Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

TGFβ1-mediated expression and alternative splicing of Fibronectin Extra Domain A in human podocyte culture

Tarunkumar Hemraj Madne*, Mark Edward Carl Dockrell

Southwest Thames Institute for Renal Research, Renal Unit, St Helier Hospital, Wrythe Lane, Carshalton, Surrey, SM5 1AA, St Georges, University of London. London, United Kingdom

Correspondence to: tarun2madne@yahoo.co.in

Received October 13, 2017; **Accepted** February 15, 2018; **Published** February 28, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.3.4

Abstract: Alternative splicing is a fundamental phenomenon to build protein diversity in health and diseases. Extra Domain A+ Fibronectin (EDA+Fn) is an alternatively spliced form of fibronectin protein present in the extra cellular matrix (ECM) in renal fibrosis. Podocytes are spectacular cell type and play a key role in filtration and synthesise ECM proteins in renal physiology and pathology. TGFβ1 is a strong stimulator of ECM proteins in renal injury. In this study, we have investigated alternative splicing of EDA+ Fn in human podocytes in response to TGFβ1. We have performed western blotting and immunofluorescence to characterise the expression of the EDA+Fn protein, real-time PCR for RNA expression and RT-PCR to look for alternative splicing of EDA+Fn in conditionally immortalised human podocytes culture.We used TGFβ1 as a stimulator and SB431542 and SRPIN340 for inhibitory studies. In this work, for the first time we have demonstrated in human podocytes culture EDA+Fn is expressed in the basal condition and TGFβ1 2.5ng/ml induced the Fn mRNA and EDA+Fn protein expression demonstrated by real-time PCR, western blotting and immunofluorescence. TGFβ1 2.5ng/ml induced the alternative splicing of EDA+Fn shown by conventional RT-PCR. Studies with ALK5 inhibitor SB431542 and SRPIN340 show that TGFβ1 induced alternative splicing of EDA+Fn was by the ALK5 receptor and the SR proteins. In human podocytes culture, alternative splicing of EDA+Fn occurs at basal conditions and TGFβ1 further induced the alternative splicing of EDA+Fn via ALK5 receptor activation and SR proteins. This is the first evidence of basal and TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes culture.

Key words: Extra Domain A; Fibronectin; Podocyte; TGFβ1; Alternative splicing.

Introduction

Alternative splicing is a fundamental phenomenon to build protein diversity and targeting this phenomenon is an eminent approach in regulating the distribution of key proteins in health and diseases. Splicing is a complex process and mainly categorised as constitutive splicing and alternative splicing. In constitutive splicing, all the introns are removed and all the exons are joined to form a mature messenger RNA. Alternative splicing leads to formation of different isoforms of proteins from a single gene (1). The alternative splicing is a typical type of splicing where splicing occurs in different patterns in which exons could be included or excluded to form mature mRNA with a different combination of exons leading to the formation of different proteins after translation (2). Alternative splicing process involves the formation of spliceosome: a large complex composed of five ribonucleoproteins (RNPs), various small nuclear RNA and as many as 150 proteins. This complex recognises the exons and introns and carries out splicing in a complex manner which leads to the formation of mature RNA of different types. The recognition and selection of splicing sites are determined by parameters such as exon length and the strength of splicing signals. The exons and introns other than splicing sites contain the exon enhancer sites and exon silencer sites. The exon enhancer sites are polypurine rich sites and recognised by a group of proteins known as SR (serine-arginine) proteins (3). The SR proteins are serine-arginine rich proteins which facilitate the exon inclusion (2,4–6).

Fibronectin (Fn) is a prominent component of the extracellular matrix (ECM). Fn is associated with different functions such as cell adhesion, cellular migration, growth, differentiation and cell signalling (7). Fn protein is a dimer made of two identical monomer subunit each of nearly 250 kDa connected via disulphide bridge at the c-terminal end. Fn is a glycoprotein contains 4-9 % of carbohydrates which is either N-linked or O-Linked with Type III repeats (8). On the basis of solubility, Fn is categorised in two types; cellular Fn produced by fibroblasts, endothelial cells, epithelial cell, mesangial cells. Plasma Fn produced largely by hepatocytes which circulate in plasma (8,9). Although Fn is a single gene product, it could exist in about 20 different isoforms due to alternative splicing. Fn gene contains different alternative splicing sites named as extra domain A (EDA), extra domain B (EDB), IIICS and V. The EDA and EDB exhibit exon skipping and code for Type III repeat which leads to the formation of EDA and EDB Fn. The EDA and EDB segments can be either fully retained or excluded by alternative splicing (10). The cellular Fn is a mixture of both excluded and retained EDA and EDB segments whereas plasma Fn does not contain any EDA or EDB segment (8,11).

