CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

MAGI-2 Is Critical for the Formation and Maintenance of the Glomerular Filtration Barrier in Mouse Kidney

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Accepted for publication
June 26, 2014.

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Membrane-associated guanylate kinase inverted 2 (MAGI-2) is a tight junction protein in epithelial tissues. We previously reported the detailed expression patterns of MAGI-2 in mouse tissues, including kidney podocytes, based on results obtained from Venus knock-in mice for *Magi2* locus. In the present study, homozygous deletion of the *Magi2* gene in mice caused neonatal lethality, which was explained by podocyte morphological abnormalities and anuria. Immunohistological analysis showed that loss of MAGI-2 function induced a significant decrease in nephrin and dendrin at the slit diaphragm of the kidney, although other components of the slit diaphragm were unchanged. Furthermore, nuclear translocation of dendrin was observed in the podocytes of the MAGI-2-null mutants, along with enhanced expression of cathepsin L, which is reported to be critical for rearrangement of the actin cytoskeleton in podocytes. Expression analysis of the null mutants showed that loss of MAGI-2 function induces abnormal expression of various types of adhesion-related molecules. The present study is the first to demonstrate that MAGI-2 has a critical role in maintaining the functional structure of the slit diaphragm and that this molecule has an essential role in the functioning of the kidney filtration barrier. (*Am J Pathol* 2014, 184: 2699–2708; <http://dx.doi.org/10.1016/j.ajpath.2014.06.019>)

Membrane-associated guanylate kinase inverted 2 (MAGI-2) is a multi-domain scaffolding protein containing multiple PDZ and WW domains. Although their function is not fully understood, these domains work as key components in the binding to proline-rich and arginine-rich proteins. *In vivo*, the MAGI-2 molecule is expected to have an important function in neuronal tissues, such as brain, where it is highly expressed. Consistently, MAGI-2 [alias synaptic-scaffolding molecule (S-SCAM)] has been found to localize to the postsynaptic density area of the spine.¹ Furthermore, MAGI-2 has been reported to bind to *N*-methyl-D-aspartate receptor (a glutamate receptor) and is localized to both excitatory and inhibitory synapses,² both observations supporting a putative neuronal role for MAGI-2.

The knockout mouse is a powerful tool for elucidating molecular function *in vivo*. Mice lacking MAGI-2 exhibit abnormal elongation of dendritic spines, suggesting that this protein has an important role during morphogenesis of neurons.³ However, particular attention needs to be given to

Magi2 gene expression, because the protein molecule has three splicing variants: MAGI-2 α , MAGI-2 β , and MAGI-2 γ .⁴ Knockout mice lacking MAGI-2 exhibit loss of function of only the α variant; the other two splicing forms remain intact. Thus, the functions mediated by the other splicing variants of MAGI-2 remain unknown.

To understand the *in vivo* functions of MAGI-2, we previously studied a mutant mouse having a Venus fluorescence reporter cassette inserted in exon 6 at the *Magi2* locus (Venus knock-in mouse for *Magi2*) via the homologous recombination technique. Because exon 6 of *Magi2* is common to all three variants of the protein, we can use Venus fluorescence to visualize expression of all three splicing variants of MAGI-2. Venus is a mutant form of the

Supported in part by Ministry of Education, Culture, Sports, Science and Technology of Japan Grants-in-Aid for Scientific Research 20380058 and 23380055 (K.N.) and 23591201 (K.A.).

Disclosures: None declared.

enhanced yellow fluorescent protein, with a 30-fold greater intensity than the original form.⁵ Because of this increased fluorescence of Venus, we were able to detect the detailed tissue distribution of MAGI-2.⁶ In brief, strong MAGI-2 expression was observed in the brain, with moderate expression in the testis and kidney. Given that exon 6 is common to all three splicing variants, insertion of the Venus reporter cassette into exon 6 is expected to cause loss of function of all forms of MAGI-2 protein. With this idea in mind, we generated homozygous crosses with the Venus knock-in mouse. We observed interesting phenotypes indicative of unknown *in vivo* functions of MAGI-2 protein.

Materials and Methods

Generation of *Magi2*^{-/-} Mice

Construction of the targeting vector and generation of *Magi2* (<http://www.ncbi.nlm.nih.gov>; GenBank accession number NM_001170746) knockout mice were performed as described previously.⁷ All animal studies were conducted with the authorization of the Institutional Animal Care and Use Committee of Tohoku University.

