

The Zebrafish Model to Study the Role of microRNAs in Glomerular Function and Disease

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Abstract

microRNAs (miRs) are non-coding small RNAs that play an important role in posttranscriptional regulation of gene expression. Recent studies indicate that miRs are also mediators in different disease processes. Here we review how the zebrafish model can be used to study the role of miRs in glomerular function and disease.

Microinjections of miR mimics were done in zebrafish eggs and larvae. A transgenic zebrafish line which expresses a green fluorescent plasma protein was used to investigate protein loss through the glomerular filtration barrier. Electron microscopy analysis revealed the level and degree of glomerular damage after miR overexpression. MiR-143-3p seems to be important for glomerular glycocalyx as overexpression of miR-143-3p leads to down regulation of versican, proteinuria and damage on the endothelial and epithelial side of the glomerular filtration barrier.

In contrast, overexpression of miR-378a-3p by injection of a specific miR mimic caused proteinuria, edema, podocyte effacement and thickening of the glomerular basement membrane. These findings could be rescued by co-injections of a nephronectin construct with a mutated 3'UTR region where the miR could not bind. Thus, miR mimics can be used in the zebrafish model to study the role of miRs involved in glomerular diseases.

Keywords: microRNAs; Zebrafish; Glomerulus; Glomerular diseases; Nephronectin; Glycocalyx

The zebrafish physiology of many organs including the kidney is very similar to that of human beings. Zebrafish develop from a fertilized egg to free-swimming larvae in only 48 h. The zebrafish larvae's correlate for the human kidney is the pronephros consisting of two nephrons with glomeruli fused at the embryo mid-line ventral to the dorsal aorta [1] (Figure 1a).

The glomerular filtration barrier of the pronephros is ultra-structurally almost indistinguishable from a mammalian glomerulus with interdigitating podocyte foot processes, a thin glomerular basement membrane and a fenestrated glomerular endothelium [2]. These structures can be found in 120 h old zebrafish larvae on ultra-structural level (Figures 1b and 1c). To generate these pictures zebrafish larvae were fixed in solution D and embedded in EPON. Ultrathin sectioning from head to tail was performed with a microtome until the glomerular region was found and sections transferred onto copper slit grids that were stained with uranyl acetate and lead citrate. Imaging was done with a transmission electron microscope.

The pronephros tubular epithelium is composed of two proximal tubule segments, a proximal straight tubule as well as the early and late distal tubus [3]. The tubular system expresses a brush border of microvilli on the apical side for reabsorption processes (Figure 1d). The glomerular filtration of the zebrafish pronephros begins around 48 h post fertilization (hpf) [4]. Taking into consideration the structural and functional similarities and the fact that zebrafish gene expression can easily be influenced by specific knockdown and overexpression techniques using microinjections of morpholinos (MOs), mRNAs, microRNAs (miRs), small RNAs or CRISPR/Cas9 technology, the zebrafish serves as an ideal model to study glomerular diseases [5]. We and others successfully used the zebrafish model to screen for novel genes involved in kidney diseases [6-8]. On the other hand, gene mutations identified from human genetic studies can be verified as causative factors of proteinuric kidney disease in the zebrafish model.

An upcoming field of research interest is the role of miRs in disease processes. MiRs regulate gene expression posttranscriptional by binding

to the 3'-untranslated region (3' UTR) of a target mRNA and inhibiting translation [9,10]. Different miRs have been found to be enriched in human kidneys include miR-192, miR-194, miR-204, miR-215 and miR-216 [11]. MiRs are also essential for podocyte homeostasis [12-15] and can be secreted in body fluids [16,17].

To antagonize the function of miRs, several antagomirs have been engineered and are commercially available. On the contrary, miR effects can be enhanced with miR mimics, which are chemically modified short double-stranded RNA sequences.

Delivery of miRs in the zebrafish larvae system can be accomplished by microinjection of miR mimics in one-to-four cell stage or at 48 hpf by cardinal vein (c.v.) injection. After injections in egg stage the miR mimic is equally distributed in all zebrafish cells during later cell division. The c.v. injection at 48 h post fertilization (hpf) allows a miR overexpression at a time when glomerular function and vascular system of the zebrafish larvae are already developed.

We established a functional screening for loss of plasma proteins in zebrafish larvae as a hint for proteinuria assay based on the clearance of a ~78 kDa vitamin D-binding protein fused to enhanced green fluorescent protein (DBP-eGFP) fluorescent dextran expressed in a transgenic zebrafish line (Tg[l-fabp:DBP:EGFP]). The expressed fluorescent protein can be monitored over the retinal vessel plexus of the zebrafish. Loss of circulating fluorescent plasma proteins is a hint for leakiness of the glomerular filtration barrier. Electron microscopy analysis of zebrafish

