***Jobst-Schwan et al.*, "Steroid treatment exacerbates nephrotic syndrome in a CRISPR/Cas9 knockout zebrafish model of *magi2a*"**

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Supplemental Material S1: COMPLETE METHODS

Zebrafish experiments were performed in *Danio rerio*, strain *l-fabp*: VDBP-GFP (AB). All national and institutional guidelines for the care and use of laboratory animals were followed. The zebrafish experiments were approved by the Boston Children's Hospital (BCH) Institutional Animal Care and Use Committee (IACUC). A complete description of the husbandry and environmental conditions in housing for the fish used in these experiments is available as a collection in protocols.io (dx.doi.org/10.17504/protocols.io.mrjc54n).

Generation of zebrafish mutant lines by CRISPR/Cas9

Target selection and sgRNA generation

Single guide RNA (sgRNA) targets were selected using the CHOPCHOP online tool v1 following their ranking algorithm.[1] The algorithm takes into account all potential off-target sites that differ in up to 2 nucleotides, GC-content and presence of a guanine residue in the last position before the Protospacer Adjacent Motif (PAM) sequence, since these factors influence the efficiency of sgRNA binding and Cas9 cleavage.[2] Targets were chosen in early exons to potentially introduce early frameshift mutations to maximize loss of function of the protein. sgRNAs were generated by *in-vitro* transcription from oligonucleotide based templates using the MEGAscript T7 Transcription Kit (Ambion).[2] Since sgRNA activity is higher if two guanine bases follow the T7 promotor,[2] template sequences were modified accordingly if necessary (**Suppl. Table S1**). The resulting change of one or two nucleotides at the 5' end of the gRNA results in a higher indel frequency [2] and does not reduce specificity.[3]

Microinjection, mutation analysis and breeding

2 µl of sgRNA stock (500 ng/µl) were mixed with 2 µl of recombinant Cas9 protein (1 µg/µl, PNA Bio, Thousand Oaks, CA) and incubated on ice for at least 10 min to allow formation of the sgRNA/Cas9 complex. 2 nl of the injection mix was injected intracellularly in one-cell stage zebrafish embryos using glass needles and a micromanipulator. DNA was extracted from 10 pooled injected embryos and an uninjected control group at 48 hpf using the HotShot protocol.[4] Mutagenesis was determined by a T7 endonuclease assay as described before.[5] Positive clutches (F0 generation) were raised to adulthood and outcrossed against wildtype fish. Germline transmission was also determined by the T7 endonuclease assay. Positive clutches (F1 generation) were raised to adulthood and genotyped individually. Fish carrying the same mutation were pooled being the founders of the heterozygous stable knock out line.

Edema and survival analysis

Embryos were generated by timed breedings and kept in fish water containing 0.002% methylene blue until 24 hours post fertilization (hpf) followed by fish water only. Larvae were transferred to rotifer feeding solution at 5 days post fertilization (dpf), the solution being changed every other day. The dishes were monitored daily until 22 dpf. Edema phenotype contained whole body edema with ascites and perorbital edema (**Fig 1A-B, Fig 3A**) and scoring of the edema phenotype was performed in a dorsal view of the larvae as follows: 1) periorbital edema defined as at least 3 out of 4 clear “bulges” in front or behind the eye 2) ascites defined as extension of the transversal larval diameter

with a clear fluid filled space between skin and the opaque body axis. The clear character of both phenotypes disappears in older larvae (>12 dpf) as they develop a continuous layer of pigmentation. Upon onset of edema, larvae were isolated and followed further until reaching the endpoint for survival. The endpoint for survival was reached when minimal residual cardiac activity without visible blood flow in the tail vein was observed. Larvae that reached the endpoint were euthanized immediately using Tricaine (0.4–0.8 mg/ml) to minimize distress. The DNA was extracted individually from dead larvae and the genotype was confirmed by Sanger sequencing.

Proteinuria assay

Embryos were generated by timed breedings as described above. At 6 dpf, larvae were sorted for edema phenotype, transferred to fresh fish water, and housed individually in 60 µl fish water for 24 h. Water samples were snap frozen and the larvae were genotyped individually.

Acetone precipitation

6 water samples per genotype were pooled and four times the pooled sample volume of cold (-20°C) Acetone was added. Tubes were vortexed, incubated at -20°C for 1 h, and centrifuged at 15,000 x *g* (4°C) for 10 min. Acetone was removed by pipetting and pellets were dried for 20 min at room temperature. Pellets were dissolved in 15 µl 1x SDS-sample buffer.

SDS-PAGE

Reconstituted samples were cooked at 95°C for 10 min and cooled on ice for 5 min. SDS-PAGE was performed on Bis-Tris gradient gels (4-12%) according to standard

procedures. Samples were loaded on a 1 mm gel with 15 chambers to concentrate the sample in a small gel volume.

