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Jagged/Notch signalling is required for a subset of TGFβ1 responses in human kidney epithelial cells

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ABSTRACT

The Jagged/Notch pathway has been implicated in TGFβ1 responses in epithelial cells in diabetic nephropathy and other fibrotic conditions *in vivo*. Here, we identify that Jagged/Notch signalling is required for a subset of TGFβ1-stimulated gene responses in human kidney epithelial cells *in vitro*. TGFβ1 treatment of HK-2 and RPTEC cells for 24 h increased Jagged1 (a Notch ligand) and Hes1 (a Notch target) mRNA. This response was inhibited by co-incubation with Compound E, an inhibitor of γ-secretase (GSI), an enzyme required for Notch receptor cleavage and transcription regulation. In both cell types, TGFβ1-responsive genes associated with epithelial–mesenchymal transition such as E-cadherin and vimentin were also affected by γ-secretase inhibition, but other TGFβ1 targets such as connective tissue growth factor (CTGF) and thrombospondin-1 (THBS1) were not. TGFβ1-induced changes in Jagged1 expression preceded EMT-associated gene changes, and co-incubation with GSI altered TGFβ1-induced changes in cell shape and cytoskeleton. Transfection of cells with the activated, cleaved form of Notch (NICD) triggered decreased expression of E-cadherin in the absence of TGFβ1, but did not affect α-smooth muscle actin expression, suggesting differential requirements for Notch signalling within the TGFβ1-responsive gene subset. Increased Jagged1 expression upon TGFβ1 exposure required Smad3 signalling, and was also regulated by PI3K and ERK. These data suggest that Jagged/Notch signalling is required for a subset of TGFβ1-responsive genes, and that complex signalling pathways are involved in the crosstalk between TGFβ1 and Notch cascades in kidney epithelia.

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1. Introduction

TGFβ1 is the primary cytokine mediator of fibrotic kidney disease [1,2]. Canonical Smad signalling in response to TGFβ1 involves phosphorylation of Smad2/3 which then dimerise with Smad4, translocate to the nucleus and bind to specific promoters to regulate the transcription of TGFβ-responsive genes [3]. Many other signal transduction pathways have been implicated in TGFβ-induced fibrosis in kidney and other organs, including PI3K/Akt, p38MAPK, NFκB and RhoA [4–8]. In kidney, TGFβ1 has been reported to induce epithelial–mesenchymal transition leading to increased myofibroblast accumulation and scarring of kidney [9,10]. The process of EMT is characterised by disassembly of epithelial cell adherens junctions, changes in cell shape, degradation and invasion of the basement membrane and myofibroblast migration [11]. *In vitro*, EMT can be tracked by detecting reduced E-cadherin and ZO-1 expression in the adherens and tight junctions, together with increased expression of α-smooth muscle actin and vimentin myofibroblast markers [12,13]. Although a large volume of data supports a role for TGFβ1-induced EMT in kidney disease *in vivo* [14–16], recent data from Duffield and colleagues has suggested that alternative cell types such as pericytes may also contribute to myofibroblast formation in kidney fibrosis [17].

Notch proteins are cell surface transmembrane receptors that control multiple developmental processes in metazoan development [18,19]. Notch signalling involves intercellular binding of Notch receptors with Jagged/Delta ligands on adjacent cells [19,20]. Activation of Notch receptor triggers the release of Notch intracellular domain (NCID) via the action of the γ-secretase protease, a member of the presenillin family [19]. Released NICD then translocates to the nucleus where it interacts with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h) and LAG-1) [21]. Notch/CSL both represses and activates transcription via the recruitment of chromatin remodelling complexes containing either histone deacetylases or histone acetylase proteins [18]. Notch receptor cleavage (via the action of γ-secretase) is required for nephrogenesis and normal podocytes/proximal tubule formation in the developing mouse kidney [22,23].

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Elements of the Notch signalling pathway, including Jagged1, Notch1 and 2, Hes1 and Hey1 have been identified as TGFβ1-responsive genes in diverse epithelia [24–26]. Zavadil and colleagues identified that inhibition of Jagged/Notch signalling using RNAi blocked TGFβ1-induced EMT progression in keratinocytes [27], suggesting a potential role for Notch signalling in fibrosis. *In vivo*, elevated Jagged/Notch signalling has been identified in a range of different fibrotic diseases of the kidney, liver and lung [25,26,28–30]. Levels of Jagged1 and Hes1 are also elevated in human diabetic nephropathy [26]. In transgenic mice, overexpression of the Notch intracellular domain (NICD) in podocytes triggers glomerulosclerosis [31,32]. Therapeutic interventions using inhibitors of Notch receptor cleavage (γ-secretase inhibitors, GSI) and soluble Notch ligands alleviate renal failure in mice, suggesting that pharmacological targeting of Notch may be of benefit in the treatment of human kidney disease [31–33].

In the present study, we interrogate the signalling pathways triggered by TGFβ1 that regulate Jagged/Notch and EMT-associated gene expression in human kidney proximal tubule epithelial cells. We identify that Smad3, PI3K and ERK signalling are involved in TGFβ1-mediated stimulation of Jagged1 gene expression. Additionally, our data suggests that a subset of TGFβ1-regulated genes require Notch signalling, data which advances our understanding of the role of Notch signalling in the kidney.

2. Materials and methods

2.1. Cell culture and transfection

Human proximal tubule epithelial cells (HK-2) were cultured in Dulbecco's modified Eagle's medium (DMEM-F12, Sigma) supplemented with 10% (vol/vol) fetal calf serum (GIBCO), 2% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine, EGF (10 μg/ml), triiodothronine (40 ng/ml), hydrocortisone (36 ng/μl), and 1% ITS (Sigma) at 37 °C in 95% air–5% CO_2. Primary human renal proximal tubule epithelial cells (RPTEC) were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 10% (v/v) fetal calf serum, 2% penicillin/streptomycin, 1% L-glutamine, EGF (10 μg/ml) and 1% ITS, at 37 °C in 95% air and 5% CO_2. Prior to exposure to TGFβ minus/plus GSI, cells were cultured in minimum starvation media containing only antibiotics and L-glutamine.

For TGFβ1 treatments, cells were cultured in six-well plates and treated with vehicle (PBS) or 5 ng/ml recombinant human TGFβ1 (PromoCell) for 24 h. Pharmacological inhibitors LY294002, PD98059, SB431542 and SIS3 were added at 1 μM concentration 60 min prior to treatment with TGFβ1. The γ-secretase inhibitor (Compound E, Alexis) was added together with TGFβ1 at either 0.03 nM, 0.3 nM, 3 nM or 30 nM for 24 h.

For HK-2 transfections, cells were cultured in six-well plates and transfected at 70% confluency. For transfections, 1 μg of empty vector, 1 μg pCMX-Notch IC and 1 μg RPMS-1 were used. Transfections were carried out using OptiMeM (GIBCO) and Fugene 6 Transfection Reagent (Roche) at a 3:1 Fugene:DNA ratio. Twenty four hours after transfection, the medium was replaced and cells were exposed to TGFβ1 for a further 24 h.

