

Insulin receptor substrate 2 and FoxO3a signalling are involved in E-cadherin expression and transforming growth factor- β 1-induced repression in kidney epithelial cells

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Keywords

E-cadherin; FoxO transcription factor; insulin receptor substrate; kidney; transforming growth factor- β

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(Received 21 April 2011, revised 7 July 2011, accepted 18 July 2011)

doi:10.1111/j.1742-4658.2011.08261.x

Insulin receptor substrate (IRS) proteins comprise a family of adaptor molecules that integrate extracellular signals from insulin and other ligands to intracellular effectors such as phosphoinositide 3-kinase and mitogen-activated protein kinase. The predominant forms of IRS protein in humans, IRS1 and IRS2, are widely expressed. Despite structural similarities, IRS1 and IRS2 display distinct signalling modalities, and mice lacking these proteins present with distinct phenotypes. Transforming growth factor (TGF)- β 1 is the primary cytokine shown to induce epithelial–mesenchymal transition. Recent data have demonstrated a role for IRS1 in TGF- β 1-induced epithelial–mesenchymal transition in lung epithelial cells. In the present study, we report data showing that TGF- β 1 signals via IRS2 in kidney epithelial cells. Small interfering RNA (siRNA)-mediated targeting of IRS2 increased E-cadherin expression, although it did not alter TGF- β 1-mediated E-cadherin repression. Phosphorylation of the downstream target of IRS2/Akt signalling, FoxO3a, was induced on Ser253 and, to a lesser extent, on Thr32. Transfection of FoxO3aThr32Ala mutant for 24 h greatly reduced FoxO3a phosphorylation on Ser253 but over-expression of FoxO3a Ser253Ala did not effect Thr32 phosphorylation, suggesting that a distinct order of phosphorylation of FoxO3a is required for physiological function in cells. Transfection of FoxO3a Ser253Ala mutant partially inhibited TGF- β 1-mediated E-cadherin repression at 24 h. Taken together, these data highlight novel roles for IRS2 and FoxO3a in the regulation of kidney epithelial cells by E-cadherin.

Introduction

Insulin receptor substrate (IRS) proteins comprise a family of adaptor molecules, with IRS1 and IRS2 acting as central integrators of insulin signalling to downstream effector molecules [1]. IRS2 is expressed in almost all mammalian tissues and cells [2]. IRS protein signalling is highly regulated by processes such as Tyr

and Ser/Thr phosphorylation/dephosphorylation and ubiquitination-mediated proteasomal degradation [3,4]. These processes control the magnitude and duration of the response to insulin at the level of the IRS protein. Chronic glucose, insulin or insulin-like growth factor (IGF)-1 exposure of various cell lines leads to

Abbreviations

CTGF, connective tissue growth factor; EMT, epithelial–mesenchymal transition; IGF, insulin-like growth factor; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA; TGF, transforming growth factor.

increased Ser/Thr phosphorylation-mediated proteasomal degradation of IRS2 induced by a mammalian target of rapamycin (mTOR)-dependent downregulation of phosphoinositide 3-kinase (PI3K)/Akt/mTOR activity [5]. Inhibition of PI3K by LY-294002 or mTOR by rapamycin blocked this degradation and led to IRS protein stabilization [5].

Transforming growth factor (TGF)- β 1 is a multi-functional cytokine that is involved in a variety of cellular processes, such as proliferation, cell survival and epithelial–mesenchymal transition (EMT) [6–8]. TGF- β 1 has been shown to induce Ser/Thr phosphorylation of IRS proteins, suggesting that IRS proteins may be important noncanonical downstream mediators of TGF- β 1 signalling [9,10]. It is widely accepted that TGF- β 1 is the chief pathogenic driver of EMT in tubular epithelial cells, alone or in conjunction with other mediators [8]. EMT contributes significantly to the development of renal fibrosis [11]. One of the earliest changes in EMT is the suppression of the epithelial marker, E-cadherin [8]. TGF- β 1 has also been shown to regulate the expression of connective tissue growth factor (CTGF), a downstream mediator of TGF- β 1-induced renal fibrosis [6]. Some studies have argued against the role of EMT as a source of myofibroblast recruitment in fibrosis *in vivo* and have pointed instead to the resident pericyte/perivascular fibroblast as the source of myofibroblasts in kidney fibrosis [12,13].

Akt is an important downstream target of TGF- β 1 and other ligands that signal via IRS2 [14,15]. TGF- β 1 induced EMT occurs in a PI3K/Akt-dependent manner in various cell lines [16,17]. Our group have reported data suggesting that TGF- β 1 signalling through the PI3K/Akt pathway drives EMT [18]. In the kidney, IRS2 plays a major role in insulin stimulation of renal proximal tubular transport and Akt phosphorylation *in vitro* [19]. A recent study identified a novel role for IRS2 in renal development and in the regulation of Akt signalling in the kidney [20]. FoxO3a, a member of the Forkhead family of transcription factors, has been identified as a major effector of TGF- β 1 [21]. Both insulin and IGF-1 induce PI3K/Akt-dependent phosphorylation of FoxO3a in several cell types, which leads to its nuclear exclusion, cytoplasmic retention and eventual ubiquitination-dependent proteasomal degradation [22,23]. Data obtained in a study by Kato *et al.* [21] have identified the TGF- β 1/Akt/FoxO3a pathway in mesangial cell dysfunction in renal disease. This pathway may be relevant to the pathogenesis of tubulointerstitial fibrosis and EMT in the kidney. Furthermore, recent data demonstrate a role for the TGF- β 1/Akt/FoxO3a

pathway in which TGF- β 1 was found to be a critical regulator, whereby TGF- β 1-mediated Akt activation led to the inhibition of FoxO3a via nuclear exclusion in leukaemia-initiating cells [24].

