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Original Research

TGFβ1-mediated PI3K/Akt and p38 MAP kinase dependent alternative splicing of fibronectin extra domain A in human podocyte culture

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Abstract: Alternative splicing is an important gene regulation process to distribute proteins in health and diseases. Extra Domain A+ Fibronectin (EDA+Fn) is an alternatively spliced form of fibronectin (Fn) protein, present in the extra cellular matrix (ECM) and a recognised marker of various pathologies. TGFβ1 has been shown to induce alternative splicing of EDA+Fn in many cell types. Podocytes are spectacular cell type and play a key role in filtration and synthesise ECM proteins in renal physiology and pathology. In our previous study we have demonstrated expression and alternative splicing of EDA+Fn in basal condition in human podocytes culture. TGFβ1 further induced the basal expression and alternative splicing of EDA+Fn through Alk5 receptor and SR proteins. In this study, we have investigated TGFβ1 mediated signalling involved in alternative splicing of EDA+Fn in human podocytes. We have performed western blotting to characterise the expression of the EDA+Fn protein and other signalling proteins and RT-PCR to look for signalling pathways involved in regulation of alternative splicing of EDA+Fn in conditionally immortalised human podocytes culture.We have used TGFβ1 as a stimulator and SB431542, SB202190 and LY294002 for inhibitory studies. In this work, we have demonstrated in human podocytes culture TGFβ1 2.5ng/ml induced phosphorylation of Smad1/5/8, Smad2 and Smad3 via the ALK5 receptor. TGFβ1 significantly induced the PI3K/Akt pathway and the PI3K/Akt pathway inhibitor LY294002 significantly downregulated basal as well as TGFβ1 induced alternative splicing of EDA+Fn in human podocytes. In addition to this, TGFβ1 significantly induced the p38 MAP kinase signalling pathway and p38 MAP kinase signalling pathway inhibitor SB202190 downregulated the TGFβ1-mediated alternative splicing of EDA+Fn in human podocytes. The results with PI3K and p38 MAP kinase signalling pathway suggest that inhibiting PI3K signalling pathway downregulated the basal alternative splicing of EDA+Fn in human podocytes and its the inhibition of p38 Map Kinase signalling pathway which had specifically downregulated the TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes culture. Activation of TGFβ1-mediated Smad1/5/8 via Alk5 receptor suggests that TGFβ1 signalling pathway involved Alk5/Alk1 receptor axis signalling in human podocytes.

Key words: Extra Domain A; Fibronectin; Alternative splicing; Podocyte; TGFβ1; PI3k/Akt pathway; p38 MAP Kinase pathway.

Introduction

Alternative splicing is a widespread gene regulation process occurring in the wide range of organisms. Alternative splicing leads to formation of different isoforms of proteins from a single gene (1). Extra Domain A + Fibronectin (EDA+Fn) is an alternatively spliced isoform of Fibronectin (Fn) which has been shown to be expressed in various pathologies and serve as a marker of early pathologies (2–6). In many studies, it has been demonstrated that TGFβ1 induced the alternative splicing of EDA+Fn. TGFβ1 expression in glomeruli and tubulointerstitium is demonstrated to be associated with the increased expression of EDA+Fn in diseases related to an accumulation of ECM (7). In chronic glomerulonephritis and diabetic nephropathy, it has been shown that overexpression of TGFβ isoforms 1, 2, 3 are associated with the up-regulation of EDA+Fn expression (8). Studies in tubular epithelial cells show that TGFβ1 induces up-regulation of Fn and EDA+Fn in cell lysates as well as supernatants (9). TGFβ1 mediated up-regulation of EDA+Fn induces the fibroblast differentiation and thus leads to upregulation of ECM proteins (10). TGFβ1 shown to induce alternative splicing of EDA+Fn in PTEC cells by SR proteins and thus involved in tubulointerstitial fibrosis (11). Hepatocyte growth factor has been previously demonstrated in MDCK cells to regulate alternative splicing of EDA+Fn in comparison to TGFβ1 (12).

Podocytes are spectacular cell type which plays a critical role in filtration. Podocytes form the filtration barrier in glomerulus along with endothelial cells and glomerular basement membrane (GBM) and involve in the continuous remodelling of GBM. In previous studies, it has been demonstrated that TGFβ1 significantly induces the Fn gene expression in conditionally immortalised human podocytes culture (13). In conditionally immortalised mouse podocytes TGFβ1 induces the Fn gene expression and protein expression (14).

TGFβ1 is a multifunctional cytokine that mediates diverse cellular functions in a cell type and context dependent manner. TGFβ1 imparts its effects by activating a distinct range of signalling pathway (15-16). TGFβ1 mediates its signalling by binding to its receptors which in turn recruits different proteins. The members of the TGFβ superfamily proteins binds to two distinct receptors types known as type II and type I receptors, both are required for signal transduction. There are five type II

receptors and seven type I receptors (ALK1 to -7) present in mammals. The well-studied TGFβ1 signalling pathway is the Smad protein signalling pathway (16). TGFβ1 binds to its serine-threonine kinase receptor type II. Two molecules of each type II and type I receptors form hetero-tetrameric complexes. In this complex, the type II receptor then trans-phosphorylates the type I receptors. The type I receptor then recruits and phosphorylates different transcriptional Smad proteins.