2.2. TaqMan PCR

RNA extraction was carried out using Trizol reagent (BioSciences) according to the manufacturer's protocol. RNA concentration was determined using a Nanodrop spectrophotometer and cDNA was synthesised using Superscript II Reverse Transcriptase (Invitrogen). TaqMan PCR was carried out using the following human real-time probes (Applied Biosystems): E-cadherin (Hs00170423_m1), Vimentin (Hs00185584_m1), CTGF (Hs00170014_m1), THBS-1 (Hs00170236_m1), Jagged1 (Hs00164982_m1) and Hes1 (Hs00172878_m1). Generated products were then analysed on an ABI Prism 7700 sequence detection system. Data were calculated using the ΔΔCt method and normalised to 18S levels.

2.3. Protein harvesting and Western blotting

For protein analysis, cells were harvested on ice in RIPA buffer containing 50 mM Tris–HCl, pH 7.4, 1% (vol/vol) Nonidet P-40, 0.25% (wt/vol) sodium deoxycholate, 150 mM NaCl and 1 mM EDTA, supplemented with 1× protease inhibitor cocktail (Sigma), 2 μM microcystin (Alexis Biochemicals), 10 μM Benzamidine, 1 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium vanadate and fresh 1 mM phenylmethylsulfonyl fluoride. Cells were incubated on ice for 20 min, vortexing every 5 min. Cells were then centrifuged at 14,000 rpm at 4 °C for 20 min to remove cell debris. Protein concentration was determined using Bradford reagent (Bio-Rad) and read on a plate reader using SoftPro Max software at an absorbance of 595 nm. Proteins were resolved using either 7.5% or 12% SDS-PAGE and then transferred to a polyvinylidene diflouride membrane (PVDF, Immobilon P, Millipore) at 110 V for 75 min. Membranes were blocked with 3% (wt/vol) milk (made up in TBS-T [10 mM Tris–HCl pH 7.4, 150 mM NaCl and 0.1% Tween 20]) for 60 min at RT. Membranes were then incubated with the following antibodies: polyclonal Jagged1 (1:1000, Santa Cruz), monoclonal E-Cadherin (1:1000, BD Transduction Laboratories), monoclonal α-SMA (1:1000, Sigma), and monoclonal β-actin (1:10,000, Sigma) diluted in 3% milk overnight at 4 °C. The following day membranes were washed with TBS-T for three 10-min washes, before incubation with either HRP-coupled anti-rabbit (Cell Signaling) or anti-mouse (Cell Signaling) secondary antibodies at 1:2000 dilution in 3% milk for 1 h at RT. Membranes were washed with TBS-T for three 10-min washes. Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was then used, along with an X-ray film, to reveal the reactive bands.

2.4. Immunofluorescence

HK-2 cells plated on 8-well chamber slides (Nunc) were treated with TGFβ1 and γ-secretase inhibitor Compound E added as previously described. After 24 h, the culture medium was aspirated and the slides were washed with PBS and fixed with 3.7% paraformaldehyde. Cells were permeabilised with 0.1% Triton-X 100 (Sigma) and blocked with 5% goat serum (Sigma) for 1 h. The slides were incubated overnight with primary antibodies to E-cadherin (Abcam), α-smooth muscle actin (Sigma), each at a concentration of 1:100 in 5% goat serum. The next day, slides were washed with PBS and incubated with secondary anti-mouse/rabbit Texas Red conjugated antibody (Molecular Probes) at a concentration of 1:200 in 5% goat serum. Slides were washed in PBS and incubated with 1:200 Alexa Fluor 488 phalloidin (Invitrogen) in PBS for 30 min, followed by Hoechst dye (Invitrogen), at 1:1000 in PBS for 2 min. Two drops (10–15 μl) of ProLong Antifade Gold (Invitrogen) was added to each well, prior to placing coverslips. Slides were visualised with a Zeiss AxioVision Imager M2 upright fluorescence microscope using AxioVision 4.8 software. Multi-dimensional images were acquired from replicate slide wells and representative images are presented.

3. Results

Previous data from our laboratory and others identified upregulation of Jagged1 gene expression as a feature of TGFβ-mediated renal fibrosis and diabetic kidney disease [25–27]. Global gene expression analysis using Affymetrix gene arrays identified Jagged1 as the most significantly upregulated gene in response to 24 h TGFβ1 treatment in HK-2 cells [26]. To assess the sensitivity of Jagged/Notch-mediated gene expression, human kidney epithelial cells (HK-2) were incubated with a range of increasing TGFβ1 concentrations for 24 h. Dose-dependent changes in the expression of genes associated with TGFβ1

induced renal fibrosis were then compared to alterations in Jagged/Notch pathway genes. Increasing concentrations of TGFβ1-induced a decrease in the levels of E-cadherin mRNA, and an increase in the amount of vimentin mRNA, changes previously identified as integral to TGFβ-induced epithelial–mesenchymal transition *in vitro* (Fig. 1 top panels, [9]). Levels of connective tissue growth factor (CTGF) and thrombospondin-1 (THBS1) also increased in a concentration-dependent manner (Fig. 1 middle panels). Jagged1 mRNA, as well as the Jagged/Notch target gene Hes1 also increased in HK-2 cells treated with TGFβ1 (Fig. 1 bottom panels). Marked differences in the responsiveness of each gene to TGFβ1 were detected, with 2-fold increases in vimentin expression and 8–10-fold increases in CTGF, THBS1 and Jagged1 mRNA detected. The EC_{50} of TGFβ1 for each individual gene analysed was broadly similar, with values between 2.1 and 4.2 ng/ml calculated (Fig. 1). Based on these data, all further experiments were performed using 5 ng/ml TGFβ1 for 24 h to ensure comparable responses.

Activation of Jagged/Notch signalling requires cleavage of the Notch receptor by γ-secretase, liberating a Notch receptor intracellular fragment (NICD) which translocates to the nucleus and regulates gene transcription [19]. To assess whether Notch receptor processing was required for TGFβ1-induced changes in Jagged1 and Hes1, HK-2 cells were incubated with increasing concentrations of a γ-secretase inhibitor (GSI). The robust increases in Jagged1 and Hes1 target gene levels triggered by TGFβ were inhibited by GSI in a concentration-dependent manner, with IC_{50} values of 0.76 and 2.9 nM respectively (Fig. 2A, B). Inhibition of TGFβ1-induced Jagged1 by GSI was also observed at the protein level (Fig. 2C, D). GSI-mediated inhibition of Jagged1 and Hes1 RNA induction in the absence of TGFβ1 suggested that in Jagged/Notch signalling an autocrine signalling loop may exist to regulate target gene expression in these cells (Suppl. Fig. 1). Similar induction of Jagged1 and sensitivity to GSI were seen in primary renal proximal tubule epithelial cells (RPTEC) treated with TGFβ1 (Suppl. Fig. 2). Thus, TGFβ-mediated activation of Jagged1 pathway gene expression requires Jagged/Notch receptor activation and cleavage in two independent kidney epithelial cell lines.

