Original Paper

Overexpression of TGF- β Inducible microRNA-143 in Zebrafish Leads to Impairment of the Glomerular Filtration Barrier by Targeting Proteoglycans

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Key Words

Versican • Syndecan • Glycocalyx • micro-RNA-143 • Podocytes • Zebrafish • Proteinuria • Glomerulus

Abstract

Background: TGF- β is known as an important stress factor of podocytes in glomerular diseases. Apart from activation of direct pro-apoptotic pathways we wanted to analyze micro-RNA (miRs) driven regulation of components involved in the integrity of the glomerular filtration barrier induced by TGF- β . Since miR-143-3p (miR-143) is described as a TGF- β inducible miR in other cell types, we examined this specific miR and its ability to induce glomerular pathology. **Methods:** We analyzed miR-143 expression in cultured human podocytes after stimulation with TGF- β . We also microinjected zebrafish eggs with a miR-143 mimic or with morpholinos specific for its targets syndecan and versican and compared phenotype and proteinuria development. **Results:** We detected a time dependent, TGF- β inducible expression of miR-143 in human podocytes. Targets of miR-143 relevant in glomerular biology are syndecans and versican, which are known components of the glycocalyx. We found that syndecan 1 and 4 were predominantly expressed in podocytes while syndecan 3 was largely expressed in glomerular endothelial cells. Versican could be detected in both cell types. After injection of a miR-143 mimic in zebrafish larvae, syndecan 3, 4 and versican were significantly downregulated. Moreover, miR-143 overexpression or versican knockdown by morpholino caused loss of plasma proteins, edema, podocyte effacement and endothelial damage. In contrast, knockdown of syndecan 3 and syndecan 4 had no effects on glomerular filtration barrier. **Conclusion:** Expression of versican and syndecan isoforms is indispensable for proper barrier function. Podocyte-derived miR-143 is a mediator for paracrine and autocrine cross talk between podocytes and glomerular endothelial cells and can alter expression of glomerular glycocalyx proteins.

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Introduction

The glomerular filtration barrier (GFB) of the kidney is a complex structural and functional unit of podocytes, glomerular basement membrane (GBM) and endothelial cells. The apical side of podocytes and endothelial cells is coated with a special layer called the glycocalyx [1, 2]. The glomerular glycocalyx is composed of proteoglycans, glycoproteins and glycolipids that among additional functions enhance the charge-selective properties of the glomerular filter [3, 4]. There is an interconnection between functionality of the GBM and the expression of glycocalyx proteins by podocytes [5].

Reports of the thickness of the glycocalyx have varied between 50 and 300 nm resulting from different staining protocols and functional status of the dynamic glycocalyx [6].

In general, the endothelial glycocalyx is thicker than that of podocytes and contains more glycoproteins that originate from the circulation [7].

Podocytes produce glycocalyx proteoglycans such as glypicans and syndecans [8, 9] and deletion of the proteoglycans in mice results in failure of both foot process and filtration slit formation [10].

Regulation of the glycocalyx occurs in a healthy as well as in a disease state. The mechanisms how impairment of the glycocalyx leads to proteinuria remains only partially understood.

One way to regulate gene expression is through the interaction with micro-RNAs (miRs). MiRs induce gene silencing by binding to the 3'UTR of its targeted mRNAs. Usually one miR has several targets. MiRs have been reported to regulate the proteoglycans in melanoma and smooth muscle cells [11, 12]. Moreover, it has been shown that miRs play a significant role in glomerular diseases [13, 14].

Others and we have described a role for transforming growth factor- β (TGF- β) in podocytopathies and glomerular diseases [15-17]. MiR-143-3p (miR-143) has been found to be upregulated after stimulation with TGF- β [18]. TGF- β is known to regulate versican and syndecans in different cell lines [11, 12]. Moreover, miR-143 targets syndecan-1 to repress cell growth in melanoma [19].

However, miR-143 regulated glomerular glycocalyx proteoglycan expression and its function has not been described before.

