Podocytes regulate the glomerular basement membrane protein nephronectin by means of miR-378a-3p in glomerular diseases

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The pathophysiology of many proteinuric kidney diseases is poorly understood, and microRNAs (miRs) regulation of these diseases has been largely unexplored. Here, we tested whether miR-378a-3p is a novel regulator of glomerular diseases. MiR-378a-3p has two predicted targets relevant to glomerular function, the glomerular basement membrane matrix component, nephronectin (NPNT), and vascular endothelial growth factor VEGF-A. In zebrafish (Danio rerio), miR-378a-3p mimic injection or npnt knockdown by a morpholino oligomer caused an identical phenotype consisting of edema, proteinuria, podocyte effacement, and widening of the glomerular basement membrane in the lamina rara interna. Zebrafish vegf-A protein could not rescue this phenotype. However, mouse Npnt constructs containing a mutated 3′UTR region prevented the phenotype caused by miR-378a-3p mimic injection. Overexpression of miR-378a-3p in mice confirmed glomerular dysfunction in a mammalian model. Biopsies from patients with focal segmental glomerulosclerosis and membranous nephropathy had increased miR-378a-3p expression and reduced glomerular levels of NPNT. Thus, miR-378a-3p–mediated suppression of the glomerular matrix protein NPNT is a novel mechanism for proteinuria development in active glomerular diseases.

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The glomerular filtration barrier is composed of 3 structural layers: the fenestrated endothelium, the glomerular basement membrane (GBM), and podocytes. Impairment of any single layer can have secondary effects on the other layers, ultimately leading to loss of function and proteinuria. In many cases, the initial cause of the disease and the functional interplay of GBM components during the disease process remain elusive.

MicroRNAs (miRs) regulate genes and are known to influence development, cell division, differentiation, and apoptosis.[1] Therefore, they may be promising novel candidates in the study of glomerular diseases.[2] Noncoding molecules 21 to 23 nucleotides in length, miRs bind the 3′-untranslated region (3′ UTR) of a target mRNA and inhibit translation.[3] Those enriched in human kidneys include miR-192, miR-194, miR-204, miR-215, and miR-216.[4] Furthermore, miRs are essential for podocyte homeostasis,[5–8] and it was recently shown that focal segmental glomerulosclerosis (FSGS) induces the expression of miR-193a in podocytes.[9] Moreover, miRs can be secreted in body fluids and therefore possibly could be biomarkers for various glomerular diseases,[10,11] potentially serving as early noninvasive diagnosis tools.

Recently, Kahai *et al.*[12] demonstrated that miR-378a-5p targets NPNT in osteoblasts. Nephronectin (npnt) is an extracellular matrix protein localized in the GBM.[13] However, the function and turnover of npnt in the GBM are largely unexplored. After a common stress model for cultured human podocytes, transforming growth factor-β (TGF-β) stimulation, we found miR-378a-3p upregulation. As -5p and -3p miRs of the same family often have the same targets,[14–16] we speculated that miR-378a-3p would suppress NPNT.

In this study, we show that miR-378a-3p suppresses npnt in zebrafish and murine models as well as in human glomerular diseases, and knockdown of npnt leads to podocyte dysfunction and GBM disorganization. These findings emphasize the importance of podocyte/GBM interplay in glomerular disease.

RESULTS

miR-378a-3p is upregulated in stressed podocytes and targets NPNT

Transforming growth factor-β (TGF-β) is known to be important in the development of progressive podocyte diseases.[17,18] MiR-378a-3p was significantly upregulated in cultured human podocytes after TGF-β stimulation. This increase was time dependent ($P < 0.01$) (Figure 1a). On the other hand, miR-378a-5p expression was not altered by TGF-β (data not shown). Nevertheless, TGF-β stimulation decreased both NPNT protein and mRNA expression in cultured human podocytes in a time-dependent manner. This decrease was directly inverse to the miR-378a-3p increase (Figure 1b and c). Furthermore, NPNT mRNA expression was significantly higher in cultured human podocytes compared with other glomerular cell types ($P < 0.01$) (Figure 1d).

To further analyze the potential interaction between miR-378a-3p and NPNT, podocytes were transfected with pre-miR-378a-3p oligonucleotide (miR-378a-3p mimic). This led to suppression of NPNT protein (Figure 1e and Supplementary Figure S1) compared with control. In contrast, transfection of cultured human podocytes with a chemically modified, single-stranded oligonucleotide that binds and inhibits miR-378a-3p (miR-378a-3p inhibitor) increased NPNT mRNA. However, when used in the presence of TGF-β, the miR-378a-3p inhibitor had no effect on NPNT mRNA (Figure 1f), suggesting that TGF-β also suppresses NPNT by a mechanism independent of miR-378a-3p.

