Knockdown of the Hypertension-Associated Gene 

NOSTRIN Alters Glomerular Barrier Function in Zebrafish

(Danio rerio)

Torsten Kirsch,* Jessica Kaufeld,* Ron Korstanje, Dirk M. Hentschel, Lynne Staggs, Frank Bollig, Michaela Beese, Patricia Schroder, Lisa Boehme, Hermann Haller, Mario Schiffer

Abstract—Hypertension is one of the major risk factors for chronic kidney disease. Using quantitative trait loci analysis, we identified the gene of the F-BAR protein NOSTRIN in the center of an overlapping region in rat and human quantitative trait loci that are associated with hypertension. Immunohistochemical analysis revealed a predominantly podocytic expression pattern of NOSTRIN in human and mouse glomeruli. Further, NOSTRIN colocalizes with cell–cell contact–associated proteins β-catenin and zonula occludens-1 and interacts with the slit-membrane–associated adaptor protein CD2AP. In zebrafish larvae, knockdown of nostrin alters the glomerular filtration barrier function, inducing proteinuria and leading to ultrastructural morphological changes on the endothelial and epithelial side and of the glomerular basement membrane of the glomerular capillary loop. We conclude that NOSTRIN expression is an important factor for the integrity of the glomerular filtration barrier. Disease-related alteration of NOSTRIN expression may not only affect the vascular endothelium and, therefore, contribute to endothelial cell dysfunction but might also contribute to the development of podocyte disease and proteinuria. (Hypertension. 2013;62:726-730.) ● Online Data Supplement

Key Words: glomerular filtration barrier ■ hypertension ■ podocytes ■ proteinuria ■ zebrafish

Hypertension belongs to the most severe health issues worldwide, and persistent hypertension is one of the leading causes of chronic kidney disease. Because the pathogenesis of hypertension is complex and multifactorial, deciphering the underlying mechanisms is a complex struggle. The establishment of specific mouse and rat strains with genetically determined hypertension as well as extensive genetic screens in humans allowed for narrowing the search for possible candidate genes.1,2

Using a cross between Dahl salt-sensitive and Lewis rats, Garrett et al3 were able to link a chromosomal region on chromosome 3 with differences in blood pressure. This analysis was confirmed and refined using congenic lines originating from these same strains.4 Overlapping quantitative trait loci were later found for blood pressure in a cross between hypertensive and Lyon normotensive rats.6 Moreover, linkage between premature hypertension and a region on chromosome 2 was shown in a human family in Kyrgyzstan.7 This region is, in part, orthologous to the rat chromosome 3. The common overlapping region of 7.6 Mb was defined by the 2 markers D3Chm63 and D3Rat180. There are 88 annotated genes within this region (Ensembl Rnor_5.0), including NOSTRIN. The linkage data relative to rat chromosome 3 are summarized in Figure S1 in the online-only Data Supplement.

NOSTRIN was originally described by Zimmermann et al8 as an F-BAR domain–containing protein involved in shuttling of endothelial NO synthase. Recently, the same group demonstrated that knocking down NOSTRIN in zebrafish resulted in impaired vasculogenesis and, probably by impaired fibroblast growth factor (FGF) signaling, NOSTRIN-deficient mice develop disordered vascular patterning in the postnatal retina.9 We showed that distribution of tight junctions in the blood–brain barrier capillaries of stroke-prone spontaneously hypertensive rats was altered, and that this finding was accompanied by changes in the transcript level of NOSTRIN.10 These data demonstrate that NOSTRIN might carry far more cellular functions beyond NO signaling. We here report on the role of NOSTRIN in the kidney with a special emphasis on the glomerular expression of NOSTRIN.

Methods

An expanded Materials and Methods section can be found in the online-only Data Supplement.

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Results

NOSTRIN Is Expressed in Glomerular Podocytes

Immunostainings with a polyclonal antibody directed against a peptide localized near the C terminus of rodent NOSTRIN revealed that NOSTRIN is expressed in peritubular capillaries and glomerular structures (Figure 1A). Double stainings with podocytic and endothelial markers showed that glomerular NOSTRIN expression could be assigned to podocytic structures (Figure 1B). A strong colocalization of NOSTRIN and the endothelial cell marker thrombomodulin could be observed in vascular structures outside the glomerulus (data not shown). Immunostainings of human kidney sections with an antibody directed against human NOSTRIN confirmed podocytic distribution pattern in the glomerulus (Figure 1C).

