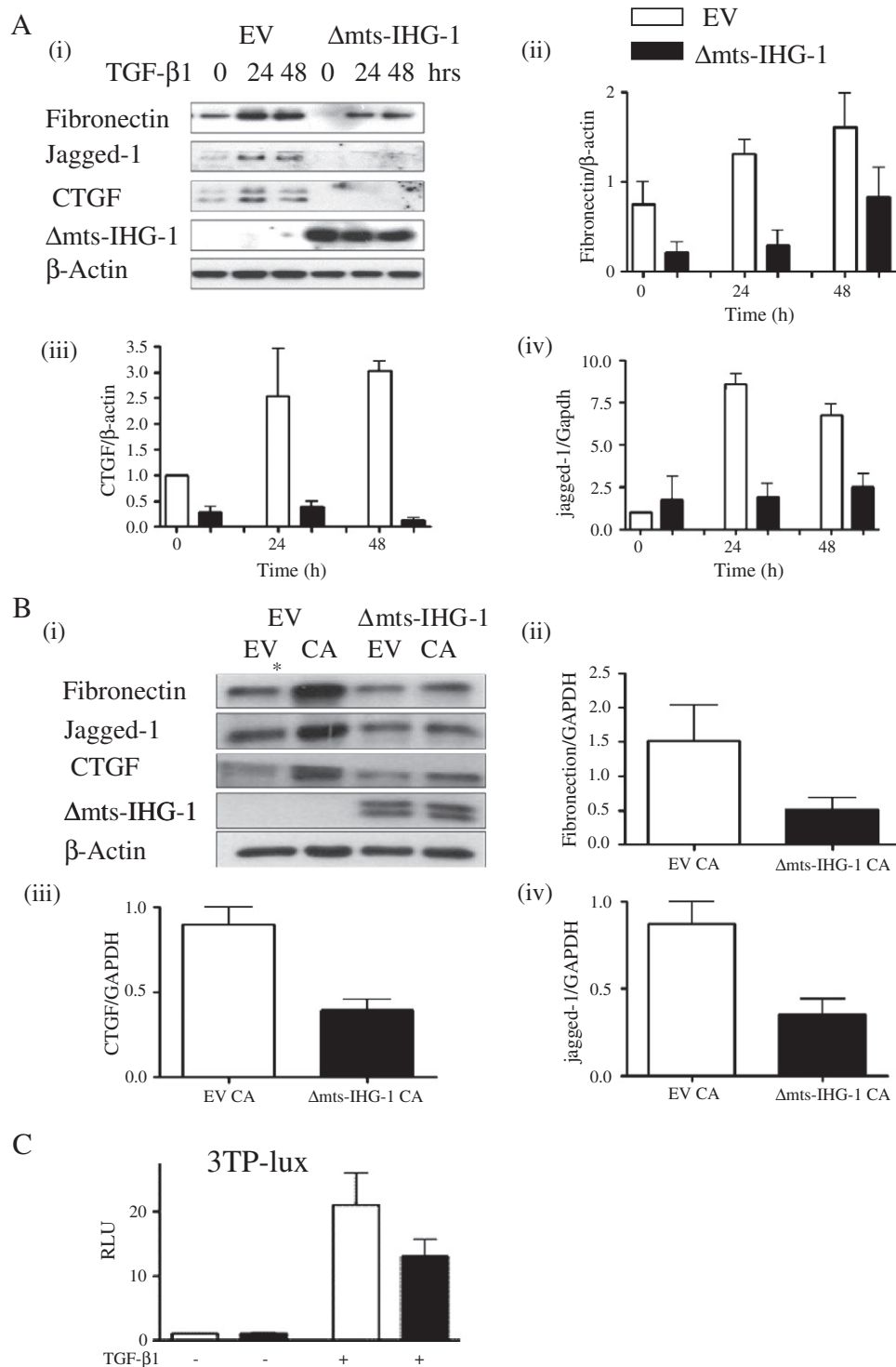


Fig. 4. Δ mts-IHG-1 inhibits TGF- β 1 profibrotic responses. A. Protein extracts were prepared from stably transfected empty vector (EV) and Δ mts-IHG-1 HK-2 cells $\pm$ TGF- β 1 (5.0 ng/ml) stimulation for times indicated. Shown are (i) representative western analyses of these extracts probed for V5, 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 $\pm$ 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 Δ mts-IHG-1 cell lines that had been transiently transduced with lentivirus overexpressing T β R1 (constitutively active (CA)) or EV control lentivirus. Experimental analysis and presentation of results are as in Fig. 4A. C. EV and Δ mts-IHG-1 HK-2 cells $\pm$ TGF- β 1 (5.0 ng/ml) stimulation for times indicated co-transfected with the 3TP-lux reporter and pRL-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- β 1-induced transcriptional responses, loss of mitochondrial localisation leads to increases in Smad7 expression and inhibition of TGF- β 1 induced profibrotic responses.

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

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

suggesting that Δ mts-IHG-1 modulates TGF- β 1 responses independently of R-Smad phosphorylation. Δ mts-IHG-1 did not alter shuttling of phosphorylated R-Smads to the nucleus following TGF- β 1

stimulation (Fig. 5B & C). Although Δ mts-IHG-1 inhibited TGF- β 1 mediated transcription from a PAI promoter reporter construct (3TP-lux) (Fig. 4C) it did not alter responses to TGF- β 1 from a Smad3 responsive