Primers

The primers used for *Magi2* genotyping were forward (F1) 5'-AATAAAAATAGCTGCTTTGAGGACAGGGAG-3', reverse (R1) 5'-GTCAAATAGAACCCACAGGGATGACAAAGA-3', and reverse (R3) 5'-ATAGACGTTGTGCTGTTGTAGTTGTACTC-3'. The primers used for *Magi2* RT-PCR and quantitative real-time PCR were forward 5'-ACAAAGCCTGAGGAGAACGA-3' and reverse 5'-CCAGCCATATGGAAGCTCAT-3'. Other primers were as follows: *Rplp0* (alias *Arbp*) (<http://www.ncbi.nlm.nih.gov>; GenBank accession number NM_007475) forward 5'-TGTGTGTCTGCAGATCGGGTAC-3' and reverse 5'-CTTTGGCGGGATTAGTCGAAG-3'; *Nphs1* (<http://www.ncbi.nlm.nih.gov>; GenBank accession no. NM_019459) forward 5'-GCCACCACCTTCACACTGAC-3' and reverse 5'-AGACCACCAACCGCAAAGAG-3'⁸; and *Ctst* (<http://www.ncbi.nlm.nih.gov>; GenBank accession no. NM_009984) forward 5'-GTGGACTGTTCTCACGCTCA-3' and reverse 5'-TATCCACGAACCTGTGTCA-3'.⁹

Antibodies

The following primary antibodies were used for immunohistochemistry or immunoelectron microscopy: rat anti-MAGI-2 antibody (dilution 1:100; antibody raised as the product between PDZ2 and PDZ3), guinea pig anti-nephrin antibody (1:100; GP-N2; Progen Pharmaceuticals, Darra, Australia), rabbit anti-nephrin antibody [1:1000; provided by Dr. Kan Katayama (Karolinska Institute, Stockholm, Sweden)], rabbit anti-dendrin antibody (1:50; provided by K.A.),¹⁰ rabbit anti-WT-1 antibody (1:25; sc-192; Santa

Cruz Biotechnology, Dallas, TX), mouse anti-cathepsin L monoclonal antibody (1:50; ab6314; Abcam, Cambridge, UK), mouse anti-synaptopodin monoclonal antibody (1:10; G1D4; Progen Pharmaceuticals), rabbit anti-podocin antibody (1:300; provided by K.A.),¹⁰ rabbit anti-CD2AP antibody [1:300; provided by Dr. Andrey Shaw (Washington University School of Medicine, St. Louis, MO)], rat anti-podocalyxin antibody (1:50; KR064; TransGenic, Kumamoto, Japan), rabbit anti-ZO-1 antibody (1:100; 40-2200; Life Technologies, Carlsbad, CA), mouse anti- β catenin monoclonal antibody (1:100; BD Transduction Laboratories 610154; BD Biosciences, San Jose, CA), rabbit anti-claudin-5 antibody (1:100; ab53765; Abcam), and rabbit anti-claudin-1 antibody (1:200; ab15098; Abcam). The following secondary antibodies were used for immunohistochemistry or immunoelectron microscopy: Alexa Fluor 488-, 555-, or 594-conjugated secondary antibodies (1:300 to 1:500; Life Technologies), 5 nm gold-conjugated goat anti-guinea pig IgG (1:100; EM.GAG5; BBI Solutions, Cardiff, UK), and 5 nm gold-conjugated anti-rabbit IgG (1:100; EM.GAR5; BBI Solutions).

Genotyping

A 5-mm section cut from the tip of the mouse tail was digested in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 5 mmol/L dithiothreitol, 0.5 mmol/L spermidine, 1% SDS with 0.2 mg/mL proteinase K] overnight at 58°C. After lysis, solubilized DNA was concentrated by isopropanol precipitation, rinsed with 70% ethanol, dried, and re-extracted with Tris-EDTA buffer. For Southern blot analysis, 3 μ g of genomic DNA extracted from the tail was digested with HindIII endonuclease and loaded onto 1% agarose gels. These DNA samples were subjected to electrophoresis and then were transferred to Biodyne B nylon transfer membranes (Pall, Port Washington, NY). The membranes were hybridized to a ³²P-labeled probe. The 3'-probe was obtained by digestion with an endonuclease and was labeled with an Amersham Megaprime DNA labeling system (GE Healthcare, Little Chalfont, UK) with [³²P]-dCTP. For PCR analysis, TaKaRa Taq polymerase (Takara Bio, Otsu, Japan) and primers as described above were used. The wild-type allele yields a PCR amplicon of 550 bp with primers F1 and R1; primers F1 and R3 generate a 730-bp product from the knockout allele.