NOSTRIN Interacts With Cell Contact–Associated Proteins in Human Podocytes

To further characterize NOSTRIN expression in podocytes, we performed double stainings of NOSTRIN with the cell contact–associated proteins β-catenin and zonula occludens-1 in cultured human podocytes. As depicted in Figure 2A, NOSTRIN displayed a close colocalization with β-catenin and zonula occludens-1 in regions of cell–cell contacts (Figure 2A). Coimmunoprecipitation experiments demonstrated that NOSTRIN interacts with cell–cell contact–specific proteins β-catenin, CD2AP, and caveolin-1 (Figure 2B).

Knockdown of NOSTRIN Induces a Renal Phenotype in Zebrafish

To prove that NOSTRIN is relevant for the integrity of the glomerular filtration barrier, we analyzed functional effects of NOSTRIN loss on the kidney in vivo in larval zebrafish. Although rat or human NOSTRIN is only 44% homologous to their zebrafish equivalent, the different functional domains remain conserved between the different species indicating that the zebrafish NOSTRIN orthologue might have similar functions (Figure 3A). After injection of 100 to 250 μmol/L nostrin-specific morpholinos, zebrafish embryos developed an edematous phenotype as depicted in Figure 3B. This phenotype includes a general edema of the body with accentuated pericardial effusion, yolk sac edema, and an arched back. The cardiovascular system seemed to develop normally. Embryos were less active in their movement and less likely to hatch spontaneously. In contrast, embryos that were injected with control morpholino at equivalent concentrations did not develop a phenotype and showed normal hatching rates.

Nostrin Knockdown in Zebrafish Leads to Proteinuria

To visualize the glomerular loss of high molecular weight proteins, we used a wt1b-GFP transgenic zebrafish11 that expresses green fluorescence in the fused glomeruli and in the proximal tubules of the pronephros. In NOSTRIN knockdown fish, a 70-kDa rhodamine-labeled dextran was injected at 72 hours post fertilization into the cardinal vein. Forty-eight hours post injection, the extravasation of rhodamine-labeled dextran could be detected within the wt1b-GFP–tagged proximal tubular cells of the morphants (Figure 4A) indicating leakage of the high molecular weight dextran through the filtration barrier and reuptake in the proximal tubular cells.

To further analyze and to quantify the proteinuria, we measured loss of fluorescence in the vasculature using a transgenic zebrafish line Tg(l-fabp:DBP-eGFP) expressing a GFP–tagged vitamin D binding protein (DBP) with a molecular weight of 78 kDa under the control of the l-fabp (liver-type fatty acid binding protein promoter). Injection of nostrin morpholinos resulted in a significant loss of fluorescence activity in the retinal blood vessels of morphants 96 hours post fertilization to 144 hours post fertilization, whereas fluorescence activity remained stable or even increased in control morpholino–injected larvae (Figure 4B). These results could be confirmed by dot-blot analysis of secreted DBP-enhanced green fluorescent protein (Figure 4C).

Knockdown of NOSTRIN Affects Glomerular Ultrastructure

To determine the structural integrity of the filtration barrier in the nostrin morphant fish, we analyzed glomeruli of 120
hours postfertilization embryos by transmission electron microscopy (Figure 5). In contrast to the normal glomerular ultrastructure of control-injected fish (Figure 5A), we detected severe ultrastructural changes in the podocyte structure with effacement of the podocyte foot processes and swelling of the endothelium in the morpholino-injected fish (Figure 5B and 5C). In addition, we found severe alterations of the basement membrane that appeared thickened and in some areas even split and disrupted. These functional and ultrastructural data demonstrate a disturbed barrier function of the zebrafish pro-nephros after knockdown of *nostrin*.

### Discussion

Patients with nondiabetic chronic kidney disease (as defined with an estimated glomerular filtration rate (eGFR) of <60 mL/min per 1.73 m² or proteinuria) and coexistent hypertension have an increased risk of cardiovascular events and death. In turn, coexistent hypertension is responsible for the progression of chronic kidney disease with accelerated segmental or global glomerulosclerosis that may be superimposed on the phenotype of the underlying kidney disease.

In this context, identification of genes and gene products that are linked to structural changes of the kidney remains one of the key questions in the analysis of renal impairment during hypertension. Analysis of overlapping quantitative trait loci represents a powerful tool to narrow down the endless list of potential candidate genes in the development of hypertension and cardiovascular disease. Applying quantitative trait loci analyses, we identified *NOSTRIN* within the overlapping regions linked to blood pressure–related traits in rats and in a kyrgyz family, suggesting a mechanistic role for *NOSTRIN* in hypertension-associated end organ failure.

