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Overexpression of heterogeneous nuclear ribonucleoprotein F stimulates renal Ace-2 gene expression and prevents TGF-β1-induced kidney injury in a mouse model of diabetes

Chao-Sheng Lo1 · Yixuan Shi1 · Shiao-Ying Chang1 · Shaaban Abdo1 · Isabelle Chenier1 · Janos G. Filep2 · Julie R. Ingelfinger3 · Shao-Ling Zhang1 · John S. D. Chan1

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Abstract

Aims/hypothesis We investigated whether heterogeneous nuclear ribonucleoprotein F (hnRNP F) stimulates renal ACE-2 expression and prevents TGF-β1 signalling, TGF-β1 inhibition of Ace-2 gene expression and induction of tubulo-fibrosis in an Akita mouse model of type 1 diabetes.

Methods Adult male Akita transgenic (Tg) mice overexpressing specifically hnRNP F in their renal proximal tubular cells (RPTCs) were studied. Non-Akita littermates and Akita mice served as controls. Immortalised rat RPTCs stably transfected with plasmid containing either rat Hnrnpf cDNA or rat Ace-2 gene promoter were also studied.

Results Overexpression of hnRNP F attenuated systemic hypertension, glomerular filtration rate, albumin/creatinine ratio, urinary angiotensinogen (AGT) and angiotensin (Ang) II levels, renal fibrosis and profibrotic gene (Agt, Tgf-β1, TGF-β receptor II [Tgf-βrII]) expression, stimulated antifibrotic gene (Ace-2 and Ang 1–7 receptor [MasR]) expression, and normalised urinary Ang 1–7 level in Akita Hnrnpf-Tg mice as compared with Akita mice. In vitro, hnRNP F overexpression stimulated Ace-2 gene promoter activity, mRNA and protein expression, and attenuated Agt, Tgf-β1 and Tgf-βrII gene expression. Furthermore, hnRNP F overexpression prevented TGF-β1 signalling and TGF-β1 inhibition of Ace-2 gene expression.

Conclusions/interpretation These data demonstrate that hnRNP F stimulates Ace-2 gene transcription, prevents TGF-β1 inhibition of Ace-2 gene transcription and induction of kidney injury in diabetes. HnRNP F may be a potential target for treating hypertension and renal fibrosis in diabetes.

Keywords ACE-2 · Akita mice · Angiotensinogen · Diabetes · Heterogeneous nuclear ribonucleoprotein F · Hypertension · Renal fibrosis · TGF-β1

Abbreviations

ACR · Albumin/creatinine ratio
AGT · Angiotensinogen
Ang · Angiotensin
BW · Body weight
DN · Diabetic nephropathy
EMSA · Electrophoretic mobility shift assay
ESRD · End-stage renal disease
hnRNP F · Heterogeneous nuclear ribonucleoprotein F
KAP · Kidney-specific androgen-regulated protein
KW · Kidney weight
MasR · Angiotensin 1–7 receptor
Diabetic nephropathy (DN), a leading cause of end-stage renal disease (ESRD), accounts for ~50% of all ESRD cases [1, 2]. While glomerulopathy is a hallmark of early renal injury in DN [3], tubulointerstitial fibrosis and tubular atrophy are major features of late-stage DN and are closely associated with loss of renal function [4–7]. The mechanisms underlying tubulointerstitial fibrosis, however, are incompletely understood. TGF-β1 is considered to be the most potent inducer of fibrogenesis [8]. Indeed, patients and animal models with type 1 or 2 diabetes have significantly elevated serum and urinary TGF-β1 levels [9–11] as well as heightened TGF-β1 mRNA and protein expression in glomeruli and the tubulointerstitium [12–16].