DBF-staining

The sensitive negative stain by 4′,5′-Dibromofluorescein (DBF) was adapted from Yu et al.[6] Directly after electrophoresis, the protein gel was stained for 10 min in DBF solution (containing 0.3% DBF, 40% Methanol, 10% Glycerol, 50 µM $ZnCl_2$). The gel was rinsed in distilled water to get rid of excess staining solution and developed in 100 mM NaAc-HAc buffer solution (pH 4.6) for 30 min. The gel was washed 3x 5 min in distilled water (which improved the sensitivity significantly) and imaged immediately on a ChemiDoc MP imager (Biorad, Hercules, CA) with the standard filter in place.

Immunoblotting

magi2a$^{+/-}$ zebrafish were incrossed and offsprings were sorted for edema phenotype at 6 dpf. Larvae were euthanized using Tricaine. The tail was cut off for DNA extraction. Remaining tissue was pooled according to phenotype, and protein was extracted from these pools. Sanger sequencing confirmed genotypes with only *magi2a*$^{-/-}$ larvae in the edema pools. Only pools that contained no homozygous larvae were used as controls in the immunoblots. SDS-PAGE was performed on Bis-Tris gradient gels (4-12%) according to standard procedures and proteins were transferred to a nitrocellulose mebrane using the iBlot system (Thermo Fisher, Waltham, MA). After blocking with SuperBlock T20 (TBS) (Thermo Fisher, Waltham, MA) membranes were stained with a biotinylated polyclonal rabbit MAGI2 antibody (ARP61404_P050, Aviva Systems Biology, San Diego, CA) targeting a N-terminal region of human MAGI2 (aa 223-282) over night at 4°C, followed by incubation with streptavidin-HRP complex (DY998, R&D

systems, Minneapolis, MN). For loading control, membranes were stained with a monoclonal mouse GAPDH antibody (sc47724, Santa Cruz Biotechnology, Dallas, TX) over night at 4°C, followed by incubation with an HRP-tagged donkey anti-mouse antibody (sc-2314, Santa Cruz Biotechnology, Dallas, TX).

Densitometry

Densitometry for DBF staining and immunoblotting was performed in Image Lab 4.1 (Biorad, Hercules, CA).

qPCR

RNA from whole individual zebrafish larvae at 6 dpf was extracted using the RNeasy Micro kit (Qiagen, Germantown, MD). Larvae where euthanized, and the tail was clipped for Sanger based genotyping. The rest of the larva was lysed in RLT lysis buffer by vigorous pipetting, and frozen at -80°C until the genotype was determined. Lysates from 7 larvae were pooled per genotype and RNA was extracted according to the manufacturer's instructions. cDNA was generated from 1 ug of RNA using the ProtoScript II First Strand cDNA Synthesis Kit (NEB, Ipswich, MA) according to the manufacturer's instructions. qPCR was performed with iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA) on a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. qPCR for *magi2a* was performed with primers spanning the exon-exon junction of exon 4 and 5 (forward primer: CAAGTCCGTCAGCAACATGG, reverse primer: TAGGGGCATCTGTGGAGGTC), and normalized by *gaph* expression (pre-tested primers, assay ID: qDreCED0021000, Biorad).

Whole mount in situ hybridization (WISH)

WISH was performed in larvae of different ages (4 dpf and 6 dpf) following a WISH standard protocol.[7] Antisense and sense probes for *magi2a* and *magi2b* were amplified from zebrafish poly-A larval cDNA with primers containing a T7 (antisense probe) or SP6 (sense probe) promoter. We used the DIG RNA Labeling Kit (SP6/T7) (11175025910; Roche, Westborough, MA) to transcribe an antisense (T7) and a sense probe (SP6). Forward (GAGATTTAGGTGACACTATAGCCCCCGAAGAATGCAAGGAA) and reverse (GAGTAATACGACTCACTATAGGGAAGAACAAACTGTGCCTCCTCT) primers for *magi2a* were designed as described before.[8] Probes for *magi2b* were designed by using the following forward (GAGATTTAGGTGACACTATAGCATACGTGACAACCTCTACC) and reverse (GAGTAATACGACTCACTATAGGGGGTTCCTCTCTGAATGAC) primer.

Larvae were euthanized using Tricaine, and fixed at indicated stages in 4 % paraformaldehyde (PFA) overnight at 4°C, dehydrated through a series of increasing concentration of methanol and stored at -20°C.