Additional TGFβ1-mediated gene expression changes in HK-2 cells were then assessed in HK-2 cells minus/plus GSI. Interestingly, differential sensitivity to GSI was displayed by TGFβ1-regulated genes, where increasing concentrations of GSI inhibited TGFβ1-mediated changes in E-cadherin, α-SMA and vimentin mRNA (Fig. 3A–C). Lower concentrations of GSI appeared to have a bigger effect on α-SMA mRNA levels (Fig. 3B). In contrast, γ-secretase inhibition had a minimal effect on TGFβ1-induced increases in CTGF and THBS1 gene expression (Fig. 3D, E). TGFβ1-stimulated expression of Grem1, a gene implicated in renal disease [34,35], was also insensitive to GSI up to 30 nM (Fig. 3F). Again, similar trends were detected in RPTEC cells, with TGFβ1-induced changes in E-cadherin

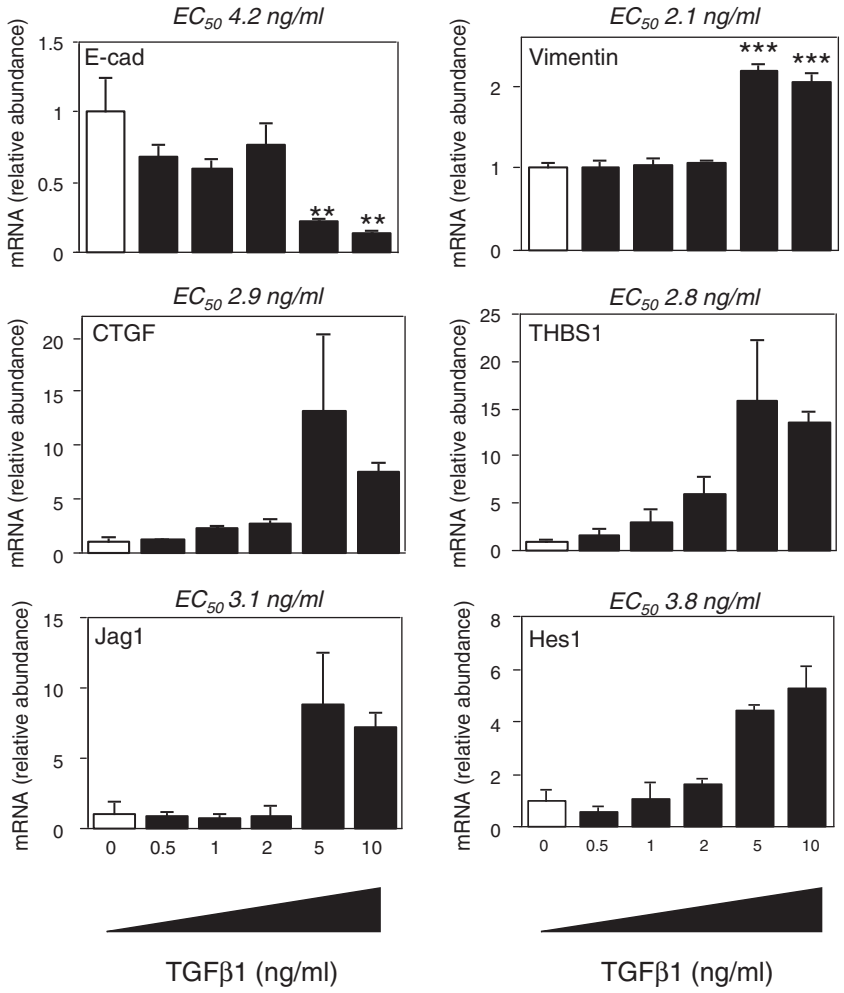

Fig. 1. TGFβ1 induces concentration-dependent gene responses in HK-2 cells. HK-2 cells were treated with TGFβ1 at concentrations of 0.5, 1, 2, 5 and 10 ng/ml for 24 h (filled bars). Control cells (empty bars) were treated with vehicle. Cells were harvested for RNA and changes in the levels of E-cadherin (E-cad), vimentin, connective tissue growth factor (CTGF), thrombospondin-1 (THBS-1), Jagged1 (Jag1) and hairy and enhancer of split 1 (Hes1) were determined using TaqMan PCR. Gene levels were normalised to control 18S, and experiments were carried out in triplicate. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test or Student's unpaired t-test. Statistical differences shown are compared to control vehicle group (0 ng/ml TGFβ1). $^{**}p<0.01$, $^{***}p<0.001$. EC_{50} values were calculated using Prism software.

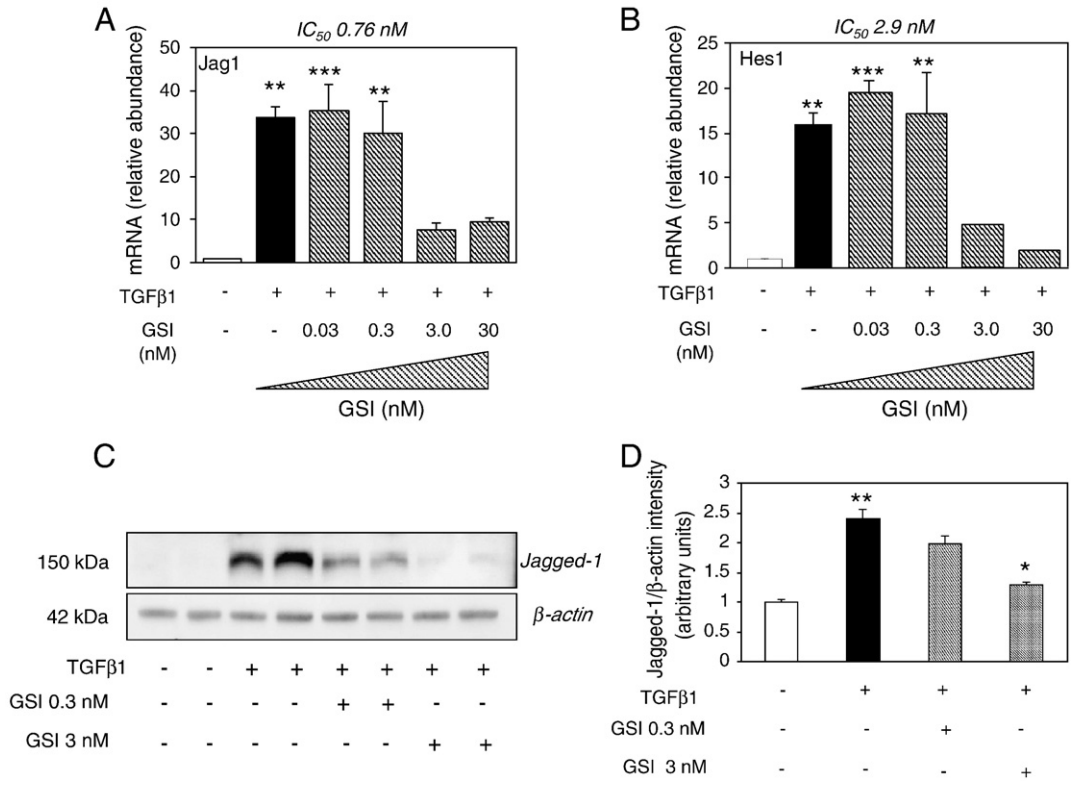

Fig. 2. TGFβ1-stimulation of Jagged1 expression requires Notch receptor cleavage. A, B. HK-2 cells were treated with vehicle (empty bars) or 5 ng/ml TGFβ1 (filled bars) for 24 h in the presence of γ-secretase inhibitor (GSI) at 0.03, 0.3, 3 or 30 nM (striped bars). DMSO was included in the vehicle and TGFβ1-treated cells as a negative control. Cells were harvested for RNA and quantitative TaqMan PCR was carried out. mRNA levels were normalised to control 18S, and experiments were carried out in triplicate. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. Statistical differences shown are compared to control group unless otherwise indicated. $^{**}p<0.01$, $^{***}p<0.001$. IC_{50} values were calculated using Prism software. C. HK-2 cells were treated with 5 ng/ml TGFβ1 for 24 h in the absence or presence of GSI at 0.3 nM or 3 nM concentrations. DMSO was used as a negative control. Protein lysates were extracted and separated by SDS-PAGE on a 7.5% gel, and probed with the Jagged1 antibody. β-Actin was used as a loading control. Experiments were carried out in duplicate and repeated n = 3 times. D. Densitometry was carried out using Scion Image and Jagged1 band intensity was normalized to β-actin. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. Values for TGFβ1-treated cells were compared to vehicle control (empty bar), and values for TGFβ1 + GSI (striped bars) were compared to TGFβ1 + DMSO (filled bar). $^{**}p<0.01$, $^{***}p<0.001$.