Here we demonstrate that miR-143-3p (miR-143) is upregulated in cultured human podocytes after stimulation with TGF- β . Versican and several syndecan isoforms are the major glycocalyx components among miR-143 targets. We describe the direct effects of versican and syndecan knockdown as well as overexpression of miR-143 in a zebrafish model for proteinuria.

We hypothesize that a tight regulation of versican and syndecan is important for proper glomerular function. Moreover, we give first hints for a cross talk between glomerular cells by miRs that regulate glycocalyx proteins.

Materials and Methods

Cell culture

Culture conditions of conditionally immortalized human podocytes were described previously [20]. Podocytes were proliferated under permissive conditions at 33° C. When cultivated at 37° C, the SV40 T-antigen was inactivated for cell differentiation. Culture medium for human podocytes was RPMI 1640 Medium (Roth) with 10% fetal calf serum (FCS; PAA Laboratories) 1% Penicillin/ Streptomycin and 0.1% Insulin. Human glomerular endothelial cells (Clonetech) were cultivated on endothelial cell basal media (EBM™-2; CC-3156, Lonza; Fisher). This medium was added with endothelial cell growth medium that contains 0.1% hEGF, 0.1% hydrocortisone, 0.4% hFGF-b, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% heparin, 2% FBS and 0.1% GA. Culture Conditions were 37° C and 5% CO₂ air atmosphere.

Human podocytes were cultured in FCS-reduced (1%) medium overnight. Then, cells were stimulated with 5 ng/ml TGF- β , 30 μ M D-glucose, 30 μ M L-glucose or 100 μ g/ml advanced glycated end product (AGE).

Cells were harvested at 12h, 24h and 48h after stimulation to run quantitative real-time PCR for miR-143. Unstimulated cells served as control at each time point. Human glomerular endothelial cells (Clonetechn) were cultivated on endothelial cell basal media (EBM™-2; CC-3156, Lonza; Fisher). This medium was added with endothelial cell growth medium that contains 0.1% hEGF, 0.1% hydrocortisone, 0.4% hFGF-b, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% heparin, 2% FBS and 0.1% GA.

MiR target prediction and protein similarity between human and zebrafish

MiRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/search.php>), FINDTAR3 (<http://bio.sz.tsinghua.edu.cn/>) and DIANA Tools (<http://diana.imis.athena-innovation.gr>) were used to predict miR targets. MiR-network analysis was done with the help of miRTarBase. FINDTAR3 (<http://bio.sz.tsinghua.edu.cn/>) and NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to look for binding affinity between miRs and mRNAs. Protein similarity between human (*H. sapiens*) and zebrafish (*D. rerio*) was found with the help of NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, <http://www.ncbi.nlm.nih.gov/homologene>) and emboss_needle alignment algorithm (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Q-PCR

Purification of total RNAs including miRs from cells and cell-free urine was done with miRNeasy Kit (QIAGEN) according to the manufacture's protocol. For miR PCR, we used TaqMan® MicroRNA Assays from life technologies according to the manufactures protocols. Program for reverse transcription was 3 min 16°C, 30 min 42°C and 5 min 85°C. Real-time PCR parameters were the following: 10 min at 95°C and 40 cycles of 15 seconds at 95°C following 1 minute at 60°C.

For mRNA targets, we used sybr green-based real-time PCR with the following protocol: 1 minute at 95°C followed by 35 cycles of 10 seconds at 95°C, 10 seconds at 60°C and 10 seconds at 72°C followed by 5 seconds at 95°C and 1 min at 65°C. Individual samples were run in triplicate. Sequences of miR target primers are given in Table 1.

Zebrafish experiments

Zebrafish strains: wild-type AB (ZIRC, Eugene, OR), transgenic Tg(l-fabp:DBP-EGFP) and transgenic wt1b:EGFP. All strains were grown and mated at 28.5° C and embryos were kept and handled in standard E3 solution as previously described [21]. MO and miR mimics (Table 1) were injected in one- to four-stage or 48 hpf in the cardinal vein of the zebrafish larvae using a Nanoject II injection device (Drummond Scientific, Broomall, PA). Morpholinos were ordered from GeneTools (Philomath, OR). MiR mimics were ordered from life technologies (mirVana® miRNA mimic, negative control and mirVana® miRNA mimic, hsa-miR-143-5p). Injections were carried out in injection buffer (100 mM KCl, 0.1% phenol red). At 48 hpf remaining chorions were manually removed. The animal protocol was approved by the MDI Biological Laboratory IACUC (#11-02).