Smad proteins are categorised in three subclasses which are receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads). Regulatory Smads are further divided into two subclasses Smad2 and Smad3 which are activated by TGFβ1 type I receptors (ALK5) and Smad1/5/8 activated by bone morphogenetic receptors. Smad1 is also activated by TGFβ1 type I receptor (ALK1) in endothelial cells (15,17,18). After activation, these R-Smads then forms complex with Smad4 (Co-Smad) and this complex then translocates to the nucleus and regulates the transcription of a target gene by binding to promoter region (18,19).

The Smad2 and Smad3 pathway are called as TGFβ mediated canonical signalling pathway whereas Smad1/5/8 are called as TGFβ mediated non-canonical Smad signalling pathway (20). TGFβ1 has been shown to mediate a vast range of cellular functions such as growth, proliferation, apoptosis, ECM production, migration and adhesion (21). The regulation of all these functions is critical for the healthy development and functioning of cells. TGFβ1 mediates these functions by regulating a diverse range of signalling pathways. Disrupted regulation of these signalling pathways leads to various pathologies. TGFβ1 has been reported to regulate signalling pathways independent of Smads such as MAP kinase signalling and PI3K/Akt signalling pathway (19,22,23).

Mitogen-activated protein (MAP) kinases are superfamily of intracellular signalling molecules which operates through different subfamilies of proteins namely the extracellular signal-regulated protein kinases (ERK1, ERK2 and ERK5); the c-jun NH2-terminal kinases (JNK1, JNK2, JNK3) and p38 (-α,-β,-γ and -δ) (24). The p38 MAP kinase pathway plays a vital role in cellular processes such as inflammation, differentiation and apoptosis. MAP kinase pathway can be activated by growth factors such as TGFβ1, EGF and external stimuli such as mechanical stress, osmotic shock etc. The p38 MAP kinase pathway has been shown to regulate the Fn expression in transformed human kidney cells by our group (25). I have investigated the p38 MAP Kinase pathway in human podocytes in response to TGFβ1 and its role in the regulation of EDA+Fn expression in human podocyte culture by using SB202190, a potent chemical inhibitor of p38 MAP Kinase pathway. SB202190 is a pyridinyl imidazole, which has been shown to inhibit the p38α and p38β through competing with ATP (1) and thus inhibits the phosphorylation of p38 MAP kinase (26). SB202190 2.5μM has been shown to downregulates the Fn expression in human kidney cells by our group (25).

The PI3K pathway has been implicated in a diverse range of cellular activities like cell proliferation, survival, adhesion, cytoskeleton remodelling etc. Many studies show that the PI3K pathway is activated by TGFβ1 in mesangial cells and epithelial cells and prevents apoptosis (27–29). PI3K pathway has been shown to regulate the TGFβ1 mediated alternative splicing of EDA+Fn in mouse fibroblast (30). PI3K pathway has been demonstrated to be involved in the TGFβ1 mediated alternative splicing of EDA+Fn in human PTEC cells (11). I have investigated the PI3K/Akt kinase signalling pathway in the regulation of alternative splicing of EDA+Fn in human podocyte culture by using LY294002, a potent chemical inhibitor of the PI3K signalling pathway (31–33). LY294002 is an ATP-competitive inhibitor and has been shown to inhibit alternative splicing of EDA+Fn at a 5μM concentration in PTEC cells by our group (11).

In our previous study we have demonstrated expression and alternative splicing of EDA+Fn in basal condition in human podocytes culture. TGFβ1 further induced the basal expression and alternative splicing of EDA+Fn through Alk5 receptor and SR proteins. In this paper, we have investigated TGFβ1 mediated Smad signalling pathway, p38 MAP Kinase signalling pathway and PI3K signalling pathways in the regulation of EDA+Fn expression in human podocytes culture.

Materials and Methods

Cell culture

Conditionally immortalized human podocyte cell culture retrovirally transfected by temperature sensitive SV40 large T-antigen (Developed by Dr Moin Saleem, a kind gift from Jochen Raiser) were cultured as monolayer at the permissive temperature of 33°C in a humidified atmosphere of 5% CO_2 and 95% air, with RPMI 1640 medium supplemented with heat-inactivated 10% Fetal Calf Serum, L-glutamine (2mM)-penicillin (100U/ml)-streptomycin (100μg/ml) antibiotics, 5mM D-Glucose and insulin (5μg/ml)-transferrin (5μg/ml)-sodium selenite (5ng/ml). The medium was changed every alternate day. Confluent cells were passaged by aspirating the media and incubating with trypsin–EDTA solution (trypsin (5 g/l), Na2-EDTA (2 g/l), NaCl (8.5 g/l) for 4 minutes at 33°C temperature. Trypsin was neutralised with normal culture medium and cells were centrifuged at 350 x g for 6 min at room temperature. The cell pellet was re-suspended in fresh culture medium. Viable cells were counted with 0.4% trypan blue dye exclusion method and were seeded at a density of 10,000 cells/cm^2. For experimental studies, podocytes were grown for 4 days at 33°C (permissive condition) and then at 37°C for 14 days (Non-permissive condition). All the experiments were conducted on overnight serum starved 80-90% confluent culture of terminally differentiated podocytes passages between 3 and 25.