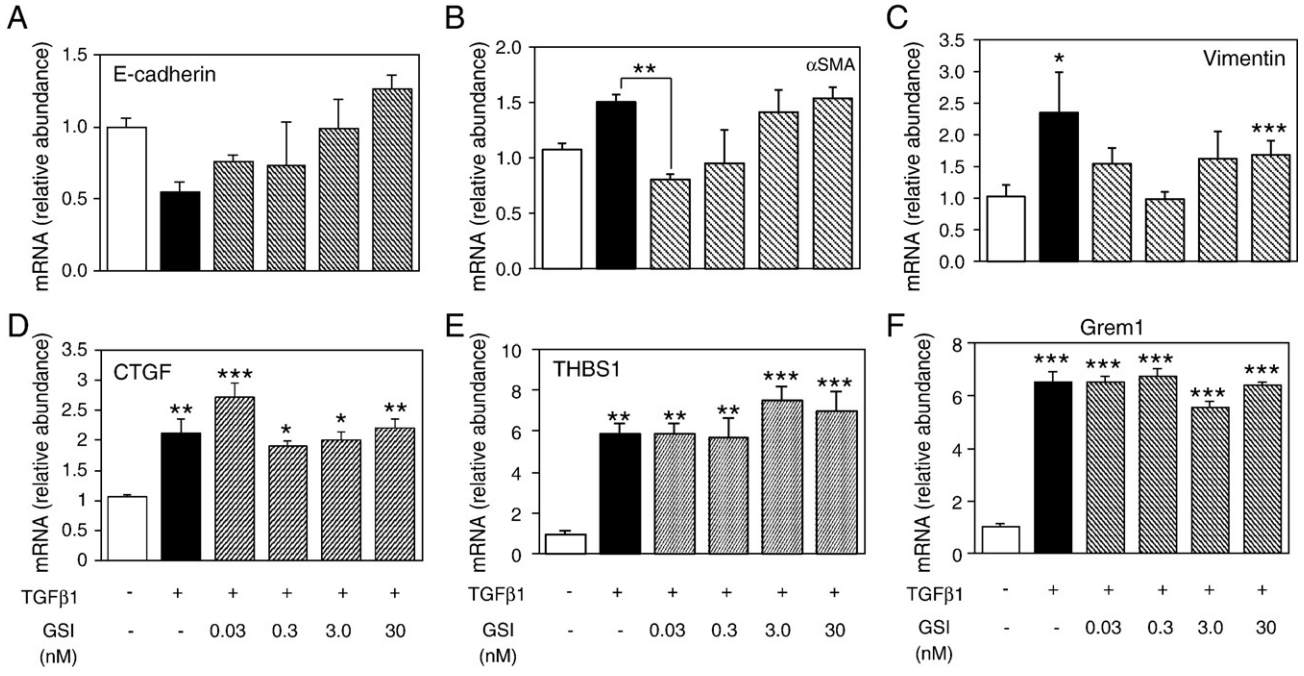

Fig. 3. Notch receptor cleavage is required for a subset of TGFβ1 responsive genes. HK-2 cells were treated with vehicle (empty bars) or 5 ng/ml TGFβ1 for 24 h minus/plus GSI at 0.03, 0.3, 3 or 30 nM concentrations (striped bars), with DMSO as a negative control (filled bars). Cells were harvested for RNA and changes in the levels of the indicated genes were analysed. Levels of each gene were normalised to control 18S, and experiments were carried out in triplicate. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. Statistical differences shown are compared to vehicle control group, unless otherwise indicated (Fig. 3B). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

and α-SMA inhibited by GSI, with no significant inhibition of CTGF RNA levels seen (Suppl. Fig. 3A–C). GSI incubation caused little or no inhibition of baseline RNA levels for all four genes tested without TGFβ1, suggesting that the GSI effect is specific for TGFβ1-mediated gene induction, rather than baseline transcription per se (Suppl. Fig. 1C–F). Significantly, upregulation of Jagged1 and Hes1 mRNA at the 12 h time-point in TGFβ1-treated HK-2 cells preceded changes in EMT-associated gene targets such as E-cadherin and α-SMA (Fig. 4). These data suggest that Notch receptor cleavage and pathway activation are required for a subset of TGFβ1-mediated gene expression changes in HK-2 epithelial cells.

To examine the phenotypic consequence of Jagged/Notch inhibition on TGFβ1-induced EMT, HK-2 cells were stained with antibodies against α-SMA and E-cadherin, both well established protein markers of EMT [13,15]. TGFβ1 treatment of HK-2 epithelial cells triggered the formation of an intermediate cell phenotype, with increased α-SMA, loss of E-cadherin as well as actin rearrangement and increased stress fibres visible (Fig. 5A, B, left panels). Co-incubation of cells with increasing concentrations of GSI prevented TGFβ1-induced changes in α-SMA expression and re-established punctuate E-cadherin staining at the nucleus and cell–cell junctions (Fig. 5A, B), and also reverted the EMT-like changes in actin cytoskeleton (Fig. 5A, 30 nM, 20×). These data suggest that previously observed effects of Jagged/Notch signalling on TGFβ1-induced mRNA changes are also present at the protein level, regulating cell phenotype changes associated with TGFβ1-induced EMT.

Notch1 is one of over 60 reported substrates of γ-secretase in cells [19]. To more specifically address whether cleavage of Notch1 receptor affects TGFβ1-regulated genes, a "pre-cleaved" version of the Notch receptor (NICD) and a negative regulator of NICD/CBF-1 transcription (RPMS1 [21,36]) were utilised (Fig. 6). Transfection of HK-2 cells with NICD increased both Jagged1 and α-SMA protein expression in the absence and presence of TGFβ1 (Fig. 6A–C). TGFβ1-induced mRNA levels for Jag1 and α-SMA were also increased in NICD-transfected cells (Suppl. Fig. 4). NICD had little or no effect on E-cadherin protein or RNA expression, suggesting that Notch receptor cleavage is necessary, but not sufficient for E-cadherin downregulation (Fig. 6A, D and Suppl. Fig. 4C). Transfection of the RPMS1 inhibitor of Notch1-mediated gene transcription only modestly reduced TGFβ1-induced increases in Jagged1 and α-SMA, with little effect on E-cadherin expression levels (Fig. 6). These changes were also reflected at the mRNA level (Suppl. Fig. 4). These data suggest that diverse TGFβ1 gene targets display different modes of regulation by elements of the Jagged1/Notch signalling cascade.

