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Research article

Targeting alternative splicing of fibronectin in human renal proximal tubule epithelial cells with antisense oligonucleotides to reduce EDA+ fibronectin production and block an autocrine loop that drives renal fibrosis

Mysore Keshavmurthy Phanish [a,b], Felicia Heidebrecht [a], Michaela Jackson [c], Frank Rigo [c], Mark Edward Carl Dockrell [a,b,*]

[a] SWT Institute for Renal Research, Renal Unit, St Helier Hospital, Epsom and St Helier University Hospitals NHS Trust, London, UK
[b] St Georges' University of London, London, UK
[c] IONIS Pharmaceuticals, 2855, Gazelle Ct, Carlsbad, CA 92010, USA

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ABSTRACT

TGFβ1 is a powerful regulator of fibrosis; secreted in a latent form, it becomes active after release from the latent complex. During tissue fibrosis, the EDA + isoform of cellular fibronectin is overexpressed. In pulmonary fibrosis it has been proposed that the fibronectin splice variant including an EDA domain (FN EDA+) activates latent TGFβ. Our work investigates the potential of blocking the 'splicing in' of EDA with antisense oligonucleotides to inhibit TGFβ1-induced EDA + fibronectin and to prevent the cascade of events initiated by TGFβ1 in human renal proximal tubule cells (PTEC).

Human primary PTEC were treated with TGFβ1 for 48 h, medium removed and the cells transfected with RNase H-independent antisense oligonucleotides (ASO) designed to block EDA exon inclusion (ASO5). The efficacy of ASO to block EDA exon inclusion was assessed by EDA + fibronectin RNA and protein expression; the expression of TGFβ, αSMA (α smooth muscle actin), MMP2 (matrix metalloproteinse-2), MMP9 (matrix metalloproteinse-9), Collagen I, K Cadherin and connexin 43 was analysed.

Targeting antisense oligonucleotides designed to block EDA exon inclusion in fibronectin pre mRNA were effective in reducing the amount of TGFβ1 -induced cellular EDA + fibronectin RNA and secreted EDA + fibronectin protein (assessed by western immunoblotting and immunocytochemistry) in human proximal tubule cells in an *in vitro* cell culture model. The effect was selective for EDA + exon with no effect on EDB + fibronectin RNA and total fibronectin mRNA.

Exogenous TGFβ1 induced endogenous TGFβ, αSMA, MMP2, MMP9 and Col I mRNA. TGFβ1 treatment for 48h reduced the expression of K-Cadherin and increased the expression of connexin-43. These TGFβ1-induced pro-fibrotic changes were attenuated by ASO5 treatment. 48 h after the removal of exogenous TGFβ, further increases in αSMA, MMP2, MMP9 was observed; ASO5 significantly inhibited this subsequent increase. ASO5 treatment also significantly inhibited ability of the cell culture medium harvested at the end of the experiment (96h) to stimulate SMAD3 reporter cells. The role of endogenous TGFβ1 was confirmed by the use of a TGFβ receptor inhibitor.

Our results demonstrate a critical role of FN EDA+ in a cycle of TGFβ driven pro-fibrotic responses in human PTEC and blocking its production with ASO technology offers a potential therapy to interrupt this vicious circle and hence limit the progression of renal fibrosis.

1. Introduction

Fibrosis is characterised by loss of epithelial cells, qualitative and quantitative changes of the interstitial extracellular matrix (ECM), such as de novo expression of fibronectin splice variant FN extra domain A (EDA + or EIIIA) [1–3].

In common with other extracellular matrix proteins FN EDA+ is a component of tissue scaffolding and a regulator of cell behaviour. In

* Corresponding author. SWT Institute for Renal Research, Renal Unit, St Helier Hospital, Epsom and St Helier University Hospitals NHS trust, London, SM5, UK.
E-mail addresses: m.phanish@nhs.net (M.K. Phanish), mark.dockrell@nhs.net (M.E.C. Dockrell).

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cancer, EDA + fibronectin acts as central organiser of ECM proteins and mediates crosstalk between the tumour microenvironment and epithelial cells [4]. Studies in mouse models of fibrosis have shown the importance of FN EDA+ in the development of lung fibrosis: FN EDA+ is required for lung fibroblasts to acquire myofibroblast phenotype and subsequently develop fibrosis after bleomycin challenge [5,6]. TGFβ1 is a powerful pleiotropic growth factor. Its activity is controlled at many levels including gene expression and maintenance in a latent, inactive form. In order to exert its pro fibrotic effects, TGFβ has to be released from the latent complex in which it is secreted. The activation of latent TGFβ by FN EDA + has been proposed as a mechanism contributing to progression of lung fibrosis [6–8].

Although there is strong evidence of FN EDA + expression in kidney disease in human biopsies and in vivo models [9,10], little is known about the role of EDA+ in renal fibrogenesis. During the processing of the pre-mRNA constitutive splicing involves the removal of introns and the joining of the remaining exons to form messenger RNA. Alternative splicing, is a regulated process that leads to the inclusion of different sets of exons in the mRNA sequence, resulting in multiple mRNA transcripts and hence multiple proteins, potentially differing in structure and function, from a single gene[11–13]. The EDA exon flanks the cell binding domain (RGDS) of fibronectin molecule and EDA peptide segment alone has been shown to be sufficient to induce myofibroblast differentiation and proliferation via binding to integrins [14]. In vitro, It has also been reported that EDA segment binds to Toll-like receptor 4 and therefore, likely to have a role to play in renal inflammation [15].

The majority of genes are alternatively spliced, and splicing is often dysregulated in

pathological conditions which make it an attractive therapeutic target [16–18].

The fibronectin gene contains 3 alternatively spliced regions (EDA, EDB and IIICS), resulting in more than 20 different isoforms. FN EDA+ (also known as EIIIA FN), a form of FN expressed in embryogenesis and disease, results from the inclusion of the alternatively spliced EDA (EIIIA) exon [7,19,20]. Cellular fibronectin proteins containing the EDA domain are insoluble, present as dimers or multimers on cell surface and once secreted into the extracellular matrix they interact with matrix components, cellular receptors and other proteins [20].

Selective regulation of alternative splicing could be of significant therapeutic value [21]. The use of RNase H-independent antisense oligonucleotides (ASO) has shown promise in this field [22,23]. Traditionally ASO bind the target mRNA and induce its degradation by RNase H, however RNase H-independent ASOs work by steric blockage of pre mRNA sequences that are essential for splicing regulation and normally bound by splicing proteins [24,25]. The steric block ASOs are widely used to modulate alternative splicing in order to exclude or retain a specific exon and we used this technology to achieve exon skipping of EDA exon from fibronectin pre mRNA.

The proximal tubule is traditionally considered the site of initiation of tubular interstitial fibrosis by production of profibrotic markers in a TGFβ-dependent manner. Work from Bonventre and colleagues have produced evidence that proximal tubule damage and the associated expression of fibronectin can precede glomerulosclerosis [26]. More recently, favourable clinical outcomes relating to inhibition of sodium-glucose transporter receptor 2 (SGLT2) on the proximal tubule has produced a "tubulocentric" perspective in diabetic kidney disease [27].

In this study, we evaluate the efficacy of ASOs designed to block EDA exon inclusion in human renal proximal tubule cells, investigate whether TGFβ events and the fibrotic process are dependent on the presence of FN EDA+, explore the role of activation of latent TGFβ in renal proximal tubular cells and determine if antisense technology can be used *in vitro* to modulate the alternative splicing of the exon EDA of fibronectin.

2. Materials and methods

2.1. Cell culture

PTECs (Primary Human Proximal Tubule Epithelial Cells, Lonza, Basel, Switzerland) were cultured according to the manufacturer's recommendations (37^0C in supplemented media with 0.25 % FCS). The cells were treated with 2.5 ng/ml TGFβ in 0.1 % BSA in serum free medium for 48h. The treatment medium was removed and cells were transfected with 100 nM antisense oligonucleotides [negative control (NC) or targeting ASO, ASO5)] in 2 μL Lipofectamine 2000/mL Opti-MEM (*Life Technologies*). After 24h, medium was removed and cells allowed to recover for 24h in growth medium. The outcomes were assessed at 48h and 96h. The post TGFβ cell model for assessing the effect of targeting ASO (ASO5) on TGFβ1 induced fibrotic outcomes is shown in Fig. 2G.