Stimuli and inhibitors

Serum-starved differentiated podocytes at 37°C were treated with TGFβ1 2.5ng/ml or vehicle (0.1% w/v BSA).And for inhibitory studies serum-starved differentiated podocyte cells were pre and co-treated with ALK5 inhibitor SB431542 (10μM), p38 MAP kinase inhibitor SB202190 (2.5 μM and 5μM) and PI3K/Akt pathway inhibitor LY29004 (5μM) with or without TGFβ1 (2.5ng/ml) compared with vehicle (0.1% w/v BSA) for indicated period of time.

Western Blot Analysis

After the treatment, cells were washed once with ice-cold 1 x PBS and lysed by cell scraper in 70µl ice-cold lysis buffer Tris/HCl (20mM), NaCl (150mM), 1 % (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM EDTA, phosphatase inhibitor cocktail (1x) and protease inhibitor cocktail (2x). Cell lysates were kept on ice for 15min and centrifuged at 10000 x g at 4°C for 10 min to remove cell debris. Supernatants containing protein were collected and the pellet containing cell debris was discarded. Supernatants containing proteins were either stored at -80°C for future use or was subjected to protein quantification. Total cellular protein concentration was determined using the colorimetric BCA (bicinchoninic acid) protein assay kit following manufacturer protocol.

The protein samples were prepared for western blotting under denaturing and reducing condition by heating at 70°C for 10min in a solution containing NuPAGE LDS Sample buffer (1x) and NuPAGE Sample Reducing Agent (1x). Equal amounts of cellular proteins were subjected to SDS-PAGE. Proteins were transferred onto PVDF membrane for 4h in NuPAGE transfer buffer at 30 V using the XCell II Blot Module (Life Technology). After transfer the blots were washed with TBS-T buffer [Tris-buffered saline/20mM Tris/HCl, 150mM NaCl and 0.1% (v/v) Tween 20) 5% (w/v)] on 3D gyratory rocker for 15 min. Blots were then blocked with TBS-T fat-free milk 5% (w/v) for 60min. Blots were washed for 10min x 3times with TBS-T. Blots were incubated with appropriate primary antibodies (Table 1) either in TBS-T with 5% (w/v) BSA (rabbit polyclonal antibodies) or in TBS-T with 5% (w/v) fat-free milk (Mouse Monoclonal antibodies) at 4°C overnight. Blots were washed for 10min x 3times with TBS-T buffer on a 3D gyratory rocker. Blots were incubated with horseradish peroxidase HRP-labelled secondary antibody for 1h at room temperature at 3D gyratory rocker and developed with ECL Prime enhanced chemiluminescence western blotting detection system and visualised with Hyperfilm ECL photographic film developed by MI-5 X-ray film processor (VWR). Blots were stripped and re-probed for housekeeping tubulin protein as a loading control.

Western blot films were imaged by ImageQuant 300 Imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

RNA extraction, reverse transcription and PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen UK Ltd) using manufacturer's protocol. All buffers for RNA extraction were provided as part of extraction kit. All disposable plastic wares were purchased as DNase and RNase free. Total cellular RNA concentration was then quantified by measuring the absorbance at 260nm and quality was measured by determining 260 to 230 ratio using NanoDrop Spectrophotometer. The extracted RNA was subjected to Reverse Transcription (RT) to synthesise cDNA. RT was performed to synthesise cDNA using Reverse Transcriptase system (Applied Biosystem, Foster City, CA, USA). The cDNA generated after the RT-PCR was subjected to conventional PCR amplification for EDA+/-Fn. The PCR reaction was performed using the Crimson Taq Polymerase (New England Biolabs) in a total volume of 25µl and the volume of cDNA template was 1µl from 1 in 10 diluted RT reactions. Each forward and the reverse primer was used at 20µM with 1.25 U polymerase per reaction. The inclusion of EDA exon was detected using a pair of primer binding constitutively spliced exon upstream and downstream of 270bp EDA exon. The PCR leads to inclusion of EDA exon which yields two products a 104bp band fragment corresponding to EDA-Fn and a 374bp fragment corresponding to EDA+Fn detected as two separate bands after the agarose gel electrophoresis.

The primer sequence for EDA+/-Fn was
5'GGAGAGAGTCAGCCTCTGGTTCAG3' Forward,
5'TGTCCACTGGGCGCTCAGGCTTGTG3' Reverse.

PCR steps were as: initial denaturation for 5 min at 95°C and 25 cycles of 30 sec at 95°C for, annealing temperature for 30 sec at 56°C, 30 sec at 68°C and final extension for 5 min at 68°C. The PCR product is separated by 1.5% agarose gel electrophoresis and visualised with ethidium bromide staining under UV light. The separated bands of EDA+/-Fn in the agarose gel were captured by ImageQuant 300 imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

Statistical Analysis

Data handling, Statistical analysis and presentation were performed using Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism, v4.0 (GraphPad Software, Inc). Results were expressed as Mean ± SEM (standard error of the mean) of 3-6 independent experiments. Comparison between the means of 2 groups was made by Student's t-test. Comparison between the means of more than two groups was made by Analysis

Table 1. Antibody dilutions used for Western blotting.