EDA+Fn (Fn with EDA+) is expressed in wound healing, developing embryo and in pathologies such as cancer, lung fibrosis, renal fibrosis, skin fibrosis and

liver fibrosis (9). EDA+Fn is synthesised by cells such as fibroblasts, endothelial cells, epithelial cell and mesangial cells(12). The alternative splicing in Fn pre-mRNA which results in EDA exon inclusion leads to the formation of Fn Type III repeat with EDA+Fn (13). The inclusion of EDA exon has been shown to be influenced by the presence of exon enhancing sites and exon silencing sites. The EDA exon enhancing site is purine rich 81 nucleotide site located in the centre of EDA exon. The recognition of exon enhancing site by SR proteins greatly enhances the EDA alternative splicing. Downstream of EDA enhancing site a short stretch of the nucleotide sequence is present known as exon silencing site which silences the EDA exon inclusion. The mutation in exon silencing site has demonstrated the complete inclusion of EDA exon (14,15). EDA is present adjacent to the amino acid sequence Arg-Gly-Asp (RGD) that has a unique interaction property. The RGD site is recognised by cell surface integrin's and can regulate cellular events as adhesion, migration, differentiation and growth (8,9,11,16,17).In pulmonary fibrosis, lung fibroblast shows marked expression of EDA+Fn. EDA+Fn null mice fail to develop pulmonary fibrosis and EDA+Fn has been shown to be essential for the development of pulmonary fibrosis (18). EDA+Fn was shown to be necessary for allergen-induced lower airway fibrosis (19). These findings suggest that EDA+Fn is expressed in pathologies and could regulate events involved in the development of pathologies.

The RT-PCR studies were performed to demonstrate the inclusion of EDA exon which yields two products; a 104bp band fragment corresponding to EDA-Fn (Fn lack EDA) and a 374bp fragment corresponding to EDA+Fn detected as two separate bands after the agarose gel electrophoresis. The primer flanked the EDA exon and thus conventional PCR leads to the generation of EDA+ and EDA- bands. The difference in sizes of the two bands is equal to the size of the EDA exon (270bp). The ratio of EDA+Fn to EDA-Fn mRNA determines the EDA+Fn alternative splicing of Fn (20).

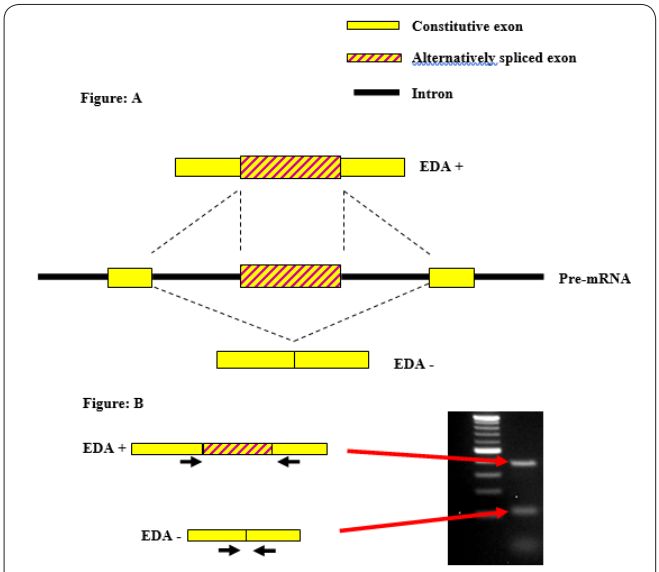

Figure A. Alternative splicing of EDA+Fn: The diagrammatic representation of mechanism of alternative splicing of EDA+Fn demonstrating inclusion and exclusion of EDA exon to form Fn with EDA and Fn without EDA respectively. Figure B, Alternative splicing of EDA+Fn: The agarose gel electrophoresis demonstrating the alternative splicing of Fn with two bands EDA+Fn and EDA-Fn. The black arrow represents the position of primers which flanks the EDA exon.

TGFβ1 is a cytokine which overexpressed during fibrosis. Overexpression of TGFβ mediates glomerulosclerosis and fibrosis by stimulating ECM proteins secretion such as collagen type I-III and IV, Fn and laminin (21) (22). 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 (23).

Podocytes are highly specialised terminally differentiated cells. Podocytes are characterised by the presence foot processes which form a narrow slit by interdigiting with foot processes of neighbouring podocyte. The narrow slits of approximately 40nm diameter are called as the slit diaphragm through which the process of filtration takes place. The endothelial cells and podocytes lie on either side of glomerular basement membrane (GBM). Endothelial cells, GBM and podocytes together make a complex barrier called as glomerulus filtration barrier (GFB). The healthy maintenance of GFB is crucial for the normal filtration. Defects in most of the components of GFB have been reported in many kidney pathologies (24-27). GBM is a dynamic structure made up of proteins actively secreted by podocytes and endothelial cells. In the adult, both podocyte and endothelial cells constantly add and assemble the matrix constituents, maintaining a hydrated mesh-like structure made up of collagen IV, laminin, entactin, agrin, perlecan. It has been recently described the presence of Fn in GBM (24). It has been shown that podocytes in culture produce Fn. However, there is a lack of knowledge about the alternative splicing of EDA+Fn in human podocytes. Thus exploring the expression and alternative splicing EDA+Fn in human podocyte could provide an improved understanding into the insight of podocyte biology in glomerular injury.