Table 1. Sequence of morpholinos and miRs

Sequence	Primer/ morpholino
5'ACCTTAATAGTAACCCATGCGC3'	Human <i>VCAN</i> forward primer
5'TGCGGAGAAATTCAGTGGTG3'	Human <i>VCAN</i> reverse primer
5'TCCTGCAGTCAGCCTCCTAT3'	Zebrafish <i>vcn</i> forward primer
5'TGGTGGTGTCTTCGTCTGTG3'	Zebrafish <i>vcn</i> reverse primer
5'GATCAAGATGGCTCTGGGGA3'	Human <i>SDC1</i> forward primer
5'CCACTTCTGGCAGGACTACA3'	Human <i>SDC1</i> reverse primer
5'CTGCTCTCGACTCCGCC3'	Human <i>SDC2</i> forward primer
5'GGCAGGAGGCTTCGTTTTCG3'	Human <i>SDC2</i> reverse primer
5'AAATCTCCCTCCACGACAGA3'	Zebrafish <i>sd2</i> forward primer
5'CAGGAGGATGAGGAAGATGG3'	Zebrafish <i>sd2</i> reverse primer
5'GAGGTGCTCGTAGCTGTGAT3'	Human <i>SDC3</i> forward primer
5'GGCTTCTGGTATGTGACGCT3'	Human <i>SDC3</i> reverse primer
5'GTGGGCGTGGTTTTCACAC3'	Zebrafish <i>sd3</i> forward primer
5'TGCCTTCGTCTTTCTTTTCA3'	Zebrafish <i>sd3</i> reverse primer
5'GTCTGGCTCTGGAGATCTGG3'	Human <i>SDC4</i> forward primer
5'TGGGGGCTTTCTTTGTAGATG3'	Human <i>SDC4</i> reverse primer
5'CTGACGATGAGGATGGCTTT3'	Zebrafish <i>sd4</i> forward primer
5'TTGTAGATGGGCGTCTTTCC3'	Zebrafish <i>sd4</i> reverse primer
5'TGACACTGGCAAACAATGCA3'	Human <i>HPRT</i> forward primer
5'GGTCCTTTTACCAGCAAGCT3'	Human <i>HPRT</i> reverse primer
5'ACAAAACACTATGCGGCTG3'	Zebrafish <i>hpert</i> forward primer
5'GTGTCCACCATGTCCTTCA3'	Zebrafish <i>hpert</i> reverse primer
3'CCTCTTACCTCAGTTACAATTATA 5'	miR-143-3p mimic
3'ACUGGACUUGGAGUCAGAAGG5'	miR-CTRL
5'CTGAAACACCCATGGGAGTGACAT 3'	Zebrafish <i>vcana</i> -MO
5'ACATTGCTGAATATACTCACAGCCA3'	Zebrafish <i>sd3</i> -MO
5'TCTCTTCAATACAAGTAGCCGAGT3'	Zebrafish <i>sd4</i> -MO
5'CCTCTTACCTCAGTTACAATTATA3'	Zebrafish <i>CTRL</i> -MO

Zebrafish assay for proteinuria

Tg(l-fabp:DBP:EGFP) zebrafish which express a green fluorescent vitamin D binding protein fused with the enhanced green fluorescent protein (*DBP-EGFP*) under the control of the liver-type fatty acid binding protein (*l-fabp*) promoter were used to screen for proteinuria in zebrafish larvae. The green fluorescence of the fish can easily be seen in the eye vessels of the fish under light microscopy. The *DBP-eGFP* fusion protein has a molecular weight of approximately 78 kDa. If the glomerular filtration barrier is impaired, the fish loses plasma proteins and the eye fluorescence decreases. Injected morpholinos and miRNA was titrated in dose response experiments. Between 20 and 40 fish were alive in each group 120 hpf. Results shown depict a representative experiment out of three independent injection experiments performed.