Canonical TGFβ-pathway signalling involves TGFβ type II/I receptor-mediated phosphorylation of Smad2/3, which then dimerise with Smad4 and translocate to the nucleus to drive gene transcription [3]. Other signalling pathways such as the PI3K/Akt and MAPK pathways are also activated by TGFβ in different cells [4–6]. To examine whether canonical Smad signalling was involved in TGFβ1-mediated Jagged1 upregulation, HK-2 cells were incubated with both a TGFβ1 receptor Ser/Thr kinase antagonist (SB431542) and a specific Smad3 inhibitor (SIS3, [37]). Induction of Jagged1 protein by TGFβ1 was abolished by the pretreatment of cells with both SB431542 and SIS3 (Fig. 7A, B). TGFβ1-induced phosphorylation of Smad3 was also significantly reduced by both of these inhibitors (Fig, 7A, B), suggesting that activation of Smad signalling is required for Jagged1 induction in response to TGFβ1. Inhibitors of non-canonical TGFβ signalling pathways such as PI3K/Akt (LY294002) and ERK (PD98059) also inhibited Jagged1 upregulation in response to TGFβ1 (Fig. 7C, D). However, this inhibition was less dramatic than that detected with the TGFβ1 receptor/Smad3 inhibitors (Fig. 7A, B). These data suggest that both canonical Smad signalling and non-canonical PI3K/Akt and ERK signalling contribute to TGFβ1-induced upregulation of Jagged1 in HK-2 epithelial cells.

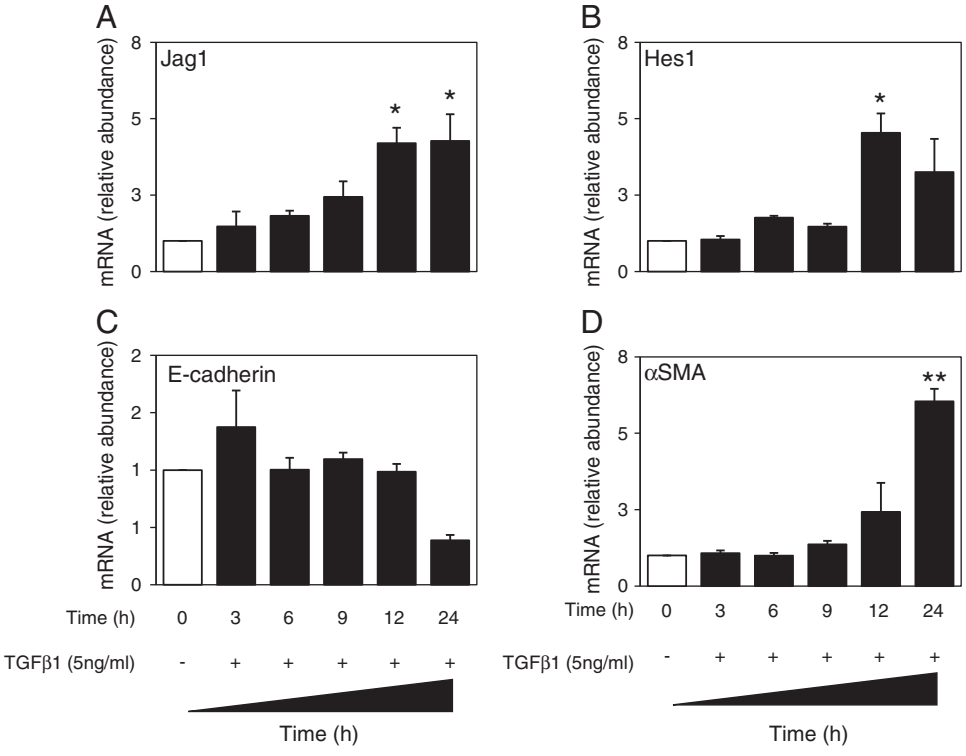

Fig. 4. Jagged1 induction precedes EMT-associated gene changes in kidney epithelial cells. HK-2 cells were treated with vehicle (empty bars) or TGFβ1 (5 ng/ml) for the indicated times. RNA was isolated and changes were in gene expression measured via real-time PCR. RNA levels for Jag1, Hes1, E-cadherin and α-SMA were normalised to 18S and analysed using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. Experiments were carried out three times in triplicate. Statistical differences shown are compared to vehicle control for each gene. *$p<0.05$, **$p<0.01$.