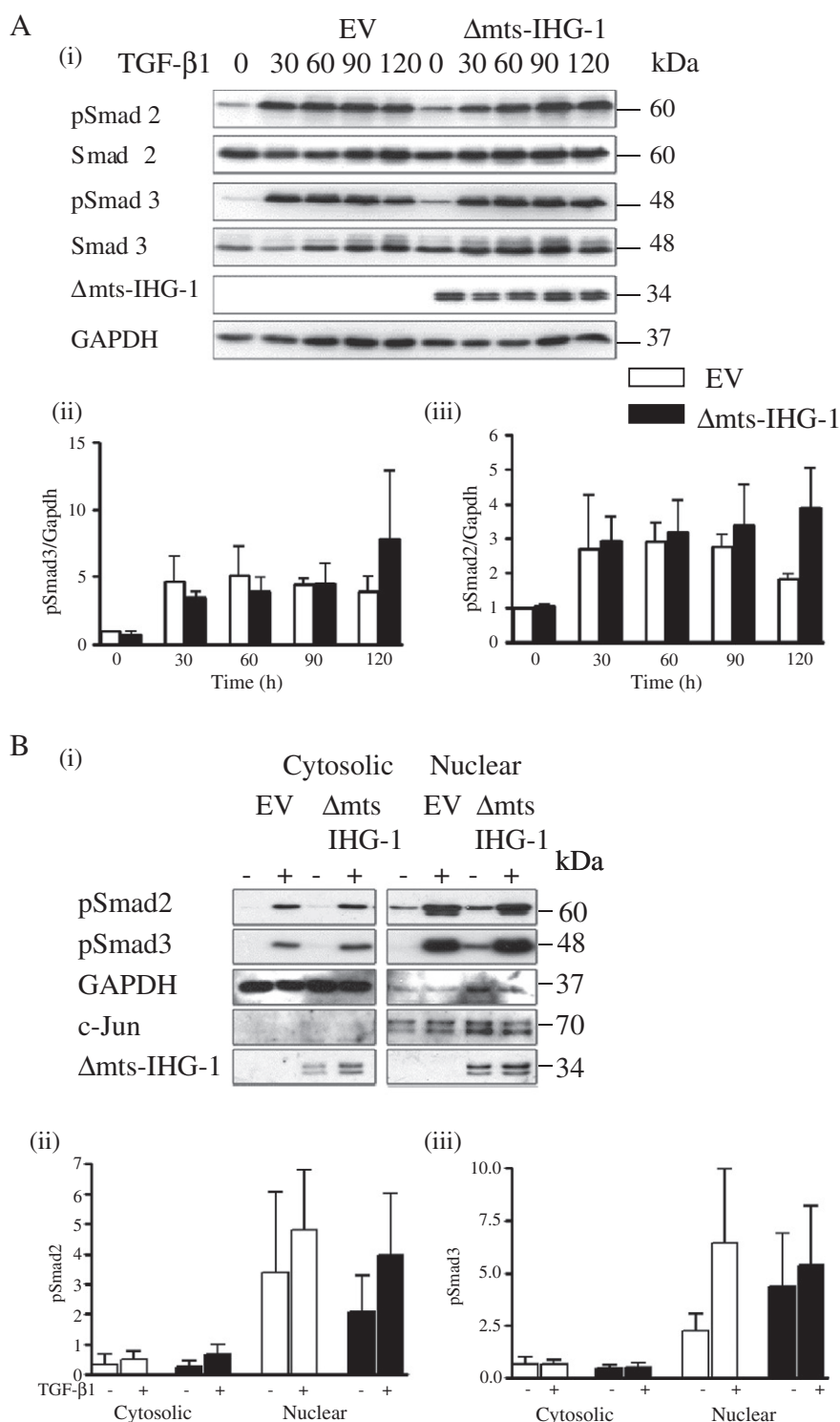
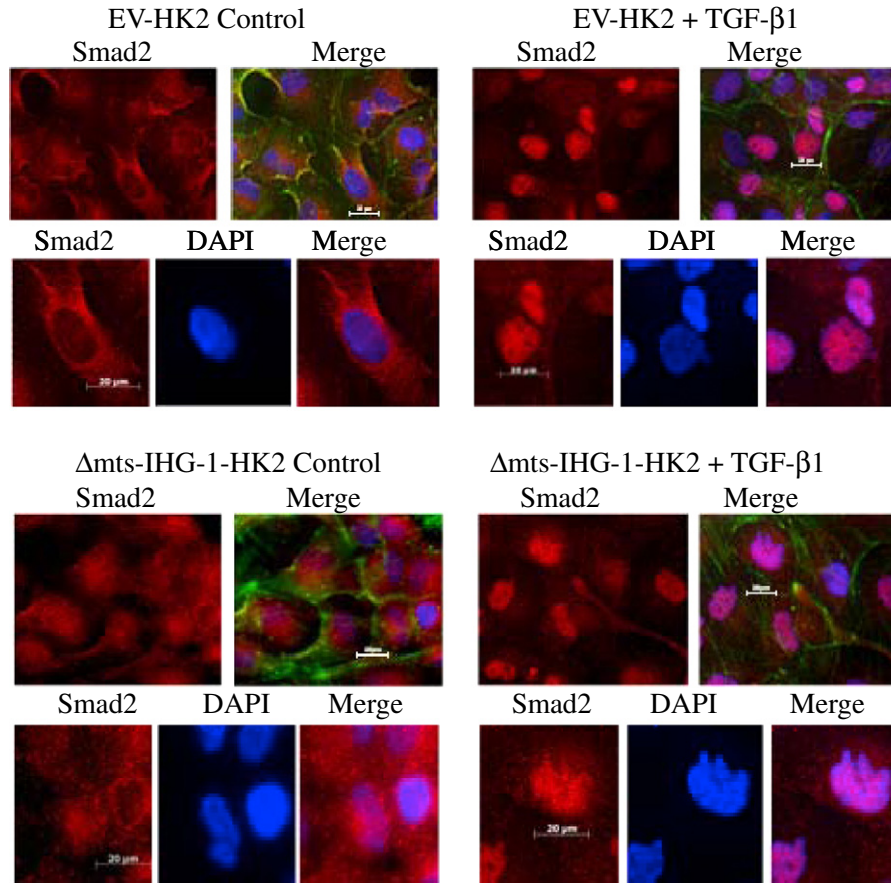
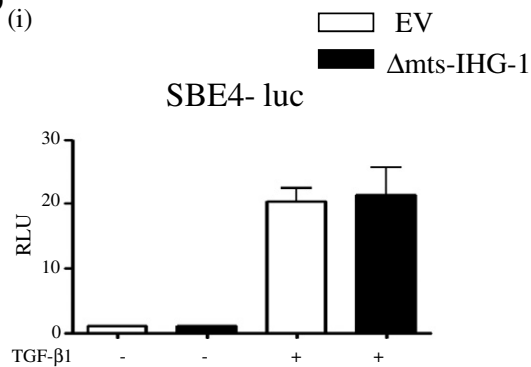