For ALK5 inhibition cells were treated for 48 h with TGFβ1 and for further 48h with 10 μM SB431542 in growth medium with 0.1 % DMSO and 0.1 % BSA.

Human primary proximal tubule cells from three donors were used: 4F1374 (Donor 1), 7F4025 (Donor 2) and 7F4110 (Donor 3). For dual luciferase assay HKC8 cells were cultured as previously described [28]. HKC8 cells were a kind gift from Prof. Racusen (John Hopkins University, Baltimore, USA) [28].

2.2. Screening of targeted ASOs to prevent inclusion of EDA + exon and subsequent experiments to study the effect of targeting ASO on TGFβ1 induced pro-fibrotic outcomes in human PTECs

20 targeting ASOs were designed and screened for their efficacy at the IONIS laboratories, California. A positive control ASO targeting PTEN gene was used in initial screening experiments and a negative control ASO, in addition to targeting ASOs was used in all subsequent experiments. After screening of 20 ASOs targeting fibronectin splicing at the EDA exon, 6 were chosen for subsequent experiments based on their efficacy of which 4 targeted both human and mouse fibronectin gene whilst other two targeted only human fibronectin gene. The sequences of these 6 selected ASOs along with their chemistry is given in supplementary files, Table S1. As our plan was to perform in vivo experiments in mice in the future, we selected ASOs that were effective against both human and mouse fibronectin gene. The efficacy of ASOs to prevent EDA + exon inclusion was assessed in two models: Model 1 (Fig. 2A): Transfections with targeting ASOs was performed for 4h followed by overnight recovery and TGFβ1 treatment for 24h. Model 2 (Fig. 2B): Cells were treated with TGFβ1 for 24h followed by 4h of transfection with targeting ASO and overnight recovery before cell lysis.

To investigate the effect of ASO5 (targeting ASO) on TGF β1 induced pro-fibrotic effects and the role of FN EDA+ in TGFβ-driven fibrotic process, we used the following *in vitro* "post TGFβ model" of primary human PTECs: cells were first treated with 2.5 ng/ml TGFβ1 for 48 h to induce the fibrotic process and later, after removing the exogenous TGFβ, transfected for 24h with splicing-modulating antisense (selected after screening of 20 targeting ASOs) as simulation of therapeutic intervention after the onset of the disease. After further 24 h of recovery the experiment was terminated. All analyses shown were done at the end point of the experiment (96h) unless otherwise stated.

2.3. PCR

RNA was isolated using the RNeasy Kit (Qiagen). An additional ethanol precipitation was performed (1/10 vol 5M NaCl and 3 vol pure ethanol were added, RNA was precipitated at −80 °C for 1 h, samples were centrifuged at 10 000g for 20 min at 4 °C, washed in 75 % ethanol and air-dried). 1 μg total RNA was reverse-transcribed according to the manufacturer's protocol (High-Capacity Reverse Transcription Kit, *Applied Biosystems*).

For quantitative real-time PCR the following probes were used: fibronectin (Hs00287359_m1), α-Smooth Muscle Actin (Hs00559403_m1), MMP2 (Hs01548727_m1),

MMP9 (Hs00234579_m1), Collagen 1A1 (Hs00164004_m1), p21 (Hs00355782_m1) and the endogenous control large ribosomal protein P0 (Hs04189669_g1). All probes and PCR reagents were from *Applied Biosystems*. The gene expression levels were determined using

the comparative Ct method. If results from cells from different donors were combined, the values of each donor were indexed on the own negative control treatment, to correct for variability in basal levels of gene expression between different donors.

For simultaneous detection of both splice variants of fibronectin at EDA exon a pair of primers binding constitutively spliced exons upstream and downstream of the EDA exon were used (forward primer 5′GGAGAGAGTCAGCCTCTGGTTCAG 3′ and reverse primer 5′ TGTCAACTGGGCGCTCAGGCTTGTG 3′). The PCR reaction was performed using the.

Crimson Taq Polymerase (*New England Biolabs*). The primers were used at 0.4uM, polymerase 1.25U per reaction and the volume of the cDNA template was 1 μl from a 1 in 10

diluted RT reaction. The PCR amplification program was: initial denaturation 5 min at 95 °C, 5 and 26 cycles of: 30 s 95 °C, 30 s 56 °C, 30 s 68 °C followed by a final extension for 5 min at 68 °C. PCR products were separated on a 2 % agarose gel, images were acquired in 16-bit TIFF format with the gel documentation software *ImageQuant 300 Capture (GE Healthcare)* with an appropriate exposure time to avoid overexposure. The bands were analysed using the *ImageQuant TL* software (*GE Healthcare*) with rolling ball background correction.

2.4. Western blotting

Medium was removed and cells lysed in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 % Triton X, 0.5 % sodium deoxycholate, 0.1 % SDS; pH 7.2) supplemented with protease and phosphatase inhibitors (Roche). The protein concentration was determined by BCA assay (*Thermo Scientific*). Gel electrophoresis and transfer of proteins to PVDF membrane was performed according to the manufacturer's protocol. The membrane was blocked in 5 % milk in TBST and incubated for 1h with primary antibodies against EDA + Fn (*Abcam*. clone IST-9) and 45 min with HRP-labelled secondary antibody (*Sigma-Aldrich*). Blots were incubated in the enhanced chemiluminescence solution (*GE Healthcare*), exposed on Amersham Hyperfilm ECL and developed. Images of the films were acquired with the software *ImageQuant 300 Capture (GE Healthcare)*.

2.5. Immunofluorescence

Cells were fixed in 3.7 % paraformaldehyde, blocked with 5 % goat serum in 0.2 % Triton X in PBS and incubated over night with primary antibody to EDA + Fn (1:100), K-Cadherin (1:50), αSMA (1:100), Connexin 43(1:100). The secondary antibody 1:200 (Alexa Fluor® 555 Goat Anti-Mouse IgG, *Molecular Probes*) was added together with 1 μg/mL DAPI and Phalloidin (*Molecular Probes*) 1:50. Cells were analysed and images acquired and processed with the software *NIS Elements* (*Nikon*). Contrast was adjusted for purposes of illustration for all pictures using the same parameter settings.

2.6. Dual luciferase assay

PTEC were treated with TGFβ1 and ASO or SB431542 as described above. For ASO experiments the culture media after transfection and after recovery were used immediately for treating HKCs. For ALK5 inhibition experiments the culture media were removed from PTEC and used directly to treat HKCs. HKC8 were transfected with 1 μg/mL CAGA (x4) Smad3 responsive reporter construct and 10 ng/mL of pRL-CMV Renilla (*Promega*). The transfection was carried out for 24 h in OptiMEM medium (*Gibco*) with 4uL FuGENE (*Promega*)/mL OptiMEM according to the manufacturer's protocol. After transfection, cells were treated with the culture media from PTEC for 24 h, lysed and luciferase activity measured using the Dual-Luciferase® Reporter Assay System (*Promega*) according to the manufacturer's instructions and the luminescence was read with.

FLUOstar OPTIMA (*BMG Labtech*). CAGA (4) Smad3 responsive reporter plasmid was a kind gift from Prof. Donald Fraser (Cardiff, UK).

2.7. LDH

The LDH release was determined using the *CytoTox 96® Non-Radioactive Cytotoxicity Assay* (*Promega*) according to the manufacturer's instructions.

2.8. Secreted total fibrillar collagen

The total fibrillar collagen secreted in the medium was measured using a Sirius Red total

Collagen Detection Kit (*Chondrex Inc.*) according to the manufacturer's recommendations.

2.9. MTS assay

The MTS assay was performed using the *CellTiter 96® AQueous Non-Radioactive Cell*

Proliferation Assay (MTS) (Promega) as indicated in the manufacturer's protocol. For the quantitative analysis of results, the readings were correlated to an equivalent cell number. A calibration curve for this was acquired and results from the MTS assay were only used for the linear range of the curve. EGFR inhibitor AG1478 was used at a concentration of 5 μM.

2.10. Statistical analysis

The data presented are results from at least three independent experiments. The results section and the figure legends describe the 'n' numbers. The bar charts represent mean and standard deviation or standard error of mean. Statistical analysis was performed with the *GraphPad Prism* software using a 2-tailed paired T-test for comparison of 2 groups or ANOVA with multiple comparisons for comparing more than two groups. A p value < 0.05 was considered significant and the P values are given on each bar chart.