Target protein	Size(KDa)	Antibody	Dilution	Cat. no	Supplier
EDA+ Fn	220	Mouse monoclonal	1:1000	ab 6328	Abcam
pSmad2	60	Rabbit monoclonal	1:1000	3108	Cell signaling
pSmad3	52	Rabbit monoclonal	1:1000	9520	Cell signaling
pSmad1/5/8	60	Rabbit polyclonal	1:1000	9511	Cell signaling
pP38	43	Rabbit polyclonal	1:1000	9211	Cell signaling
pAkt (Phospho-Akt-Ser473 antibody)	60	Rabbit polyclonal	1:1000	9271	Cell signaling
Antimouse IgG(Secondary Ab)	-	Rabbit	1:40	A9044	Sigma-Aldrich
Antirabbit IgG(Secondary Ab)	-	Rabbit	1:1000	7074	Cell signaling
α/β-Tubulin	55	Rabbit polyclonal	1:1000	2148	Cell signaling

of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test. P value <0.05 was considered as significant.

Results

Characterisation of TGFβ1-mediated canonical Smad signalling pathway in human podocyte culture

Podocytes were allowed to differentiate, serum starved overnight and treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of pSmad2, pSmad3 and tubulin as housekeeping protein. TGFβ1 significantly induced the pSmad2 and pSmad3 phosphorylation as compared to vehicle (Figure 1).

Characterisation of TGFβ1-mediated non-canonical Smad signalling pathway in human podocyte culture

Podocytes were grown till differentiation, serum starved overnight and treated with TGFβ1 2.5ng/ml compared with vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of pSmad1/5/8 and tubulin as housekeeping protein. TGFβ1 significantly induced the pSmad1/5/8 phosphorylation as compared to vehicle (Figure 2).

TGFβ1-mediated Smad signalling by TGFβ Type I receptor ALK5 in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days and serums starved overnight. Podocytes were pre-incubated (1h) and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and SB431542 (10μM), SB431542 (10μM) and compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of pSmad3 and pSmad1/5/8 and tubulin as housekeeping protein. The SB431542 significantly downregulated TGFβ1-induced pSmad3 (Figure 3, Panel A) and pSmad1/5/8 phosphorylation (Figure 3, Panel B) as compared to vehicle.

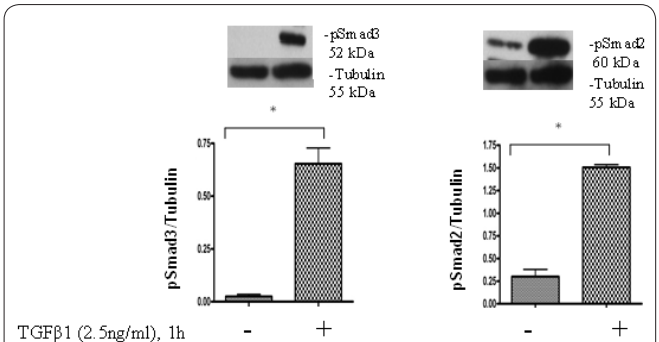

Figure 1. The TGFβ1 mediated phosphorylation of pSmad3 (Panel A), pSmad2 (Panel B) indexed to tubulin as housekeeping protein. Serum-starved differentiated podocytes were treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Student's t-test was performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

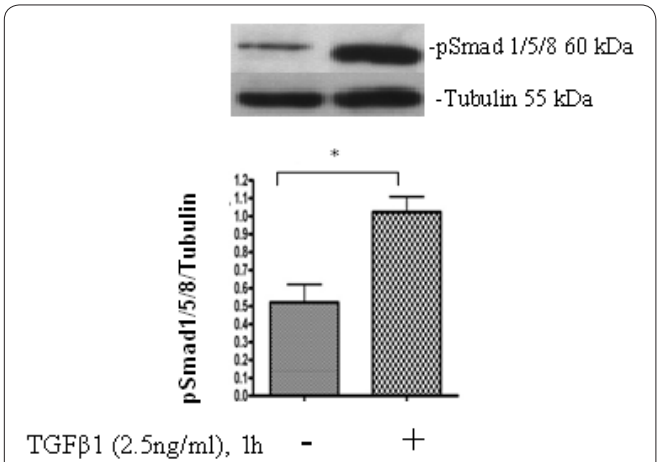

Figure 2. The TGFβ1 mediated pSmad1/5/8 phosphorylation indexed to tubulin as housekeeping protein. Serum-starved differentiated podocytes were treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Student's t-test was performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

Characterisation of TGFβ1-mediated PI3K/Akt pathway in human podocyte culture

Podocytes were allowed to differentiate for 14 days, serum starved overnight and treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of pAkt and tubulin as housekeeping protein. TGFβ1 treatment induced the pAkt phosphorylation as compared to vehicle (Figure 4).