Fig. 5. Δ mts-IHG-1 does not alter Smad phosphorylation, translocation or ability to bind SBE. A. Protein extracts were prepared from EV or Δ mts-IHG-1 HK-2 cell lines $\pm$ 5 ng/ml TGF- β 1 stimulation at times indicated. Shown are (i) western analyses of these extracts probed for V5, pSmad2, Smad2, pSmad3, Smad3 and GAPDH. Alterations in protein expression for (ii) pSmad2, (iii) pSmad3 were measured using densitometric analysis. The results shown are means $\pm$ 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- β 1 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 $\pm$ s.e.m. of at least three independent experiments. Differences in means are not significant. C. EV or Δ mts-IHG-1 HK-2 cell lines $\pm$ 5 ng/ml TGF- β 1 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 Δ mts-IHG-1 HK-2 cells $\pm$ TGF- β 1 (5.0 ng/ml) stimulation for times indicated co-transfected with the (i) SBE-luc and pRL-CMV or (ii) SBE-Smad7 and pRL-CMV. Luciferase assay experimental analysis and presentation of results are as in Fig. 1B. Differences in means for (ii) are significant ($P < 0.05$).

C



D (i)



(ii)

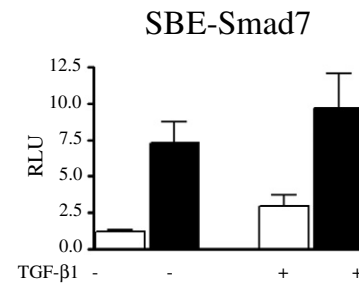


Fig. 5 (continued).

SBE4-luc [31] minimal promoter reporter construct (Fig. 5D(i)) suggesting that Δ mts-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- β 1 in mammalian cell lines (i.e. HeLa, Mv1Lu/L17, and 293 cells). Δ mts-IHG-1 significantly increased basal activity from the mutated Smad7 promoter SBE-Smad7 [32] in renal epithelial cells which was unaltered following TGF- β 1 stimulation. Surprisingly TGF- β 1 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. Δ mts-IHG-1 increases nuclear Smad7 expression and requires Smad7 to inhibit TGF- β 1 responses

Δ mts-IHG-1 was found to localise to both cytosolic and nuclear fractions (Fig. 6A). Δ mts-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 Δ mts-IHG-1 inhibits TGF- β 1 signalling by increasing Smad7 expression in the nucleus. To determine whether Smad7 expression was necessary for Δ mts-IHG-1 to modulate TGF- β 1 mediated transcriptional responses, we investigated the effects of short hairpin microRNA (shRNAmir) induced loss of Smad7 expression on the ability of Δ mts-IHG-1 to inhibit TGF- β 1 transcriptional responses.