The present study aimed to identify a potential role for the TGF- β 1/IRS2/FoxO3a pathway in the regulation of E-cadherin and other EMT-associated genes in renal proximal tubular epithelial cells. The data obtained suggest that IRS2 expression appears to repress basal E-cadherin expression, although it is not required for TGF- β 1-induced repression of E-cadherin. Foxo3a Ser253 phosphorylation is regulated by prior FoxO3a phosphorylation on Thr32 and is important for the repression of E-cadherin in HK-2 cells.

Results

A range of human cells, corresponding to the major cell types in the kidney, were probed for IRS2 expression. IRS2 was detected in human proximal tubule epithelial cells (HK-2, renal proximal tubule epithelial cells), primary mesangial cells and podocytes, as well as in transformed embryonic kidney cells (HEK293) (Fig. 1A). Expression in HK-2 cells was particularly strong, so these cells were used to further investigate IRS2 signalling. Growth factor deprivation of HK-2 cells for 24 h reduced the overall IRS2 level, as well as IRS2 Tyr phosphorylation and mobility on SDS/PAGE (Fig. S1). Treatment of HK-2 cells with insulin restored IRS2 levels and increased IRS2 pTyr signalling and protein mobility, suggesting a dynamic regulation of IRS2 protein levels and signalling in HK2 cells (Fig. S1). TGF- β 1 has previously been shown to induce fibrosis-like changes in kidney epithelial cells via Smad and other signalling pathways [7,25]. To determine the role of IRS2 in TGF- β 1 signalling in HK-2 cells, siRNA targeting was used to reduce IRS2 expression. Optimized siRNA transfection of HK-2 cells reduced IRS2 mRNA by 55% and IRS2 protein by 65% compared to scrambled control (Fig. 1B–D). No changes in IRS1 expression were detected in IRS2 siRNA-treated cells (data not shown). This system was therefore used to evaluate the role of IRS2 in TGF- β 1 signalling in kidney epithelial cells *in vitro*.

Treatment of HK-2 cells with TGF- β 1 induced a shift in IRS2 mobility, potentially as a result of increased Ser/Thr phosphorylation, because no increase in pTyr signalling was observed in TGF- β 1-treated cells (Fig. 2, 'control +'); see also Fig. S1B. TGF- β 1-induced retardation of the remaining IRS2 protein was more evident in IRS2 siRNA-treated cells (Fig. 2). The effect of reduced IRS2 expression on

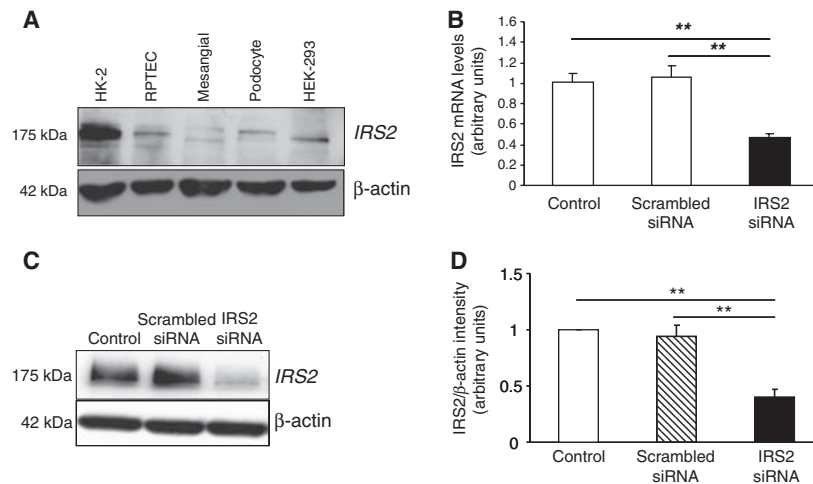


Fig. 1. Expression and knockdown of IRS2 in kidney cells. (A) Protein lysates from HK-2 and renal proximal tubule epithelial cells, primary human mesangial cells, AB 8/13 podocyte cells or human embryonic kidney cell lines probed by western blotting with IRS2 antibody. β -actin was used as a loading control. (B) HK-2 cells were reverse transfected for 48 h with control, siRNA scrambled or siRNA targeting IRS2 (20 μ M). RNA was extracted and TaqMan PCR performed using specific oligonucleotides for human IRS2. Samples were normalized to 18S control. Data are plotted as the mean $\pm$ SEM ($n = 4$ for all groups). Statistical analysis was performed using one-way analysis of variance with post-hoc Tukey–Kramer comparison tests ($**P < 0.01$). (C) Protein was extracted from siRNA-transfected HK-2 cells and probed with IRS2 antibody after SDS/PAGE. β -actin was used as a loading control. (D) Band intensities from western blotting were quantified using SCION IMAGE software (Scion Corp., Frederick, MD, USA). The intensity ratio of IRS2/ β -actin is shown with values for the control set at 1. The results are representative of four independent experiments.