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) and SRPK1 inhibitor SRPIN340 (10μM) 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 10min 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 30V 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 15min. 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 as (EDA+ Fn 220kDa, ab6328-Abcam, 1:1000, Mouse monoclonal), (Total Fn 220kDa, sc- 9068-SantaCruz biotechnology, 1:2000, Rabbit Polyclonal), (α/β-Tubulin, 55kDa, 2148-Cell signalling, 1:1000, Rabbit Polyclonal) 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 1.25U 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 were: EDA+/-Fn 5'GGAGAGAGTCAGCCTCTGGTTCAG3' Forward, 5'TGTCCACTGGGCGCTCAGGCTTGTG3' Reverse. 18s RNA 5'GTAACCCGTTGAACCCCATT3' Forward, 5'CCATCCAATCGGTAGTAGCG3' Reverse.

PCR steps were as: initial denaturation for 5min at 95°C and 25 cycles of 30sec at 95°C for, annealing temperature for 30sec at 56°C, 30 sec at 68°C and final extension for 5min 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).

The 1:10 diluted cDNA synthesised after RNA extraction and reverse transcription PCR was used to study gene expressions by real-time PCR. Real-time PCR was performed by using TaqMan custom made FAM/MGB probes labelled TaqMan gene expression assay supplied by Applied Biosystems (Foster City, CA, USA). Each assay contains pre-formulated primers and TaqMan FAM/MGB probes in a 20x concentration and was supplied for 250 reactions at a 20μl reaction volume. Pre-optimized TaqMan gene expression assays containing FAM/MGB dye-labeled probes were used for the expression analysis of target gene of interest. TaqMan endogenous control for GAPDH gene as custom made primer and probe sets labelled with VIC/MGB reporter dye was used as a housekeeping gene to normalise the gene expression supplied by Applied

Biosystems. To present the relative gene expression, the widely used comparative C_T method also referred to as the $2^{-\Delta\Delta C}$ method was used.

The primer sequence were: Fibronectin 5' CGGTGGCTGTCAGTCAAG 3' Forward, 5' AAACCTCGGCTTCCTCCATAA 3' Reverse. GAPDH 5' ACAACTTTGGTATCGTGGAAGG 3' Forward, 5' GCCATCACGCCACAGTTTC 3' Reverse.

Immunocytochemistry

After the experiment, the medium was removed, and cells were washed with the warm 1xPBS. Cells were fixed with paraformaldehyde (3.7% in PBS) and 0.2% Triton X-100 (permeabilizing agent) for 20min at room temperature. Cells were then washed three times with the 1xPBS. Cells were blocked with blocking buffer (1xPBS, 5% serum derived from the same species in which the secondary antibody was raised) for 1h at room temperature. Cells were washed three times with 1xPBS. Cells were incubated with primary antibodies as (EDA+ Fn 220kDa, ab6328-Abcam, 1:1000, Mouse monoclonal), (Synaptopodin 100kDa, sc-5049-Santa-Cruz biotechnology, 1:100, Rabbit polyclonal) diluted in primary antibody dilution buffer [1xPBS, 0.2% Triton X-100] overnight at 4°C. Next day cells were washed three times with 1x PBS and incubated with FITC-labelled secondary antibody as (Antimouse IgG Secondary Ab-Rabbit, 1:40, A9044-Sigma-Aldrich), (Antirabbit IgG Secondary Ab-Rabbit, 1:1000, 7074-Cell signalling) diluted in 1xPBS, 0.2% Triton X-100 mixed with the DAPI (nuclear stain) for 60min at room temperature in the dark. Cells were washed three times with 1xPBS and then mounted with glycerol. Cells were examined with a fluorescence microscope (Nikon Eclipse Ti-S) using appropriate excitation wavelength at 40x magnification. Plates were stored at 4°C in the dark.

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

Results

TGFβ1-mediated gene expression of Fn in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days. Overnight serum starved podocytes cells were treated with TGFβ1 2.5ng/ml and vehicle (0.1 % w/v BSA) for 24h. Cells were lysed and RNA extraction was done. Real-time PCR was performed to look for Fn gene expression and indexed with GAPDH gene expression as the housekeeping gene. In human podocyte culture, TGFβ1 2.5ng/ml significantly induced the Fn gene expression as compared to vehicle (0.1 % w/v BSA) at 24h (Figure 1).

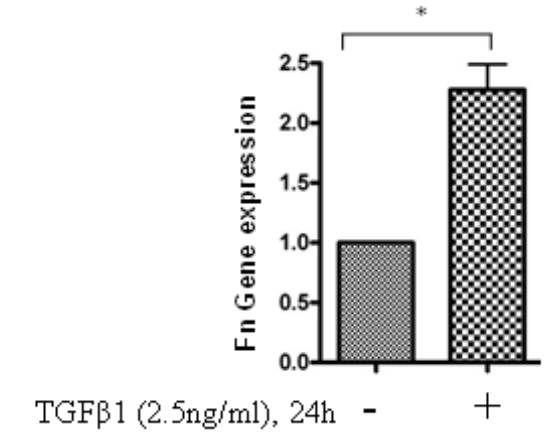

Figure 1. Fn gene expression indexed to the housekeeping gene GAPDH in podocytes. Podocytes were treated with TGFβ1 2.5ng/ml for 24h and compared with vehicle (0.1 % w/v BSA). The Student's t-test is performed and the graph represents as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

TGFβ1-mediated EDA+/-Fn mRNA expression shown by RT-PCR in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days, serums starved overnight and treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1% w/v BSA) for 24h. Cells were lysed and RNA was extracted. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA expression (Figure 2). TGFβ1 significantly increased EDA+/-Fn mRNA ratio as compared to vehicle. These results suggest that TGFβ1 induced the alternative splicing of EDA+Fn in human podocytes culture.