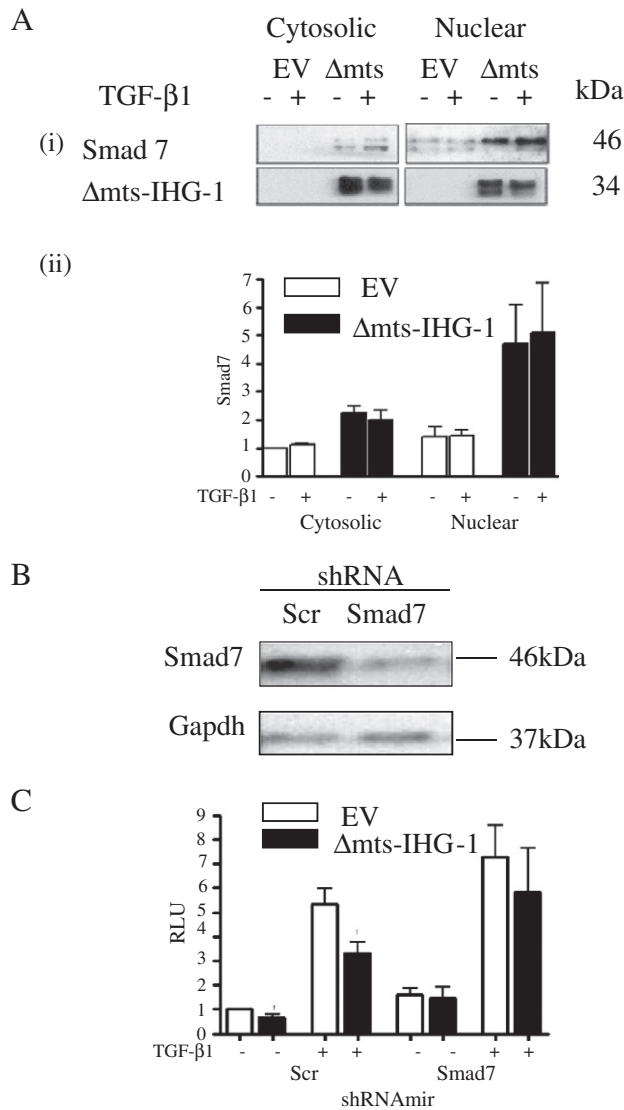


Fig. 6. Δmts-IHG-1 needs Smad7 to inhibit TGF-β responses. **A.** Cytosolic and nuclear extracts were prepared from EV and Δmts-IHG-1 cell lines following stimulation with 5 ng/ml TGF-β1 for 60 min. Shown are western analyses of these extracts probed for Smad7 and V5. Alterations in protein expression 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 RNAir (Fig. 6B). Δmts-IHG-1 over-expression significantly inhibited TGF-β1 mediated expression from 3TP-lux when transfected with a plasmid expressing scrambled shRNAir (Fig. 6C). In contrast in cells transfected with shRNAir 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.

Δ mts-IHG-1 increased expression of Smad7 mRNA, protein and promoter activity and inhibited TGF- β 1 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. Δ mts-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 Δ mts-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- β 1 target genes. When Smad7 expression was knocked down by shRNA, Δ mts-IHG-1 no longer altered TGF- β 1 transcriptional responses indicating that Δ mts-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- β 1 signal transduction. Expression of Δ mts-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. Δ mts-IHG-1 failed to inhibit TGF- β 1 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]. Δ mts-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 Δ mts-IHG-1 still poses difficulties for delivery. Our future aim is to determine the regions within Δ mts-IHG-1 that are necessary to induce the expression of Smad7 and thus develop a more readily deliverable therapeutic. We have renamed Δ mts-IHG-1 as ITA1 (inhibitor of TGF- β 1 activity 1) and propose ITA1 as a novel biotherapeutic for the treatment of fibrotic renal disease.

Acknowledgements

This work was funded by the Science Foundation Ireland (J.C., S. M^cC., U.B., F.M., C.G., M.M, grant numbers SFI/06/IN.1/B114 and SFI/RFP06/BIC304), Wellcome Trust (F.M., C.G., M.M.), the Health Research Board (B.G., F.H. D.H.) and the Government of Ireland Programme for Research in Third Level Institutions. E.B. was a recipient of an Irish Research Council studentship. This work was presented in part at the American Society of Nephrology (ASN) annual meeting 2010, San Diego, USA. We thank Catherine Moss for technical

assistance in real-time PCR in this study. We acknowledge receipt of reagents from Dr. Rik Derynck (University of California, San Francisco, CA), Dr Fang Liu (Rutgers University, Piscataway, NJ), Dr. Joan Massague (Sloan Kettering Institute, New York, NY), Dr Ralf Janknecht (University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma) and Dr Bert Voglestein (John Hopkins University, Baltimore, MD).