3. Results

3.1. Expression of EDA+ and EDA-splice variants in human proximal tubule epithelial cells (Fig. 1)

Primary and transformed (HKC-8) human Proximal Tubule Epithelial Cells (PTEC) express EDA+ and EDA- Fibronectin determined by RT-PCR using primer pairs to detect both EDA+ and EDA-bands within same PCR reaction (Fig. 1). The expression of EDA + fibronectin was higher in the transformed HKC-8 cells compared to primary PTEC in basal conditions.

3.2. Screening of ASOs to assess efficacy in human proximal tubule cells (Fig. 2)

Among the ASOs screened, ASO5 was found to be the most effective for reducing the amount of EDA + mRNA in human proximal tubule cells in basal and TGFβ1 stimulated conditions in two cell models tested (Model 1: ASO given before TGFβ1 treatment; Model 2: ASO given after TGFβ1 treatment) (Fig. 2A and B). ASO5 demonstrated greater reduction in EDA+/EDA-ratio in the presence of TGFβ1 (Fig. 2C and E) and it was efficacious in human primary PTEC lines from two different donors

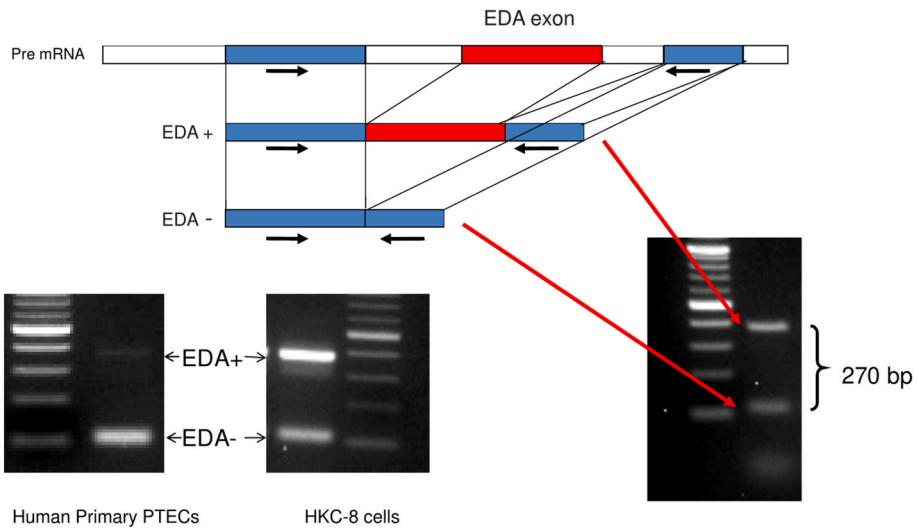

Fig. 1. The expression of EDA+ and EDA-fibronectin mRNA in human proximal tubule epithelial cells Conventional RT-PCR to detect both splice variants in the same PCR reaction demonstrated two bands representing EDA+ and EDA-fibronectin mRNA separated by 270 bp. Both human primary and transformed (HKC-8) human tubule epithelial cells expressed EDA+ and EDA-fibronectin with higher levels of EDA + mRNA expression noted in transformed cells under basal conditions.

(Fig. 2D). In addition, the effect of 6 selected ASOs on secreted EDA + fibronectin protein was assessed by Western blotting (Fig. 2F). Based on results obtained from these screening experiments, ASO5 was selected for all further experiments. The experimental plan for subsequent experiments to investigate the effect of ASO5 on TGFβ1-induced pro-fibrotic outcomes is shown in Fig. 2G.

3.3. ASO5 is a specific modulator of fibronectin splicing at the EDA exon (Fig. 3)

Given post TGFβ, ASO5 decreases the EDA + to EDA-splice variants ratio significantly without altering the splicing at the EDB exon (Fig. 3, a1 and a2). The change in the ratio results from both a decrease in EDA + mRNA and an increase in EDA-mRNA (Fig. 3, a3) whereas the level of total fibronectin mRNA is unchanged (Fig. 3b). The levels of EDA protein are also significantly reduced (Fig. 3c).

3.4. ASO5 ameliorates morphological changes induced by TGFβ and improves cell viability (Fig. 4)

The morphology of the cells treated with targeting ASO (ASO5) or negative control ASO (NC) at the end point of the experiment (96h) was assessed microscopically to study the effect of reducing EDA + fibronectin production.

TGFβ1 treatment for 96h resulted in marked cell loss (4a, panel 2) which was ameliorated by ASO5 treatment (Fig. 4a, panel 4). ASO5 treatment resulted in significant reductions in LDH release induced by TGFβ1 (Fig. 4c). Treatment with ASO5 resulted in partial restoration of cortical localization of f-actin as showed by phalloidin staining (green) (arrows in Fig. 4b) and a marked reduction in EDA + fibronectin deposition (red) (Fig. 4D).

3.5. ASO5 reduces levels of TGFβ-induced pro-fibrotic outcomes (Figs. 5 and 6)

To assess possible benefits of knocking down EDA + fibronectin in a fibrotic environment, ASO5 effects on gene expression levels of TGFβ-activated genes in a post TGFβ model were determined. To exclude potential influence of the genetic background, primary cells from three different donors were analysed. ASO5 significantly reduced the mRNA levels of αSMA, MMP2 and MMP9 in TGFβ treated cells (Fig. 5a) and the profiles of single donors were similar (Supplementary Fig. S2). However, for Collagen 1a1 the levels of gene activation with TGFβ were extremely variable in different donors (Fig. 5 b1), and the effect of ASO5 was not consistent (Supplementary Fig. S2). An additional analysis of secreted total fibrillary collagen in showed a significant reduction with ASO5 (Fig. 5 b2). TGFβ1 treatment resulted in loss of proximal tubular junctional protein K-Cadherin and this loss was partially restored by ASO5 (Fig. 6). The expression of connexion 43, a gap junction protein, was increased by TGFβ1 treatment and this increase was ameliorated by ASO5 treatment (Fig. 6).

3.6. ASO5 inhibits further increase of TGFβ-induced outcomes after removal of exogenous TGFβ (Fig. 7)

We showed in Fig. 5 that at the endpoint of the post-TGFβ model, after 48 h TGFβ1 followed by 24 h of treatment with ASO5 and a further 24 h recovery (96h), the levels of αSMA,

MMP2 and MMP9 are significantly decreased compared to cells transfected with negative control antisense. Next, we asked if treatment with ASO5 reversed the effects of TGFβ, or prevented a further increase in these outcomes. In the first case, TGFβ would increase the outcomes in the first 48 h to a level that remains unchanged for the following 48 h for the negative control antisense treatment, whereas treatment with ASO5 would decrease it (Fig. 7 a1). In the second case, the level of pro-fibrotic molecules (outcomes) would continue to increase after the removal of TGFβ in cells transfected with negative control antisense, and ASO5 treatment would leave the levels unchanged (Fig. 7 a2). To answer this question, we compared the gene expression levels of αSMA, MMP2 and MMP9 after 48 h TGFβ and at the end point of the experiment (96h) (Fig. 7b).

At 48h, TGFβ significantly raised the levels of MMP2 and MMP9 compared to vehicle, notably αSMA levels were comparable at this point. After removal of TGFβ, in cells transfected with negative control antisense a further increase in all three outcomes was observed. In ASO5-transfected cells after previous TGFβ treatment the levels of the outcomes were reduced to comparable (MMP2) or significantly lower (αSMA and MMP9) levels than those observed at 48 h (Fig. 7 b). These observations support the hypothesis that ASO5 treatment prevents a further increase in the analysed outcomes (model shown in 7-a2).