TGFβ1-mediated expression of EDA+Fn protein in human podocytes culture

Podocytes were grown on 6 well cell culture plate

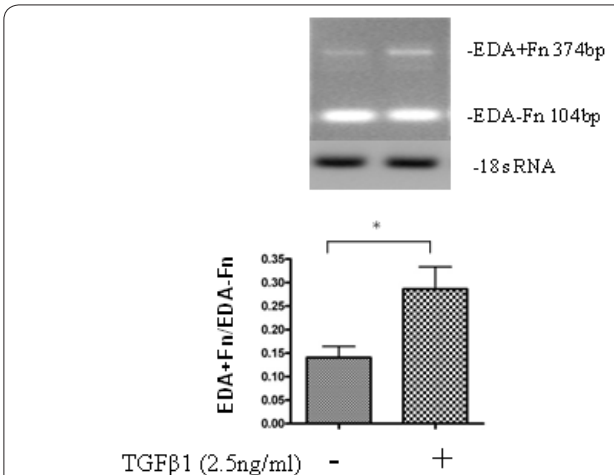

Figure 2. The EDA+Fn and EDA-Fn mRNA expression after TGFβ1 2.5ng/ml treatment compared with vehicle (0.1% w/v BSA) for 24h. Agarose gel electrophoresis picture displays RT-PCR products EDA+Fn and EDA-Fn bands. TGFβ1 significantly increased the EDA+/-Fn mRNA ratio as compared to vehicle. The graph represents the ratio of EDA+Fn to EDA-Fn mRNA bands. The Student's t-test is performed and the graph represents band density expressed as SEM with n=6 (independent experiments). P value <0.05 was considered as significant.

and allowed to differentiate for 14 days, serum starved overnight, treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1 % w/v BSA) for 72h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for EDA+Fn expression and indexed to tubulin as the housekeeping protein. TGFβ1 induced the expression of EDA+Fn protein compared with vehicle (Figure 3).

TGFβ1-mediated cellular expression of EDA+Fn in human podocyte culture

Podocytes were grown on 24 well plates and allowed to differentiate for 14 days, serum starved overnight, treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1 % w/v BSA) for 72h. Immunocytochemistry was performed to look for expression of EDA+Fn and counterstained with DAPI for nuclear localisation. Immunofluorescence pictures show the expression of EDA+Fn (red colour staining) and nuclear staining (blue colour) in human podocytes treated with TGFβ1 2.5ng/ml and compared with vehicle. TGFβ1 2.5ng/ml induced the EDA+Fn expression (Figure 4).

TGFβ1-mediated expression of Total Fn in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days. HKC8 cells were also used as positive control for Total Fn expression. Overnight serum starved podocytes and HKC8 cells were treated with TGFβ1 2.5ng/ml and compared with vehicle (0.1 % w/v BSA) at 72h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for Total Fn expression and indexed with tubulin as the housekeeping protein. In human podocyte culture, TGFβ1 2.5ng/ml did not induce the expression of Total Fn as compared to vehicle (0.1 % w/v BSA) at 72h (Figure 5, Panel A). However in HKC8 cells TGFβ1 2.5ng/ml significantly induced the expression of Total Fn as compared vehicle (0.1 % w/v BSA) at 72h (Figure 5, Panel B).

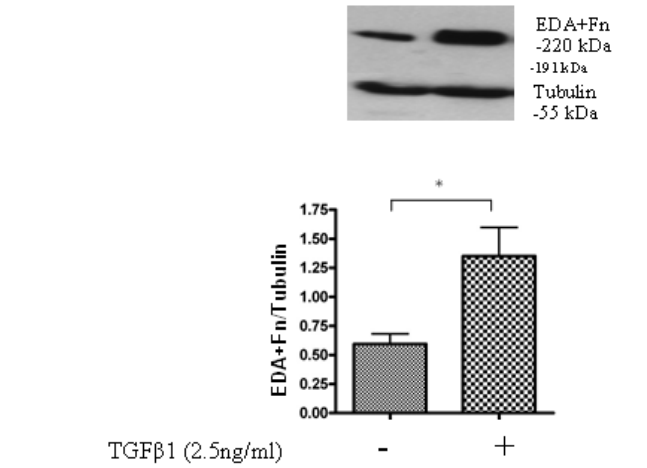

Figure 3. TGFβ1 mediated EDA+Fn protein expression indexed to the housekeeping protein tubulin. Podocytes were treated with TGFβ1 2.5ng/ml for 72h and compared with vehicle (0.1 % w/v BSA). The Student's t-test is performed and the graph represents band density expressed as SEM with n=6 (independent experiments). P value <0.05 was considered as significant.