TGFβ1-mediated PI3K/Akt pathway in regulation of EDA+Fn protein expression in human podocyte culture

Podocytes were allowed to differentiate, serum starved overnight and treated with TGFβ1 2.5ng/ml, pre (1h) and co-incubated with PI3k/Akt inhibitor LY294002 5μm, compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 72h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for expression of EDA+Fn and tubulin

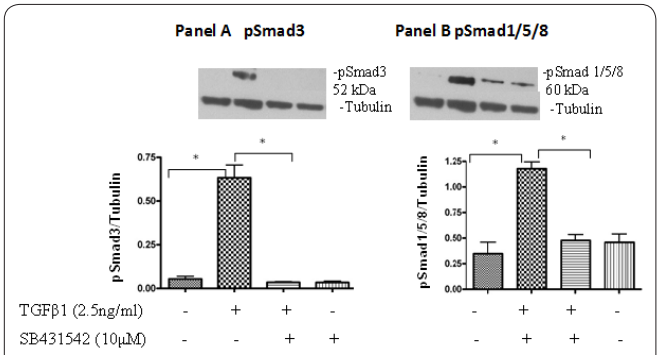

Figure 3. The pSmad3 and pSmad1/5/8 phosphorylation indexed with tubulin as housekeeping protein. Serum-starved differentiated podocytes were pre-incubated (1h) and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and SB431542 (10μM), SB431542 (10μM) and compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 1h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

as housekeeping protein. TGFβ1 induced the expression of EDA+Fn as compared to vehicle. LY294002 significantly downregulated the TGFβ1-induced EDA+Fn expression. LY294002 treatment significantly downregulated the basal EDA+Fn expression (Figure 5).

TGFβ1-mediated PI3K/Akt pathway in regulation of alternative splicing of EDA+Fn in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days and serums starved overnight. Podocytes were pre (1h) and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and PI3K/Akt pathway inhibitor LY294002 (5μM), LY294002 (5μM), compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 24h. Cells were lysed and RNA was extracted. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA ratio. TGFβ1 significantly increased EDA+/-Fn mRNA ratio as compared to vehicle. LY294002 significantly downregulated the TGFβ1-increased EDA+/-Fn mRNA ratio. LY294002 treatment significantly downregulated the basal EDA+/-Fn mRNA ratio (Figure 6).

Characterisation of TGFβ1-mediated p38 MAP kinase signalling pathway in human podocyte culture

Podocytes were allowed to differentiate for 14 days, serum starved overnight and treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of phospho-p38 and tubulin as housekeeping protein. TGFβ1 significantly induced the phospho-p38 phosphorylation as compared to vehicle (Figure 7).

TGFβ1-mediated p38 MAP kinase pathway in regulation of EDA+Fn protein expression in human podocyte culture

Podocyte cells were allowed to differentiate for 14 days, serum starved overnight and treated with TGFβ1 2.5ng/ml pre (1h) and co-incubated with MAP kinase inhibitor SB202190 2.5μm and 5μm, compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 72h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for EDA+Fn expression and tubulin as housekeeping protein. TGFβ1 significantly induced the EDA+Fn expression as compared to vehicle. MAP kinase inhibitor SB202190 (2.5μm and 5μm) significantly downregulated the TGFβ1-induced EDA+Fn expression, however, increasing the concentration of SB202190 to 5μm did not further downregulated the TGFβ1-induced EDA+Fn expression (Figure

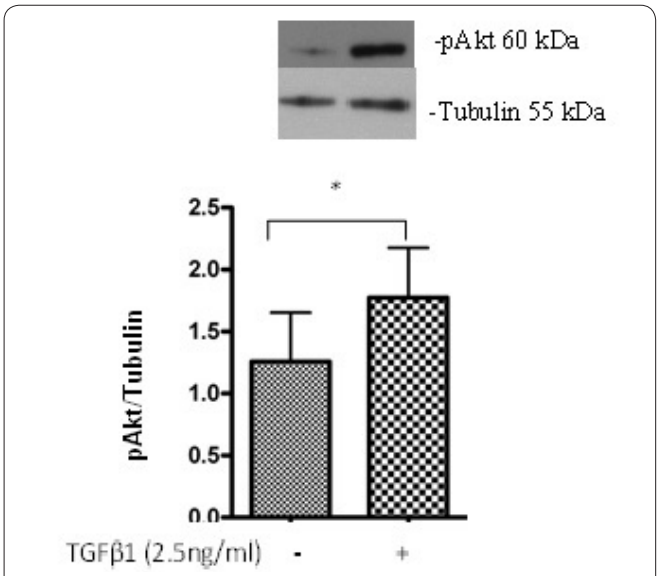

Figure 4. The pAkt phosphorylation indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 2.5ng/ml and compared with (vehicle 0.1% BSA). Student's t-test was performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

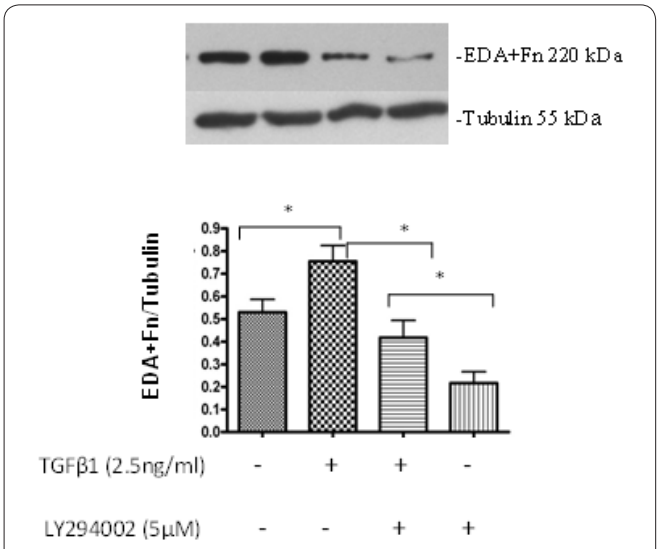

Figure 5. TGFβ1 mediated EDA+Fn expression indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 2.5ng/ml pre (1h) and co-incubated with PI3K inhibitor LY294002 5μm for 72h, compared with vehicle (0.1% w/v BSA, 0.1% DMSO). Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