Nevertheless, online target prediction tools (mirtarbase, FindTar3) identified NPNT as a potential target of miR-378a-3p. The binding site for miR-378a-3p on the NPNT mRNA is preserved in zebrafish, mice, and humans (Supplementary

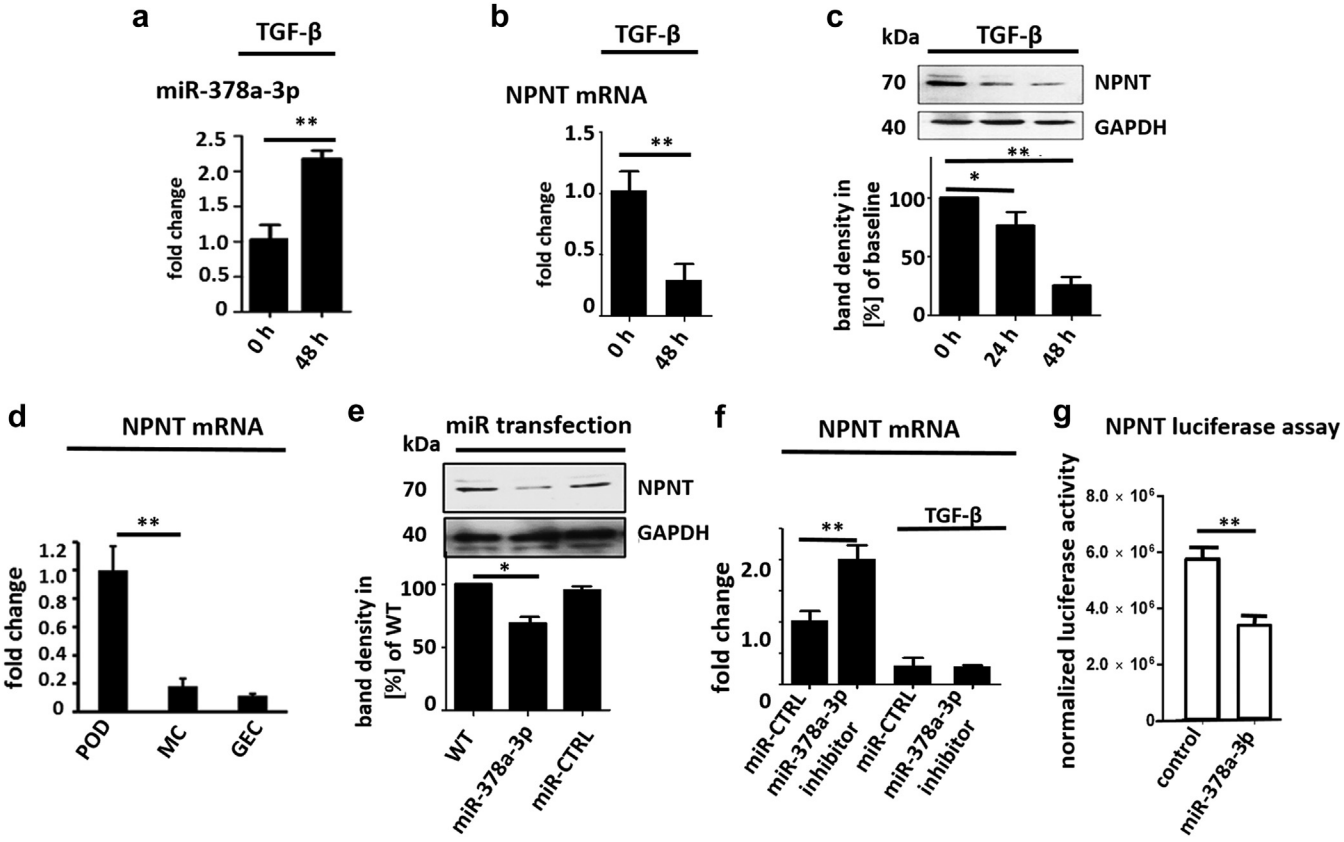

Figure 1 | MicroRNA (miR)-378a-3p regulates nephronectin (NPNT) expression in cultured human podocytes. (**a**) Real-time polymerase chain reaction (PCR) reveals induction of miR-378a-3p expression in cultured human podocytes after transforming growth factor-β (TGF-β) stimulation (5 ng/ml) at 48 hours compared with baseline. $^{**}P < 0.01$. (**b**) Real-time PCR reveals repression of NPNT mRNA in cultured human podocytes after TGF-β stimulation (5 ng/ml) at 48 hours compared with baseline. $^{**}P < 0.01$. (**c**) Western blot for NPNT in cultured human podocytes after TGF-β stimulation (5 ng/ml) compared with baseline for the time points indicated. Quantification of NPNT protein expression is given in the histogram. $^{*}P < 0.05$, $^{**}P < 0.01$. (**d**) Real-time PCR for relative NPNT mRNA expression in cultured human podocytes (POD), human mesangial cells (MC), and human glomerular endothelial cells (GEC). $^{**}P < 0.01$. (**e**) Western blot for NPNT in cultured human podocytes at baseline and 72 hours after a 4-hour transfection with an miR-378a-3p mimic (5 nM) indicates complete suppression of NPNT protein compared with control after 72 hours. Quantification of NPNT protein expression is given in the histogram. (**f**) Real-time PCR reveals upregulation of NPNT in cultured human podocytes after transfection with an miR-378a-3p inhibitor. Stimulation with TGF-β in the presence of an miR-378a-3p inhibitor decreased NPNT expression comparable to stimulation with TGF-β alone. $^{**}P < 0.01$. (**g**) Luciferase reporter assay to validate miR-378a-3p binding to NPNT was performed in human embryonic kidney (HEK) cells 293. Cells were lysed 24 hours after transfection and subsequently used for luciferase activity. Luciferase reads were normalized with β-galactosidase values. $^{**}P < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.