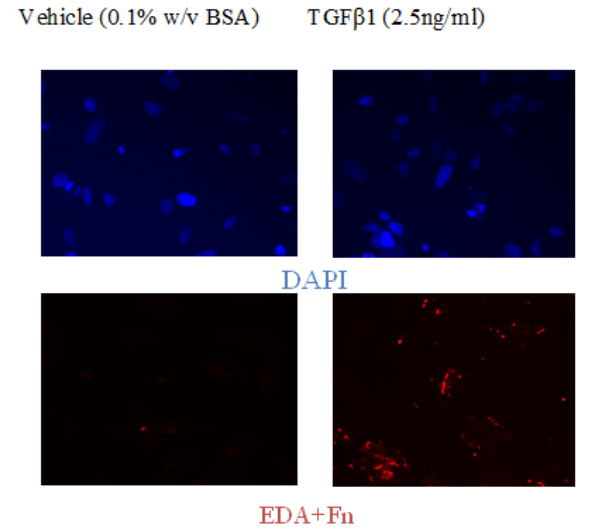

Figure 4. The immunofluorescence pictures of TGFβ1 mediated cellular localisation of EDA+Fn (red colour) with nuclear staining DAPI (blue colour) in human podocyte culture. Cells were cultured on plastic dishes treated with TGFβ1 2.5ng/ml and vehicle 0.1 % w/v BSA for 72h n=4 (independent experiments).

TGFβ1-mediated expression of EDA+/-Fn mRNA through TGFβ1 receptor ALK5 in human podocytes 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 with (0.1% w/v BSA, 0.1 % DMSO) and SB431542 (10μM) for 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 24h. Cells were lysed and RNA was extracted. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA expression. TGFβ1 significantly increased the EDA+/-Fn mRNA ratio as compared to vehicle. SB431542 significantly downregulated the TGFβ1 increased EDA+/-Fn mRNA ratio in human podocytes culture (Figure 6).

TGFβ1-mediated expression of EDA+/-Fn mRNA by SR Proteins

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days and serums starved overnight. Podocytes were pre-incubated with

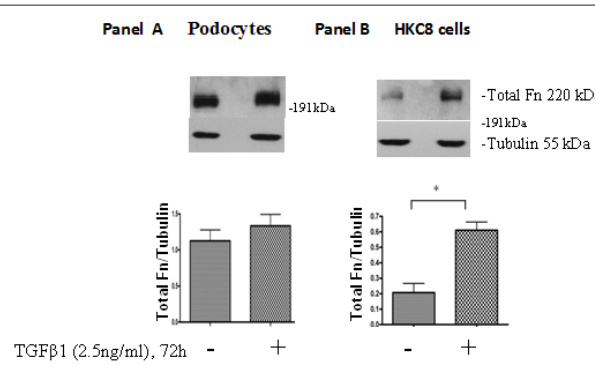

Figure 5. Expression of Total Fn indexed to the housekeeping protein tubulin in podocytes (Panel A) and in HKC8 cells (Panel B). Cells were treated with TGFβ1 2.5ng/ml for 72h and compared with vehicle (0.1 % w/v BSA). The Student's t-test is performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

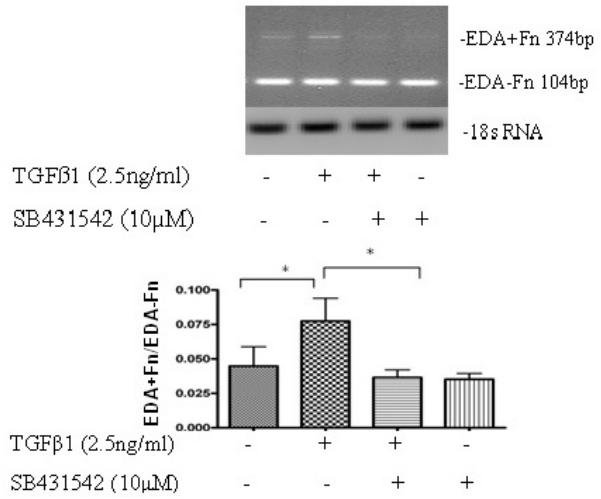

Figure 6. TGFβ1 2.5ng/ml significantly increased the expression of EDA+/-Fn mRNA ratio as compared to vehicle and this was downregulated by SB431542 (10µM) at 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

vehicle (0.1% w/v BSA, 0.1 % DMSO) and SRPIN340 (10µM) for 1h and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and SRPIN340 (10µM), SRPIN340 (10µM) 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 ratio as compared to vehicle. SRPIN340 significantly downregulated the TGFβ1 increased EDA+/-Fn mRNA ratio (Figure 7).

Discussion

The podocytes are spectacular cell type, characterised by the presence of the foot processes which interdigit with foot processes of neighbouring podocyte to form a narrow slit of nearly 30-40nm in width. GBM is a dynamic structure made up of proteins actively secreted by podocytes and endothelial cells. In the adult, both podocyte and endothelial cells constantly add and assemble the matrix constituents, maintaining a hydrated mesh-like structure made up of collagen IV, laminin, entactin, agrin, perlecan (24–27). A more detailed analysis has revealed a more complex protein make up of GBM which contains the Fn as a component (24). EDA+Fn is an alternatively spliced isoform of Fn which is expressed in wound healing, developing embryo and in pathologies such as cancer, lung fibrosis, renal fibrosis, skin fibrosis and liver fibrosis. EDA+Fn is synthesised by cells such as fibroblasts, endothelial cells, epithelial cell and mesangial cells. 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 (28). Studies in tubular epithelial cells show that TGFβ1 induces upregulation of Fn and EDA+Fn in cell lysates as well as supernatants (29). TGFβ1 mediated up-regulation of EDA+Fn induces the fibroblast differentiation and thus leads to upregulation of ECM proteins (30). TGFβ1 shown to induce alternative splicing of EDA+Fn in PTEC cells by SR proteins and thus involved in tubulointerstitial fibrosis (20).