References

- [1] M. Murphy, C. Godson, S. Cannon, S. Kato, H.S. Mackenzie, F. Martin, H.R. Brady, Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells, *J. Biol. Chem.* 274 (1999) 5830–5834.
- [2] M.R. Clarkson, M. Murphy, S. Gupta, T. Lambe, H.S. Mackenzie, C. Godson, F. Martin, H.R. Brady, High glucose-altered gene expression in mesangial cells. Actin-regulatory protein gene expression is triggered by oxidative stress and cytoskeletal disassembly, *J. Biol. Chem.* 277 (2002) 9707–9712.
- [3] M. Murphy, N.G. Docherty, B. Griffin, J. Howlin, E. McArdle, R. McMahon, H. Schmid, M. Kretzler, A. Drogue, C.E. Runyan, S.C. Hubchak, R.K. Basu, J.F. Curley, R.D. Smith, T. Hayashida, TGF-beta signal transduction in chronic kidney disease, *Front. Biosci.* 14 (2009) 2448–2465.
- [4] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor beta in human disease, *N. Engl. J. Med.* 342 (2000) 1350–1358.
- [5] S. Itoh, M. Landstrom, A. Hermansson, F. Itoh, C.H. Heldin, N.E. Heldin, P. ten Dijke, Transforming growth factor beta1 induces nuclear export of inhibitory Smad7, *J. Biol. Chem.* 273 (1998) 29195–29201.
- [6] A. Nakao, M. Afrakhte, A. Moren, T. Nakayama, J.L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N.E. Heldin, C.H. Heldin, P. ten Dijke, Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling, *Nature* 389 (1997) 631–635.
- [7] H. Hayashi, S. Abdollah, Y. Qiu, J. Cai, Y.Y. Xu, B.W. Grinnell, M.A. Richardson, J.N. Topper, M.A. Gimbrone Jr., J.L. Wrana, D. Falb, The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling, *Cell* 89 (1997) 1165–1173.
- [8] S. Souchevnytskyi, T. Nakayama, A. Nakao, A. Moren, C.H. Heldin, J.L. Christian, P. ten Dijke, Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors, *J. Biol. Chem.* 273 (1998) 25364–25370.
- [9] P. Kavsak, R.K. Rasmussen, C.G. Causing, S. Bonni, H. Zhu, G.H. Thomsen, J.L. Wrana, Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation, *Mol. Cell* 6 (2000) 1365–1375.
- [10] T. Ebisawa, M. Fukuchi, G. Murakami, T. Chiba, K. Tanaka, T. Imamura, K. Miyazono, Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation, *J. Biol. Chem.* 276 (2001) 12477–12480.
- [11] C. Suzuki, G. Murakami, M. Fukuchi, T. Shimanuki, Y. Shikauchi, T. Imamura, K. Miyazono, Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane, *J. Biol. Chem.* 277 (2002) 39919–39925.
- [12] Y. Tajima, K. Goto, M. Yoshida, K. Shinomiya, T. Sekimoto, Y. Yoneda, K. Miyazono, T. Imamura, Chromosomal region maintenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-beta signaling by Smad7, *J. Biol. Chem.* 278 (2003) 10716–10721.
- [13] W. Shi, C. Sun, B. He, W. Xiong, X. Shi, D. Yao, X. Cao, GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor, *J. Cell Biol.* 164 (2004) 291–300.
- [14] S. Zhang, T. Fei, L. Zhang, R. Zhang, F. Chen, Y. Ning, Y. Han, X.H. Feng, A. Meng, Y.G. Chen, Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation, *Mol. Cell. Biol.* 27 (2007) 4488–4499.
- [15] E. Gronroos, U. Hellman, C.H. Heldin, J. Ericsson, Control of Smad7 stability by competition between acetylation and ubiquitination, *Mol. Cell* 10 (2002) 483–493.
- [16] M. Simonsson, C.H. Heldin, J. Ericsson, E. Gronroos, The balance between acetylation and deacetylation controls Smad7 stability, *J. Biol. Chem.* 280 (2005) 21797–21803.
- [17] S. Kume, M. Haneda, K. Kanazaki, T. Sugimoto, S. Araki, K. Isshiki, M. Isono, T. Uzu, L. Guarente, A. Kashiwagi, D. Koya, SIRT1 inhibits transforming growth factor beta-induced apoptosis in glomerular mesangial cells via Smad7 deacetylation, *J. Biol. Chem.* 282 (2007) 151–158.
- [18] X.H. Feng, R. Derynck, Ligand-independent activation of transforming growth factor (TGF) beta signaling pathways by heteromeric cytoplasmic domains of TGF-beta receptors, *J. Biol. Chem.* 271 (1996) 13123–13129.
- [19] E. Borgeson, N.G. Docherty, M. Murphy, K. Rodgers, A. Ryan, T.P. O'Sullivan, P.J. Guiry, R. Goldschmeding, D.F. Higgins, C. Godson, Lipoxin A and benzo-lipoxin A attenuate experimental renal fibrosis, *FASEB J.* 25 (2011) 2967–2979.
- [20] M.J. Ryan, G. Johnson, J. Kirk, S.M. Fuerstenberg, R.A. Zager, B. Torok-Storb, HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney, *Kidney Int.* 45 (1994) 48–57.
- [21] X. Yan, Y.G. Chen, Smad7: not only a regulator, but also a cross-talk mediator of TGF-beta signalling, *Biochem. J.* 434 (2011) 1–10.