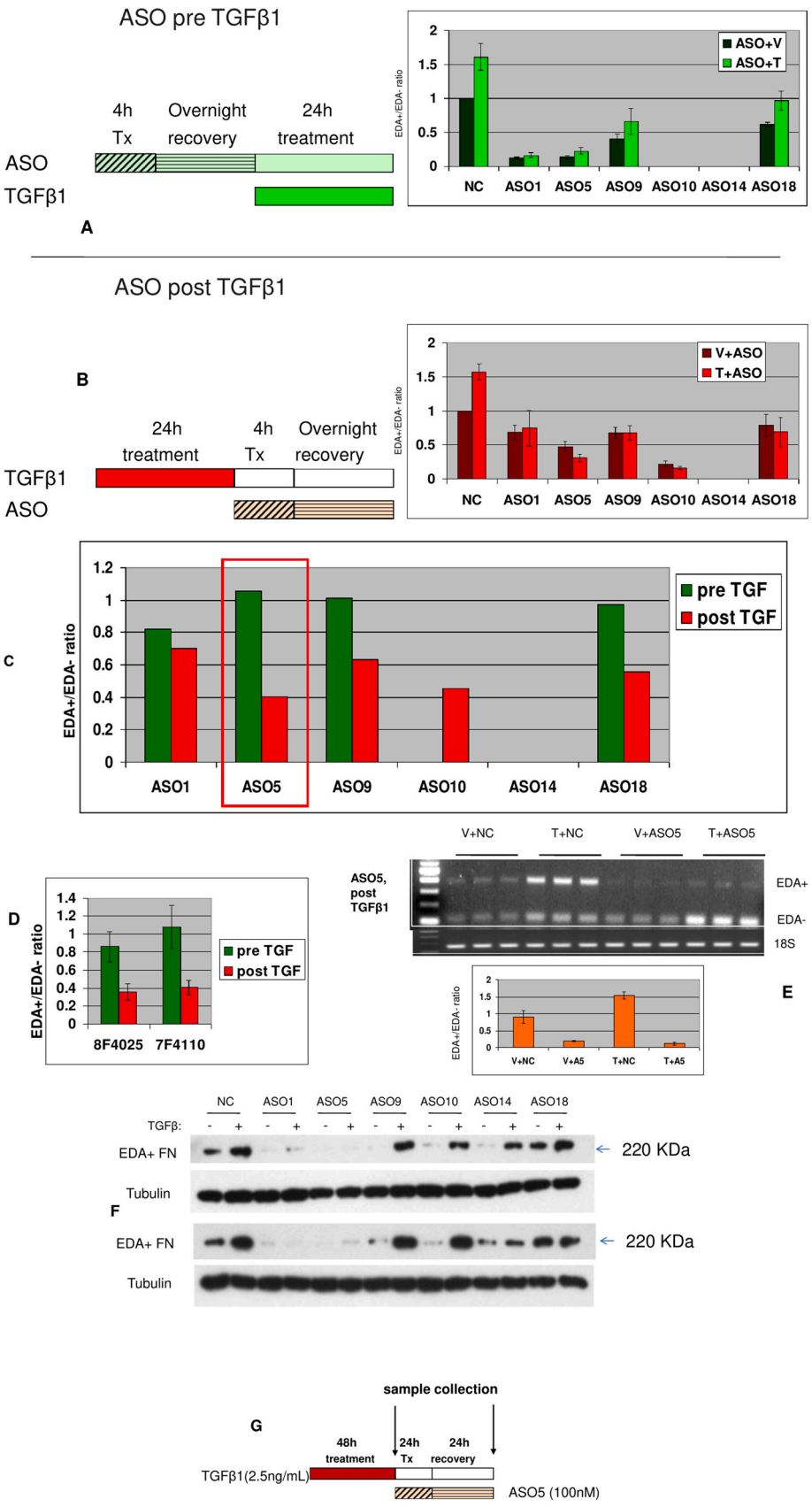

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Fig. 2. (A to G). The screening of targeting ASOs in human proximal tubule cells.Initial screening was done in basal culture conditions in transformed human PTECs (HKC-8 cells); subsequent screening was done in human primary proximal tubule cells under basal as well as stimulated conditions (TGFβ1, 2.5 ng/mL). The efficacy of the ASOs (6 ASOs screened) to reduce EDA + mRNA expression when given before (Fig. 2A) or after (Fig. 2B, C, D, E) TGFβ1 was evaluated. Fig. 2D demonstrates the efficacy of ASO5 in reducing the EDA+/EDA-mRNA ratio in primary PTECs derived from two separate donors. In addition, the effect of selected ASOs on the expression of secreted EDA + fibronectin protein in the presence and absence of TGFβ1was assessed by Western blotting (Fig. 2F). The cell model used for experiments to assess the effect of ASO5 on TGFβ1-induced fibrotic outcomes in human PTECs is shown in Fig. 2G.Tx = transfection. V=Vehicle (0.1 % BSA), T = TGFβ1 (2.5 ng/mL), ASO= Targeting Antisense Oligonucleotide (100 nM), NC = negative control antisense oligonucleotide (ASO) (100 nM). TGF = TGFβ1, FN=Fibronectin.

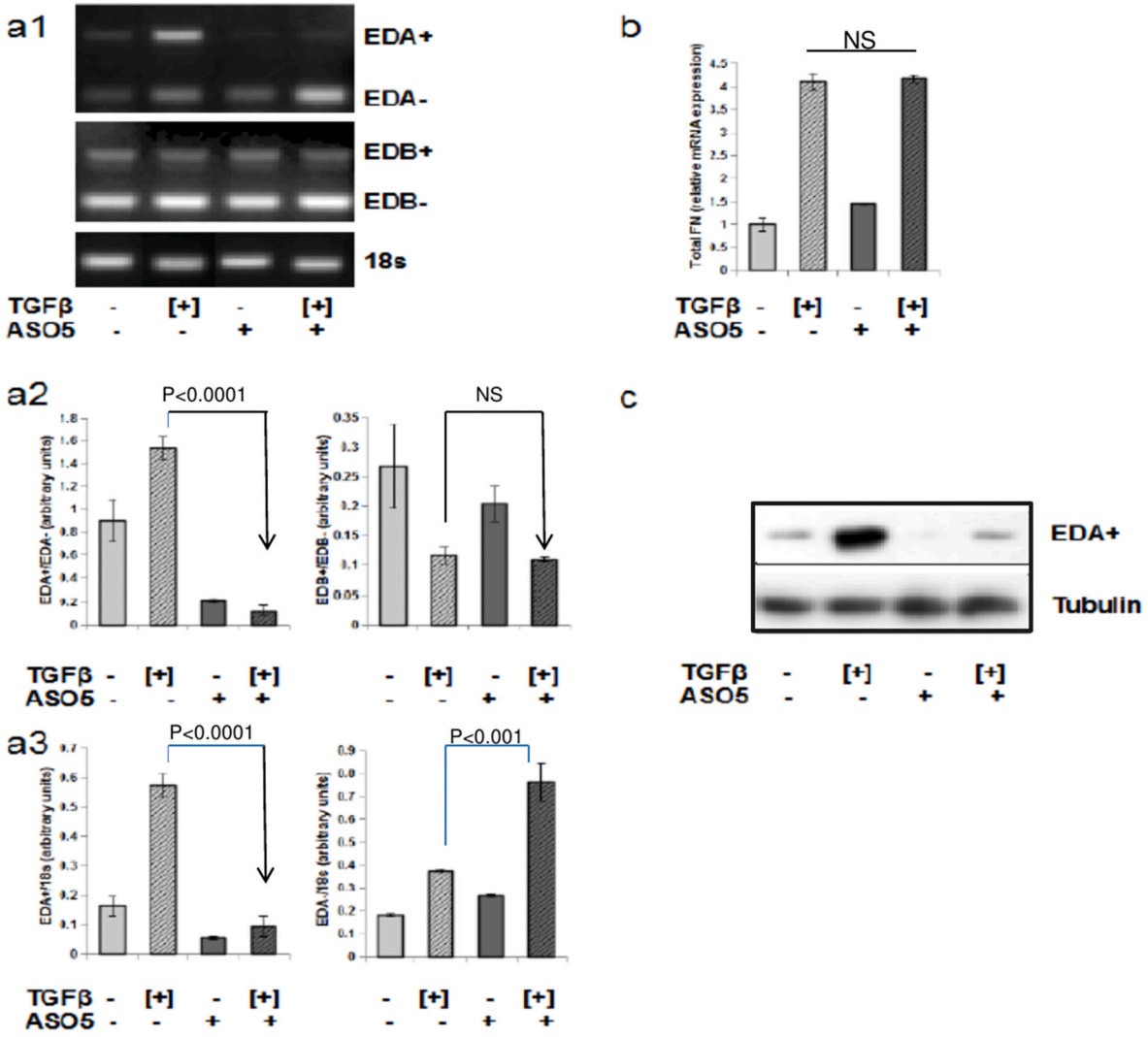

Fig. 3. ASO5 is a specific modulator of fibronectin spicing at the EDA exon.
(a1): Semiquantitative PCR for EDA ± and EDB ± using one pair of primers to detect both splice variants in the same PCR reaction.(a2): Densitometric analysis of the PCR results: Ratios of splice variants for EDA(EDA+/EDA-) and EDB (EDB+/EDB-).(a3): Analysis of single EDA splice variants (EDA+ and EDA-) normalized for 18s. Data are shown as mean ± SEM of three independent experiments.(b) Quantitative real-time PCR for total FN, mean ± SEM of three independent experiments. (c) Representative Western blot showing the effect of ASO5 treatment on the protein isoform containing EDA + domain. Square brackets indicate the presence of TGFβ for only the first 48 h of the experiment.