We previously reported that high glucose milieu enhances expression of angiotensinogen (AGT, the sole precursor of all angiotensins) through generation of reactive oxygen species (ROS) in cultured rat renal proximal tubular cells (RPTCs) [17, 18]. Rat AGT overexpression in RPTCs leads to hypertension, albuminuria and RPTC hypertrophy, and enhances TGF-β1 expression in diabetic AGT-transgenic (Tg) mice [19, 20]. Conversely, RPTC-selective overexpression of catalase or pharmacological blockade of the renin–angiotensin system (RAS) attenuates hypertension, ROS generation, kidney injury and normalised RPTC ACE-2 expression in mouse models of diabetes [21–24]. Taken together, these observations indicate that oxidative stress-induced upregulation of AGT expression and downregulation of ACE-2 expression in RPTCs, resulting in higher angiotensin (Ang)II/Ang 1–7 ratio, may be key determinants of development of hypertension and nephropathy in diabetes.

We reported that insulin inhibits high glucose stimulation of rat renal Agt gene expression via two nuclear proteins—heterogeneous nuclear ribonucleoproteins F and K (hnRNP F, hnRNP K)—that interact with the insulin-responsive element (IRE) in the Agt gene promoter [25–28], and that hnRNP F overexpression in RPTCs inhibits Agt gene expression and kidney hypertrophy in Akita Hnrnpf-Tg mice [29]. Here, we report that overexpression of hnRNP F stimulates Ace-2 gene transcription and suppresses profibrotic gene (Tgf-β1, Tgf-βrII) expression in RPTCs of Akita Hnrnpf-Tg mice. We have confirmed these changes by in vitro studies in rat RPTCs. We also show that hnRNP F overexpression prevents TGF-β1 signalling and inhibition of Ace-2 gene expression in RPTCs. Finally, we identified the putative DNA response elements (REs) in the Ace-2 gene promoter that are responsive to hnRNP F and TGF-β1.

Introduction

Diabetic nephropathy (DN), a leading cause of end-stage renal disease (ESRD), accounts for ~50% of all ESRD cases [1, 2]. While glomerulopathy is a hallmark of early renal injury in DN [3], tubulointerstitial fibrosis and tubular atrophy are major features of late-stage DN and are closely associated with loss of renal function [4–7]. The mechanisms underlying tubulointerstitial fibrosis, however, are incompletely understood. TGF-β1 is considered to be the most potent inducer of fibrogenesis [8]. Indeed, patients and animal models with type 1 or 2 diabetes have significantly elevated serum and urinary TGF-β1 levels [9–11] as well as heightened TGF-β1 mRNA and protein expression in glomeruli and the tubulointerstitium [12–16].

We previously reported that high glucose milieu enhances expression of angiotensinogen (AGT, the sole precursor of all angiotensins) through generation of reactive oxygen species (ROS) in cultured rat renal proximal tubular cells (RPTCs) [17, 18]. Rat AGT overexpression in RPTCs leads to hypertension, albuminuria and RPTC hypertrophy, and enhances TGF-β1 expression in diabetic AGT-transgenic (Tg) mice [19, 20]. Conversely, RPTC-selective overexpression of catalase or pharmacological blockade of the renin–angiotensin system (RAS) attenuates hypertension, ROS generation, kidney injury and normalised RPTC ACE-2 expression in mouse models of diabetes [21–24]. Taken together, these observations indicate that oxidative stress-induced upregulation of AGT expression and downregulation of ACE-2 expression in RPTCs, resulting in higher angiotensin (Ang)II/Ang 1–7 ratio, may be key determinants of development of hypertension and nephropathy in diabetes.

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**Table 1** Physiological measurements

|                     | WT               | Hnrnpf-Tg        | Akita       | Akita Hnrnpf-Tg |
|---------------------|------------------|------------------|-------------|-----------------|
| Blood glucose (mmol/l) | 10.8±0.64        | 11.2±0.67        | 34.5±0.71*** | 35.1±0.79***    |
| SBP (mmHg)           | 110.7±2.71       | 113.8±2.67       | 133.4±2.59** | 121.5±3.52**    |
| KW (mg)              | 398.7±16.01      | 396.9±1.936      | 550.0±27.60** | 432.7±21.97††   |
| BW (g)               | 22.6±0.16        | 22.7±0.21        | 22.3±0.36    | 22.0±0.13       |
| KW/BW ratio          | 10.5±0.57        | 11.3±0.38        | 20.7±0.54**  | 16.6±1.15**     |
| KW/TL ratio          | 17.6±0.67        | 17.4±0.78        | 24.6±1.14**  | 18.7±1.25†      |
| GFR (μl min⁻¹ g⁻¹)   | 7.3±0.44         | 8.3±0.39         | 19.8±1.61**  | 16.2±0.85**††   |
| Urinary ACR (mg/mmol)| 1.8±0.33         | 1.8±0.35         | 13.6±3.25**  | 5.8±1.07††      |
| Urinary AGT/Cre ratio (pmol/μmol) | 1,418±242.4 | 1,439±137.5 | 4,512±753.6** | 2,804±204.7†† |
| Urinary Ang II/Cre ratio (pmol/μmol) | 19.56±6.065 | 19.5±7.964 | 299.38±89.06** | 133.05±12.68**† |
| Urinary Ang I–7/Cre ratio (pmol/μmol) | 17.97±1.807 | 18.30±2.019 | 10.99±0.734† | 17.45±1.238†† |