*NOSTRIN* consists of an N-terminal F-BAR domain and a C-terminal SH3 domain that mediate plasma membrane binding and protein–protein interactions. It has been attributed to the control of endothelial nitric oxide synthase activity and has been shown to interact with dynamin-2, neural Wiskott–Aldrich syndrome protein, and caveolin-1. More recently, the same group showed that *NOSTRIN* is involved in vascular development and that loss of *NOSTRIN* resulted in impaired vascular patterning.

Here, we showed that in human and rodent glomeruli, *NOSTRIN* is expressed predominantly in epithelial podocytes and interacts with proteins such as CD2AP that are known to be essential for proper slit-membrane function. In mice, inhibition of neural Wiskott–Aldrich syndrome protein by c-mip (c-maf–inducing protein) induces heavy proteinuria and accounts for cytoskeletal disorganization and effacement of foot processes in podocytes. Similar pathological mechanisms, for example, reorganization of the podocytic cytoskeleton and proteinuria, have also been described for cleavage of dynamin by cathepsin-L, and overexpression of mutant dynamin that is resistant to cathepsin-L cleavage circumvented proteinuria and foot process retraction. As both

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**Figure 2.** NOSTRIN interacts with junction-associated proteins in cultured human podocytes. **A**, Double stainings of NOSTRIN with the junction-associated proteins β-catenin (top) and zonula occludens-1 (ZO-1; bottom) revealed a close colocalization at cell–cell contact regions. **B**, Coimmunoprecipitation experiments showed that NOSTRIN interacts with β-catenin, the slit-membrane–associated protein CD2AP and caveolin-1. IB indicates immunoblotting; IP, immunoprecipitation; and L, podocyte lysate. Scale bar, 25 µm.

**Figure 3.** Morpholino-induced knockdown of nostrin induced an edematous renal phenotype in zebrafish. **A**, The zebrafish orthologue of NOSTRIN displays similar domain structures compared with rat and human NOSTRIN indicating similar functional activities of the orthologue. **B**, After injection of 100 to 250 mmol/L nostrin morpholino, the embryos developed a phenotype as shown (a and c, control morpholino injection; b and d, nostrin morpholino injection).
proteins represent potential interaction partners of NOSTRIN, it is conceivable that NOSTRIN functions as an adaptor molecule controlling correct spatial localization of dynamin and neural Wiskott–Aldrich syndrome protein and, therefore, modulates podocyte architecture. However, NOSTRIN is described to interact with fibroblast growth factor-1 receptor (FGFR1) and the small G-protein Rac1. Rac1 is known to modulate cell adhesion and migration through cytoskeletal reorganization processes. More recently, a study demonstrated that Rac1 is necessary for recruitment of the adaptor protein CD2AP to

Figure 4. Proof of filter leakiness in wt1b transgenic fish. A, The wt1b-GFP fish displays a green fluorescent pronephros (a, a’). After injection of a red fluorescent high molecular weight dextran (b, b’), uptake of red fluorescent dextran in green fluorescent tubular cells can be monitored (c, c’). B, Intravascular fluorescence loss after knockdown of nostrin is measured over the retinal blood vessel plexus in Tg(l-fabp:DBP-eGFP) fish after control morpholino injection (Ctrl; top) and in mild phenotypes (P1/P2) of Nostrin knockdown fish. Fluorescence levels of individual fish were monitored over time and displayed as percentage of baseline in mild phenotypes (P1/P2) and severe edematous phenotypes (P3/P4) at 96, 120, and 144 hpf. C, Dot-blot analysis of secreted green fluorescent protein–tagged serum proteins after knockdown of nostrin collected in the embryo raising medium of Ctrl, P2-, P3- and P4-phenotype knockdown fish.

Figure 5. Ultrastructural analysis of kidney structures in nostrin knockdown fish. A, In control fish ultrastructural analysis reveals normal foot process architecture (a and b). Tubular cells display intact brush borders (c). B, In NOSTRIN knockdown fish with a mild phenotype (P1/2) glomerular basement membrane is affected and foot processes show partial effacement (a and b). C, In knockdown fish with a severe phenotype (P3/4), massive endothelial damage and complete effacement of podocyte foot processes could be seen (a and b). In both knockdown phenotypes tubular cell structure appears normal, indicating a glomerular (podocyte) phenotype in the knockdown fish.
regions of cell–cell contacts where it modulates proper junction formation in epithelial cells. These data propose a model, in which NOSTRIN, Rac1, and CD2AP form a complex that modulates correct formation of the glomerular slit membrane.