- [24] A.C. Chung, X.R. Huang, L. Zhou, R. Heuchel, K.N. Lai, H.Y. Lan, Disruption of the Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice, *Nephrol. Dial. Transplant.* 24 (2009) 1443–1454.
- [25] H.Y. Chen, X.R. Huang, W. Wang, J.H. Li, R.L. Heuchel, A.C. Chung, H.Y. Lan, The protective role of Smad7 in diabetic kidney disease: mechanism and therapeutic potential, *Diabetes* 60 (2011) 590–601.
- [26] P.F. Hsieh, S.F. Liu, T.C. Lee, J.S. Huang, L.T. Yin, W.T. Chang, L.Y. Chuang, J.Y. Guh, M.Y. Hung, Y.L. Yang, The role of IL-7 in renal proximal tubule epithelial cells fibrosis, *Mol. Immunol.* 50 (2012) 74–82.
- [27] F.Y. Liu, X.Z. Li, Y.M. Peng, H. Liu, Y.H. Liu, Arkadia regulates TGF-beta signaling during renal tubular epithelial to mesenchymal cell transition, *Kidney Int.* 73 (2008) 588–594.
- [28] R. Rampal, K.B. Luther, R.S. Haltiwanger, Notch signaling in normal and disease states: possible therapies related to glycosylation, *Curr. Mol. Med.* 7 (2007) 427–445.
- [29] M. Murea, J.K. Park, S. Sharma, H. Kato, A. Gruenewald, T. Niranjani, H. Si, D.B. Thomas, J.M. Pullman, M.L. Melamed, K. Susztak, Expression of Notch pathway proteins correlates with albuminuria, glomerulosclerosis, and renal function, *Kidney Int.* 78 (2010) 514–522.
- [30] F.Y. Liu, X.Z. Li, Y.M. Peng, H. Liu, Y.H. Liu, Arkadia-Smad7-mediated positive regulation of TGF-beta signaling in a rat model of tubulointerstitial fibrosis, *Am. J. Nephrol.* 27 (2007) 176–183.
- [31] L. Zawel, J.L. Dai, P. Buckhaults, S. Zhou, K.W. Kinzler, B. Vogelstein, S.E. Kern, Human Smad3 and Smad4 are sequence-specific transcription activators, *Mol. Cell* 1 (1998) 611–617.
- [32] N.G. Denissova, F. Liu, Repression of endogenous Smad7 by Ski, *J. Biol. Chem.* 279 (2004) 28143–28148.
- [33] M.T. Coughlan, D.R. Thorburn, S.A. Penfold, A. Laskowski, B.E. Harcourt, K.C. Sourris, A.L. Tan, K. Fukami, V. Thallas-Bonke, P.P. Nawroth, M. Brownlee, A. Bierhaus, M.E. Cooper, J.M. Forbes, RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes, *J. Am. Soc. Nephrol.* 20 (2009) 742–752.
- [34] J.M. Forbes, M.T. Coughlan, M.E. Cooper, Oxidative stress as a major culprit in kidney disease in diabetes, *Diabetes* 57 (2008) 1446–1454.
- [35] C. Michaeloudes, M.B. Sukkar, N.M. Khorasani, P.K. Bhavsar, K.F. Chung, TGF-beta regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells, *Am. J. Physiol. Lung Cell. Mol. Physiol.* 300 (2011) L295–L304.
- [36] R.N. Re, J.L. Cook, The mitochondrial component of intracrine action, *Am. J. Physiol. Heart Circ. Physiol.* 299 (2010) H577–H583.
- [37] M. Jullig, N.S. Stott, Mitochondrial localization of Smad5 in a human chondrogenic cell line, *Biochem. Biophys. Res. Commun.* 307 (2003) 108–113.