All data are expressed as means±SEM

* p<0.05, ** p<0.01 vs WT, † p<0.05, †† p<0.01 vs Akita mice

Blood glucose levels, following 4–5 h fasting, were determined with an Accu-Chek Performa System (Roche Diagnostics, Laval, QC, Canada). Body weight (BW) was recorded. Urine was collected and assayed for albumin/creatinine ratio (ACR) by enzyme-linked immunosorbent assays (Albuwell and Creatinine Companion, Exocell, Philadelphia, PA, USA). GFR was measured as described by Qi et al [32] as recommended by the Animal Models of Diabetic Complications Consortium (www.diacomp.org) with fluorescein isothiocyanate inulin [23, 28, 33].

Kidneys were immediately removed, then changed to serum-free DMEM, ensuring that endogenously secreted TGF-β1 would not interfere in the assay. After 45 min preincubation, active human recombinant TGF-β1 was added (considered as time 0 h) and incubated for various time periods up to 24 h. In separate experiments, RPTCs were incubated for 24 h in serum-free medium in the presence or absence of TGF-β1± various concentrations of SB431542.

**Immunohistochemical staining** Immunohistochemical staining was performed by the standard avidin-biotin-peroxidase complex method in four to five sections (4 μm thick) per kidney and three mouse kidneys per group (ABC Staining System; Santa Cruz Biotechnology [Santa Cruz, CA, USA]) [23, 24, 28, 29]. Staining was analysed under light microscopy by two independent, blinded observers. The collected images were assessed by National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/) [23, 24, 28, 29].

**Cell culture** Immortalised rat RPTCs (passages 12–18) [35] were cultured in 5 mmol/l d-glucose DMEM containing 5% FBS until they reached 60–70% confluence. The media were then changed to serum-free DMEM, ensuring that endogenously secreted TGF-β1 would not interfere in the assay. After 45 min preincubation, active human recombinant TGF-β1 [36] (0 to 10 ng/ml) was added (considered as time 0 h) and incubated for various time periods up to 24 h. In separate experiments, RPTCs were incubated for 24 h in serum-free medium in the presence or absence of TGF-β1± various concentrations of SB431542.

**Real-time quantitative PCR** Hnrnpf, Ace, Ace-2, MasR, Tgf-β1, Tgf-β3R1, Tgf-β3R2, collagen type IV, collagen type I, fibronectin 1 and β-actin mRNA expression levels in RPTs were quantified by real-time quantitative PCR (RT-qPCR) with forward and reverse primers (ESM Table 2) [23, 24, 28, 29].

**Western blotting** Western blotting (WB) was performed as described previously [23, 24, 28, 29]. The relative densities of hnrnp F, ACE, ACE-2, Ang 1–7 receptor (MasR), TGF-β1, TGF-β RI, TGF-β RII, fibronectin 1, p-Smad2/3, Smad2/3
and β-actin bands were quantified by computerised laser densitometry (ImageQuant software, version 5.1; Molecular Dynamics, Sunnyvale, CA, USA).

**Statistical analysis** The data are expressed as means±SEM. Statistical analysis was performed by the Student’s t test or one-way analysis of variance and the Bonferroni test as appropriate provided by Graphpad Software, Prism 5.0 (www.graphpad.com/prism/Prism.htm). A value of $p \leq 0.05$ was considered to be statistically significant.