3.7. EDA + fibronectin possibly acts by activating latent TGFβ to maintain fibrotic phenotype (Fig. 8)

The role of EDA + fibronectin in activating latent TGFβ has previously been discussed in animal models of lung fibrosis [4,6]; because our data suggested that blocking EDA + FN with ASO inhibits progression of a fibrotic process *in vitro* we subsequently tried to assess if a similar mechanism is present in primary human PTEC. TGFβ induces its own gene product (Fig. 8 a), which is consistent with previous work from others and our lab showing the autoinduction of TGFβ [29,30]. To assess if the events in the last 48 h of the post-TGFβ model require TGFβ receptor activation we treated cells with the ALK5 inhibitor SB431542 for 48 h after the removal of exogenous TGFβ and analysed the gene levels of αSMA, MMP2 and MMP9.

Treatment with the ALK5 inhibitor significantly reduced their expression, in a similar manner to that observed with ASO5 treatment (Fig. 8b). Visual comparison of microscope images of cells after 48h TGFβ and at the end time point of the experiment suggested that the observed increase in cell number is likely to be due to both inhibition of cell death and increase in proliferation. To investigate whether the former requires TGFβ, LDH activity was measured when cells were incubated with the ALK5 inhibitor SB431542 for 48 h after the removal

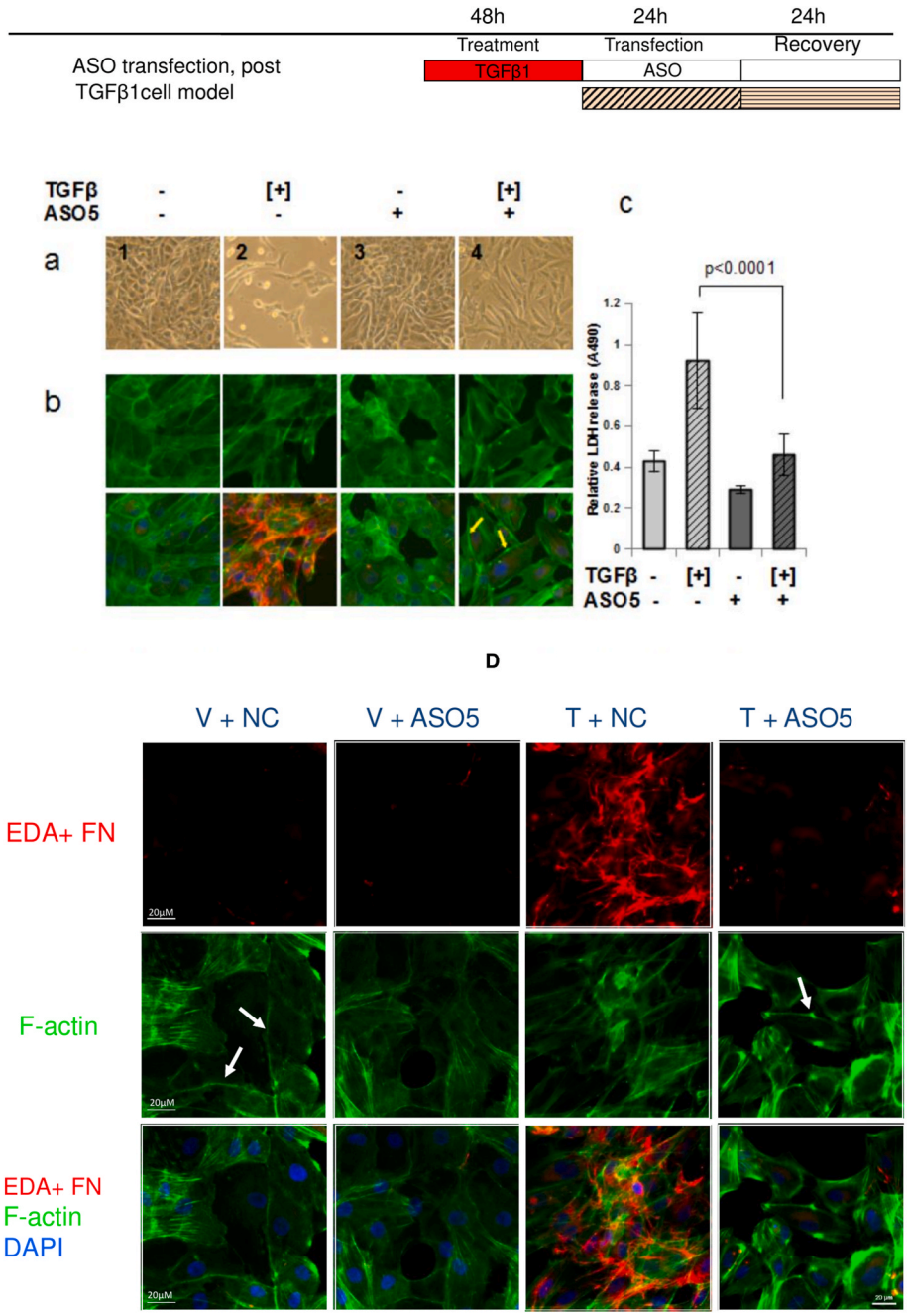

Fig. 4. ASO5 ameliorates morphological changes induced by TGFβ1, improves cell vitality and blocks TGFβ1-induced EDA + fibronectin deposition in human primary proximal tubule cells (a) Phase contrast images of human PTEC in culture: The strong loss of cells observed in cells pretreated with TGFβ (a2) is attenuated by ASO5 (a4). (b) Phalloidin staining (green) showing actin filament distribution: Treatment with TGFβ increased the formation of actin stress fibres and ASO5 treatment restored the cortical localization of actin (arrows in b, bottom panel). Immunostaining for EDA (red) shows marked increase in deposition of EDA + fibronectin in response to TGFβ (bottom 2nd panel 4b) and the effect of ASO5 treatment (Fig. 4b and d). There was marked reduction in TGFβ-induced EDA + fibronectin deposition in ASO5 treated cells (4D). Counterstaining of nuclei with DAPI (blue). Square brackets indicate the presence of TGFβ for only the first 48 h of the experiment. (c) Secreted LDH in culture medium: Cell death was quantitatively assessed by LDH release. The results show that treatment with ASO5 post TGFβ results in reduced cell death. Data are shown as mean ± SD of 5–6 independent experiments. V = vehicle (0.1 % BSA), T = TGFβ1 (2.5 ng/mL), NC = negative control ASO (100 nM), A5 = ASO5 (Targeting ASO, 100 nM). FN=Fibronectin.

of exogenous TGFβ. LDH activity showed a similar pattern as in cells transfected with ASO (Fig. 8c). To answer the question if ASO5 increases cell proliferation (or metabolic activity) independently of exogenous TGFβ and whether TGFβ receptor activation is required we treated cells with ASO5 with or without the ALK5 inhibitor without previous TGFβ stimulation. Both transfection with ASO5 or treatment with SB431542 showed an increase in MTS metabolism (Fig. 8d), but in combination no further increase was detected. Since fibronectin and EDA + domain are known to stimulate cell cycle progression and proliferation in fibroblasts [14,31,32], and TGFβ induces cell cycle arrest by upregulation of p21 gene in epithelial cells [33], we hypothesized that effects of ASO5 on proliferation are indirect by inhibiting the activation of latent TGFβ. Consequently, cells are not arrested in G1/S and remain competent to respond to mitogen factors in the culture medium, such as EGF. MTS assay in cells treated with ASO5 and EGFR inhibitor AG1478 shows no increased MTS metabolism with ASO5 if EGF pathway is blocked