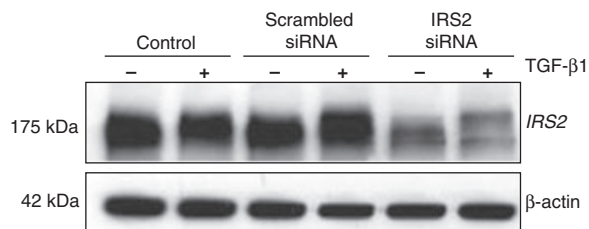


Fig. 2. TGF- β 1 induces an electrophoretic band shift in IRS2 in HK-2 cells. HK-2 cells were reverse transfected with control, siRNA scrambled or siRNA targeting IRS2 for 48 h and subsequently treated with vehicle (4 mM HCl in 0.1% BSA) or TGF- β 1 (10 ng·mL⁻¹) for 24 h. Protein lysates were probed by western blotting with IRS2 or β -actin antibody after SDS/PAGE. The results are representative of four independent experiments.

TGF- β 1-regulated gene expression was then examined. E-cadherin is a key regulator of epithelial cell structure and function, and a loss of E-cadherin is recognized as one of the first steps in the sequence of events in EMT [8]. siRNA-mediated reduction in IRS2 expression did not affect TGF- β 1-induced E-cadherin repression at either the mRNA or protein level (Fig. 3). However, IRS2 targeting significantly increased basal levels of E-cadherin mRNA and protein in HK-2 cells (Fig. 3). The fold-decrease in E-cadherin mRNA and protein induced by TGF- β 1 was therefore higher in cells transfected with IRS2 siRNA (Fig. 3B, E). Similar results

were obtained for a second TGF- β 1 regulated gene, CTGF (data not shown). These data suggest that the basal expression of E-cadherin and other genes in HK-2 cells is inhibited by signalling via IRS2, and that TGF- β 1-mediated regulation of E-cadherin and CTGF may occur independently of IRS2 signalling.

Data have been obtained that identify a signalling pathway from TGF- β 1 $\rightarrow$ PI3K $\rightarrow$ Akt leading to FoxO transcription factor inhibition via nuclear exclusion [21,26]. A potential role for IRS2 in TGF- β 1-mediated FoxO3a gene transcription was therefore assessed. TGF- β 1 treatment of HK-2 cells for up to 72 h decreased mRNA and protein levels of two FoxO3a target genes: Bim and MnSOD (Fig. 4). Three isoforms of Bim [Bim EL (extra long), Bim L (long) and Bim S (short)] were detected in HK-2 cells and levels were decreased by TGF- β 1 treatment (Fig. 4). These results suggest a loss of FoxO3a transcriptional activity as a result of TGF- β 1 stimulation in HK-2 cells, and are in agreement with previous data showing that TGF- β 1 induced Akt activity in kidney epithelial cells, leading to FoxO3a phosphorylation and inhibition [22]. The requirement for IRS2 in TGF- β 1-mediated inhibition of FoxO3a target gene expression was then examined. Basal levels of Bim and MnSOD were not significantly affected by siRNA-mediated inhibition of IRS2 (Fig. S2). TGF- β 1 stimulation reduced the expression of both Bim and MnSOD in the