Transmission electron microscopy

Zebrafish larvae were fixed in solution D overnight, washed 3x in 0.1 M cacodylate buffer and post fixed in 1% OsO₄ for 1 hours. Tissues were once again washed, dehydrated and embedded in EPON at 120 hpf (recipe/protocol from EMS, Hatfield, PA 19440, USA). Semi-thin (300 nm) and ultra-thin (90 nm) sectioning was cut with a Leica UC-6 Microtome. Sections obtained were mounted onto Formvar coated Ni slot grids (EMS). Grids were stained for 30 min in 5% uranyl acetate followed by 0.1% lead citrate for 15 min.

Statistics

We used the delta-delta cycle threshold (CT) method to normalize our cell culture miR screening data and to generate global fold changes in miR expression. Fold changes were generated with the formula fold change = $2^{-(\Delta\Delta CT)}$ with $\Delta\Delta CT = \Delta CT(\text{sample}) - \Delta CT(\text{reference})$ with $\Delta CT(\text{sample}) = CT \text{ value for sample normalized to housekeeper miR}$ and $\Delta CT(\text{reference}) = \text{mean of the miR CT value of all cell types normalized to mean CT value of housekeeper miR}$. The housekeeper of the cell culture miR screening was U6 snRNA-001973. The maximum allowable CT value in our study was set to 40. Heat map was generated with Multiple Array Viewer and Cluster analysis was done with HCL tree and Person correlation.

For qPCR fold changes were generated with the formula fold change = $2^{-(\Delta\Delta CT)}$ with $\Delta\Delta CT = \Delta CT(\text{sample}) - \Delta CT(\text{reference})$ with $\Delta CT(\text{sample}) = CT \text{ value for sample normalized to HPRT gene}$ and $\Delta CT(\text{reference}) = CT \text{ value for calibrator normalized to HPRT gene}$. All data are shown as means $\pm$ SD and were compared by ANOVA to look for statistical significance.

Results

MiR-143 is upregulated in cultured human podocytes after stimulation with TGF- β

We investigated the regulation of miR-143 in cultured human podocytes and could find a time dependent induction of miR-143 expression after stimulation with TGF- β . In contrast to that, stimulation of podocytes with other known stressors (e.g. of the diabetic milieu) like high glucose or advanced glycated end products (AGE) had no effect on miR-143 expression levels (Fig. 1 A-C). In addition, the TGF- β induced expression was cell type specific for podocytes, as we could not detect upregulation of miR-143 in other glomerular cell types like glomerular endothelial cells and mesangial cells (data not shown).

MiR-143 targets glycocalyx proteins that are expressed by podocytes and glomerular endothelial cells

Next, we compared expression of potential targets of miR-143 relevant for the glomerular filtration barrier between human and zebrafish and focused on glycocalyx components. The glycocalyx component versican is expressed in human (VCAN) and zebrafish (*vcn*) species. SDC1, 2, 3 and 4 are human isoforms of the glycocalyx protein syndecan whereas the zebrafish expresses *sdc2*, 3 and 4. VCAN, *vcn*, SDC1, SDC3, *sdc3*, SDC4 and *sdc4* are potential targets of miR-143, while SDC2 and *sdc2* have no predicted binding site for miR-143 and a zebrafish orthologue for SDC1 is not described. (Fig. 2A). The protein domains of human VCAN/ SDCs and zebrafish *vcn*/ *sdc*s are highly conserved. Human SDC2 (NP_002989.2) and zebrafish *sdc2* (NP_775330.2) share an amino acid sequence identity of 50.4% and a similarity of 63.8%. Identity between SDC3 (NP_055469.3) and *sdc3* (XP_1919756.5) is

Fig. 1. Expression of miR-143 is up-regulated in cultured human podocytes after stimulation with TGF- β . Q-PCR depicts relative expression of miR-143 in cultured human podocytes after stimulation with TGF- β (5 ng/ml) (A), 30 μ M D-glucose and L-glucose (B) or 100 μ g/ml advanced glycated end product (AGE) (C). Relative expression levels of miR-143 at different time points after stimulation are illustrated as fold changes compared to untreated cells at baseline. Significance between stimulated and untreated cells is given at each time point with n.s. not significant, * $p < 0.05$ and ** $p < 0.01$.