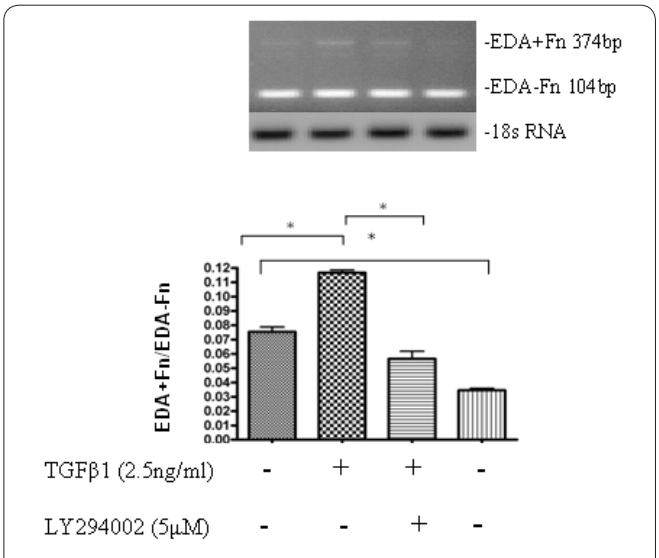

Figure 6. The TGFβ1 mediated EDA+/-Fn mRNA expression downregulated by LY294002 treatment. TGFβ1 2.5ng/ml significantly induces the expression of EDA+/-Fn mRNA ratio as compared to vehicle and this was downregulated by LY294002 (5μM) at 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

8).

TGFβ1-mediated p38 MAP kinase pathway in regulation of alternative splicing of EDA+Fn in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days and serums starved overnight. Podocytes were pre-incubated (1h) and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and pP38 MAP kinase inhibitor SB202190 (2.5μM), SB202190 (2.5μM) and compared with vehicle (0.1% w/v BSA, 0.1% DMSO) for 24h. Cells were lysed and RNA was extracted. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA ratio. TGFβ1 significantly increased the EDA+/-Fn mRNA ratio as compared to vehicle. SB202190 significantly downregulated the TGFβ1-increased EDA+/-Fn mRNA ratio (Figure 9).

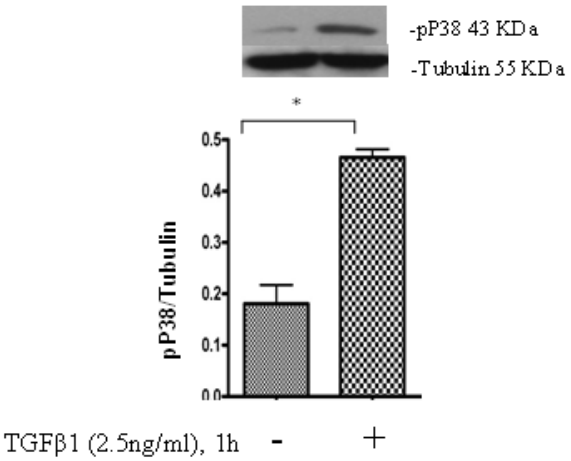

Figure 7. The TGFβ1 induced pP38 phosphorylation indexed to tubulin as housekeeping protein. Serum-starved differentiated podocytes were treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Student's t-test was performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

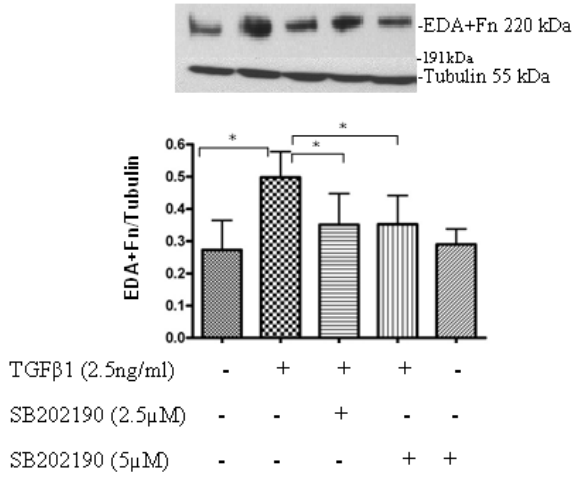

Figure 8. The TGFβ1-induced EDA+Fn expression indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 2.5ng/ml, pre (1h) and co-incubated with MAP kinase inhibitor SB202190 2.5μm and 5μm for 72h, compared with vehicle (0.1% w/v BSA, 0.1% DMSO). Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