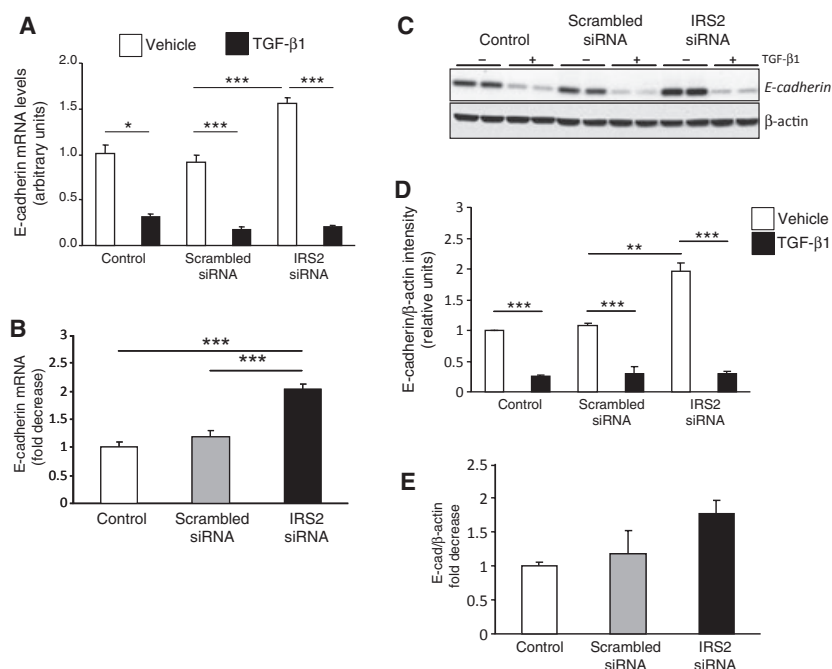


Fig. 3. E-cadherin expression is significantly increased by IRS2 knockdown. HK-2 cells were reverse transfected with control, siRNA scrambled and siRNA targeting IRS2 (20 μM) for 48 h and subsequently treated with vehicle (4 mM HCl in 0.1% BSA) or TGF- β 1 (10 ng·mL $^{-1}$) for 24 h. RNA was extracted from transfected HK-2 cells. (A) Quantitative TaqMan PCR was performed using specific oligonucleotides for human E-cadherin. Samples were normalized to the levels of the 18S control. (B) The fold change within each group was calculated between vehicle and TGF- β 1-treated levels of E-cadherin. Values for the control were set at 1. Data are plotted as the mean $\pm$ SEM ($n = 4$ for all groups). (C) Protein from transfected HK-2 cells was probed by western blotting with E-cadherin antibody after SDS/PAGE using β -actin as a loading control. (D) Band intensities were quantified using SCION IMAGE software. The intensity ratio of E-cadherin/ β -actin is shown in the graph, with values for the control set at 1. (E) The fold change within each group was calculated between vehicle and TGF- β 1-treated levels of E-cadherin, with values for the control set at 1. Statistical analysis was performed using one-way analysis of variance with post-hoc Tukey–Kramer comparison tests (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The results are representative of four independent experiments.

presence of IRS2 siRNA to a level similar to that seen with the control and scrambled siRNA (Fig. S2). These data suggest that TGF- β 1 signalling to FoxO3a and its target genes may occur independently of IRS2 *in vitro* in HK-2 cells.

In its unphosphorylated state, FoxO3a is a nuclear transcriptional regulator of genes involved in apoptosis, proliferation and the control of oxidative phosphorylation [22]. Phosphorylation of FoxO3a on two main sites (Thr32 and Ser253) by PI3K/Akt and other kinases promotes its nuclear export and retention in the cytoplasm, as well as a loss of transcriptional activity [22]. The ability of TGF- β 1 to regulate FoxO3a phosphorylation in HK-2 cells was therefore examined. TGF- β 1 induced a modest increase of endogenous FoxO3a phosphorylation on Thr32 in HK-2 cells compared to insulin (data not shown). Endogenous Ser253 phosphorylation could not be detected with the available antibodies in HK-2 cells (data not shown). Transfection of HK-2 cells with wild-type FoxO3a cDNA increased both basal and TGF- β 1-stimulated Ser253

phosphorylation after 24 h (Fig. 5). Expression of a FoxO3a Ser253Ala mutant abolished this signal, confirming that these bands correspond to Ser253 phospho-FoxO3a (Fig. 5). These data suggest that TGF- β 1 can regulate FoxO3a phosphorylation in HK-2 kidney epithelial cells.

The importance of FoxO3a phosphorylation on Thr32 and Ser253 in HK-2 cells was then examined. Preliminary data suggested that the expression of FoxO3a Ser253Ala mutant attenuated the inhibition of E-cadherin expression induced by TGF- β 1 in HK-2 cells (Fig. S3). By contrast, transfection of FoxO3a Thr32Ala had no effect on TGF- β 1-induced E-cadherin reduction (Fig. S3). To further characterize this effect, the effect of wild-type FoxO3a expression was compared with both FoxO3aThr32Ala and Ser253Ala expression in HK-2 cells. Expression of wild-type FoxO3a did not alter basal or TGF- β 1-induced decreases E-cadherin levels in HK-2 cells (Fig. 6). Elevated levels of pThr32 and pSer253 FoxO3a were detected when wild-type FoxO3a was over-expressed

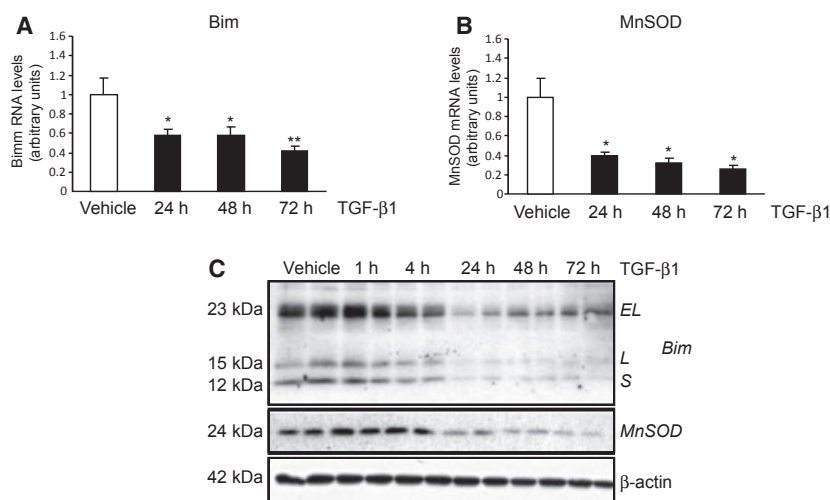


Fig. 4. TGF- β 1 decreases the expression of FoxO3a target genes Bim and MnSOD. Nonstarved HK-2 cells were treated with vehicle or TGF- β 1 (10 ng·mL⁻¹) for 0, 24, 48 and 72 h. Quantitative TaqMan PCR was performed using human specific oligonucleotides for (A) Bim and (B) MnSOD. Samples were normalized to the levels of the 18S control. Data are plotted as the mean $\pm$ SEM with values for the control set at 1 ($n = 3$ for all groups). Statistical analysis was performed using one-way analysis of variance with post-hoc Tukey–Kramer comparison tests (* $P < 0.05$, ** $P < 0.01$). (C) Protein lysates were probed Bim and MnSOD antibodies. The locations of Bim extra long (EL), long (L) and short (S) are indicated. β -actin was used as a loading control. The results are representative of three independent experiments.

