A rapid gene screening system to identify novel mechanisms for development of chronic kidney disease in zebrafish (*Danio rerio*)

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Data for genes relevant to chronic kidney disease is continually increasing in an era of microarrays, genome-wide association studies and quantitative trait locus analysis. Using mammalian models is costly, time intensive and therefore includes only a small number of test subjects. However, in our *in vivo* zebrafish model we are able to screen entirely novel genes in 4-6 weeks in hundreds of live test subjects at a fraction of the cost of a mammalian model. This system produces consistent and reliable evidence for gene relevance in chronic kidney disease; the results then provide merit for further analysis in mammalian models.

Chronic kidney disease (CKD) is a national and world health care priority. CKD is rarely detected early enough in patients, typically leads to kidney failure and frequently requires therapy through dialysis or transplantation. These therapies are extremely costly and weigh heavily on healthcare budgets. It is predicted that the global cumulative cost to treat CKD over the next decade will exceed $1 trillion. Despite the urgency and relevance, our understanding of the mechanisms of chronic kidney disease is limited and the specific molecular pathways still have to be elucidated in order to provide effective therapy. Although the list of genes suspected to play a role in the development of chronic kidney disease continues to grow, researchers have been limited to published literature searches to select the most relevant genes to investigate. The problem has been the lack of an *in vivo* model to quickly prioritize target molecules. Here we describe a rapid gene screening system based on zebrafish (*Danio rerio*) as an animal model.

Zebrafish are an ideal model to test gene deletion or overexpression strategies in kidney development since there is a fundamental conservation in the molecular anatomy between zebrafish and mammalian kidney[7]. This makes it an excellent animal model for translational research. Larval zebrafish contain a pronephric glomerulus that is comparable in structure to a mammalian nephron. Zebrafish develop from fertilized egg to free-swimming larvae with a functioning pronephros in only 48 h[1,2]. Single gene knockdown can be performed using morpholino antisense strategies[3,5,6] and its effect can be monitored in zebrafish within 2-5 days post fertilization. Generalized edema due to loss of protein in the urine is a typical hallmark of acute or chronic kidney disease. After gene knockdown by morpholino injection the first step in our screening method is monitoring the larvae up to 120 h post fertilization for the development of edema and rating on four different scales of swelling (Fig 1).

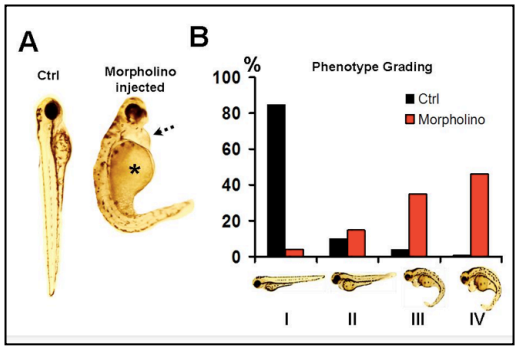

Figure 1. Edema as a sign of kidney failure in zebrafish. (A) A morpholino injected fish compared to control morpholino injection. Pericardial effusion (black arrow) and edema of the yolk sac (*) is visible. (B) Edema is graded in four stages. Stage I: no signs of edema; Stage II: mild edema; Stage III: intermediate stage of edema; Stage IV: severe edema.

For differentiation between cardial or renal origin of the observed edema we next perform the "FABP eye assay" for measuring the integrity of the glomerular filtration barrier. We use a transgenic zebrafish (*l-fabp:DBP-EGFP)* that produces a green fluorescent plasma protein (Fig 2a). The transgene expression is driven

by the FABP-liver promoter and leads to expression of a vitamin D binding protein fused with eGFP. The promoter becomes active at 72hpf and leads to an accumulation of fluorescent plasma protein that can be monitored over the retinal vessel plexus as a representative location for systemic fluorescence. In contrast to an increasing level of systemic fluorescence in the control injected fish, no accumulation is seen if the morpholino injection causes leakiness of the glomerular filtration barrier (Fig 2b). For further proof of loss of plasma protein into the urine by kidney damage, we perform an immunoblotting assay of the fish water (Fig 2c).

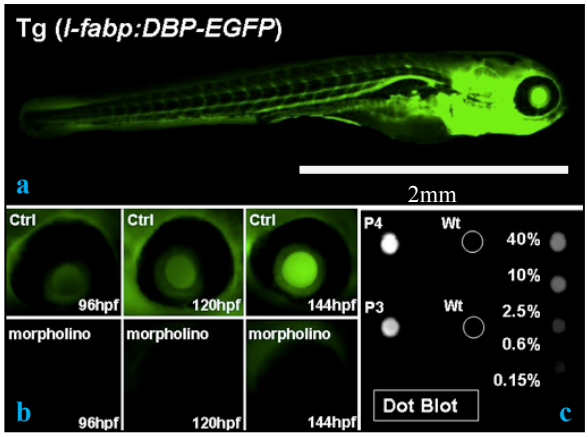

Figure 2. Eye assay and dot blot for measuring glomerular filter integrity using transgenic l-fabp:DBP-EGFP zebrafish. The l-fabp:DBP-EGFP transgenic zebrafish produces a green fluorescent plasma protein, leading to an accumulation of fluorescence that can be monitored over the retinal vessel plexus of the eye[8] (a). Pictures of the eye of the control injected group show increasing levels of fluorescence from 96hpf to 144 hpf while the retinal vessel plexus of morpholino injected fish remain dark (b). GFP lost *via* the kidney can be detected in the fish water using a dot blot immunoblotting approach (c).

After confirmation of proteinuria due to leakage of the glomerular filtration barrier, it is essential to perform a detailed ultrastructural analysis by transmission electron microscopy, since any part of the renal filter (podocytes, glomerular basement membrane or endothelial cells) can be affected. Zebrafish larvae are embedded in epon plastic blocks and cut via microtome. When the region of interest (glomerulus) is reached (Fig 3a), ultrathin sections (90 nm) are performed. Transmission electron microscopy allows visualization of the filtration barrier and analysis of injury (Fig 3b).

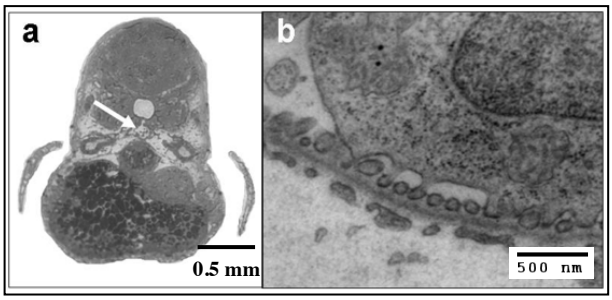

Figure 3. Ultrastructural analysis to detect defects of the glomerular filtration barrier. (a) Light microscopy of epon embedded zebrafish 120 hpf showing the glomerulus (white arrow); (b) transmission electron microscopy shows the glomerular filtration barrier under normal conditions displaying all features of a mammalian kidney with elaborate podocyte foot processes, a normal glomerular basement membrane and a fenestrated endothelium.

This zebrafish screening system produces consistent and reliable evidence for gene relevance in chronic kidney disease. It allows us to screen entirely novel genes in 4-6 weeks in hundreds of live test subjects in a cost-effective manner. In collaboration with the Jackson Lab, using this zebrafish screening model has recently prioritized a list of nearly 30 novel candidate genes for kidney damage at a fraction of the time and cost as screening exclusively in a mammalian model.

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