**Results**

**Physiological variables in Akita and Akita Hnrnpf-Tg mice**

Table 1 documents significantly higher blood glucose levels in
Akita compared with WT mice and *Hnrnpf*-Tg mice. Overexpression of hnRNP F had no effect on blood glucose levels in Akita *Hnrnpf*-Tg mice. Systolic BP (SBP), kidney weight (KW)/BW and KW/tibial length (TL) ratios, GFR and ACR were all elevated in Akita mice, compared with both WT controls and *Hnrnpf*-Tg mice. HnRNP F overexpression in RPTCs markedly attenuated these changes in diabetic Akita *Hnrnpf*-Tg mice. Furthermore, Akita mice exhibited elevated urinary AGT and Ang II levels, parallel with decreased Ang 1–7 levels, compared with WT mice. HnRNP F overexpression partially reduced urinary AGT and Ang II levels, whereas it completely normalised urinary Ang 1–7 levels—a novel finding.

**Effect of hnRNP F overexpression on AGT, ACE, ACE-2 and MasR expression in Akita *Hnrnpf*-Tg mouse kidneys** Immunostaining revealed that HnRNP F (Fig. 1a) was overexpressed in RPTCs of *Hnrnpf*-Tg and Akita *Hnrnpf*-Tg mice compared with WT and Akita mice, respectively. ACE-2 (Fig. 1b) and MasR (Fig. 1c) expression was decreased in Akita mice compared with WT controls and normalised in Akita *Hnrnpf*-Tg mice. RPTC ACE (Fig. 1d) expression did not differ between WT and *Hnrnpf*-Tg mice, whereas ACE expression was significantly higher in Akita mice than in WT controls and was not normalised in Akita *Hnrnpf*-Tg mice. WB and RT-qPCR for hnRNP F, ACE-2, MasR and ACE protein and their mRNA levels (Fig. 1e–l, respectively) confirmed these observations.

**Effect of hnRNP F overexpression on TGF-β1, TGF-β RII and TGF-β RI expression in Akita *Hnrnpf*-Tg mouse kidneys** Immunostaining of TGF-β1 (Fig. 2a) and TGF-β RII (Fig. 2b), WB of TGF-β1 (Fig. 2d) and TGF-β RII expression TGF-β1 and TGF-β2 RI expression in mouse kidneys.

Immunohistochemical staining of TGF-β1 (a), TGF-β RII (b) and TGF-β RI (c) expression in kidney sections (×200), WB (d–f) and RT-qPCR (g–i) of their respective protein and mRNA levels in freshly isolated RPTs from non-diabetic WT controls, *Hnrnpf*-Tg (F-Tg) mice, diabetic Akita mice and Akita *Hnrnpf*-Tg mice (Akita F-Tg) at week 20. Values are means±SEM corrected to β-actin, n=6. *p<0.05; **p<0.01
(Fig. 2e), and RT-qPCR of Tgf-β1 (Fig. 2g) and Tgf-βrII (Fig. 2h) mRNA expression showed significantly higher TGF-β1 and TGF-β RII expression in RPTCs of Akita mice than in WT controls and Hnrnpf-Tg mice, and they were attenuated in Akita Hnrnpf-Tg mice. In contrast, TGF-β RI expression was similar in all groups studied (Fig. 2c,f,i).

**HnRNP F overexpression suppresses renal fibrosis in Akita Hnrnpf-Tg mice** Akita mice developed renal structural damage compared with WT and Hnrnpf-Tg mice (ESM Fig. 1a, PAS staining), including tubular luminal dilatation with accumulation of cell debris, increased extracellular matrix proteins in glomeruli and tubules, and proximal tubule cell atrophy. HnRNP F overexpression markedly reversed but never completely resolved these abnormalities in Akita mice. We detected significant increases in Masson’s trichrome staining (Fig. 3a) and immunostaining for collagen type IV (Fig. 3b), fibronectin 1 expression (Fig. 3c) and collagen type I (Fig. 3d) in glomerulotubular areas in Akita mice compared with WT controls and Hnrnpf-Tg mice. These changes were reduced in Akita Hnrnpf-Tg mice. Quantification of Masson’s trichrome-stained (ESM Fig. 1b), immunostaining of collagen IV (Fig. 3e), fibronectin 1 (Fig. 3f) and collagen I (Fig. 3g), and RT-qPCR quantification of mRNA levels (Fig. 3h–j) confirmed their expression.