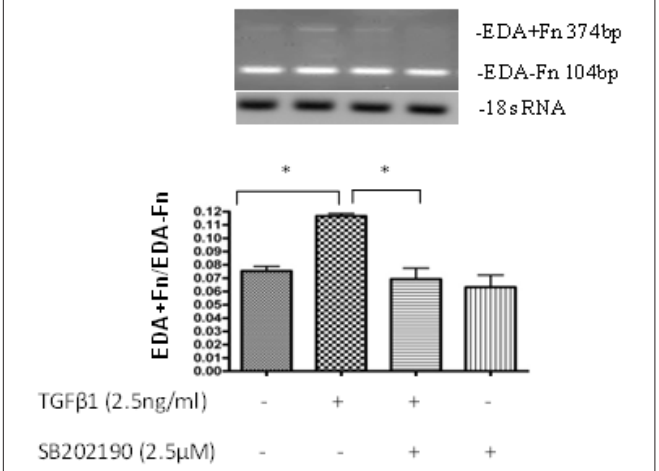

Figure 9. The TGFβ1 mediated EDA+/-Fn mRNA expression downregulated by SB202190 treatment. TGFβ1 2.5ng/ml significantly induced the expression of EDA+/-Fn mRNA ratio as compared to vehicle and this was downregulated by SB202190 (2.5μM) at 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

Discussion

TGFβ1 is multifunctional cytokine which is a key regulator of various cellular pathways in physiology and pathology. TGFβ1 induced Smad pathways are characterised as either canonical which involves Smad2 or Smad3 or as non-canonical Smad signalling pathway which involves Smad1/5/8. TGFβ1 has been shown to induce Smad-independent signalling pathways such as MAP kinase signalling pathway and PI3K/Akt signalling pathway (19,31,34). In this paper, we have investigated TGFβ1 mediated Smad signalling pathway, p38 MAP Kinase signalling pathway and PI3K signalling pathways in the regulation of EDA+Fn expression in human podocytes culture.

TGFβ1 mediated Smad signalling pathway has been shown to involved in renal fibrosis by upregulating ECM proteins expressions (22). TGFβ1 mediated Smad signalling has been demonstrated in mouse podocytes culture (35). In this studies, we have characterised TGFβ1 mediated Smad signalling in human podocytes culture. In human podocytes culture, TGFβ1 2.5ng/ml treatment for 1h significantly induced the pSmad3, pSmad2 and pSmad1/5/8 phosphorylation as compared to vehicle. These results suggest that in human podocytes TGFβ1 2.5ng/ml activates both canonical and TGFβ1 mediated non-canonical Smad signalling pathways. We have investigated the role of TGFβ1 Type I ALK5 receptor in TGFβ1 mediated activation of Smad signalling pathway using ALK5 receptor inhibitor SB431542. Results from these experiments suggest that SB431542 significantly downregulated TGFβ1 mediated Smad3 and Smad1/5/8 phosphorylation in human podocyte culture. These results suggest that TGFβ1 mediated canonical and non-canonical Smad signalling in human podocytes involved ALK5 receptor.

In endothelial cells, it has been demonstrated that TGFβ1 activates the Smad1 signalling pathway via receptor ALK1. The ALK1 and ALK5 form the hetero-

meric receptor complex. TGFβ1 enhances the ALK1 and ALK5 receptor complex formation. The ALK5 receptor has been shown to be essential for activation of TGFβ1/ALK1 mediated Smad1 signalling pathway (36-38). Thus TGFβ1 induced activation of the Smad1/5/8 signalling pathway in human podocytes culture could involve the ALK1 receptor and inhibition of ALK5 receptor by SB431542 may inhibit the ALK1 mediated Smad1/5/8 activation. Smads proteins are known to act as transcriptional factors which regulate the gene expression by regulating the transcription by directly binding to the promoter region of the gene (36). There is no published evidence demonstrating the role of Smads in the regulation of splicing. I have made few attempts to characterise TGFβ1 mediated Smad signalling events in the regulation of EDA+Fn expression in human podocytes using SIS3 and siRNA targeted to Smads (results not shown) but failed to provide any evidence of the involvement of Smad proteins in alternative splicing of EDA+Fn. TGFβ1 has also been reported to mediate pathways other than Smads associated with renal diseases such as MAP kinase pathway and PI3K/Akt pathway (37).

