Non-muscle myosin IIA is required for the development of the zebrafish glomerulus

Tobias Müller[1], Elisabeth Rumpel[1], Susanne Hradetzky[1], Frank Bollig[2], Henny Wegner[1], Antje Blumenthal[1], Andreas Greinacher[3], Karlhans Endlich[1] and Nicole Endlich[1]

[1]*Department of Anatomy and Cell Biology, Ernst-Moritz-Arndt-Universität, Greifswald, Germany;* [2]*Department of Medicine/Nephrology, Hannover Medical School, Hannover, Germany and* [3]*Department of Immunology and Transfusion Medicine, Ernst-Moritz-Arndt-Universität, Greifswald, Germany*

Mutations in the *MYH9* gene, coding for the non-muscle myosin heavy chain IIA (NMHC-IIA), are responsible for syndromes characterized by macrothrombocytopenia associated with deafness, cataracts, and severe glomerular disease. Electron microscopy of renal biopsies from these patients found glomerular abnormalities characterized by alterations in mesangial cells, podocytes, and thickening of the glomerular basement membrane. Knockout of NMHC-IIA in mice is lethal, and therefore little is known about the glomerular-related functions of *Myh9*. Here, we use zebrafish as a model to study the role and function of zNMHC-IIA in the glomerulus. Knockdown of zNMHC-IIA resulted in malformation of the glomerular capillary tuft characterized by few and dilated capillaries of the pronephros. In zNMHC-IIA morphants, endothelial cells failed to develop fenestrations, mesangial cells were absent or reduced, and the glomerular basement membrane appeared nonuniformly thickened. Knockdown of zNMHC-IIA did not impair the formation of podocyte foot processes or slit diaphragms; however, podocyte processes were less uniform in these morphants compared to controls. *In vivo* clearance of fluorescent dextran indicated that the glomerular barrier function was not compromised by zNMHC-IIA knockdown; however, glomerular filtration was significantly reduced. Thus, our results demonstrate an important role of zNMHC-IIA for the proper formation and function of the glomerulus in zebrafish.

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Correspondence: *Nicole Endlich, Institut für Anatomie und Zellbiologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Löfflerstr 23c, D-17487 Greifswald, Germany. E-mail: nicole.endlich@uni-greifswald.de*

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Non-muscle myosin II (NM-II) is critically important in cell contractility, morphology, cytokinesis, polarity, and migration.[1–5] In mammalian cells, three different genes (*Myh9, Myh10, and Myh14)* encode three different non-muscle myosin heavy chains (NMHCs), referred to as NMHC-IIA, NMHC-IIB, and NMHC-IIC.[6,7] The present work focuses on the role of NMHC-IIA. Several mutations in *MYH9* have been identified (e.g., R702, R1165, D1424, E1841, or R1933) in the past, which are responsible for a variety of autosomal-dominant, so-called MYH9-related diseases such as Epstein, Fechtner, Sebastian Syndrome, and May–Hegglin anomaly.[8–10] All of them are characterized by macrothrombocytopenia accompanied by sensoneural injury, cataracts, Döhle-like inclusions in neutrophils, and glomerular injury. In addition, clinical studies have reported a causal connection between *MYH9* mutations and glomerulosclerosis[11–14] and end-stage renal disease.[15–16] To date, 39 mutations have been found, several of which are associated with severe glomerular syndromes, such as foot process effacement of podocytes, and focal glomerular basement membrane (GBM) thickening and splitting, resulting in proteinuria and finally end-stage renal disease (reviewed by Kopp *et al.*).[17]

The molecular mechanisms responsible for these *MYH9*-related glomerular diseases are not understood. One reason for this is the lack of an appropriate animal model. NMHC-IIA knockout mice die at an early embryonic stage (E6.5–E7.5),[18] whereas heterozygous mice are viable and healthy.[19] The knockout embryos fail to organize normal germ layers, and therefore tissue-derived knockout cells are not available. Here, we use the zebrafish as a model to study the role and function of NMHC-IIA in the glomerulus. The zebrafish larvae develop a simplified kidney, the pronephros, consisting of two nephrons with a single central glomerulus.[20–23] The architecture and the function of the glomerulus of the zebrafish larvae are highly similar to the mammalian glomerulus and are therefore an accessible system to study the role of *Myh9*.[24–27] Moreover, the zebrafish is transparent and easy to manipulate. Proteins can be knocked down by microinjection of morpholinos into the fertilized eggs. Furthermore, injection of fluorescent dextran allows to study the filtration process directly in living larvae.

In this paper, we reveal the essential role of NMHC-IIA in the formation of the glomerular tuft and of the filtration barrier in the pronephros of zebrafish larvae. Moreover, by measurement of the blood clearance of microinjected fluorescent dextran with different molecular sizes in living larvae, we could determine the influence of NMHC-IIA on the filtration process *in vivo*.

RESULTS

Zebrafish NMHC-IIA (zNMHC-IIA) is expressed in podocytes and endothelial cells

For characterization of the cellular localization of zNMHC-IIA in the glomerulus, we stained cryosections of zebrafish larvae (3 d.p.f.) with an antibody against zNMHC-IIA. We found that zNMHC-IIA was expressed in podocytes (Figure 1a and d). For better identification of podocytes, we used a transgenic zebrafish line with a podocyte-specific enhanced green fluorescent protein (eGFP) expression, which is under the control of the *wt1b* promoter (*wt1b:eGFP*; Figure 1b).[28] Furthermore, zNMHC-IIA was also found in mesangial cells and endothelial cells by immunoelectron microscopy (Figure 1e and f).