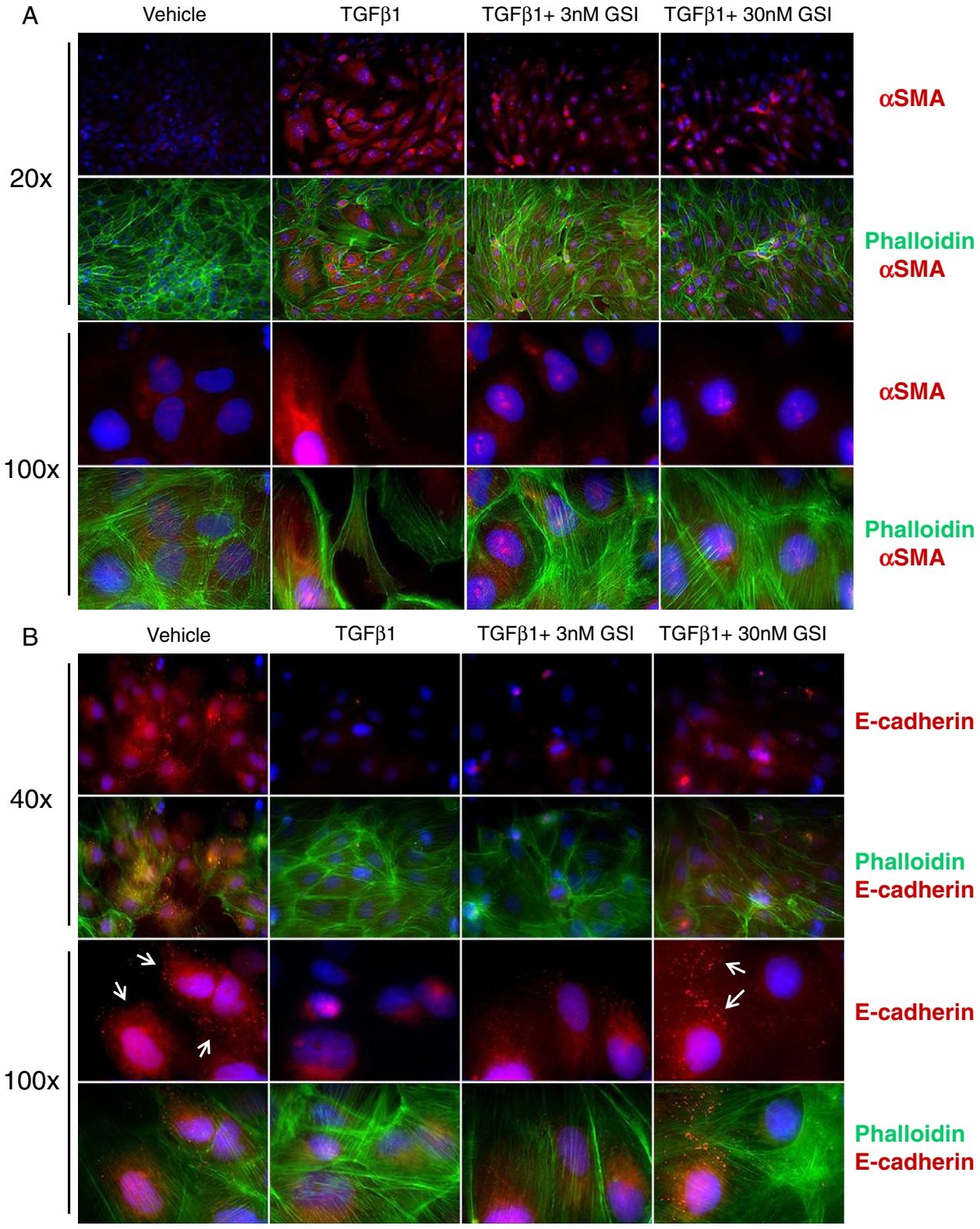

Fig. 5. γ-Secretase inhibitor inhibits TGFβ-induced changes in HK-2 epithelial cell phenotype. HK-2 cells were treated with TGFβ1 (5 ng/ml) for 24 h in the presence of vehicle (DMSO), 3 or 30 nM GSI. Cells were fixed and processed for immunofluorescence as described in Materials and methods. Cells were stained with primary antibody against α-smooth muscle actin (A, α-SMA, red) or E-cadherin (B, red), together with FITC-labelled phalloidin (green) to visualise stress fibres and DAPI (blue) to detect nuclei. Images are shown at 20× and 100× (A) and 40× and 100× (B), with and without phalloidin staining to highlight the signal from α-SMA and E-cadherin in each case. Arrows indicate staining of E-cadherin in the cell–cell junctions at 100× (B).

4. Discussion

Changes in the Jagged/Notch signalling pathway have previously been implicated in diabetic nephropathy and other fibrotic conditions of the kidney [25,27,31,32]. Using both a chemical inhibitor and cDNA transfection approach, we demonstrate that cleavage and activation of the Notch receptor is required for a subset of TGFβ1 gene responses in two distinct human kidney tubule epithelial cells (HK-2 and RPTEC).

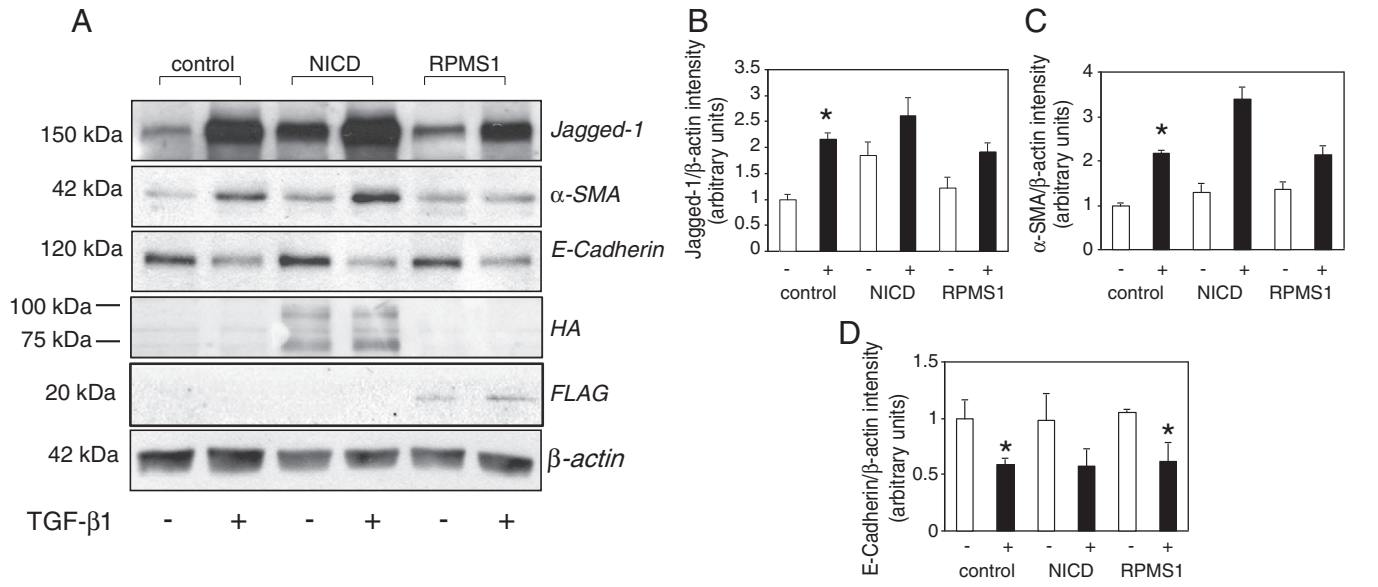

Fig. 6. Differential effects of Notch ICD and RPMS1 expression on TGFβ1-mediated EMT protein expression. HK-2 cells were transfected with empty vector (control), HA-tagged cleaved Notch receptor (NICD) or FLAG-tagged RPMS-1 transcriptional inhibitor as indicated. Cells were treated with 5 ng/ml TGFβ1 for 24 h. A. Total protein was extracted, separated by 7.5% SDS-PAGE, and probed with antibodies against Jagged1, α-SMA, E-cadherin, HA affinity tag and FLAG affinity tag. β-Actin was used as a loading control. Experiments were carried out n = 3 times. B–D. Densitometry was carried using Scion Image. Jagged1, E-cadherin and α-SMA band intensity was normalized to β-actin. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. *$p<0.05$.