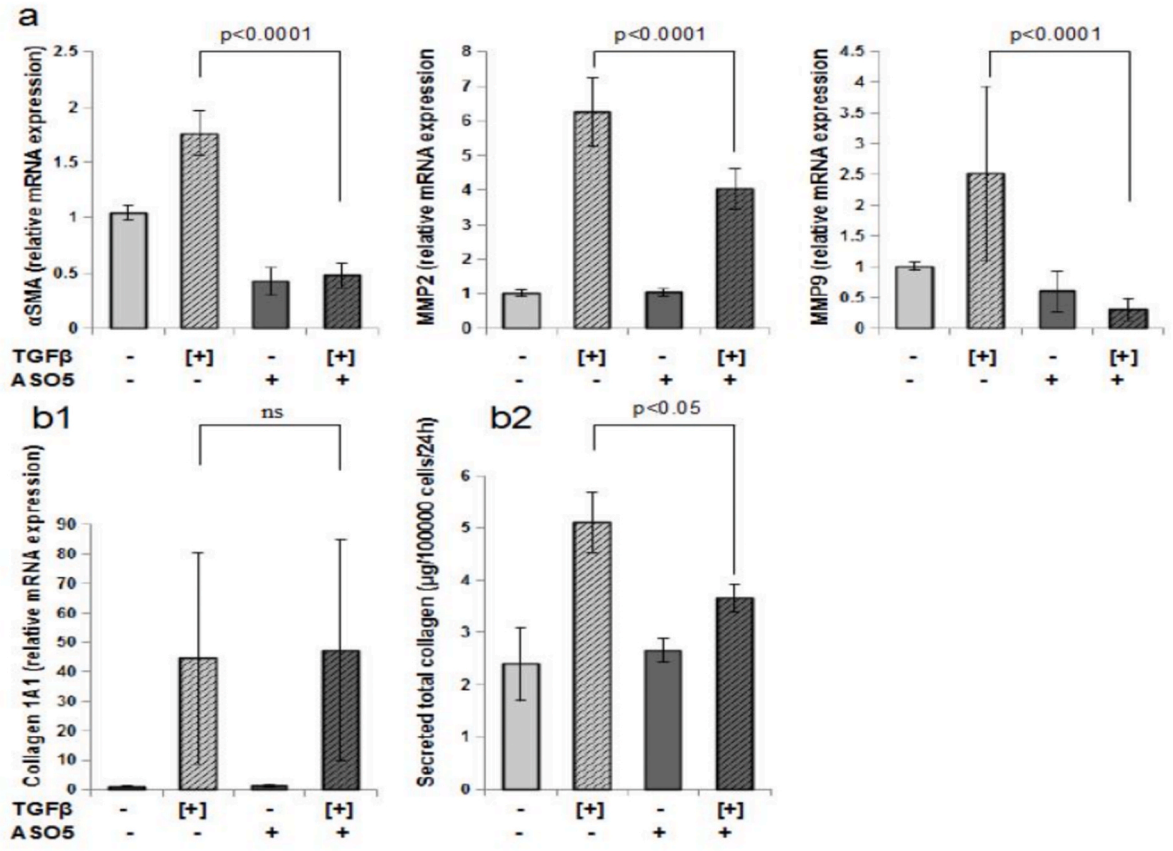

Fig. 5. ASO5 reduces levels of TGFβ-induced outcomes in human proximal tubule epithelial cells. (a). Gene expression levels for αSMA, MMP2 and MMP9 in a post TGFβ model (n = 9) (n = 3 in three different donors of primary cells)). (b1): Gene expression levels for Collagen 1a1. (b2): Levels of secreted total fibrillar collagen in a post TGFβ model. Data are shown as mean ± SD of 3 independent experiments for (b2) and mean ± SD of 9 independent experiments (n = 3 in three different donors) for (a) and (b1). Square brackets indicate the presence of TGFβ for only the first 48 h of the experiment.

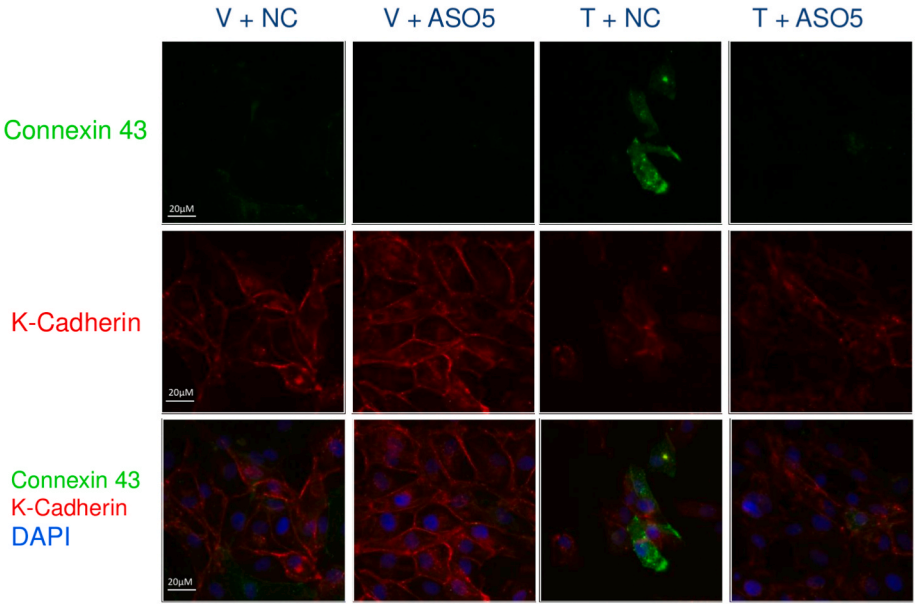

Fig. 6. The effect of ASO5 treatment on K-Cadherin and Connexin 43 expression in human proximal tubule epithelial cells. TGFβ1 treatment resulted in loss of proximal tubular junctional protein K-Cadherin and this loss was partially restored by ASO5. The expression of connexion 43, a gap junction protein, was increased by TGFβ1 treatment and this increase was ameliorated by ASO5 treatment.

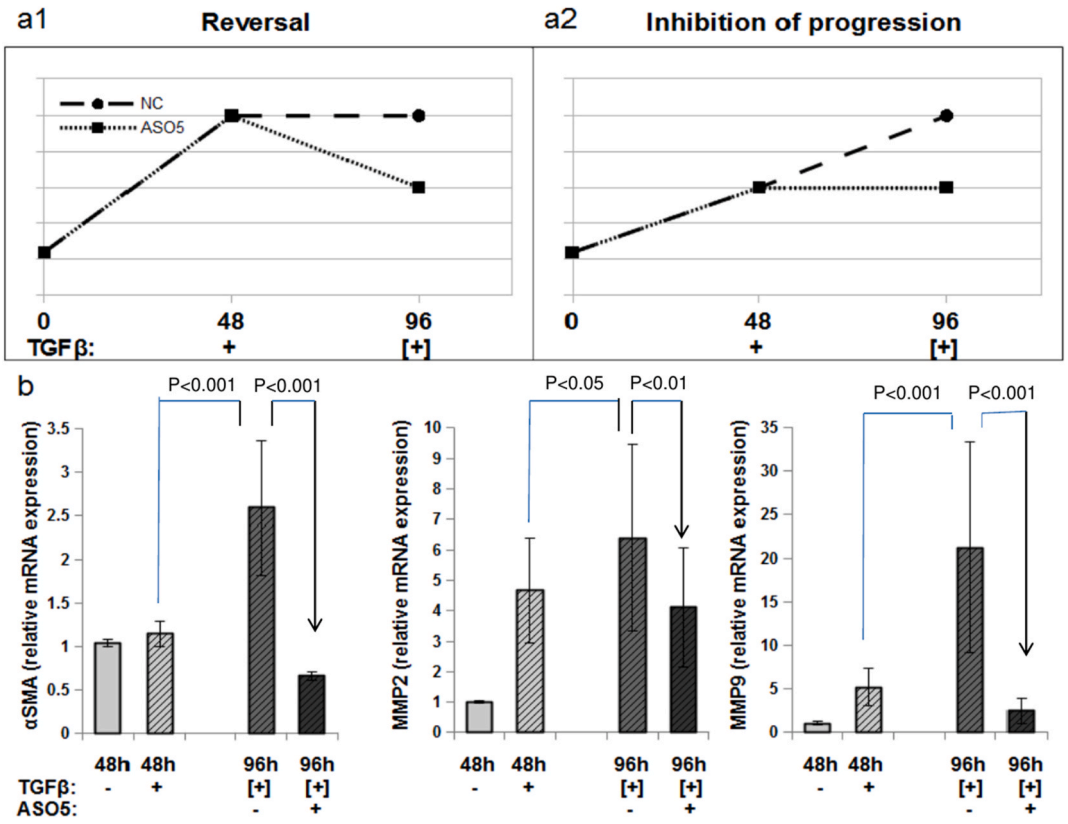

Fig. 7. ASO5 inhibits further increase of TGFβ-induced outcomes after removal of exogeneous TGFβ. (a): Theoretical models of ASO5 effect: (a1): reversal of TGFβ-induced changes in gene expression. (a2): inhibition of further increase in gene expression after removal of exogeneous TGFβ. (b): Gene expression levels for αSMA, MMP2 and MMP9 in a post TGFβ model immediately after 48 h TGFβ treatment (48 h) and at end point of experiment (96 h). Data are shown as mean ± SD of 9 independent experiments (n = 3 in three different donors of primary cells). Square brackets indicate the presence of TGFβ for only the first 48 h of the experiment.