**HnRNP F overexpression enhances Ace-2 and suppresses Agt, Tgf-βI and Tgf-βrII gene expression and protein**

![Image](Image1.png)

**Fig. 3** hNRP F overexpression attenuates renal fibrosis and profibrotic gene expression in mouse kidneys. Masson’s trichrome staining (a), immunostaining of collagen IV (Col IV) (b), fibronectin 1 (FN1) (c) and collagen I (Col I) (d) expression in kidney sections (×200); semiquantitative analysis of immunostained collagen IV (e), fibronectin 1 (f) and collagen I (g), and RT-qPCR of collagen IV (also known as Col4a1) (h), Fn1 (i) and collagen I (also known as Col1a1) (j) mRNA expression in freshly isolated RPTs from WT control mice, Hnrnpf-Tg mice (F-Tg), Akita mice and Akita Hnrnpf-Tg mice (Akita F-Tg) at week 20. Values are mean±SEM corrected to β-actin, n=6.

*p<0.05; **p<0.01
levels in rat RPTCs in vitro RPTCs stably transfected with pcDNA 3.1/Hnrnpf (RPTC-pcDNA 3.1/Hnrnpf) exhibited considerably higher levels of hnRNP F (Fig. 4a,b), lower amounts of AGT (Fig. 4a,c) and a higher amount of ACE-2 (Fig. 4a,d) than non-transfected RPTCs or RPTCs stably transfected with pcDNA 3.1 (RPTC-pcDNA 3.1).

In contrast, TGF-β1 and TGF-β RII protein levels were significantly decreased in RPTC-pcDNA 3.1/Hnrnpf compared with non-transfected RPTCs or RPTCs stably transfected with pcDNA 3.1 (RPTC-pcDNA 3.1) (p<0.01) (Fig. 4e,f,g, respectively). TGF-β R1 protein level was similar in non-transfected RPTCs, RPTC-pcDNA 3.1 or RPTC-pcDNA 3.1/Hnrnpf (Fig. 4h).

RT-qPCR of Hnrnpf, Agt, Ace-2, Tgf-β1, Tgf-βR1 and Tgf-βR1 mRNA levels confirmed these findings (ESM Fig. 2a–f).

TGF-β1 signalling and inhibition of Ace-2 gene expression in rat RPTCs TGF-β1 inhibited rat Ace-2 gene promoter activity (Fig. 5a), rat Ace-2 mRNA expression (Fig. 5b) and rat ACE-2 protein level (Fig. 5c) in a concentration-dependent manner, which was reversed by SB431542 (a TGF-β RI inhibitor) (Fig. 5d–f, respectively). Furthermore, TGF-β1 stimulated Smad 2/3 phosphorylation in a concentration- and time-dependent manner (Fig. 5g) and reversed by SB431542 (Fig. 5h). These data demonstrate that TGF-β1 inhibition of Ace-2 gene transcription is mediated, at least in part, via Smad2/3 signalling.

HnRNP F overexpression prevents TGF-β signalling, and TGF-β inhibition of Ace-2 and induction of fibrotic gene expression in RPTCs TGF-β1 had no detectable effect on hnRNP F protein levels (Fig. 6a,b). Intriguingly, hnRNP F overexpression prevented TGF-β1 stimulation of Smad 2/3 phosphorylation (Fig. 6a,c), TGF-β RI expression (Fig. 6a,d) and fibronectin 1 expression (Fig. 6a,e). HnRNP F overexpression also prevented TGF-β1-induced downregulation of MasR (Fig. 6a,f) content in RPTCs. Addition of TGF-β1 did not affect TGF-β RI expression in RPTCs (Fig. 6a,g).