Fig. 2. MiR-143 targets human and zebrafish syndecan and versican expression. (A) Illustration of binding of miR-143 seed region to versican (VCAN/ vcan) and syndecan (SDC/ sdc) isoforms in human and in zebrafish. (B) Comparison between human and zebrafish syndecan (SDC/ sdc) and versican (VCAN/ vcan) isoforms. Amino acid identity and similarity of conserved protein domains between human and zebrafish are depicted as illustrated. Syndecan 1 does not exist in zebrafish. Amino acid identity is depicted in %. Ig: immune globulin, CCP: complement control protein, SUSH: short complement-like repeat, CLECT: C-type lectin domain, EGF-CA: Calcium-binding EGF-like domain. Figures were generated with the help of NCBI tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, <http://www.ncbi.nlm.nih.gov/homologene>) and emboss_needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). (C) Q-PCR depicts relative expression of syndecan (SDC) 1, 3 and 4 as well as versican (VCAN) mRNA in cultured human glomerular endothelial cells (GEC) and podocytes (POD). Expression levels are given as fold change with standard deviation compared to human podocytes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant.

35.2% and similarity is 43%. Identity and similarity between SDC4 (NP_002990.2) and sdc4 (NP_001041614.1) are 38.1% and 52% respectively. Amino acid identity between human VCAN (NP_001119808.1) and zebrafish vcan (NP_002662132.3) is 49.8%, whereas protein similarity is 59.2% (Fig. 2B).

Fig. 3. Overexpression of miR-143 causes edema and proteinuria in zebrafish larvae. Edema quantification of zebrafish larvae at 120 hpf. (A) Representative phenotype of the larvae categorized into 4 groups: P1 = no edema, P2 = mild edema, P3 = severe edema, P4 = very severe edema. Zebrafish were injected with a miR-143 mimic (5 μ M) or miR-CTRL (5 μ M) at one to four cell stage (B) or in the cardinal vein (c.v.) at 48 hpf (C) as indicated. Left panels depict phenotype percentages with corresponding representative phenotype images. Right panels indicate results of the eye assay for proteinuria with corresponding representative fluorescent images of the retinal vessel plexus of Tg(l-fabp:DBP:EGFP) of zebrafish larvae expressing a high molecular weight fluorescent protein in the circulation at 96 hpf and 120 hpf. Bar graphs presenting maximum fluorescence intensity of the fish eye as analyzed with image J. *** $p < 0.001$, n.s. not significant; hpf: hours post fertilization. D: Q-PCR for relative *sdc2*, 3, 4 and *vcn* mRNA expression in whole zebrafish tissue at 120 hpf. Zebrafish were injected with a miR-143 mimic (5 μ M) or miR-CTRL (5 μ M) at one to four cell stage as indicated. Expression levels are compared to miR-CTRL and given as fold change. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

When we looked for VCAN and SDC mRNA expression in podocytes and glomerular endothelial cells we found that SDC1 and VCAN mRNA were expressed in both cell types. SDC, however, was mainly expressed in glomerular endothelial cells whereas SDC4 was only detectable in podocytes (Fig. 2C). Thus, podocytes and glomerular endothelial cells contribute to glycocalyx composition differentially.

MiR-143 overexpression in zebrafish larvae causes edema and loss of plasma proteins

Overexpression of miR-143 by injection of a miR-143 mimic (mirVana® miRNA mimic) in zebrafish eggs at the one to four cell stages caused a phenotype with pericardial and yolk sack edema. We quantified the zebrafish phenotype ranging from P1 = normal phenotype to P4 = very severe edema. More than 80% of miR-143 mimic injected zebrafish developed a severe (P3 = 39 %) or very severe edema phenotype (P4 = 42%, Fig. 3A).