Further evidence for a role of NOSTRIN in proper function of the podocytic filtration unit came from the zebrafish knockdown experiments where we could demonstrate that NOSTRIN deficiency results in a severe edematous phenotype that is associated with enhanced clearance of serum proteins, functional renal impairment, and loss of size selectivity of the glomerular filter. At the ultrastructural level, nostrin knockdown induced podocyte foot process effacement, swelling of glomerular endothelial cells, and, in more severe phenotypes, damage of the glomerular basement membrane. Whether the primary insult could be assigned to the podocytes or to both podocytes and endothelial cells will be subject of further examinations.

**Perspective**

We here demonstrated that NOSTRIN might evolve as a key player of mechanisms of proteinuria in hypertensive kidney disease. Further studies on regulation and function of NOSTRIN expression in the kidney and identification of possible interaction partners in podocytes are in progress in our laboratory and might pave the way for a possible novel mechanism in the development of proteinuria in cardiovascular disease.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is New?**

- Analyzing genetic mapping approaches, we identified NOSTRIN as a possible hypertension-associated gene that is expressed in glomerular podocytes and may modulate glomerular filtration.

**What Is Relevant?**

- NOSTRIN interacts with proteins necessary for proper slit-membrane function. Moreover, knockdown of NOSTRIN in zebrafish resulted in proteinuria and structural changes of glomerular filtration apparatus.

**Summary**

We conclude that NOSTRIN plays an important role for the integrity of the glomerular filtration apparatus. Altered expression of NOSTRIN may contribute to podocyte injury and the onset of proteinuria.
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KNOCKDOWN OF THE HYPERTENSION ASSOCIATED GENE NOSTRIN ALTERS GLOMERULAR BARRIER FUNCTION IN ZEBRAFISH (DANIO RERIO)

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Short title: Nostrin alters glomerular barrier in zebrafish

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Materials and Methods

Zebrafish stocks and embryos.
Zebrafish, Wildtype AB and the transgenic Tg(l-fabp:DBP-eGFP) and wt1b-GFP zebrafish line \(^1\), were grown and mated at 28.5°C. Embryos were kept and handled in standard E3 solution (5mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\), 0.33 mM MgSO\(_4\), 10-5 % methylene blue) buffered with 2 mM HEPES (Sigma-Aldrich, St. Louis, MO) as previously described in Hentschel et al.\(^2\) All zebrafish studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Zebrafish morpholino injections
Using the Nanoject II injection device (Drummond Scientific, Broomall, PA), nostrin and scrambled control morpholinos were injected into one to four-cell stage fertilized embryos at 100 to 250 µM concentrations in 4.6 nl injection buffer (20 mM Hepes, 200 mM KCl and 0.01% phenol red). Morpholino sequences were designed and ordered from GeneTools (Philomath, OR) as follows: standard control sequence - 5’-CCTCTTACCTCAGTTACAATTTATA-3’ and nostrin sequence – 5’-GGTCCTTCATCTCTCAGTCGCTGG -3’.

Fluorescence proteinuria measurements
For glomerular function studies, 48 hours post fertilization (hpf) dechorionated Tg(l-fabp:DBP-eGFP) embryos were anesthetized with a 1:20 to 1:100 dilution of 4 mg/ml Tricaine (MESAB: ethyl-3-aminobenzoate, methanesulfonate acid salt, 1% Na\(_2\)HPO\(_4\), pH 7.0) (Sigma-Aldrich) and placed in 200 µl E3 in single wells of 96 well plates (Fisher, Pittsburgh, PA) and allowed to recover. Sequential images of live fish were generated using a Zeiss inverted microscope (Axiovert 200) connected to an AxioCam MRm charge-coupled device camera, and images were taken with fixed exposure times and gain using the Axio Vision release 4.5 SP1 software package. The maximum fluorescence intensities of grayscale images of the pupil of the fish were measured using NIH’s ImageJ application and reported in relative units of brightness as previously described.\(^2\) For observation of rhodamine-labeled dextran passing the glomerular barrier and entrapment of fluorescence in the proximal tubules, we used the wt1b-GFP transgenic zebrafish line as previously described.\(^3\) The animal protocol was approved by the Mount Desert Island Biological Laboratory (MDIBL) animal care committee.