Furthermore, overexpression of hnRNP F prevented the inhibitory effect of TGF-β1 on Ace-2 protein (Fig. 6a,h) and Ace-2 mRNA (Fig. 6i) expression in RPTC-pcDNA 3.1/Hnrnpf.

Localisation of Hnrnpf- and TGF-β1 (or SMAD)-RE-RE in Ace-2 gene promoter To localise the putative DNA-RE(s) that mediate(s) the action of hnRNP F or TGF-β1 on Ace-2 gene promoter activity, plasmids containing various lengths of the rat Ace-2 gene promoter were transiently transfected into RPTC-pcDNA 3.1 or RPTC-pcDNA 3.1/Hnrnpf. The activity of pGL4.20-Ace-2 promoter (N-1,091/+83) and pGL 4.20-Ace-2 promoter (N-499/+83) exhibited respective fivefold and 12-fold increase as compared with the control plasmid, pGL 4.20 in RPTC-pcDNA 3.1 (Fig. 7a). Further deletion of nucleotides N-499 to N-241 (pGL 4.20-Ace-2 promoter [N-240/+83]) significantly reduced the rat Ace-2 promoter activity. Moreover, the activity of pGL4.20-Ace-2 promoter (N-1,091/+83) and pGL4.20-Ace-2 promoter (N-499/+83) was further increased by 1.5–2.0-fold, whereas the activity of pGL4.20-Ace-2 promoter (N-240/+83) did not increase in RPTC-pcDNA 3.1/Hnrnpf as compared with RPTC-pcDNA 3.1 (Fig. 7a). Interestingly, addition of TGF-β1 inhibited the promoter activity of pGL 4.20-Ace-2 promoter (N-1,091/+83) and did not affect the activity of pGL 4.20-Ace-2 promoter (N-499/+83) and pGL 4.20-Ace-2 promoter (N-240/+83) in RPTC-pcDNA 3.1 (Fig. 7b).
However, TGF-β1 had no inhibitory effect on the promoter activity of these constructs in RPTC-pcDNA 3.1/Hnrnpf (Fig. 7c).

In contrast, transfection of Hnrnpf siRNA significantly inhibited the promoter activity of pGL 4.20-Ace-2 promoter (N-1,091/+83) and pGL 4.20-Ace-2 promoter (N-499/+83) without affecting the activity of pGL 4.20-Ace-2 promoter (N-240/+83) in RPTC-pcDNA 3.1 (Fig. 7d). Deletion of the nucleotides N-401 to N-393 (5′-ggggagaga-3′) in the Ace-2 gene promoter markedly attenuated the promoter activity of pGL 4.20-Ace-2 promoter (N-1,091/+83) and pGL 4.20-Ace-2 promoter (N-499/+83) in RPTC-pcDNA 3.1/Hnrnpf (Fig. 7e). Interestingly, deletion of the putative proximal SMAD-RE (nucleotides N-511 to N-504 [5′-cagagaca-3′]) or distal putative SMAD-RE2 (nucleotides N-789 to N-784 [5′-gagaca-3′]) in the Ace-2 gene promoter partially attenuated whereas deletion of both REs (nucleotides N-511 to N-504 and nucleotides N-789 to N-784) completely abolished the inhibitory action of TGF-β1 on pGL 4.20-Ace-2 promoter (N-1,091/+83) activity in RPTC-pcDNA 3.1 (Fig. 7f). Furthermore, EMSA showed that the double strand DNA fragments, nucleotides N-405 to N-387 (putative proximal SMAD-RE1) and nucleotides N-797 to N-776 (putative distal SMAD-RE2) bind to the nuclear proteins from RPTCs and they could be displaced by the respective WT DNA fragments, but not by mutated DNA fragments (Fig. 7g,h, respectively). Importantly, addition of anti-hnRNP F and anti-Smad 2/3 antibody induced a supershift of the respective Hnrnpf-RE and SMAD-REs with the nuclear proteins, respectively (Fig. 7g,h).
We reported previously that overexpression of hnRNP F prevents systemic hypertension, and inhibits renal Agt gene expression and RPTC hypertrophy in diabetic Akita Hnrfp-Tg mice [29]. The present paper provides new in vivo and in vitro evidence that hnRNP F stimulates Ace-2 gene transcription via binding to the DNA-RE of the Ace-2 gene promoter, which is critical for the formation of renal Ang I–7 and subsequent expression of its antihypertensive and renoprotective actions in Akita mice [37].