(Fig. 8d). Furthermore, in the post-TGFβ model the mRNA levels of p21 are significantly reduced with ASO5 compared to negative control ASO (Fig. 8e).

To test if EDA + fibronectin activates latent TGFβ in our model we designed the following experiment: culture media from a post-TGFβ culture of primary cells were collected at the end of the experiment after ASO transfection and recovery. Cells pre-treated with TGFβ produce and secrete more latent TGFβ than cells pre-treated with vehicle and the amount of EDA + fibronectin in the medium from ASO5-transfected cells is reduced compared to negative control. If latent TGFβ was activated by EDA + fibronectin, the culture media from negative control-treated cells would contain higher levels of active TGFβ.

To assess TGFβ activity in culture media we transfected HKC8 cells with a plasmid containing a Smad3-responsive element and subsequently treated them with the cell culture media from the post-TGFβ culture of primary PTECs and the luciferase activity was determined.

Because TGFβ treatment causes apoptosis, cell death and ASO5 increases the survival of the cells (Fig. 4 a and c) the number of cells secreting latent TGFβ and EDA + fibronectin in the medium is variable. To correct for this the primary cells were lysed after collecting the recovery medium, proteins were extracted and the luciferase readings of each well were indexed on the corresponding total protein amount. The results show a decrease in luciferase activity with ASO5 treatment (Fig. 7f).

To preclude off-target effects of ASO5, we tested another ASO with a different sequence which demonstrated comparable effects on cell morphology, LDH and gene expression levels of αSMA and MMP2 (Supplementary figure S2 a, b, c). Furthermore, we confirmed the effect of EDA + knockdown on reducing TGFβ-induced outcomes by treating the cells with an EDA + blocking antibody (IST-9) post TGFβ (Supplementary figure S2 d).

4. Discussion

In this work, we demonstrate that targeting antisense oligonucleotides designed to block EDA exon inclusion in fibronectin pre mRNA are effective in reducing the amount of TGFβ1 -induced cellular EDA + fibronectin RNA and secreted EDA + fibronectin protein (assessed by western immunoblotting and immunocytochemistry) in human proximal tubule cells in an *in vitro* cell culture model. The effect is selective for EDA + exon with no effect of EDB + fibronectin RNA and total fibronectin mRNA.

TGFβ1 is known to induce the production and secretion of EDA + fibronectin in human tubule cells [34] and targeting tubule cells with antisense oligonucleotides to block EDA exon inclusion is an attractive therapeutic strategy for renal tubulo-interstitial fibrosis. Local activation of latent TGFβ is considered a key process in the development and maintenance of fibrosis [35]. Evidence from in vivo and *in vitro* studies in lung fibroblasts show that cells lacking EDA + are less capable of activating latent TGFβ, producing Collagen 1 and αSMA [1,6], with EDA + FN being required for TGFβ-induced αSMA synthesis and Collagen1 assembly [6], a pathway involving integrin α4β7 [14].

Our work demonstrates that a challenge by TGFβ1 initiates a series of pathological events in human PTEC that persist after the removal of the exogenous TGFβ. This is consistent with published work, including work from our own group in renal cells, which has described auto-induction of TGFβ as well as induction of other pro-fibrotic factors such as CTGF/CCN2 [29,30,36,37].

However, in this work we have investigated a crucial step in the TGFβ cascade by

analysing the effects of splice-switching ASO to exon EDA of fibronectin after removing the exogenous TGFβ, we could study the effects of EDA + FN on activation of autocrine TGFβ. In the "post-TGFβ" model of

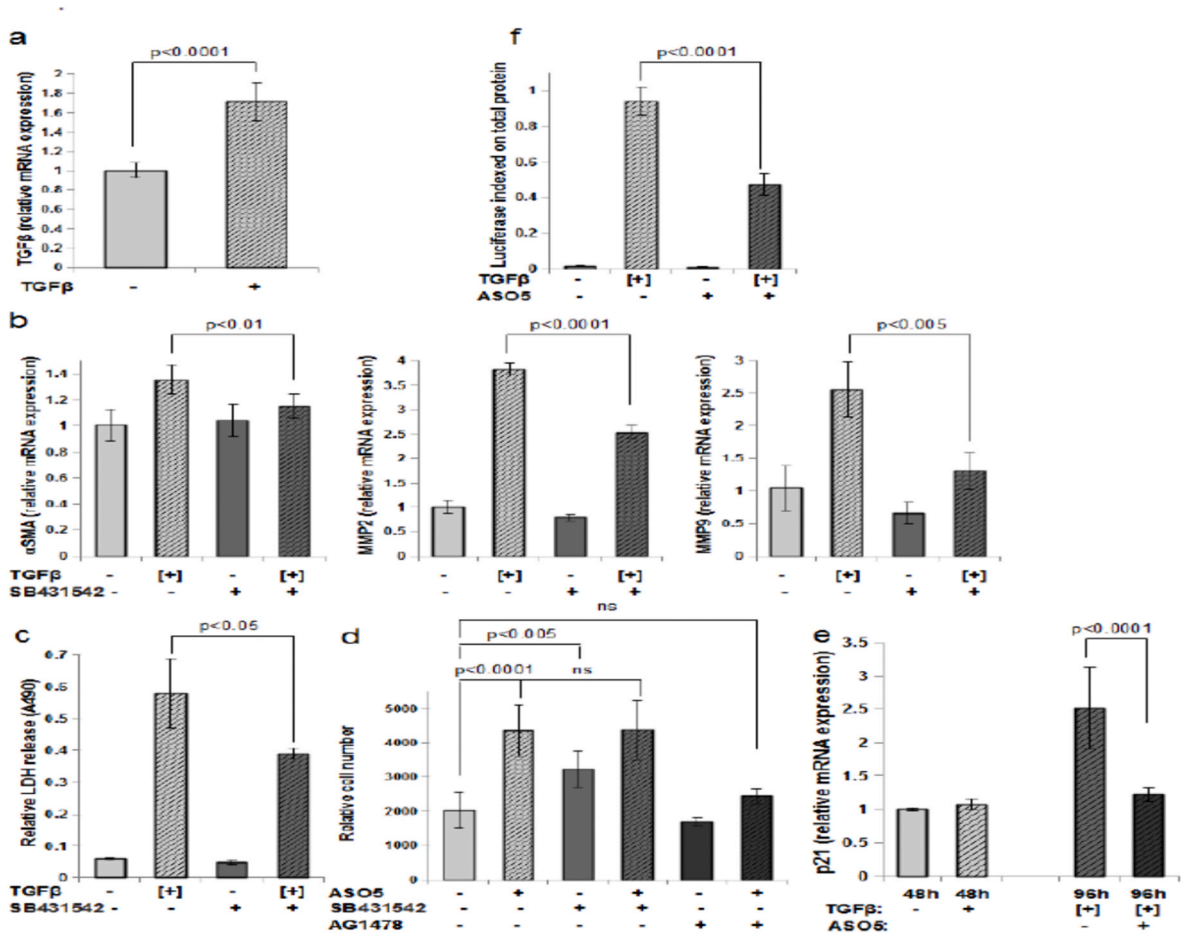

Fig. 8. EDA + Fibronectin possibly acts by activating latent TGFβ to maintain fibrotic phenotype. (a) Gene expression levels of TGFβ after treatment with 2.5 ng/ml TGFβ for 48 h (mean ± SD of 6 independent experiments). (b) Gene expression levels of αSMA, MMP2 and MMP9 after treating cells with ALK5 inhibitor SB431542 (10 μM) post TGFβ (mean ± SD of 6 independent experiments). (c) LDH release after treating cells with ALK5inhibitor SB431542 (10 μM) post TGFβ (mean ± SD of 6 independent experiments).(d) MTS assay in cells transfected with ASO5 or NC in the presence of ALK5 inhibitor SB431542 or EGFR inhibitor AG1478.(e) Gene expression levels for p21 in a post TGFβ model immediately after 48 h TGFβ treatment (48 h) and at end point of experiment (96 h). (f) Dual luciferase assay of HKC8 pre-transfected with Smad3 responsive element and treated with culture media from a post TGFβ experiment in primary PTEC. The labeling of the graph refers to the treatment of the primary PTECs whose media was used to treat the HKC8 cells. Data are shown as mean ± SD of 3 ((a), (c) and (f)), of 6 ((b) and (d)) independent experiments and for (e) as mean ± SD of 9 independent experiments (n = 3 in three different donors of primary cells). Square brackets indicate the presence of TGFβ for only the first 48h of the experiment.