Dot Blot Analysis of fish water. For dot blot analysis of fish water NOSTRIN and control morpholinos were injected into Tg(Fabp:DBP-eGFP) male X AB female embryos. Fish were transferred to 48well plates individually - one fish per well - with 500µL ERM at 72h and kept at 28°C until 168hr. Fish were graded for phenotype development removed from the wells and the fish water was stored at -20°C. Only fish water from fish with a heartbeat at 168h were used for dot blot. For the dot blot procedure 100µL fish water from 4 fish were pooled and blotted on a nitrocellulose membrane. Immunoblot was performed using anti-GFP (abcam ab290) 1:2000/anti-Rabbit 1:5000 in PBS/5%NFDM. As positive control DBP-eGFP percent of total eGFP protein extracted from an individual fish was used.
Histology and Transmission Electron Microscopy (TEM)
Morphant larval zebrafish were sampled at 120 hpf and fixed in 1.5% glutaraldehyde/1% PFA and 70 mM NaPO₄, pH 7.2. After fixation the embryos were washed three times in 0.2 M cacodylate buffer and then postfixed in 1% osmium tetroxide for one hour at room temperature. After rinsing with cacodylate buffer all specimens were dehydrated in a graded ethanol series and infiltrated and embedded with epon according to manufacturer’s protocol (Hard Plus Resin 812, Electronmicroscopy Sciences, Hatfield, PA). Thin-sections of 0.5 and 1µm were generated with a Leica RM2165 rotary microtome and stained with 0.5% toluidine blue in a 1% sodium tetraborate solution. Ultra-thin (80-100 nm, thick) sections of the kidney were cut and mounted on slot and 300 mesh grids (Luxel, Friday Harbor, WA). The sections were stained with 2% uranyl acetate in distilled water, and contrasted with lead citrate. Sections were viewed and photographed on a JEOL-1230 transmission Electron microscope (Eching, Germany).

Immunohistochemistry/Immunocytochemistry
Paraffin-sections (1.5 µm) of 3% PFA-perfusion-fixed mouse or formalin-fixed human kidney were stained with polyclonal antibodies against NOSTRIN and NEPHRIN (R&D Systems), Thrombomodulin or VE-Cadherin (clone 55-7H1 from BD Pharmingen). Specificity of antibodies was tested with non-immune serum controls. Images were analyzed by confocal microscopy. Human immortalized podocytes were fixed with acetone for 15 min at -20°C followed by methanol-permeabilization for 15 min at -20°C. Antibodies used for immunocytochemistry were anti-β-Catenin and anti-ZO-1 (BD Biosciences). Confocal images were taken using a Leica DM IRB microscope with a TCS SP3 AOBS scan head equipped with argon and krypton laser beams and a 405 nm laser. Micrographs were obtained using a HCX PL APO 63x1.4 numerical aperture objective.

Immunoprecipitation
Cells were lysed in ice-cold IP-buffer (20mM TRIS pH 8.0, 137mM NaCl, 10% Glycerol, 1% Nonidet P-40, 2mM EDTA) containing protease and phosphatase inhibitor cocktail tablets (Roche) and protein amount was determined with a BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany). Antibodies were covalently linked to ProteinA/G-magnetic beads (NEBiolabs) and added to 500 µg of pre-cleared cell lysates for 1 hour at 4°C. After washing proteins were eluted with 0.1 M glycine pH 2.5 and neutralized with 0.5 volumes of 1M TRIS pH 9.0, separated by SDS PAGE electrophoresis and blotted on to a PVDF nylon membrane. Membranes were incubated with the appropriate primary antibody followed by incubation with a horseradish peroxidase- (HRP-) conjugated secondary antibody (Cell Signaling/NEBiolabs). Bands were visualized by Western Lighting chemiluminiscence reagent (Perkin Elmer, Rodgau, Germany) and quantified by densitometry using a CCD camera and Quantity One software (Biorad Laboratories, Munich, Germany).

Statistics
For comparison of more than two groups of individuals, the nonparametric Kruskal-Wallis test was used. If significant differences in between the groups were found the Mann-Whitney U test was used to calculate the difference between each pair of groups. For the in vitro data Student’s T test or Mann-Whitney U test was applied depending on the distribution of the data. p-values (2-sided) were considered significant at p<0.05.
Statistical analysis was performed using SPSS Statistics 19 (SPSS, Muenchen, Germany) or GraphPad Prism Software (San Diego, California).

References


S1: Schematic summary of the linkage data at the NOSTRIN locus. Three blood pressure-related QTL have been mapped in the rat by Garrett et al\textsuperscript{4}, Moreno et al\textsuperscript{5}, and Bilusic et al\textsuperscript{6} and have been named BP177, BP152, and KIDM13, respectively, according to the Rat Genome Database (www.rgd.mcw.edu). The proximal region of rat chromosome 3 is in part orthologous to human chromosome 2q (indicated in grey), while the distal region is homologous to human chromosome p11. Linkage for hypertension with human chromosome 2q has been reported by Kalmyrzaev et al\textsuperscript{7}. The NOSTRIN coding region is located within the confidence intervals of all these mapped loci.