Knockdown of zNMHC-IIA causes edema

We identified the zebrafish *Myh9* ortholog by searching the zebrafish genome (Ensemble zebrafish genome assembly). The zebrafish *Myh9* gene coding for the 1754 amino-acid zNMHC-IIA protein is located on chromosome 6 on position 8,452,022. Homologs exist in mice and humans, with alignment scores of 82.6% and 83.3%, respectively.

To study the effects of selective zNMHC-IIA knockdown, we used a morpholino antisense oligonucleotide (*zMyh9*-MO) targeting the 5′-untranslated region of the *zMyh9* mRNA (transcript ID: ENSDART00000011353; protein ID: ENSDARP00000021727) including the start codon, preventing *zMyh9* mRNA translation. To test the efficiency of the knockdown, a western blot using an antibody against zNMHC-IIA was performed, showing a reduced signal in *zMyh9*-MO morphants as compared with larvae spawned from eggs injected with a control morpholino (ctrl-MO), respectively (Supplementary Figure S1 online). Injection of *zMyh9*-MO (1 mmol/l) into fertilized eggs at the two-cell stage resulted in pronounced pericardial and yolk sac edema (41.3 ± 4.4% at 4 d.p.f., $n = 4074$ larvae in 27 independent experiments; Figure 2c–e). Furthermore, we observed heart-folding defects often associated with a 40% slower heart rate as compared with the controls (data not shown). A few larvae showed a slight dorsal bending and shortening of the body axis after 3 d.p.f. The knockdown of zNMHC-IIA did not lead to a significant increase in mortality of larvae at 5 d.p.f. (29.4 ± 3.6%) as compared with ctrl-MO larvae

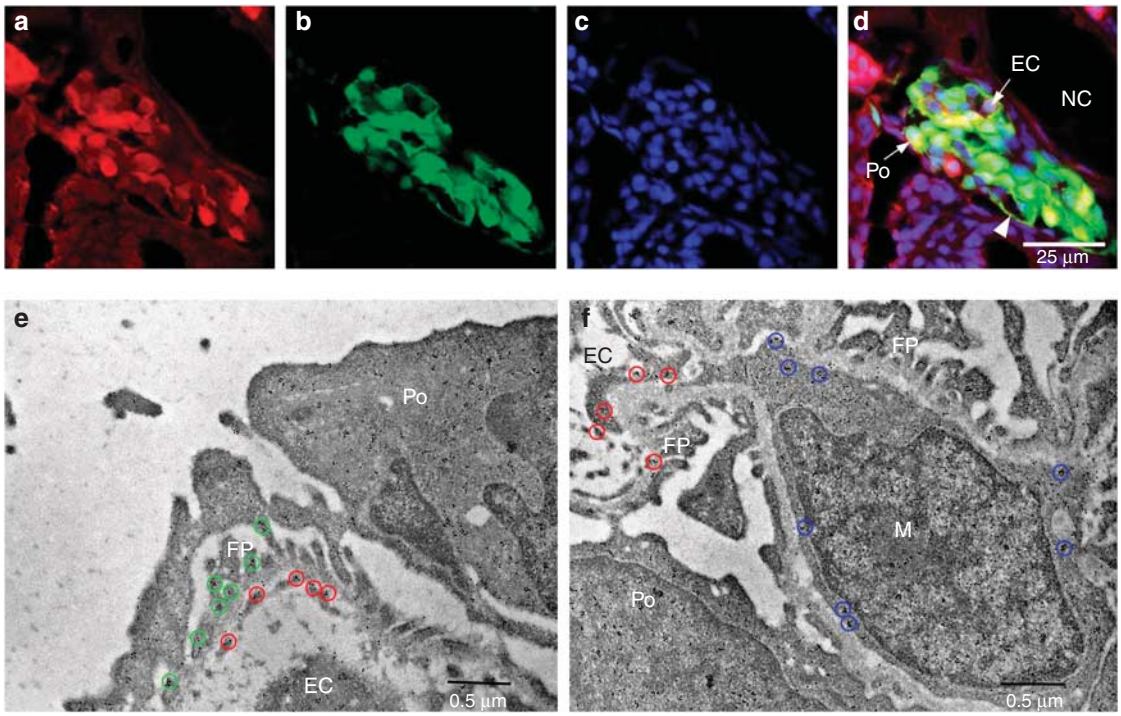

Figure 1 | Localization of zebrafish NMHC-IIA (zNMHC-IIA) in the pronephros of zebrafish larvae. Cryosections (20 µm) of zebrafish larvae (3 d.p.f.) injected with control morpholinos were immunostained with an antibody against zNMHC-IIA (red in **a**). Podocytes are strongly stained in the cell body and processes identified by the podocyte-specific expression of eGFP (green in **b**). To identify all nuclei, the sections were stained with DAPI (blue in **c** and **d**). The arrow (Po) in **d** (overlay of **a**–**c**) shows the cell body of a podocyte. The arrowhead marks podocyte processes. Immunogold staining of zebrafish larvae (**e**–**f**) showed a staining for NMHC-IIA in podocytes (green circles), in mesangial cells (blue circles), and in endothelial cells (red circles). DAPI, 4′,6-diamidino-2-phenylindole; EC, endothelial cell; eGFP, enhanced green fluorescent protein; FP, podocyte foot processes; M, mesangial cell, NC, notochord; NMHC, non-muscle myosin heavy chain; Po, podocyte.