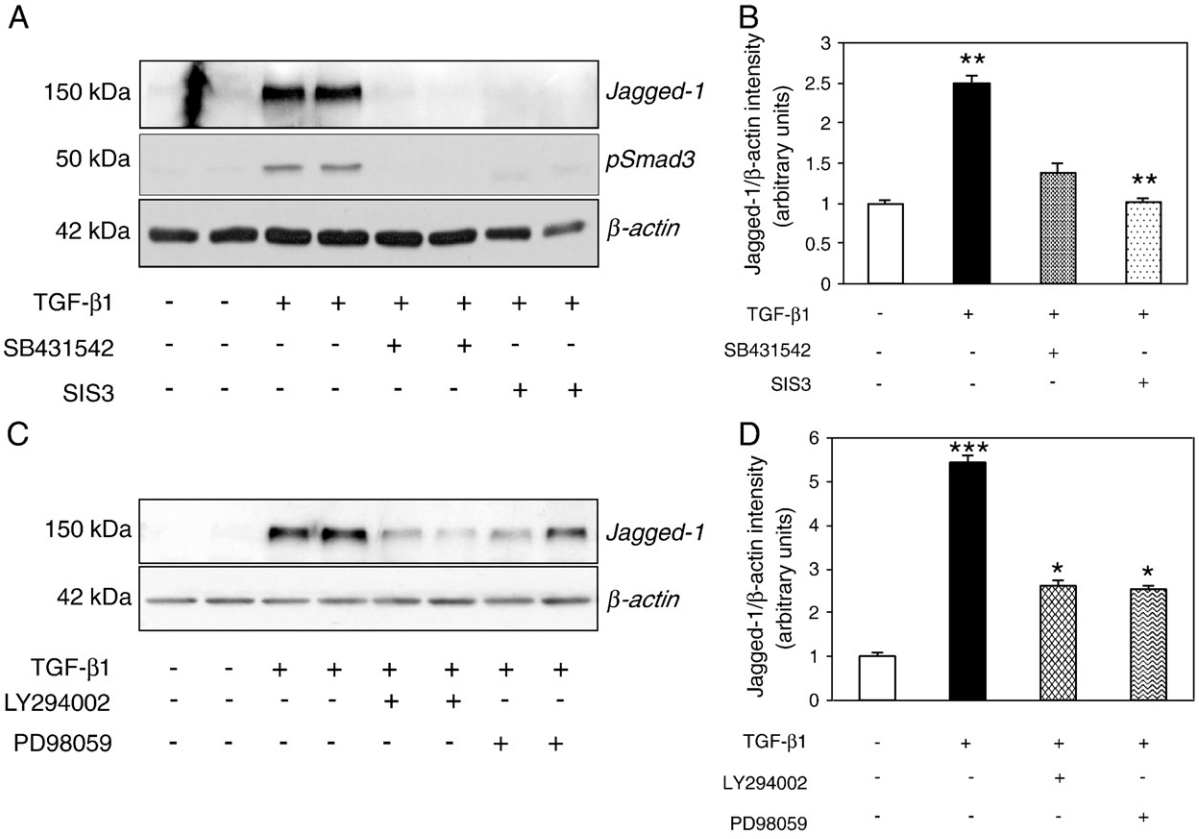

Fig. 7. Both canonical and non-canonical TGFβ1 signalling pathways regulate Jagged1 expression. HK-2 cells were treated with vehicle (empty bars) or 5 ng/ml TGFβ1 (filled bars) for 24 h. Cells were co-incubated with either the TGFβ1 receptor inhibitor SB431542 or the Smad3 inhibitor SIS3 (A, B), or the PI3K inhibitor LY294002 or the MAPK inhibitor PD98059 (C, D) at 10 μM concentration, added 1 h before TGFβ1. DMSO was added to both vehicle and TGFβ1-treated cells as a negative control. Protein lysates were separated by 7.5% SDS-PAGE, and probed with Jagged1 or phospho-Smad3 antibody. β-Actin was used as a loading control. Experiments were carried out n = 3 times. B, D. Densitometry was carried out using Scion Image and Jagged1 band intensity was normalized to β-actin. Statistical analysis was carried out using one-way ANOVA with post hoc Tukey–Kramer multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Both Jagged1 and the Jagged/Notch target gene Hes1 are exquisitely sensitive to γ-secretase inhibition, which prevents Notch receptor cleavage and activation. Upregulation of Jagged1 and Hes1 mRNA precede EMT-associated gene changes in response to TGFβ1 in HK2 cells. TGFβ1 induction of Jagged1 requires both canonical Smad3 phosphorylation and non-canonical PI3K/ERK activation. These data suggest that TGFβ1-mediated regulation of the Jagged/Notch signalling pathway in epithelial cells is a complex process that may involve autoregulation at the transcriptional level. A model summarising our data is presented in Fig. 8.

Intercellular interactions between Jagged/Delta and Notch2 [38] on adjacent cells play a key role in renal development, specifically in podocyte and proximal tubule formation [22,23,39]. A role for this signalling pathway has also been identified in autoimmunity, liver fibrosis and pancreatic cancer [28,29,31,32,40,41]. Jagged1 was initially identified as a TGFβ1 target gene in keratinocytes [24] and HK-2 kidney epithelial cells [25]. Levels of multiple components of the Notch pathway including Jagged1, proteolytically cleaved activated Notch receptor (NICD) and Hes1 were elevated in both animal models of diabetic nephropathy and human patient biopsies in both glomerular [31] and tubular kidney compartments [26]. In our hands, Jagged1 was the highest upregulated mRNA in human kidney proximal tubule epithelial cells (HK-2) treated with TGFβ1 for 24 h [26], with Hes1 upregulation also observed under these conditions (Fig. 1). Our data identified that TGFβ1-induced changes in Jagged1 and Hes1 were sensitive to inhibition of Notch receptor cleavage with γ-secretase inhibitor in both HK-2 and RPTEC cell lines (GSI, Fig. 2; Suppl. Figs. 2, 3). This is in contrast to data from keratinocytes, where GSI incubation did not inhibit TGFβ1-induced upregulation of Hey1, a Notch target related to Hes1 [27]. Differences in epithelial cell lines (kidney epithelial cells versus keratinocytes) and distinct GSI inhibitors (Compound E versus GSI-X) used may explain the disparity in these data. The earlier induction of Jagged1 at 12 h in response to TGFβ1 versus 24 h for EMT-associated genes such as α-SMA (Fig. 4) supports the idea that activation of Jagged/Notch signalling impinges on TGFβ1-induced EMT-like changes in kidney epithelial cells. Overexpression of the transcriptionally active Notch intracellular domain (NICD) also drove Jagged1 expression in the absence and presence of TGFβ1 (Fig. 6A, B). These data suggest that TGFβ1 induction of Jagged1 ligand and Hes1 target gene expression requires activation of the Jagged/Notch signalling cascade in kidney epithelial cells, which may form part of an autoregulatory loop limiting Jagged1/Hes1 expression to cells also expressing activated Notch receptor.

TGFβ1-mediated EMT-associated decreases in E-cadherin were also reversed by 24 h co-incubation with GSI in HK-2 and RPTEC cells,