In this work for the first time we demonstrated in human podocytes culture the pathological isoform of Fn, EDA+Fn is expressed at the basal condition, and TGFβ1 significantly induced the expression of EDA+Fn. The results from these studies describe that TGFβ1 2.5ng/ml induced the Fn mRNA expression in human podocytes culture demonstrated by real-time PCR. TGFβ1 2.5ng/ml at 24h induced the alternative splicing of EDA+Fn by increasing the EDA+/-Fn mRNA ratio in human podocytes culture shown by conventional RT-PCR. TGFβ1 2.5ng/ml induced the EDA+Fn protein expression at 72h in human podocytes culture demonstrated by western blotting. The result from immunofluorescence experiment shows that TGFβ1 induced the sub-cellular localisation of EDA+Fn in human podocytes culture. Studies with ALK5 inhibitor SB431542 suggest that TGFβ1 induced alternative splicing of EDA+Fn was by the ALK5 receptor. Studies with SRPIN340 suggest that TGFβ1 induced alternative splicing of EDA+Fn involves the SR proteins.

These results suggest that TGFβ1 mediates the transcription of Fn pre-mRNA, the inclusion of EDA+ in Fn pre-mRNA by alternative splicing and expression of EDA+Fn in human podocytes culture. This finding is consistent with the previous findings where TGFβ1 induces the EDA+Fn expression in human PTEC cells and fibroblast cells (20,31). However, this is the first evidence for this event in human podocytes.

In our studies, in human podocytes culture at basal condition produces a high level of Total Fn protein. TGFβ1 2.5ng/ml slightly induces further Total Fn protein expression but statistically not significant. However HKC8 cells produce a low level of Total Fn and TGFβ1 2.5ng/ml induced the Total Fn protein expression significantly. In previous studies, it has been demonstrated that TGFβ1 5ng/ml significantly induces the Fn gene expression in conditionally immortalised human podo-

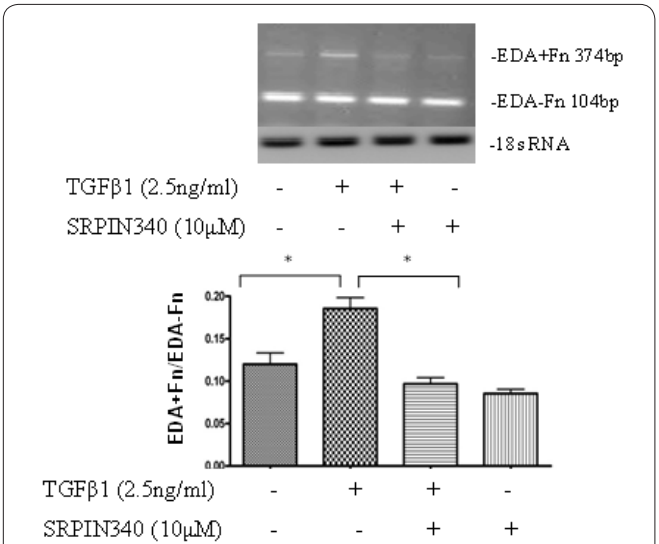

Figure 7. TGFβ1 2.5ng/ml significantly increased the EDA+/-Fn mRNA ratio as compared to vehicle and this was downregulated by SRPIN340 (10µM) at 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

cytes culture (32). In conditionally immortalised mouse podocytes TGFβ1 2ng/ml induces the Fn gene expression and protein expression (33). Thus the possible reason for no significant TGFβ1 mediated induction of Total Fn protein in my experiments could be the cell type difference. These results suggest that human podocytes culture at basal condition produces a high level of Total Fn and low level of EDA+Fn and TGFβ1 2.5ng/ml did not further induces Total Fn protein expression but induces alternative splicing of EDA+Fn and thus EDA+Fn protein expression.

It has been shown in studies that TGFβ signalling involves the interaction of transcriptional factors with cell-type-specific master transcriptional factors. These master transcriptional factors are present in high concentrations which help the binding of transcriptional factors such as Smad3 to the gene to be transcribed and thus regulate cell-type-specific transcription of genes. In embryonic stem cells Smad3 occupy the genome with master transcriptional factor Oct4, in myotubes, Smad3 occupy the genome with master transcriptional factor Myod1 and in pro-B cells, Smad3 occupy genome with master transcriptional factor PU.1 (34). In each of these cells, Smad3 is directed by a master transcriptional factor present in that cell type and transcribe the cell-type specific genes. This explains the TGFβ mediated cell type-specific regulation of transcription of genes (34).