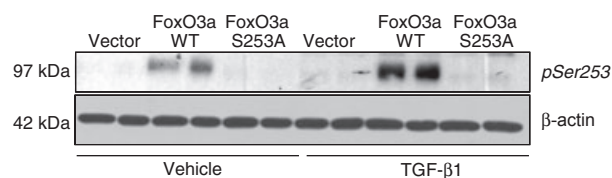


Fig. 5. TGF- β 1 induces phosphorylation of FoxO3a on Ser253. HK-2 cells were transiently transfected with 5 μ g of control plasmid, FoxO3a wild-type and FoxO3a-S253A mutant for 24 h. Transfected cells were then stimulated with vehicle or TGF- β 1 (10 ng·mL⁻¹) for 24 h. Protein lysates were probed with FoxO3a phosphoSer253 antibody. β -actin was used as a loading control. The results are representative of three independent experiments.

(Fig. 6). Expression of FoxO3a Thr32Ala decreased the intensity of Thr32 phosphorylation and, unexpectedly, also Ser253 phosphorylation, suggesting that FoxO3a phosphorylation on Ser253 requires concomitant phosphorylation on Thr32. Transfection of FoxO3a Ser253-Ala reduced Ser253 phosphorylation to basal levels, although it did not significantly affect FoxO3a Thr32 phospho-levels, suggesting that the phosphorylation of FoxO3a on Ser253 requires prior or concomitant phosphorylation on Thr32, but not *vice versa* (Fig. 6A). Importantly, expression of the FoxO3a Ser253Ala mutant inhibited TGF- β 1-induced decreases in E-cadherin compared to wild-type FoxO3a controls (46.8% versus 24.5% reduction; FoxO3a wild-type versus FoxO3a S253A) (Fig. 6B). These data suggest that

FoxO3a Ser253 phosphorylation is important for TGF- β 1-mediated regulation of E-cadherin, and potentially other EMT-related genes in kidney epithelial cells.

Discussion

In the present study, we have: (a) identified a novel link between TGF- β 1 and IRS2 in HK-2 kidney epithelial cells; (b) shown that siRNA-mediated knock-down of IRS2 increases E-cadherin expression; (c) demonstrated a requirement for FoxO3a phosphorylation on Thr32 to achieve full Ser253 phosphorylation; and (d) identified a role for phosphorylation of FoxO3a on Ser253 in TGF- β 1-mediated repression of E-cadherin expression in HK-2 cells. A summary of these findings is presented in Fig. 7.

Treatment of HK-2 cells with TGF- β 1 induced a retardation of IRS2 migration on SDS/PAGE, suggesting the phosphorylation of this protein (Fig. 2). We could not detect increased Tyr phosphorylation of IRS2 in response to TGF- β 1 in HK2 cells, suggesting that the retardation of IRS2 on SDS/PAGE is a result of Ser/Tyr phosphorylation (Fig. S1B). Previous studies have reported a high abundance of Ser and Thr residues in IRS proteins and have demonstrated heavy phosphorylation of IRS proteins *in vitro* and *in vivo* [27,28]. To our knowledge, this is the first demonstration that TGF- β 1 can signal via IRS2 in kidney epithelial cells. Other

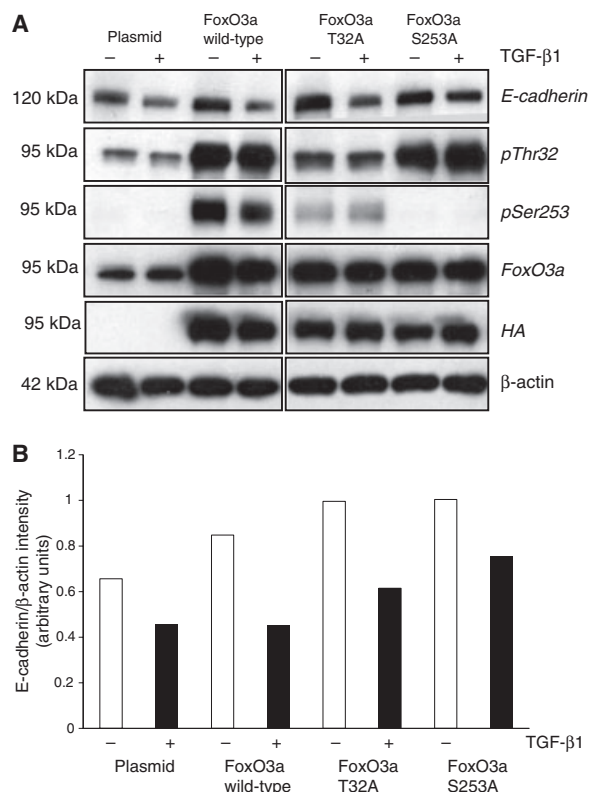


Fig. 6. FoxO3a-S253A mutant expression blunts TGF-β1-mediated E-cadherin downregulation in kidney epithelial cells. (A) HK-2 cells were transiently transfected with 5 μg of control plasmid, haemagglutinin (HA)-tagged FoxO3a wild-type, HA-FoxO3a-T32A mutant or HA-FoxO3a-S253A mutant for 24 h. Transfected cells were then stimulated with vehicle or TGF-β1 (10 ng·mL⁻¹) for 24 h. Protein lysates probed with antibodies raised against FoxO3a phosphoThr 32, phospho-Ser253, E-cadherin, total FoxO3a, HA epitope and β-actin. The results are representative of three independent experiments. (B) Band intensity ratios for E-cadherin were normalized to β-actin and plotted for each plasmid and treatment.