human primary PTEC we showed that reducing the expression of EDA + FN reduces cell death and expression of TGFβ target genes αSMA, MMP2 and MMP9 after the removal of exogenous TGFβ1. Similar results were observed in cell cultures where ALK5 inhibitor was added post-TGFβ, which suggests the involvement of a second wave of TGFβ signalling. Indeed, the result of the experiments illustrated in Fig. 8f showing that conditioned culture media from post-TGFβ culture treated with ASO5 induced less activation of the CAGA(x4) Smad3 responsive reporter construct further supported this and confirmed the role of EDA + FN.

The effects on Collagen1 mRNA were not consistent among cells obtained from different donors. It is known that the levels of Collagen 1α1 precursor mRNA in activated fibroblasts are regulated by both transcriptional and posttranscriptional mechanisms, including modulation of mRNA stability [38,39]. In donor 3, ASO5 treatment significantly decreased the levels of Collagen 1a1 mRNA, whereas no decrease in seen in the other two donors. (Supplementary Fig. S2). The degree of induction of mRNA by exogenous TGFβ at 48 h was variable with >30-fold in donor 1, about 19-fold in donor 2 and 7.5-fold in donor 3 (data not shown). The lack of ASO5 effect in donors where exogenous TGFβ massively increases mRNA levels could be because the reduction in EDA + mRNA is minimal after 96 h.

It is interesting to note that the use of our antisense did have a significant effect on the formation of fibrillar collagen even though the effect on the mRNA was not. This might imply that there are multiple pathways at work. Transcriptional as well as post translational regulation of collagen 1 production and deposition has been described [38,39]. It is possible that the induction of collagen mRNA is not necessarily dependent on the autoinduction of TGFβ but perhaps by some other downstream signal such as CTGF which may remain unaffected by this antisense treatment. We hypothesise that in our cell model of human proximal tubule epithelial cells, EDA + fibronectin affects fibrillar collagen production through transcriptional as well as post translational mechanisms; the requirement of EDA + fibronectin in collagen matrix formation has been previously described and it is also possible that EDA + fibronectin interferes with collagen endocytosis promoting its deposition in extracellular space.

The EDA + fibronectin has been shown to stimulate cell proliferation and migration in fibroblasts and epithelioid cells derived from Chinese hamster ovaries through activation of α4β7 Integrin and ERK signalling [40]. In human renal tubule epithelial cells, however, ASO5 to reduce EDA + fibronectin increased cell number. ASO5 and ALK5 inhibitor treatment did not show additive effects on cell proliferation suggesting that EDA + FN and TGFβR are in the same pathway. After 48 h TGFβ treatment no significant change in p21 mRNA was observed, but the

further increase in gene expression at 96 h is significantly reduced with ASO5 (Fig. 8 e). Evidence from the literature shows that in colon carcinoma cells the cell cycle regulation by exogenous vs. autocrine TGFβ uses different pathways, with p21 being a more sensitive regulator of cell cycle than CDK4.

Whereas p21 can be strongly induced by autocrine TGFβ, CDK4 can only be activated by potent exogenous TGFβ signalling [40]. These results together with our data demonstrating inhibition of TGFβ induction of cell-cycle inhibitor P21 by ASO5 support the hypothesis that the increase in proliferation with ASO5 treatment is more likely to be a result of blocking latent TGFβ activation and consequent reduction in cell cycle arrest (and possibly reduced apoptosis as TGFβ is known to induce apoptosis in epithelial cells) rather than a direct effect of reduction in EDA + FN protein. In concordance with this, the EGFR inhibitor blocked the ASO5 effect on proliferation, showing that ASO treatment does not act directly on proliferation but rather preserve the competence of cells to respond to EGF by avoiding cell cycle arrest.

The role of TGFβ in fibrogenesis is well established and targeting it in renal disease was proposed as long ago as 1997 By Border & Noble [41]. However, global knock-out is not an attractive therapy. Alternative splicing is dysregulated in some pathological conditions, being a more selective process for pharmacological targeting [16–18]. The idea of targeting EDA + domain is not new, its value as a therapeutic target has been discussed previously [2]. To our knowledge it has not yet been considered in renal disease. Previous work from our lab has shown that the regulation of FN splicing at EDA exon in human PTEC involves activation of PI3K/SRp40 by TGFβ [34], but PI3K or SRp40 knock out are no more attractive than TGFβ knockdown.

The renal tubulo-interstitial fibrosis is a final common pathway for the progression of kidney disease and proximal tubules play a crucial role in initiation and progression of fibrosis through secretion of growth factors, inflammatory cytokines, extracellular matrix proteins and by undergoing epithelial-mesenchymal *trans*-differentiation (EMT). Redirection of alternative splicing can be achieved using RNase H-independent antisense oligonucleotides [11,22,42] and this may allow for a highly targeted strategy and the proximal tubule represents an excellent target for ASO therapy [43,44]. In this work, we have demonstrated that production of EDA fibronectin (RNA and secreted protein) in tubule cells in basal and TGFβ1 stimulated conditions can be reduced by ASOs designed to prevent 'splicing in' of EDA + exon. The EDB+ and total fibronectin expression remains unaltered. We then investigated the effect of targeting ASO on TGFβ1 induction of pro-fibrotic events in tubule cells. α SMA [45], MMP2[46], MMP9 [47] and connexin 43 [48] are all markers and mediators of renal fibrosis. The induction of all of these four proteins was inhibited by ASO treatment. In addition, TGFβ1 induced loss of specific proximal tubular junctional cadherin, K Cadherin (a key initial step of EMT) was inhibited by ASO treatment. These results demonstrate the ability of specific ASO designed to reduce the production of EDA + fibronectin in reducing pro-fibrotic events induced by TGFβ1 in tubule cells. Whether the reduction in tubulointerstitial EDA + FN would impair 'wound healing' in a damaged kidney and the role of EDA + fibronectin in inflammatory response and repair in acute kidney injury and chronic kidney disease remains to be investigated, and whether alternative pro-fibrotic factors such as CTGF/CCN2 or PDGF would continue to drive fibrosis is not yet known. *In vivo* experiments in mouse models of renal fibrosis will be required to answer these questions.

5. Conclusions

Antisense oligonucleotides designed to target and inhibit 'splicing in' of EDA exon of fibronectin mRNA in human renal proximal tubule cells result in selective inhibition of EDA + fibronectin production while leaving the total fibronectin unaffected. In our cell model, targeting ASO (ASO5) resulted in reductions in TGFβ induced pro-fibrotic outcomes (and improved cell survival) which persisted post TGFβ removal confirming the role of EDA + fibronectin in sustaining TGFβ effects in chronic phase. Based on our results, we believe that targeting the splicing of EDA + FN in the renal tubule cells will reduce the deposition of EDA + fibronectin, reduce the amount of active TGFβ in the tubulointerstitial tissue and reduce tubulointerstitial fibrosis and possibly glomerulosclerosis [26].

CRediT authorship contribution statement

Mysore Keshavmurthy Phanish: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Felicia Heidebrecht:** Methodology, Investigation. **Michaela Jackson:** Validation, Resources, Methodology, Investigation. **Frank Rigo:** Resources, Project administration, Methodology. **Mark Edward Carl Dockrell:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2024.114186.

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