TGFβ1 mediates its effect by binding to its receptors which in turn recruits different proteins. The members of the TGFβ superfamily binds to two distinct receptors types known as type II and type I receptors (ALK receptors). SB431542 is a small chemical molecule has been shown to inhibit the ALK5 receptor activity and widely used to block TGFβ1 mediated effects in cell culture studies. TGFβ1 has been shown to induce the ECM matrix proteins including Fn and leads to podocytes dedifferentiation (32). Herman-Edelstein shows that SB431542 inhibits the angiotensin-induced Fn gene expression in human podocytes culture (32). To establish TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes through receptor ALK5, I have used SB431542 to inhibit the ALK5 receptor. The results of this experiment show that the SB431542 significantly downregulated the TGFβ1 induced EDA+/-Fn mRNA ratio The result from this experiment suggests that TGFβ1 induced alternative splicing of EDA+Fn was through receptor ALK5.

Alternative splicing has been shown to be regulated by serine-arginine rich proteins (SR). The alternative splicing of Fn in human PTEC has been shown to be regulated by SR proteins (20). SRPIN340 is a chemical inhibitor and has been shown to inhibit selectively serine–arginine protein kinase 1 (SRPK1), a protein kinase that phosphorylates proteins containing serine-arginine-rich domains (35,36). SRPIN340 has been shown to inhibit the alternative splicing of VEGF in human podocyte culture (35,36). I have used SRPIN340 to investigate the SR proteins mediated regulation of TGFβ1 induced alternative splicing of EDA+Fn in human podocyte culture. The results of this experiment show that the SRPIN340 significantly downregulated the TGFβ1 induced EDA+/-Fn mRNA ratio. This result suggests that TGFβ1 induced alternative splicing of EDA+Fn was regulated by the SR proteins in human podocytes in culture. SR proteins are the family of splicing regulators proteins. TGFβ1 has been shown to induce different SR proteins such as SRp20, SRp30, SRp40, SRp55 and SRp75. SRp30 and SRp20 have been demonstrated to regulate the alternative splicing of Fn (6). SRp40 has been shown to regulate alternative splicing of EDA+Fn in human PTEC cells (20).

In Summary in human podocytes culture, alternative splicing of EDA+Fn occurs at basal conditions and TGFβ1 further induced the alternative splicing of EDA+Fn via ALK5 receptor activation and SR proteins were involved in the alternative splicing of EDA+Fn. This is the first evidence of basal and TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes culture.

Human podocytes are involved in production of ECM proteins in physiology and pathology. In this study we have demonstrated in human podocytes culture, alternative splicing of EDA+Fn occurs at basal conditions and TGFβ1 further induced the alternative splicing. Thus targeting alternative splicing of EDA+Fn either by targeting TGFβ1 mediated signalling or by SR proteins involved in alternative splicing could be an interesting approach to regulate alternative splicing of EDA+Fn in human podocytes in culture.

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.

Funding- Maharashtra Government of India.

Authors' contributions-

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

Dr Mark Edward Carl Dockrell- Lead supervisor.

Acknowledgements- Dr Phanish Mysore and Dr Iain Macphee for their technical guidance.

References

1. Kim E, Goren A, Ast G. Alternative splicing: current perspectives. Bioessays 2008; 30(1):38–47.

2. Kelemen O, Convertini P, Zhang Z, Wen Y, Shen M, Falaleeva M, et al. Function of alternative splicing. Gene 2012; 514(1):1–30.

3. Risso G, Pelisch F, Quaglino A, Pozzi B, Srebrow A. Regulating the regulators: serine/arginine-rich proteins under scrutiny. IUBMB Life 2012; 64(10):809–16.

4. Lynch KW. Regulation of alternative splicing by signal transduction pathways. Adv Exp Med Biol 2007; 623:161–74.

5. Blaustein M, Pelisch F, Srebrow A. Signals, pathways and splicing regulation. Int J Biochem Cell Biol 2007; 39(11):2031–48.

6. Hallgren O, Malmström J, Malmström L, Andersson-Sjöland A, Wildt M, Tufvesson E, et al. Splicosomal and serine and arginine-rich splicing factors as targets for TGF-β. Fibrogenesis Tissue Repair 2012; 5(1):6.

7. Gorin Y, Block K, Hernandez J, Bhandari B, Wagner B, Barnes JL, et al. Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 2005; 280(47):39616–26.