HnRNP F, a member of the pre-mRNA-binding protein family [38] regulates gene expression at both the transcriptional and post-transcriptional levels. Indeed, hnRNP F engages in alternative splicing of various genes [39–41] and associates with TATA-binding protein, RNA polymerase II, nuclear cap-binding protein complex and various transcriptional factors.[42, 43]

The Akita mouse is an autosomal-dominant model of spontaneous type 1 diabetes in which the Ins2 gene is mutated. Akita mice develop hyperglycaemia and systemic hypertension, leading to cardiac hypertrophy, left ventricular diastolic dysfunction, glomerulosclerosis and enhanced oxidative stress in RPTs, closely resembling those observed in patients with type 1 diabetes [44, 45].

Our study provides evidence for a novel mechanism for hnRNP F lowering of SBP: inhibition of intrarenal Agt gene expression and RAS activation, concomitant with upregulation of the ACE-2/Ang I–7/MasR axis. Indeed, our results show that hnRNP F overexpression inhibited renal AGT and Agt mRNA expression (ESM Fig. 1 c–e), lowered urinary AGT and Ang II levels and normalised urinary Ang I–7 levels.

We consistently observed decreased renal ACE-2 expression in Akita mice as previously reported [23, 24]. Decreased ACE-2 expression also has been reported in male streptozotocin (STZ)-induced diabetic mice [46], STZ-induced diabetic rats [47, 48] and human type 2 diabetic kidneys [49, 50].

The precise mechanism by which hnRNP F overexpression leads to upregulation of renal Ace-2 and MasR gene expression in diabetes remains unclear. One possibility is that hnRNP F binds to putative Hnrfp-RE(s) in the Ace-2 and MasR gene promoters, subsequently enhancing Ace-2 and MasR gene transcription. This possibility is supported by our findings that hnRNP F considerably augments the activity of an Ace-2 gene promoter and that the Hnrfp siRNA and deletion of the putative Hnrfp-RE markedly reduced the rat Ace-2 gene promoter activity in RPTCs. Furthermore, the biotinylated-labelled Hnrfp-RE specifically bound to RPTC nuclear proteins and the addition of anti-hnRNP F antibody yielded a supershift of biotinylated-labelled Hnrfp-RE binding with nuclear proteins in EMSA. These data demonstrate that hnRNP F binds to the putative Hnrfp-RE and stimulates Ace-2 gene transcription. Of note, hnRNP F is not specific for

**Discussion**

The present report identifies a novel mechanism by which hnRNP F prevents hypertension and kidney injury in diabetic Akita mice, i.e. hnRNP F stimulation of renal Ace-2 gene transcription and mitigation of the inhibitory effect of TGF-β1 on Ace-2 gene transcription.

