Investigation of the role of novel gene HPS3 and microRNA 636 in proteinuria on zebrafish (*Danio rerio*)

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As part of the study of proteinuria as a key symptom of many chronic kidney diseases, the relevance of novel gene HPS3 and microRNA 636 for the glomerular filtration barrier (GFB) is investigated by injecting morpholino oligonucleotides and microRNA mimics in zebrafish larvae to cause a knockdown of HPS3 (Hermansky-Pudlak-Syndrome Gene 3) and the target genes of microRNA 636. To understand the relevance of the genes, we examined the phenotype, the fusion of glomeruli and the development of proteinuria in injected zebrafish. Thus far our results indicate that knockdown of HPS3 and overexpression of microRNA 636 effect the GFB through causing proteinuria and fish with edema.

Based on an up-regulation of the microRNA 636 in the urine of patients suffering from Alport's syndrome[1], a rare but serious kidney disease that includes proteinuria as part of a complex of severe symptoms, one could identify HPS3 as a target of miRNA[2] 636 through next generation sequencing. HPS3 is one of nine identified human HPS-causing genes[3]. We hypothesized that novel gene HPS3 and the microRNA 636 play a role in proteinuria.

To evaluate the hypothesis we injected three morpholinos[4,5] in transgenic GFB-labeled zebrafish larvae: a morpholino to knockdown HPS3, a microRNA 636 mimic and a control morpholino without binding potential to make sure not the injection process itself is harmful. In addition we used uninjected wild type to consider the egg quality. To assess the damage the absence of the genes have for the glomerular filtration barrier we did a phenotype analysis5, sorting the fish in four main categories due to edema in heart and yolk sack, after 72 hours post fertilization (hpf). The eye-fluorescence assay[5,6] after 96 hpf and 120 hpf is used to investigate the fluorescence level in the retinal vessels of the zebrafish to later be able to evaluate the condition of the filtration barrier. The used transgenic zebrafish Tg(l-fabp:DBP:EGFP) express a vitamin D binding protein fused with the enhanced green fluorescent protein (DBP-EGFP). If the filtration barrier is damaged, the fluorescence level in the retinal vessels due to the loss of DBP-GFP will be reduced comparable to the loss of human albumin in proteinuria. In addition to the phenotype- and eye-fluorescence assay we look at the blood flow after 48 hpf as a control experiment to distinguish between a potential cardiac phenotype and a phenotype based on an impaired filtration barrier. Moreover the wt1b-assay[5] can be used to control whether the glomeruli are fused and the development of the kidney was influenced by the injected morpholino. This assay is performed 48 hpf with injected transgenic wt1b-fab-fish.

The analysis of the eye-fluorescence assay reveals for both, HPS3 and microRNA 636, a significant difference of maximum fluorescence in the retinal vessels between the control fish and fish injected with the morpholino (Fig 1 and 2). The data show that the injection occurs in a loss of fluorescence due to a lower concentration of GFB in the blood. Those results confirm our considerations that the knockdown of HPS3 and overexpression of microRNA 636 can cause damage to the filtration barrier and therefore create proteinuria. With the phenotype-analysis we could show that for the microRNA 636 the extent of edema in heart and yolk sack is dose depending: the higher the injected concentration, the stronger the phenotype. Especially in the range between 5 μM and 15 μM a change of concentration has a huge impact on the phenotype. While for 5uM injected microRNA 636 mimic mostly healthy fish without edema could be observed, for a injection with 15 μM the number of fish with a very strong edema is dominating. As well for HPS3 a dose dependence could be determined. Moreover the phenotypeanalysis revealed that the knockdown of HPS3 can cause very strong developmental problems for 25 μM and 50 μM (Fig 3). Since the ratio of fish with a very distinct phenotype and fish with no phenotype differs strongly in the different experiments, further research is necessary to be able to publish reproducible data

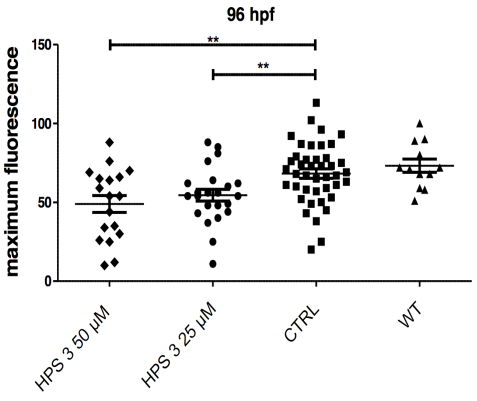

Figure 1. Analysis of the eye-fluorescence assay for HPS3. The data show a significant difference in the fluorescence level for both injected HPS3-morpholinos in comparison to the control.

for HPS3. What we could show with not only the phenotype- and eye-fluorescence assay but also our control assays is that a very high concentration has a toxic effect on the fish and causes developmental delays (seen in the wt1b-assay), the stagnation of the blood flow and a severe phenotype.

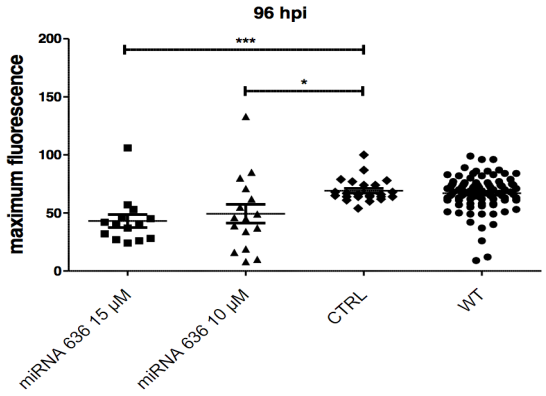

Figure 2. Analysis of the eye-fluorescence assay 96 hours post injection for microRNA 636. Fluorescence levels for the 15 μM injection and a significant difference for the 10 μM injection were significantly different than controls.

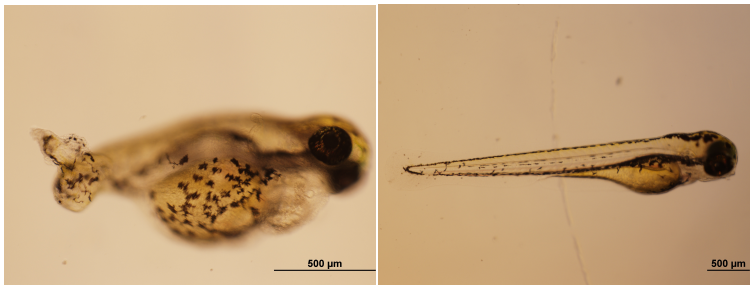

Figure 3. Phenotype-pictures for HPS3 25 μM. The left picture shows a strong phenotype for a fish that did not have blood flow after 48 hpf. The right picture shows a fish injected with the same concentration but that in comparison had blood flow after 48 hpf and does not show any edema in heart or yolk

Our next step will be to use electron microscopy to better understand the impact of the knockdown of HPS3 and overexpression of microRNA 636 on the glomerular filtration barrier. Electron microscopy enables us to understand which particular part of the glomerular filtration barrier is damaged by the absence of the genes.

This research was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103423 and 1P20GM104318.

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