8. Pankov R. Fibronectin at a glance. J Cell Sci 2002; 115(20):3861–3.
9. To WS, Midwood KS. Plasma and cellular fibronectin: distinct and independent functions during tissue repair. Fibrogenesis Tissue Repair 2011; 4(1):21.
10. Barnes VL, Musa J, Mitchell RJ, Barnes JL. Expression of Embryonic Fibronectin Isoform EIIIA Parallels -Smooth Muscle Actin in Maturing and Diseased Kidney. J Histochem Cytochem 1999; 47(6):787–97.
11. Mao Y, Schwarzbauer JE. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. Matrix Biol 2005; 24(6):389–99.
12. Losino N, Waisman A, Solari C, Luzzani C, Espinosa DF, Sassone A, et al. EDA-containing fibronectin increases proliferation of embryonic stem cells. PLoS One 2013 J; 8(11):e80681.
13. Pankov R. Fibronectin at a glance. J Cell Sci 2002; 115(20):3861–3.
14. Muro AF, Caputi M, Pariyarath R, Pagani F, Buratti E, Baralle FE. Regulation of fibronectin EDA exon alternative splicing: possible role of RNA secondary structure for enhancer display. Mol Cell Biol 1999; 19(4):2657–71.
15. Muro AF, Iaconcig A, Baralle FE. Regulation of the fibronectin EDA exon alternative splicing. Cooperative role of the exonic enhancer element and the 5′ splicing site. FEBS Lett 1998; 437(1–2):137–41.
16. Baelde HJ, Eikmans M, van Vliet AI, Bergijk EC, de Heer E, Bruijn JA. Alternatively spliced isoforms of fibronectin in immune-mediated glomerulosclerosis: the role of TGFbeta and IL-4. J Pathol 2004; 204(3):248–57.
17. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci 2010 15;123(Pt 24):4195–200.
18. Muro AF, Moretti F a, Moore BB, Yan M, Atrasz RG, Wilke C a, et al. An essential role for fibronectin extra type III domain A in pulmonary fibrosis. Am J Respir Crit Care Med 2008 Mar 15; 177(6):638–45.
19. Olsen AL, Sackey BK, Marcinkiewicz C, Boettiger D, Wells RG. Fibronectin extra domain-A promotes hepatic stellate cell motility but not differentiation into myofibroblasts. Gastroenterology 2012; 142(4):928–937.e3.
20. Phanish MK, Heidebrecht F, Nabi ME, Shah N, Niculescu-Duvaz I, Dockrell MEC. The regulation of TGFβ1 induced fibronectin EDA exon alternative splicing in human renal proximal tubule epithelial cells. J Cell Physiol 2015; 230(2):286–95.
21. Li JH, Huang XR, Zhu H-J, Johnson R, Lan HY. Role of TGF-beta signaling in extracellular matrix production under high glucose conditions. Kidney Int 2003; 63(6):2010–9.
22. Kanwar YS. TGF-β and renal fibrosis: a Pandora's box of surprises. Am J Pathol 2012; 181(4):1147–50.
23. Yamamoto T, Noble NA, Cohen AH, Nast CC, Hishida A, Gold LI, et al. Expression of transforming growth factor-beta isoforms in human glomerular diseases. Kidney Int 1996; 49(2):461–9.
24. Byron A, Randles MJ, Humphries JD, Mironov A, Hamidi H, Harris S, et al. Glomerular cell cross-talk influences composition and assembly of extracellular matrix. J Am Soc Nephrol 2014; 25(5):953–66.
25. Patrakka J, Tryggvason K. Molecular make-up of the glomerular filtration barrier. Biochem Biophys Res Commun 2010; 396(1):164–9.
26. Gunwar S, Ballester F, Noelken ME, Sado Y, Ninomiya Y, Hudson BG. Glomerular Basement Membrane: identification of a novel disulfide-cross-linked network of 3, 4, and 5 chains of type iv collagen and its implications for the pathogenesis of alport syndrome. J Biol Chem 1998; 273(15):8767–75.
27. Miner JH. The glomerular basement membrane. Exp Cell Res 2012; 318(9):973–8.
28. Shihab FS, Yamamoto T, Nast CC, Cohen AH, Noble NA, Gold LI, et al. Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. J Am Soc Nephrol 1995;6(2):286–94.
29. Viedt C, Bürger a, Hänsch GM. Fibronectin synthesis in tubular epithelial cells: up-regulation of the EDA splice variant by transforming growth factor beta. Kidney Int 1995; 48(6):1810–7.
30. Kohan M, Muro AF, White ES, Berkman N. EDA-containing cellular fibronectin induces fibroblast differentiation through binding to alpha4beta7 integrin receptor and MAPK/Erk 1/2-dependent signaling. FASEB J 2010; 24(11):4503–12.
31. White ES, Sagana RL, Booth AJ, Yan M, Cornett AM, Bloomheart CA, et al. Control of fibroblast fibronectin expression and alternative splicing via the PI3K/Akt/mTOR pathway. Exp Cell Res 2010; 316(16):2644–53.
32. Herman-Edelstein M, Thomas MC, Thallas-Bonke V, Saleem M, Cooper ME, Kantharidis P. Dedifferentiation of immortalized human podocytes in response to transforming growth factor-β: a model for diabetic podocytopathy. Diabetes 2011; 60(6):1779–88.
33. Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y. Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. Am J Pathol 2008; 172(2):299–308.
34. Mullen AC, Orlando D a, Newman JJ, Lovén J, Kumar RM, Bilodeau S, et al. Master transcription factors determine cell-type-specific responses to TGF-β signaling. Cell 2011; 147(3):565–76.
35. Dong Z, Noda K, Kanda A, Fukuhara J, Ando R, Murata M, et al. Specific inhibition of serine/arginine-rich protein kinase attenuates choroidal neovascularization. Mol Vis 2013; 19(July 2012):536–43.
36. Nowak DG, Amin EM, Rennel ES, Hoareau-Aveilla C, Gammons M, Damodoran G, et al. Regulation of vascular endothelial growth factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis. J Biol Chem 2010; 285(8):5532–40.