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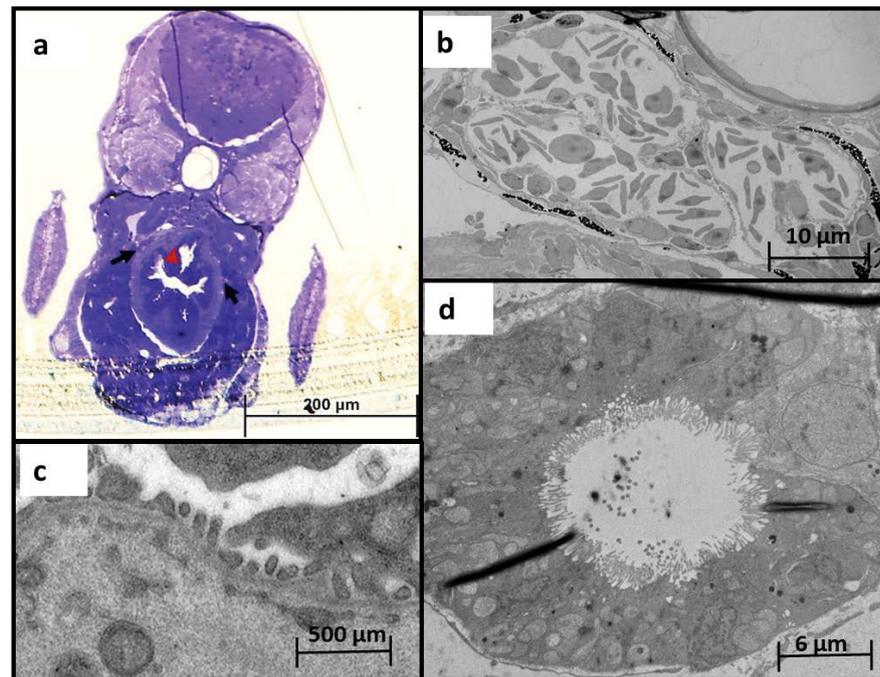


Figure 1: Zebrafish pronephros at 120 hpf. Semi thin section of the zebrafish at the pronephros region (a). Transmission electron microscopy picture of the glomerulus (b), the glomerular filtration barrier (c) and the tubular system with brush border (d). hpf: Hours Post Fertilization.

pronephros allows the investigation of the ultra-structural correlate of protein loss.

We recently investigated the role of two different miRs for glomerular function and disease in the zebrafish model: miR-143-3p was found to be up regulated in cultured human podocytes after stimulation with TGF-beta as a cell-stress model. Different glomerular glycocalyx proteins like versicans (vcan) and syndecans (sdc) are predicted targets of miR-143-3p (target scan, miRtarBase). We demonstrated that miR-143-3p overexpression by injection of a specific miR-143-3p mimic in zebrafish egg stage leads to a significant reduction of vcan and sdc RNA isoforms in 120 days old zebrafish larvae. Moreover, miR-143-3p overexpression caused a nephrotic phenotype with generalized edema, loss of plasma proteins, podocyte effacement, glomerular endothelial cell swelling and loss of glomerular endothelial fenestration.

Comparing the functional and ultra-structural glomerular changes of miR-143-3p overexpression to MO based knockdown of miR-143-3p target genes, vcan appeared to have the highest *in vivo* relevance because glomerular damage induced by miR-143-3p overexpression was comparable to that seen after vcan knockdown alone.

We hypothesize that podocyte-derived miR-143-3p might function as a mediator for glomerular crosstalk between podocytes and glomerular endothelial cells and act in a paracrine as well as autocrine manner [18].

Another miR that was cell-type specifically up regulated in cultured human podocytes after stimulation with TGF-beta was miR-378a-3p. miR-378a-3p was described to target nephronectin (NPNT) in osteoblasts [19]. Mass spectrometry-based proteomics of extracellular glomerular matrix extracts in humans identified that NPNT expression is specifically localized in the GBM [20]. NPNT also is a ligand of integrin $\alpha 8$ - $\beta 1$ during kidney development [21-23]. miR-378a-3p was up regulated in cultured human podocytes after stimulation with

TGF-beta as well as in urine samples and glomeruli from patients with idiopathic membranous glomerulonephritis and focal segmental glomerulosclerosis compared to control and other glomerular diseases. We verified that miR-378a-3p suppresses NPNT/npnt expression after transfection of cultured human podocytes with a miR-378a-3p mimic as well as after miR-378a-3p mimic injection in zebrafish egg stage.

Npnt knockdown in zebrafish larvae by either injection of npnt-MO or miR-378a mimic resulted in generalized edema and detection of injected 70-kDa dextran in the water.

Podocyte effacement and thickening of the glomerular basement membrane were the ultra-structural correlates for edema and proteinuria in both npnt-MO and miR-378a-3p mimic-injected zebrafish. Vegf-Aa is another glomerular target of miR-378a-3p. However, the injection of a recombinant zebrafish vegf-Aa protein was unable to rescue the phenotype caused by miR-378a-3p mimic injection.

Further experiments with two different mouse Npnt constructs confirmed the effect of miR-378a-3p on npnt. The cRNA of a murine Npnt+mu3' construct containing the functional domains of Npnt together with a mutated 3' UTR region was able to prevent proteinuria and edema due to miR-378a-3p mimic injection. In contrast, zebrafish co-injected with miR-378a-3p mimic and a cRNA of a murine Npnt+3' construct containing the functional domains of Npnt together with the 3' UTR region developed edema and loss of plasma proteins.

The pathologic role of miR-378a-3p was further confirmed in a mouse model with serial intraperitoneal injections or a miR-378a-3p mimic leading to similar phenotypes as in zebrafish larvae. The clinical relevance of miR-378a-3p was shown by the detection of upregulated miR-378a-3p in urine samples as well as glomeruli of kidney biopsies of patients with idiopathic membranous glomerulonephritis and focal segmental glomerulosclerosis [24].

Due to the nature of miRs that often have multiple targets; we cannot rule out completely that the observed phenotypes in the zebrafish after miR-143-3p and miR-378a-3p over expression were due to knockdown of other targets than vcan and sdc5 a.e. npnt.

However, the similar phenotype after specific MO injections together with the failed rescue experiment with proteins of other glomerular targets of the miR as well as rescue experiments with constructs with mutations in the miR binding region highly support the important role of our investigated targets.

Thus, the zebrafish serves as a model to study the glomerular filtration barrier. miR-378a-3p-mediated suppression of NPNT and miR-143-3p mediated down regulation of glycocalyx proteins might play a role in glomerular disease. It is suggestive that many other miRs might play a role for glomerular function and the zebrafish model is a useful tool to investigate their role easily.

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