studies have demonstrated that TGF-β1 can induce IRS2 phosphorylation, leading to growth inhibition of Mv1Lu mink lung epithelial cells [10]. The mechanism of this phosphorylation and the candidate kinases responsible remain to be identified. TGF-β1 decreased E-cadherin expression in HK-2 cells within 24 h of exposure and siRNA targeting IRS2 affected the levels of E-cadherin at the mRNA and protein level (Fig. 3). Knockdown of IRS2 increased the basal level of the epithelial marker, E-cadherin; however, subsequent exposure to TGF-β1 decreased the level of E-cadherin to a level similar to that seen in the control and scrambled groups (Fig. 3). These data suggest that TGF-β1 signalling via IRS2 may not be involved in the inhibition of E-cadherin expression in human proximal tubular epithelial cells. HK-2 cells also express IRS1, which was not affected by IRS2

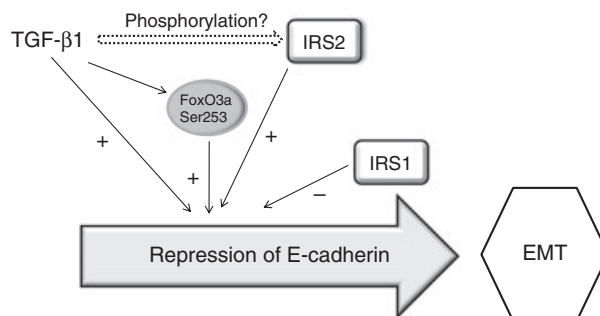


Fig. 7. Schematic of suggested mechanism of E-cadherin promoter regulation by IRS proteins and FoxO3a. Repression of E-cadherin is an early and integral component of TGF-β1-induced EMT. Data from our group and others have shown that IRS1 and IRS2 can positively and negatively affect the repression of E-cadherin expression. TGF-β1 inhibition of E-cadherin expression appears to involve FoxO3a phosphorylation on Ser253, potentially via the Akt pathway. The clear links between TGF-β1 and IRS2 phosphorylation and signalling remain to be determined.

siRNA transfection (data not shown). Akt is a downstream effector molecule of insulin and TGF-β1 signalling. IRS proteins mediate many physiological functions of insulin and IGF-1, including the inhibition of apoptosis, through the direct activation of the PI3K/Akt cascade [29]. Previous studies have demonstrated that TGF-β1 induction of EMT in various cells lines is dependent on PI3K/Akt activity, which has a negative effect on E-cadherin expression [16–18]. The data obtained in the present study suggest that IRS2 may also be a negative regulator of E-cadherin, via Akt signalling. Knockdown of IRS2 using siRNA removes this inhibition, leading to an increase in the basal level of E-cadherin (Fig. 3). However, TGF-β1 over-rides this effect in HK-2 cells by inducing decreased E-cadherin levels (Fig. 3). We hypothesize that canonical TGF-β1 signalling via Smad2/3 is the dominant pathway involved in the inhibition of E-cadherin expression, and that this pathway is not affected by IRS2 knockdown. Our data shed new light on the function of IRS2 in providing an inhibitory constraint for E-cadherin expression, which is released in the presence of TGF-β1 in kidney epithelial cells.

There are five IRS proteins expressed in humans: IRS1, IRS2 and IRS4, IRS5 and IRS6 [27,30]. Knock-out mouse studies have demonstrated that, despite structural similarities, each IRS protein has distinct signalling modalities [31–33]. Recent results obtained by Shi *et al.* [34] showed for the first time that TGF-β1 induces EMT via IRS1 signalling in A549 and Mv1Lu lung epithelial cells, and that IRS1 functions as a critical suppressor of TGF-β1-induced EMT. siRNA-mediated

knockdown of IRS1 reduced E-cadherin expression in a study by Shi *et al.* [34], inducing EMT-like changes in the absence of IRS1. Interestingly, siRNA-mediated knockdown of IRS2 increased E-cadherin expression and had no effect on TGF- β 1 induced EMT (Fig. 3). Importantly, treatment of HK-2 cells with TGF- β 1 did not induce a mobility shift in IRS1, in contrast to IRS2 (Fig. 2; data not shown). With the usual caveats about cell line-specific effects in place, the data of the present study, together with those of Shi *et al.* [34], identify novel differences between IRS1 and IRS2 signalling in the maintenance of epithelial cell phenotypes *in vitro*. The signalling diversity of IRS proteins suggested above is supported by the fact that IRS1 was shown to be a suppressor (and IRS2 a positive regulator) in breast cancer metastasis and EMT [35].