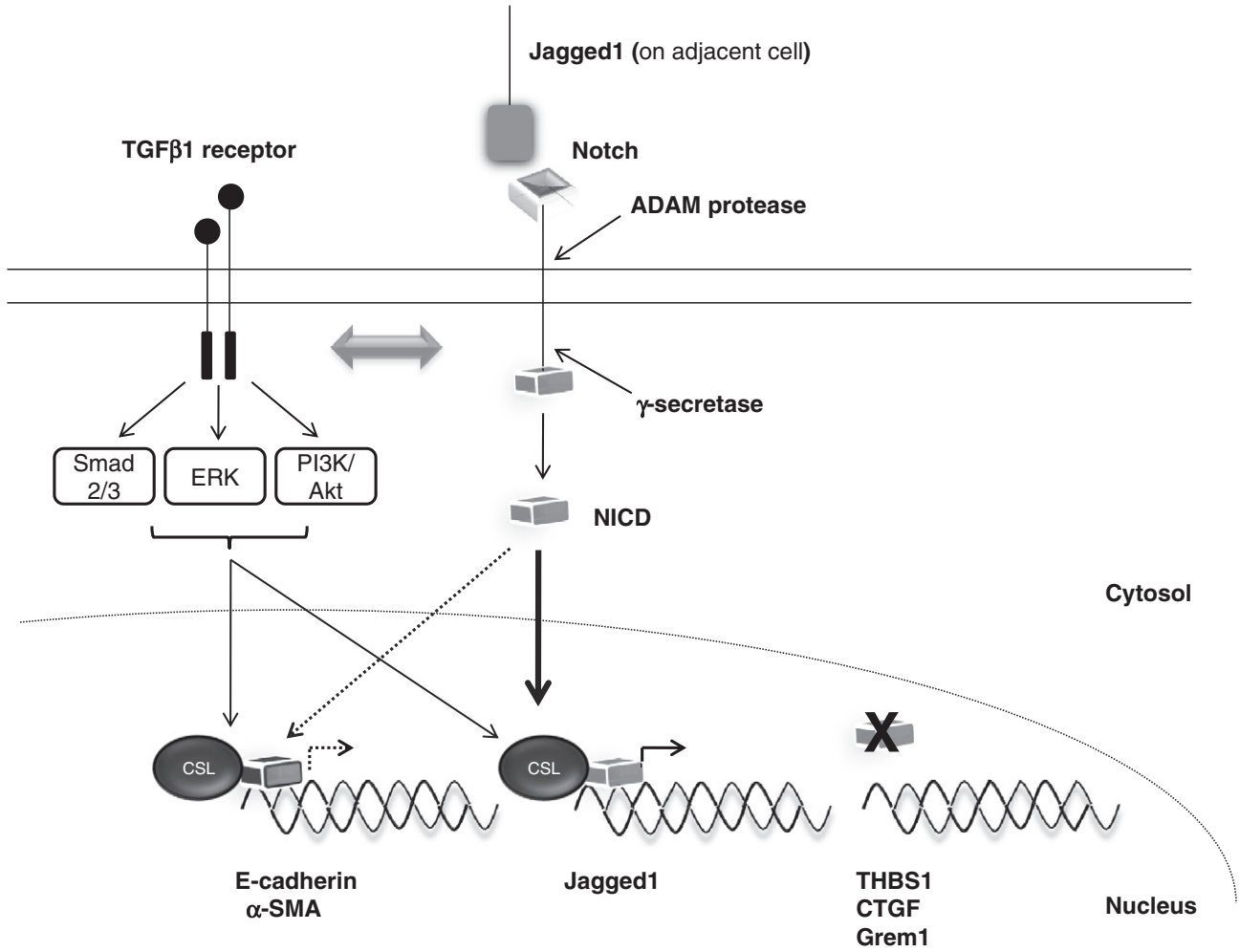

Fig. 8. Proposed mechanism of Notch-mediated regulation of TGFβ1 gene responses in kidney epithelial cells. Activation of TGFβ receptors triggers activation of multiple pathways including canonical Smad2/3 phosphorylation, PI3K/Akt and MAPK. Intercellular signalling involving Jagged/Delta ligands and Notch receptors on adjacent cells triggers ADAM-protease mediated cleavage of the extracellular domain of Notch, and intracellular liberation of the Notch intracellular domain (NICD) which translocates to the nucleus and binds to the CSL transcriptional complex (containing the RBPj transcription factor) to regulate gene transcription. TGFβ1-responsive genes such as E-cadherin and Jagged1 require Notch receptor cleavage for transcriptional regulation. Other genes such as thrombospondin-1 (THBS1), CTGF and Grem1 appear to be activated by TGFβ1 independent of Notch receptor influence. Further complexity of regulation may exist where NICD is sufficient to activate TGFβ1 targets such as Jagged1 (solid arrow) but not other genes such as E-cadherin (dashed arrow). Crosstalk between the two pathways is indicated by the double-headed arrow.

similar to previous data observed in keratinocytes [27]. Other genes associated with TGFβ-induced cytoskeletal changes in EMT such as α-SMA were also sensitive to GSI at different concentrations (Fig. 3B, C; Suppl. Fig. 3B). Clear changes in HK-2 cell phenotype were observed with TGFβ1, with actin rearrangement, increased stress fibres and a more "fibroblast"-like cell shape evident (Fig. 5). Increased staining for α-SMA at the lamellopodial extrusions and decreased E-cadherin staining at cell–cell junctions clearly suggested EMT-like changes in these cells (Fig. 5). GSI treatment reversed these effects on α-SMA expression, E-cadherin staining and overall "intermediate" cell phenotype (Fig. 5). However, TGFβ-induced increases in genes not involved in disassembly of adherens junctions such as CTGF, THBS1 and Grem1 were insensitive to inhibition by GSI (Fig. 3D–F). These data suggest that Notch signalling may play a more pronounced role in the regulation of a subset of TGFβ1-regulated genes involved in EMT. Proteolytically cleaved Notch (NICD) shuttles to the nucleus where it binds to CSL proteins, triggering transcriptional derepression of CSL-targeted genes in a complex manner [19]. Indeed, over-expression of NICD induced Jagged1 and α-SMA expression in HK-2 cells in the absence and presence of TGFβ1 (Fig. 6A, B; Suppl. Fig. 4). The lack of robust changes in E-cadherin mRNA and protein levels in cells transfected with NICD is surprising, and can possibly be explained by NICD being necessary but not sufficient for E-cadherin transcriptional repression, but necessary and sufficient for Jagged1 and α-SMA transcriptional activation. Transfection of RPMS1, a negative regulator of Notch IC via binding to the CBF-1 co-repressor complex [36], had minimal effects on gene expression minus/plus TGFβ1 (Fig. 6). While transfection efficiency is likely to be an issue here, the inability of RPMS-1 to abolish TGFβ1-mediated effects on Jagged1, α-SMA and E-cadherin supports the concept that Notch signalling is only one element of an intricate mechanism of TGFβ1-mediated transcriptional regulation. Recent data reporting the role of miRNA-192 in regulating TGFβ1-induced EMT and fibrosis in DN support a complex model of transcriptional regulation downstream of TGFβ1 in renal epithelial cells [42].

Multiple downstream signal transduction pathways are activated by TGFβ1, including canonical Smad signalling, PI3K, RhoA, NFκB and MAPK (reviewed in [43]). TGFβ1-induced increases in Jagged1 were completely Smad3 dependent and partially PI3K/MEK dependent (Fig. 7). These data are consistent with previous observations demonstrating that genetic deletion of Smad3 abolishes TGFβ1-induced Hey1 and Jagged1 induction in primary mouse tubular epithelial cells [27]. Bioinformatic analysis of the predicted Jagged1 promoter (3 kb upstream of the transcription start site) identified Smad3/Smad4 binding sites in human, mouse and rat sequences suggesting that direct regulation of Jagged1 transcription occurs via Smad3/4 promoter binding (data not shown). Previous data from our laboratory and others showed that TGFβ1-induced changes in EMT-associated proteins such as E-cadherin and α-SMA were also PI3K/Akt dependent in kidney epithelial cells [5], as well as mammary epithelial cells [4]. Previous authors have identified a crosstalk between Notch signalling and Akt, where increased Akt activity in lymphoblasts leads to resistance to GSI-mediated Notch inhibition and T-cell apoptosis [44]. Additionally, Akt has been shown to directly interact with Smad3 preventing its phosphorylation and nuclear accumulation and inhibiting TGFβ1-induced apoptosis [45,46]. Thus, a complex sequence of signalling events and interactions involving Notch, Smad proteins and PI3K/Akt mediate epithelial cell responses to TGFβ1.

The requirement for Jagged/Notch activity for TGFβ1-mediated expression of Jagged1 and EMT-associated genes such as E-cadherin suggests that these genes contain NICD/CSL target sequences in their promoter regions. MatInspector analysis identified RBPJ (a component of the CSL complex) transcription factor binding sites in both human Jagged1 and Hes1, but not CTGF or THBS1. This site was not predicted in human E-cadherin promoter, and only Hes1 contained conserved RBPJ sites in human, mouse and rat promoters (data not shown). Others have demonstrated that Notch activation drives the expression of transcription factors such as Snail and Slug which directly regulate genes such as E-cadherin to induce EMT in mammary and kidney epithelial cells [47,48]. We predict that TGFβ1 target genes sensitive to GSI may contain CSL recognition sequences in their promoters, compared to TGFβ1 target genes such as CTGF and THBS1 which may not contain these CSL sites. Future approaches using bioinformatics and chromatin immunoprecipitation using NICD/CSL antibodies will interrogate this hypothesis. Together with data from other authors using different epithelial cells such as primary human proximal tubule (PTEC), keratinocytes and primary human breast epithelia our results suggest that Notch signalling may impinge on specific gene responses involved in TGFβ-induced EMT [24,27,47,48]. Future experiments will elucidate the exact mechanisms of Notch-mediated regulation of TGFβ1 gene transcription in epithelial cells.

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