To investigate whether the edema phenotype was caused by proteinuria we used our established zebrafish proteinuria model in a transgenic Tg(l-fabp:DBP:EGFP) zebrafish that expresses a 78 kDa green fluorescent plasma protein that can be monitored in the retinal

Fig. 4. Knockdown of syndecan 2 and versican causes edema and proteinuria in zebrafish larvae. (A) Edema quantification of zebrafish larvae at 120 hpf. Zebrafish were injected with a specific morpholinos for syndecan 3 (sdc3-MO, 100 μ M), syndecan 4 (sdc4-MO, 100 μ M), versican (vcan-MO, 100 μ M), a scrambled control (CTRL-MO, 100 μ M) or a combination of syndecan 3 (sdc3-MO, 100 μ M) and syndecan 4 (sdc4-MO, 100 μ M) at the one to four cell stage as indicated. The edema phenotypes of the larvae were categorized into 4 groups: P1 = no edema, P2 = mild edema, P3 = severe edema, P4 = very severe edema. (B) Corresponding fluorescent images of the retinal vessel plexus of Tg(l-fabp:DBP:EGFP) of zebrafish larvae expressing a high molecular weight fluorescent protein in the circulation. Scatter graph presents maximum of circulating fluorescence intensity in the fish eye at 120 hpf, analyzed with image J. *** $p < 0.001$, n.s. not significant; hpf: hours post fertilization.

plexus of the fish [22, 23]. In miR-143 injected zebrafish, plasma protein fluorescence was significantly lower compared to control indicating loss of this protein from the circulation (Fig. 3A).

To rule out that the observed phenotype and proteinuria after miR-143 overexpression were due to miR induced developmental defects of the kidney we injected the miR-143 mimic in the cardinal vein of the zebrafish at 48 hours post fertilization (hpf). At this time zebrafish pronephros is already filtering. After cardinal vein (c.v.) injection of miR-143 no proteinuria or defect in zebrafish morphology was observed at 96 hpf. However, at 120 hpf miR-143 c.v. injected zebrafish also developed pericardial effusions, yolk sac edema and significant loss of plasma proteins (Fig. 3B).

To investigate to which extent the mRNA expression of vcan and sdc isoforms are affected by miR-143 overexpression in zebrafish we performed Q-PCRs from whole zebrafish at 120 hpf. We discovered that miR-143 injection significantly decreased expression of sdc3, sdc4 and vcan mRNA, but had no significant effect on sdc2 expression (Fig. 3C). These results are in line with the computer algorithm based target scan of miR-143 (Fig. 2A).

Vcan is indispensable for the integrity of the GFB in zebrafish larvae

To explore the differential role of vcan and different sdc isoforms in the glomerulus we performed knockdown experiments by injection of specific splice donor morpholinos (MO) in zebrafish embryos. Knockdown of vcan caused an edema phenotype in zebrafish at 120 hpf. In contrast, sdc3 and sdc4 knockdown had no effect on zebrafish phenotype (Fig. 4A). As described above, we quantified proteinuria with our transgenic Tg(l-fabp:DBP:EGFP) zebrafish. Vcan-MO injection resulted in significant loss of plasma proteins in zebrafish, whereas plasma proteins were retained in the blood plasma after sdc3 and sdc4 knockdown indicating an intact filtration barrier. In addition, a combined knockdown of sdc3 and sdc4 by injecting sdc3-MO and sdc4-MO together had no effect on phenotype or plasma protein loss (Fig. 4B). Efficiency of target knockdown after morpholino injections in zebrafish eggs was confirmed by Q-PCR (Fig. 5A).