The FoxO family of transcription factors are phosphorylated on Ser and Thr residues in response to insulin and other growth factor-mediated activation of Akt [22,36]. This leads to their nuclear export, cytoplasmic localization via 14-3-3 binding and reduced transcription of target genes such as Bim and MnSOD [22] (Fig. 4). FoxO3a has been identified as an effector of TGF- β 1 [21] and Kato *et al.* [21] suggest that, in mesangial cells, decreased FoxO3a transcriptional activity as a result of TGF- β 1 may lead to the progression of diabetic nephropathy. However, TGF- β 1/FoxO3a signalling appears to be cell-type specific and possibly disease state-specific [37]. The data of the present study demonstrate that TGF- β 1 decreases FoxO3a-regulated gene expression in HK-2 cells, as previously shown in mesangial cells [21]. However, knockdown of IRS2 did not affect TGF- β 1 regulation of FoxO3a regulated genes, suggesting that TGF- β 1/FoxO3a signalling may occur independently of IRS2 activity in HK-2 cells (Fig. S2). It is likely that TGF- β 1/FoxO3a signalling may engage canonical Smad2/3 signalling in these and other cells. In addition, a direct link between TGF- β 1 and activation of PI3K/Akt has been suggested, without IRS protein involvement [38]. Importantly, these data identify a novel role for IRS2 in TGF- β 1- and insulin-mediated regulation of longevity and dauer formation in *Caenorhabditis elegans* and potentially more complex organisms [39,40].

The data obtained in the present study demonstrate that the disruption of FoxO3a Ser253 phosphorylation resulted in E-cadherin levels that were higher than those of cells transfected with wild-type FoxO3a and other mutant constructs (Fig. 6A). These data demonstrate that Ser253 may be involved in TGF- β 1-mediated decreased E-cadherin and, by extension, EMT in HK-2 cells. Brunet *et al.* [22] showed that a Ser253Ala mutant GST-FOXO3a fusion protein was not phosphorylated

by Akt. The major site of phosphorylation of the Forkhead domains *in vitro* appears to comprise this Ser253 site, which is located in the DNA-binding domain, and has previously been suggested as a key regulator of FoxO3a function [22,41] (Fig. 5). In HK-2 cells, transfection of Thr32Ala FoxO3a did not dramatically alter TGF- β 1-induced E-cadherin repression, although it did trigger a marked decrease in FoxO3a Ser253 phosphorylation (Fig. 6A). These data suggest that concomitant Thr32 phosphorylation is required for full Ser253 phosphorylation of FoxO3a. By contrast, mutation of Ser253 did not affect Thr32 phosphorylation, suggesting that a precise order of phosphorylation is involved in FoxO3a regulation in cells. Mutation of Ser253Ala attenuated TGF- β 1-induced decreases in E-cadherin expression (Fig. 6). Importantly, despite the reduced levels of FoxO3a Ser253 phosphorylation seen in cells transfected with the Thr32 mutant, only a modest inhibition of TGF- β 1-induced decreases in E-cadherin was seen (Figs 6A and S3). Why would mutation of FoxO3a Ser253Ala affect TGF- β 1-mediated inhibition of E-cadherin? One possibility is that mutation of Ser253 prevents the Akt-dependent nuclear export of FoxO3a. This may lead to altered TGF- β 1-mediated changes in Twist expression and Zeb-1/Snail recruitment to the E-cadherin promoter, which are pathways that have been described in other studies [42,43]. A role for FoxO3a in EMT has previously been suggested by Belguise *et al.* [44] in breast cancer, where it was shown that ectopic expression of a constitutively active FoxO3a over-rode the TGF- β 1-mediated invasive phenotype and induced a more epithelial phenotype, inducing E-cadherin at the mRNA and protein levels. Expression of the constitutively active FoxO3a also led to a potent activation of the E-cadherin promoter in various cell types, whereas dominant negative FoxO3a reduced the epithelial phenotype and induced mesenchymal gene expression [44]. This finding is supported by our novel data obtained in HK2 cells indicating that EMT-like changes may be prevented by blocking FoxO3a phosphorylation on Ser253, thereby allowing active FoxO3a to remain in the nucleus to function and prevent TGF- β 1 downregulation of E-cadherin in kidney epithelial cells.

Materials and methods

Cell culture and transfection

Immortalized human kidney epithelial cells (HK-2) from American Type Culture Collection (ATCC, PO Box 1549, Manassas Virginia, 20108-1549 USA) were cultured and maintained in DMEM-F12 Hams (Sigma, St Louis, MO, USA) supplemented with L-glutamine (2 mM), penicillin

(100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹), epidermal growth factor (10 ng·mL⁻¹), hydrocortisone (36 ng·mL⁻¹), tri-iodothyronine (4 pg·mL⁻¹) and insulin-transferrin-selenium (5 µg·mL⁻¹) (Sigma) at 37 °C in 95% air and 5% CO₂. Renal proximal tubule epithelial cells and mesangial cells were maintained in MCDB-131 (Gibco, Gaithersburg, MD, USA) supplemented with L-glutamine (2 mM), penicillin (100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹) and 10% fetal bovine serum. Podocyte cells were maintained in RPMI-1640 (Gibco) and supplemented with L-glutamine (2 mM), penicillin (100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹) and insulin-transferrin-selenium (5 µg·mL⁻¹). HEK-293 cells were maintained in MEM (Sigma), supplemented with L-glutamine (2 mM), penicillin (100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹) and 10% fetal bovine serum.