Larvae were rehydrated in graded methanol series, washed in PBS and permeabilized with proteinase K (20ug/ml), followed by washes in 4% PFA and PBS. Larvae were transferred in hybridization mix (HM; 50% Formamide, 5x SSC, 0.1% Tween, 50 ug Heparin, 500 ug/ml tRNA) for 2 hours at 70°C and incubated in 100 ng of labelled RNA probe at 70°C overnight. The following day, larvae were washed with decreasing concentration of 2x SSC at 70°C and afterwards incubated in blocking buffer for several hours. Thereafter, larvae were incubated with anti-Dig antibody (1:10,000 in blocking

buffer, 11093274910; Roche, Westborough, MA) overnight at 4°C, washed in PBST (0.1 % Tween) and incubated with alkaline Tris buffer (ATB) (100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20). The staining solution was prepared with NBT and BCIP (S3771, Promega, Madison, WI) according to manufacturer's instructions and added to the ATB. Larvae were incubated with staining solution for several hours until the staining reaction was sufficient and washed several times at room temperature with PBST (0.1 % Tween) to stop the staining reaction. Stained larvae were kept in 100% glycerol at 4°C in the dark before imaging.

Drug treatment experiments

Embryos were generated by timed breedings and kept in fish water containing 0.002% methylene blue until 24 hpf followed by fish water only. Embryos were dechorionated mechanically with dissection forceps at 48 hpf, and distributed randomly in treatment groups. Drugs or vehicle control were added to the fish water at 48 hpf and changed every other day. Larvae were monitored twice a day until 9 dpf (8 dpf for Tacrolimus and Cyclosporine). Larvae presenting with edema were euthanized, and DNA was extracted followed by Sanger sequencing.

Dexamethasone (Sigma, St. Louis, MO), Prednisolone (Medisca, Plattsburgh, NY), Tacrolimus (Medisca, Plattsburgh, NY) and Cyclosporine A (Medisca, Plattsburgh, NY) were dissolved in DMSO. Fish water of treatment groups and vehicle control contained 0.001% DMSO each. ROCK inhibitor Y-27632 (STEMCELL Technologies,Cambridge, MA) and lysophosphatidic acid (LPA; Santa Cruz Biotechnology, Dallas, TX) were dissolved in phosphate buffered saline (PBS). Fish water of treatment groups and

vehicle control contained 0.1% PBS each. RHO Activator II (Cytoskeleton, Denver, CO) was dissolved in water and treatment groups were compared to untreated controls.

Imaging

Larval imaging was performed on a Leica M205 FA stereoscope equipped with a Leica DFC 300 FX camera (Leica Microsystems, Wetzlar, Germany) at 5 and 21 dpf.

Electron microscopy

Zebrafish larvae were euthanized using Tricaine (0.4–0.8 mg/ml). The tail was separated for DNA extraction. The body was fixed in 5.0% glutaraldehyde 2.5% paraformaldehyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) for 24h at 4°C, washed in 0.1M cacodylate buffer and postfixed with 1% Osmiumtetroxide (OsO_4)/1.5% Potassiumferrocyanide($KFeCN_6$) for 1 hour, washed in water 3x and incubated in 1% uranyl acetate in maleate buffer for 1hr followed by 3 washes in maleate buffer and subsequent dehydration in grades of alcohol (10min each; 50%, 70%, 90%, 2x10min 100%). The samples were then put in propyleneoxide for 1 hr and infiltrated ON in a 1:1 mixture of propyleneoxide and TAAB 812 Resin mixture (Marivac Canada Inc. St. Laurent, Canada). The following day the samples were embedded in TAAB 812 Resin mixture and polymerized at 60 degrees C for 48 hrs. Ultrathin sections (about 80 nm) were cut on a Reichert Ultracut-S microtome, picked up on to copper grids stained with lead citrate and examined in a JEOL 1200EX Transmission electron microscope equipped with an AMT 2k CCD camera.

Light microscopy and staining

Zebrafish larvae were euthanized using Tricaine (0.4–0.8 mg/ml). The tail was separated for DNA extraction and the body was fixed in 4% paraformaldehyde in PBS for 24h at 4°C. After 3 washes in PBS, samples were decalcified in 0.5 mM EDTA for 3 days at room temperature. The tissue was dehydrated in an ethanol row followed by 2 incubations in 100% xylene, and incubated in paraffin over night at 60°C with consecutive embedding. Transverse 8 µm sections were obtained on a Leica RM2255 microtome (Leica Microsystems, Wetzlar, Germany). Resin embedded samples were processed as indicated above for EM, but 0.5 µm sections were obtained. H&E and toluidine blue staining were performed according to standard procedures. Imaging was performed on a Nikon Eclipse N*i* compound microscope equipped with a Nikon DS-Fi2 camera (Nikon Instruments, Melville, NY).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism® (version 7.00; GraphPad Software, Inc, La Jolla, CA). Significance was calculated using unpaired t-test (two-tailed) and a standard confidence interval of 95% to perform proteinuria assay, and determine areas of glomerular surface and nuclei in μm^2. Significance was calculated using unpaired one-way ANOVA with multiple comparisons and a standard confidence interval of 95% for qPCR. Post hoc analysis was performed according to Tukey. Kaplan-Meier-blots for onset of edema and survival were analyzed by Log-rank (Mantel-Cox) test (standard confidence interval of 95%).

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