Fig. 5. Quantification of target knockdown after versican and syndecan morpholino injection and fusion of zebrafish glomeruli at 48 hpf after versican and syndecan knockdown. (A) Q-PCR for relative sdc 3, 4 and vcan mRNA expression in whole zebrafish tissue at 120 hpf. Zebrafish were injected with syndecan 3 morpholino (sdc3-MO, 100 µM), syndecan 4 morpholino (sdc4-MO, 100 µM), versican morpholino (vcan-MO, 100 µM) or control morpholino (CTRL-MO, 100 µM) at one to four cell stage as indicated. Expression levels are compared to miR-CTRL and given as fold change. ** $p < 0.01$, *** $p < 0.001$. (B) Pictures illustrate glomerular fusion of transgenic zebrafish that expresses eGFP under the control of the wt1b promotor (wt1b:EGFP zebrafish) at 48 hpf. Zebrafish were injected with miR-143 mimic (miR-143, 5 µM) or miR control (miR-CTRL, 5 µM), syndecan 3 morpholino (sdc3-MO, 100 µM), syndecan 4 morpholino (sdc4-MO, 100 µM), versican morpholino (vcan-MO, 100 µM) or control morpholino (CTRL-MO, 100 µM) as indicated. Right pictures depicts glomerular fusion in fluorescent channel. Scale bar: 200 µm. Left pictures illustrates a merged picture of bright field and fluorescent channel. Scale bar: 200 µm.

We performed experiments to rule out that the observed phenotypes after sdc and vcan knockdown as well as miR-143 overexpression were due to delay in glomerular development:

During zebrafish pronephros development, the glomerular structures fuse at the midline around 48 hpf [24, 25]. We confirmed timely fusion of the glomeruli after miR-143 overexpression as well as after sdc and vcan knockdown with the use a transgenic zebrafish (wt1b:eGFP zebrafish) that expresses eGFP under the control of the wt1b promotor [26] (Fig. 5B).

Podocyte effacement and glomerular endothelial cell damage is the ultrastructural correlate for proteinuria after miR-143 overexpression

To investigate the structural root cause of proteinuria we analyzed the zebrafish glomeruli at 120 hpf by transmission electron microscopy (Fig. 6A). We classified podocyte ultrastructure as normal, partial and complete effacement and quantified corresponding areas in the glomeruli (Fig. 6B). To investigate glomerular endothelial cell morphology we quantified endothelial cell fenestrations along the GBM (Fig. 6C). Per condition, a total stretch of at least 50 µm of GBM was analyzed. MiR-143 overexpression by miR-143 mimic injection in zebrafish eggs caused podocyte effacement, glomerular endothelial cell swelling and loss of endothelial fenestration. Glomerular damage was less severe when miR-143 was overexpressed at 48 hpf by c.v. injection of the miR-143 mimic.

When miR-143 targets were knocked down separately by specific MO injection ultrastructural changes were noted as follows: Vcan-MO injection caused podocyte

Fig. 6. Glomerular damage after miR-143 overexpression and knockdown of syndecan and versican in zebrafish larvae. (A) Transmission electron microscopy pictures of the pronephros of zebrafish larvae at 120 hpf after injection with a syndecan 3 morpholino (sdc3-MO, 100 μ M), syndecan 4 morpholino (sdc4-MO, 100 μ M), versican morpholino (vcan-MO, 100 μ M) or control morpholino (CTRL-MO, 100 μ M), as well miR-143 mimic (miR-143, 5 μ M) or miR control (miR-CTRL, 5 μ M). White arrows illustrate podocyte effacement. Black arrows illustrates loss of endothelial cell fenestration. (Aa) Normal, partial and complete effacement of podocytes was quantified in the different groups. (Ab) Quantification endothelial cell fenestration per μ m GBM. *** $p < 0.001$, n.s. not significant.

effacement as well as glomerular endothelial cell swelling with loss of fenestration. The glomeruli of sdc3-MO and sdc4-MO injected zebrafish, however, were comparable to CTRL-MO injected fish.

Discussion

The anatomical structures of the GFB in the kidney consist of the fenestrated endothelium, the GBM and the interdigitating foot processes of podocytes. In addition, the endothelial cell surface layer and the podocytes are covered by a highly sialylated and negatively charged glycocalyx.

Since its discovery the functional role of the glomerular glycocalyx for the integrity of the GFB has become increasingly evident [27]. Impairments in the glycocalyx can cause proteinuria even when the other cellular components of the glomerular filter are intact. Cases of nephrotic syndrome without ultrastructural changes despite presence of nephrotic syndrome have been described in the literature [28].

The direct involvement of podocyte proteoglycans for the proper function of the GFB was further documented by the development of nephrotic syndrome in the absence of these proteins [8, 29-31].