- [38] U.I. Heine, J.K. Burmester, K.C. Flanders, D. Danielpour, E.F. Munoz, A.B. Roberts, M.B. Sporn, Localization of transforming growth factor-beta 1 in mitochondria of murine heart and liver, *Cell Regul.* 2 (1991) 467–477.
- [39] L. Pang, T. Qiu, X. Cao, M. Wan, Apoptotic role of TGF-beta mediated by Smad4 mitochondria translocation and cytochrome c oxidase subunit II interaction, *Exp. Cell Res.* 317 (2011) 1608–1620.
- [40] C. Mitchell, M.A. Robin, A. Mayeuf, M. Mahrouf-Yorgov, A. Mansouri, M. Hamard, D. Couton, B. Fromenty, H. Gilgenkrantz, Protection against hepatocyte mitochondrial dysfunction delays fibrosis progression in mice, *Am. J. Pathol.* 175 (2009) 1929–1937.
- [41] W. Zhao, S.S. Chen, Y. Chen, R.A. Ahokas, Y. Sun, Kidney fibrosis in hypertensive rats: role of oxidative stress, *Am. J. Nephrol.* 28 (2008) 548–554.
- [42] W. Zhao, T. Zhao, Y. Chen, R.A. Ahokas, Y. Sun, Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats, *Mol. Cell. Biochem.* 317 (2008) 43–50.
- [43] A. Willaert, S. Khatiri, B.L. Callewaert, P.J. Coucke, S.D. Crosby, J.G. Lee, E.C. Davis, S. Shiva, M. Tsang, A. De Paepe, Z. Urban, GLUT10 is required for the development of the cardiovascular system and the notochord and connects mitochondrial function to TGFbeta signaling, *Hum. Mol. Genet.* 21 (6) (Mar 15 2012) 1248–1259.
- [44] M. Jain, S. Rivera, E.A. Monclus, L. Synenki, A. Zirk, J. Eisenbart, C. Feghali-Bostwick, G.M. Mutlu, G.R. Budinger, N.S. Chandel, Mitochondrial reactive oxygen species regulate transforming growth factor-beta signaling, *J. Biol. Chem.* 288 (2013) 770–777.
- [45] X.M. Meng, X.R. Huang, J. Xiao, A.C. Chung, W. Qin, H.Y. Chen, H.Y. Lan, Disruption of Smad4 impairs TGF-beta/Smad3 and Smad7 transcriptional regulation during renal inflammation and fibrosis in vivo and in vitro, *Kidney Int.* 81 (2012) 266–279.
- [46] G. Brodin, A. Ahgren, P. ten Dijke, C.H. Heldin, R. Heuchel, Efficient TGF-beta induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter, *J. Biol. Chem.* 275 (2000) 29023–29030.
- [47] X. Yan, Z. Liu, Y. Chen, Regulation of TGF-beta signaling by Smad7, *Acta Biochim. Biophys. Sin.* 41 (2009) 263–272.
- [48] H. Fukasawa, T. Yamamoto, A. Togawa, N. Ohashi, Y. Fujigaki, T. Oda, C. Uchida, K. Kitagawa, T. Hattori, S. Suzuki, M. Kitagawa, A. Hishida, Down-regulation of Smad7 expression by ubiquitin-dependent degradation contributes to renal fibrosis in obstructive nephropathy in mice, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 8687–8692.
- [49] M. Landstrom, N.E. Heldin, S. Bu, A. Hermansson, S. Itoh, P. ten Dijke, C.H. Heldin, Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells, *Curr. Biol.* 10 (2000) 535–538.
- [50] T. Miyake, N.S. Alli, J.C. McDermott, Nuclear function of Smad7 promotes myogenesis, *Mol. Cell. Biol.* 30 (2010) 722–735.
- [51] R.J. Akhurst, A. Hata, Targeting the TGFbeta signalling pathway in disease, *Nat. Rev. Drug Discov.* 11 (2012) 790–811.