HK-2 cells were plated at 2×10^5 cells/well, 24 h before transfection. Cells were transfected with 5 µg of control plasmid, FoxO3a wild-type or FoxO3a mutant constructs (generous gifts from Dr Anne Brunet, Stanford University, Palo, Alto, CA, USA) using OPTI-MEM® (Gibco) and Eugene® 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) at a lipid/DNA ratio of 5 : 2. Cells were incubated with DNA-lipid complexes for 24 h, after which transfection medium was removed and replaced with full HK-2 media containing diluent (4 mM HCl in 0.1% BSA) or TGF-β1 (10 ng·mL⁻¹) for 24 h. Transfected cells were identified based on immunoblotting by using antibodies to the haemagglutinin-epitope tag located at the N-terminus of the FoxO3a constructs.

IRS2 siRNA targeting

HK-2 cells (50 000 cells·mL⁻¹) were reverse transfected in six-well plates with siRNA scrambled (20 µM) (nontarget 2; Applied Biosystems, Foster City, CA, USA) and siRNA targeting IRS2 (20 µM) (Applied Biosystems) using 100 µL of Opti-MEM® (Gibco) and 1.17 µL of lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA) per millilitre of media. A 2 mL volume was sufficient for each well. HK-2 media used was without penicillin or streptomycin. Cells were incubated with RNA-lipid complexes for 48 h, after which the transfection media was removed and replaced with HK-2 media treated with diluent (4 mM HCl in 0.1% BSA) or TGF-β1 (10 ng·mL⁻¹) for 24 h. Cells were lysed and separated on SDS/PAGE and probed by western blotting with specific antibodies, as described below.

TaqMan real-time PCR

Total RNA was extracted from cells using Trizol® Reagent (Invitrogen) in accordance with the manufacturer's instructions. The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). TaqMan PCR was car-

ried out using human FAM-labelled real-time probes (Applied Biosystems): IRS2 (Hs00275843_s1), E-cadherin (Hs00170423_m1), CTGF (Hs00170014_m1), Bim (Hs00708019_s1) and MnSOD (Hs00167309_m1). Generated products were analyzed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Data were expressed using the $\Delta\Delta C_T$ method and normalized to 18S levels.

Protein extraction, immunoblotting and immunoprecipitation

Cultured cells were lysed in radioimmunoprecipitation assay buffer supplemented with 50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, supplemented with 1 × protease inhibitor cocktail (Sigma), 1 mM NaF, 40 mM β-glycerophosphate, 2 µM microcystin, 1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated on ice for 15 min, vortexed every 5 min, and centrifuged at 20 000 g for 15 min at 4 °C to pellet the cell debris. Protein extracts (20 µg) were then resolved using 7.5%, 10% or 12% (v/v) SDS/PAGE depending on the protein molecular weights. Proteins were then transferred onto polyvinylidene difluoride membrane (Immobilon P; Millipore, Billerica, MA, USA) for 75 min at 110 V and incubated in blocking buffer NaCl/Tris-Tween (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) supplemented with 3% (w/v) skimmed milk for 60 min. Membranes were then incubated with antibodies diluted in 3% milk in NaCl/Tris-Tween overnight at 4 °C: IRS2 (dilution 1 : 500; Millipore), IRS1 (dilution 1 : 1000; Cell Signaling Technology, Beverly, MA, USA), phospho-FoxO3a (Ser253) (dilution 1 : 1000; Cell Signaling), phospho-FoxO3a (Thr32) (dilution 1 : 1000; Millipore), total FoxO3a (dilution 1 : 1000; Cell Signaling), MnSOD (dilution 1 : 1000; Millipore), Bim (dilution 1 : 500; ProSci, Poway, CA, USA), E-cadherin (dilution 1 : 2500; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and β-actin (dilution 1 : 25 000; Sigma). After three washes for 10 min in NaCl/Tris-Tween, the membranes were incubated with anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-linked secondary sera (Cell Signaling) at a dilution of 1 : 5000 in NaCl/Tris-Tween containing 3% milk for 60 min. Reactive bands were revealed using enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and X-ray film. Densitometry was carried out using IMAGEJ software (NIH Image, Bethesda MD, USA). Band intensities were calculated and divided by the corresponding β-actin intensity. Ratios were plotted as arbitrary intensity units.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was carried out using INSTAT software (GraphPad Software Inc., San Diego, CA, USA). Analysis of variance

with a post-hoc Tukey–Kramer multiple comparison test or Student's unpaired *t*-test was used to identify significant differences, where appropriate. *P* < 0.05 was considered statistically significant.

Acknowledgements

We thank Dr Anne Brunet (Stanford University, Palo Alto, CA, USA) for the generous gift of FoxO3a wild-type and mutant constructs. Collaborators in the UCD Diabetes Research Centre are acknowledged for their helpful discussions. This work was supported by an Irish government Embark Postgraduate Research award (RS/2005/18) to R.M.C. and D.P.B., as well as a Science Foundation Ireland RFP award (07/RFP/BIMF22) to D.P.B. F.B.H. is supported by a Health Research Board Ireland fellowship. D.P.B. is currently supported by Action Medical Research UK SP4582 grant and DEL Northern Ireland.

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Supporting information

The following supplementary material is available:

Fig. S1. Effect of insulin and TGF- β 1 on IRS2 Thr phosphorylation in HK-2 cells.

Fig. S2. Knockdown of IRS2 does not alter the expression levels of FoxO3a target genes.

Fig. S3. Transfection of FoxO3a Ser253Ala mutant inhibits TGF- β 1-mediated decreases in E-cadherin expression.

This supplementary material can be found in the online version of this article.

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