What remains unclear, however, is how the structure and components of the glycocalyx are regulated under physiological and pathophysiological conditions. In this report we investigated the role of specific glomerular glycocalyx proteins which are regulated through a TGF- β inducible miR and give evidence for a glomerular cell cross talk that might influence

glycocalyx protein expression in disease and foster proteinuria development. Our hypothesis is supported by the following evidence:

First, we identified miR-143 as predominately expressed in podocytes after TGF- β stimulation compared to other renal cell types in culture indicating a cell specific stress response. This is well in line with other reports where miRs were upregulated under mechanical stress in podocytes or nephrotic diseases [14, 32, 33].

Secondly, miR-143 targeted versican and syndecan isoform expression in human podocytes and we demonstrated that miR-143 overexpression leads to a significant reduction of vcan and sdc isoforms in zebrafish. Although these downstream targets of miR-143 have been described in other cell lines [11, 12], regulation of these proteoglycans by miR-143 in the glomerulus has not been reported previously.

Moreover, we showed that miR-143 overexpression caused a nephrotic phenotype with generalized edema, loss of plasma proteins, podocyte effacement, glomerular endothelial cell swelling and loss of glomerular endothelial fenestration. When we characterized VCAN and SDC isoform expression in glomerular endothelial cells and podocytes, we can demonstrate differential expression for these proteoglycans in the podocyte and endothelial glycocalyx layers.

In zebrafish, vcan knockdown affected the endothelial and epithelial side of the glomerulus. These results are in line with our data from cultured cells where VCAN mRNA was expressed in human glomerular endothelial cells and podocytes. Previous findings by other groups also detected VCAN expression in both cell types [8, 9].

Besides being a known glomerular glycocalyx component, VCAN also seems to have a structural role for podocytes and endothelial cells since its ablation caused podocyte effacement and glomerular endothelial damage [7-9].

Vcan appears to have the highest *in vivo* relevance with respect to glomerular changes of the zebrafish proteoglycans targeted by miR-143 we observed. Glomerular damage induced by miR-143 overexpression was comparable to that seen after vcan knockdown alone.

Podocytes and glomerular endothelial cells produce different SDC isoforms. However, the knockdown of zebrafish sdc3 and sdc4 alone or in combination has no effect on GFB function or podocyte phenotype. Similarly, Liu et al. stated that podocyte foot processes, slit diaphragms, GBM and glomerular endothelium were structurally normal in *Sdc4*^{-/-} mice [34]. Even though endothelial cell fenestration per μ m GBM was significantly reduced in sdc3-MO compared to CTRL-MO injected zebrafish larvae sdc3-MO injected fish maintained a functional filtration barrier. Even when sdc3 and sdc4 were knocked down together the glomerular filtration barrier was intact. The reason for this might be that other proteoglycans compensate for the loss of sdc3 and sdc4.

Proteoglycans like syndecans or perlecan carry heparan sulphate side chains. Similar results were seen with perlecan – another important proteoglycan of the glycocalyx. Here the charge component of perlecan could be compensated by other proteoglycan and preserve barrier function [35].

Versican probably seems to have a more specific role than just adding charge and cannot be relapsed by other proteoglycans.

Recently, miR-143 and miR-145 have been described in a paracrine communication between other cells. *Hergenreider et al.* showed that shear stress is able to induce miR-143 in endothelial cells that controls target gene expression in nearby smooth muscle cells [36].

We hypothesize that miR-143 functions as a mediator for glomerular crosstalk between podocytes and glomerular endothelial cells by affecting the glomerular glycocalyx. Podocyte derived miR-143 might act in a paracrine as well as autocrine manner by targeting proteoglycans in glomerular endothelial cells as well as podocytes.

In summary, these data indicate that glomerular glycocalyx proteins are regulated by miR143 and that miR-143 may be a novel mediator of TGF- β induced glomerulonephropathy. An increased podocyte miR-143 expression results in down regulation of vcan and sdc isoforms not only in podocytes but also in glomerular endothelial cells causing functional and structural impairments of the GFB.

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Disclosure Statement

The authors declare no competing or financial interests.

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