Fig. 6 hnRNP F overexpression prevents TGF-β1 signalling, stimulation of profibrotic gene and inhibition of ACE-2 expression in rat RPTCs. (a) Immunoblotting of hnRNP F, Smad2/3 phosphorylation, TGF-β RI, TGF-β RI, fibronectin 1 (FN1), MasR and ACE2 levels in naive RPTCs, RPTC-pcDNA 3.1 or RPTC-pcDNA 3.1/Hnrfp in the presence or absence of TGF-β1 (2 ng/ml) after 24 h culture. Quantification of the level of hnRNP F (b), Smad2/3 phosphorylation (c), TGF-β RI (d), fibronectin 1 (e), MasR (f), TGF-β RI (g), ACE-2 (h) and Ace-2 mRNA (i). Values are mean±SEM, n=3. Similar results were obtained in three independent experiments. *p<0.05; **p<0.01
Fig. 7 Identification of Hnrnpf-RE and SMAD-RE in the Ace-2 gene promoter. (a) Luciferase activity of the plasmid containing various lengths of Ace-2 gene promoter in RPTC-pcDNA 3.1 (white bars) and in RPTC-pcDNA 3.1/Hnrnpf (black bars); (b) in RPTC-pcDNA 3.1±TGF-β1 (white bars, without hTGF-β1; black bars, with 2 ng/ml hTGF-β1); and (c) in RPTC-pcDNA3.1/Hnrnpf±TGF-β1 (white bars, without hTGF-β1; black bars, with 2 ng/ml hTGF-β1); (d) in RPTC-pcDNA 3.1±Hnrnpf siRNA (white bars, treated with 50 nM hHnrnpf siRNA; black bars, treated with 50 nM hHnrnpf siRNA); cultured in normal glucose media for 24 h. (e) Promoter activity of the Ace-2 gene ±Hnrnpf-RE in RPTC-pcDNA 3.1 (white bars) and in RPTC-pcDNA 3.1/Hnrnpf (black bars) or (f)±SMAD-REs in RPTC-pcDNA 3.1 in the absence or presence of TGF-β1 (white bars, without hTGF-β1; black bars, with 2 ng/ml hTGF-β1). Values are mean±SEM, n=6. The experiments were repeated twice. *p<0.05; **p<0.01. EMSA and supershift EMSA of the putative biotinylated Hnrnpf-RE (g) and biotinylated SMAD-REs (h) with RPTC nuclear proteins±excess unlabelled WT Hnrnpf-RE or mutated Hnrnpf-REs (M1 to M4 are mutants of Hnrnpf-RE with nucleotides mutated or deleted in the binding motif as shown in ESM Table 2) or WT SMAD-RE or mutant SMAD-REs (SMAD-RE1 [M1 and M2] and SMAD-RE2 [M1 and M2] are mutants of respective SMAD-RE1 and SMAD-RE2 with nucleotides mutated in the binding motif as shown in ESM Table 2). Rabbit IgG or rabbit anti-hnRNP F or anti-Smad2/3 antisera was added to the reaction mixture and incubated for 30 min on ice before incubation with the biotinylated probe. Results are representative of three independent experiments. SS, supershift band.
Ace-2 gene expression but also affects the expression of Agt [25] and other genes [51, 52].

Currently, little is known about the mechanisms by which TGF-β1 downregulates renal Ace-2 gene expression in diabetes. Chou et al [53] reported that SB431542 inhibited high glucose and TGF-β1 inhibition of Ace-2 mRNA expression in cultured NRK-52 cells. Our findings confirm these observations. Our present studies also demonstrate that TGF-β1 inhibits the activity of pGL 4.20-rat Ace-2 promoter (N-1, 091/+83) and that deletion of putative SMAD-REs in the Ace-2 gene promoter mitigates the inhibitory effect of TGF-β1 on the Ace-2 gene promoter activity. Furthermore, biotinylated-labelled SMAD-REs bound to RPTC nuclear proteins and the addition of anti-Smad2/3 antibody yielded a supershift of labelled DNA with nuclear proteins. These data demonstrate that the inhibitory effect of TGF-β1 on Ace-2 gene transcription is mediated, at least in part, via the SMAD-REs in the Ace-2 gene promoter.

Intriguingly, hnRNP F overexpression prevented TGF-β1 signalling on Smad2/3 phosphorylation and on TGF-β1 inhibition of Ace-2 gene promoter activity in RPTCs. At present, the underlying molecular mechanism of how hnRNP F prevents TGF-β1 inhibition of Ace-2 gene transcription is not yet defined. One possibility might be that hnRNP F directly inhibits Tgf-β1r II gene expression as shown in our studies. The second possibility is that hnRNP F might interfere or prevent the interaction of Smad2/3 with other transcriptional factor(s) to inhibit Ace-2 gene transcription. Clearly, more studies are needed to define the molecular interaction of hnRNP F with Smad2/3 on Ace-2 gene transcription.

In summary, the present study suggests a major role for hnRNP F in attenuating systemic hypertension and renal fibrosis in experimental diabetes and possibly in diabetic human kidneys. Our observations raise the possibility that selective targeting of this antihypertensive and anti-fibrotic protein may represent a novel approach for preventing or reversing the pathological manifestations of DN, particularly tubular fibrosis.

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