I have investigated PI3K/Akt signalling pathway in the regulation of EDA+Fn expression and alternative splicing in human podocytes culture. The results of the experiment suggest that human podocyte culture expressed a high basal level of pAkt and TGFβ1 significantly induced the further phosphorylation of pAkt. Insulin has been shown to induce PI3K/Akt pathway (38). Growth medium contains insulin as a supplement and could induce high basal phosphorylation of pAkt. To demonstrate PI3K/Akt signalling pathway in the regulation of TGFβ1 mediated EDA+Fn expression in human podocytes culture, I have used PI3K pathway inhibitor LY294002. The results of this experiment suggest that LY294002 significantly downregulated the TGFβ1-induced EDA+Fn protein expression. LY294002 also significantly downregulated the basal EDA+Fn expression. LY294002 also downregulated the basal EDA+/-Fn mRNA ratio as well as TGFβ1-induced EDA+/-Fn mRNA ratio in human podocytes culture. These results demonstrate that inhibiting the PI3K/Akt pathway by LY294002 downregulated the basal as well as the TGFβ1-induced alternative splicing in human podocytes culture. These results suggest that in human podocyte culture PI3K pathway regulates the basal as well as the TGFβ1-induced expression and alternative splicing of EDA+Fn. These results are consistent with the results in PTEC cells where PI3K/Akt pathway has been previously demonstrated to regulate the alternative splicing of EDA+Fn (11). PI3K/Akt pathway is reported to be involved in various cellular functions like cell proliferation, cell adhesion and cell survival (31,39). It has been shown that PI3K/Akt pathway is involved in the regulation of expression of ECM proteins and thus in the regulation of fibrosis. In human primary tubular epithelial cells (PTEC), it has been demonstrated that PI3K/Akt pathway mediates the alternative splicing of EDA+Fn. In PTEC cells it has been shown that alternative splicing of EDA+Fn involves the SR proteins (11). SR proteins are phosphorylated by SRPK and Clk proteins and on phosphorylation translocate to the nucleus and regulate alternative splicing events (1,40–43). Akt has been demonstrated to modulate the activity of SRPK and Clk protein (44). Akt has also been demonstrated to directly phosphorylate the SR proteins (45). Thus PI3K/Akt pathway could regulate the alternative splicing of EDA+Fn in human podocytes by a similar mechanism.

p38 MAP kinase pathway has been demonstrated to be up-regulated in various cell types. p38 MAP kinase pathway has been shown to regulate the Fn expression in transformed human kidney cells by our group (25). p38 MAP kinase pathway has been shown to be involved in ECM proteins expression regulation in epithelial cells (46). Based on these findings I have investigated the role of TGFβ1 mediated p38 MAP kinase pathway in the regulation of EDA+Fn expression in human podocyte culture. The results from p38 MAP kinase experiment suggest that TGFβ1 induced the pP38 MAP kinase pathway in human podocytes. Chemical inhibitor SB202190 has been shown to be a potent inhibitor of p38 MAP kinase pathway (47,48). To demonstrate the p38 MAP kinase signalling pathway in the regulation of TGFβ1 mediated EDA+Fn expression in human podocytes, I have treated human podocytes with SB202190 in presence and absence of TGFβ1. The results of these experiments suggest that MAP kinase inhibitor SB202190 downregulated TGFβ1 mediated EDA+Fn protein expression. SB202190 also downregulated TGFβ1 induced EDA+/-Fn mRNA ratio shown by RT-PCR. The results from p38 MAP kinase experiment suggest that human podocytes demonstrate the p38 MAP kinase signalling pathway and inhibiting the p38 MAP kinase pathway by SB202190 downregulated the TGFβ1 mediated alternative splicing of EDA+Fn.

The alternative splicing is shown to be regulated by the SR proteins. The SR proteins are shown to be present in the cytoplasm and translocate to the nucleus on phosphorylation. The recognition of exon enhancing site by SR proteins greatly enhances the EDA alternative splicing. SR proteins are shown to be phosphorylated by several kinases such as SRPK1 and Clk (41,49,50). Nuclear transport of p38 MAP kinase has been reported (51). It could be possible that p38 Map Kinase could regulate the SR protein phosphorylation directly or indirectly by regulating SRPK1 and Clk and thus involved in SR protein-mediated alternative splicing of EDA+Fn. It has been shown that splicing factor SPF45 activation is mediated by p38 MAP kinase proteins and thus could be involved in alternative splicing (44,52). p38 MAP kinase has also been shown to phosphorylate hnRNAP1 which is involved in alternative splicing regulation (53) and thus could be involved in alternative splicing of EDA+Fn.

In summary, the key finding with Smad signalling studies in human podocytes is that podocytes exhibit Smad1/5/8 expression in addition to canonical Smads: Smad2 and Smad3 via ALK5 receptor demonstrated by using ALK5 inhibitor SB431542.

TGFβ1 mediated signalling pathway involved in alternative splicing of EDA+Fn in human podocytes were PI3K/Akt pathway and p38 MAP kinase pathway. The results with PI3K and p38MAP kinase signalling pathway suggest that inhibiting PI3K signalling pathway downregulated the basal alternative splicing of EDA+Fn in human podocytes and its the inhibition of p38 Map Kinase signalling pathway which had specifi-

cally downregulated the TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes culture.

This study describes that EDA+Fn is an alternatively spliced form of Fn and targeting alternative splicing of EDA+Fn is a key approach to regulating expression of EDA+Fn. TGFβ1 induced the EDA+Fn alternative splicing in human podocytes. p38 MAP kinase pathway and PI3K signalling pathways are potential pathways involved in regulation of alternative splicing of EDA+Fn in human podocytes. Targeting these signalling pathways could be a fascinating approach to regulating the alternative splicing and expression of EDA+Fn in podocyte in health and diseases.

Declarations

Ethics approval and consent to participate- Not applicable.

Consent for publication- Not applicable.

Availability of data and materials- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests- The authors declare that they have no competing interests.

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Authors' contributions-

Dr Tarunkumar H Madne- All research works carried by this author.

Dr Mark Edward Carl Dockrell- Lead supervisor.

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