Detection of Transcription Product

For Northern blot analysis, *Magi2* probes spanned from 2273 to 3490 nucleotides (1218 bp) in the mouse *Magi2* mRNA. mRNA preparation and Northern blot analyses were performed as described previously.⁶ For RT-PCR, cDNA synthesis was performed with SuperScript III Reverse Transcriptase according to the manufacturer's protocol (Life Technologies). cDNA synthesis for quantitative real-time RT-PCR was performed using PrimeScript RT master mix

(Takara Bio) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio) and specific primers for *Nphs1* and *Ctsl* in the a Dice real-time thermal cycler system (Takara Bio). Normalization across samples was to the average expression of the constitutive *Arbp* gene.

Measurement of Plasma Creatinine

Plasma creatinine levels were measured using a dry-chemistry autoanalyzer (DRI-CHEM 3500V; Fuji Film, Tokyo, Japan). Breathing neonatal mice were decapitated and approximately 20 μ L of blood was immediately collected with a Pipetman pipette (Gilson, Madison, WI). The blood sample was centrifuged ($700 \times g$) for 5 minutes at room temperature to prepare plasma supernatant. A 10- μ L plasma sample was further processed with the autoanalyzer according to the manufacturer's protocol.

Scanning Electron Microscopy

Kidney samples (approximately 2 mm³) were immersed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 hours. Next, the samples were postfixed in 2% OsO₄ in 0.1 mol/L phosphate buffer for 2 hours and then stained with 1% tannic acid in 0.1 mol/L phosphate buffer for 1 hour at room temperature. Tissues were dehydrated in ethanol and freeze-dried with *tert*-butyl alcohol in a freeze dryer (ES-2030; Hitachi, Tokyo, Japan). After drying, samples were coated with platinum and palladium and were visualized with a scanning electron microscope (S-4200; Hitachi) at an accelerating voltage of 15 kV.

Transmission Electron Microscopy

Samples for transmission electron microscopy were prepared as for scanning electron microscopy, up to the staining with tannic acid. After dehydration in ethanol, samples were embedded in epoxy resin (TAAB Laboratories, Reading, England). Ultrathin sections (approximately 80 nm) were cut with an ultramicrotome (Reichert-Nissei Ultracut S; Leica, Vienna, Austria) and mounted on a copper grid. The sections were stained in uranyl acetate for 1 hour and in lead citrate for 7 minutes at room temperature. Grids were viewed with a transmission electron microscope (H-7650; Hitachi) at an accelerating voltage of 100 kV.

Immunohistochemistry

The kidneys were removed, cut, and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) on ice for 15 minutes and incubated in 20% sucrose in 0.1 mol/L PBS until the tissues sank to the bottom of tube on ice. The tissues were embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan), snap-frozen in liquid nitrogen, and sectioned (5 μ m thick) in

a cryostat. Tissue sections were mounted on MAS-coated glass slides (Matsunami, Tokyo, Japan) and blocked with blocking solution (2% fetal calf serum, 2% bovine serum albumin, 0.2% fish gelatin in 0.1 mol/L PBS) for 30 minutes at room temperature. Primary antibodies diluted in blocking solution were added for 1 hour at room temperature and visualized using secondary antibodies diluted in blocking solution for 1 hour at room temperature. Sections were stained with DAPI to counterstain the nuclei, sealed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), covered, and imaged using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Histological Analysis

Kidneys were removed, cut, and fixed in 4% paraformaldehyde in 0.1 mol/L PBS on ice overnight. After dehydration, the kidneys were embedded in paraffin. Paraffin blocks were sectioned at 5- μ m or 2- μ m thickness, and stained with hematoxylin and eosin or with periodic acid–Schiff for histological evaluation by light microscopy, respectively.

Immunoelectron Microscopy

Kidney samples (approximately 2 mm³) were immersed in 4% paraformaldehyde in 30 mmol/L HEPES buffer (pH 7.4) for 2 hours on ice. After fixation, the tissues were dehydrated in ethanol and embedded in LR White resin (London Resin, Reading, UK). Ultrathin sections (approximately 100 nm) were cut with an ultramicrotome and mounted on a nickel grid. Immunostaining methods were as described above. After staining, grids were blocked in 1% bovine serum albumin in PBS and then postfixed in 2% glutaraldehyde in 0.1 mol/L PBS. Electron microscopy staining was performed with platinum blue solution (TI-Blue staining kit; Nisshin EM, Tokyo, Japan).¹¹ Grids were viewed with a transmission electron microscope (H-7650; Hitachi).

Results

Generation of *Magi2*^{-/-} Mice

We generated *Magi2* knockout mice (*Magi2*^{-/-} mice) in which exon 6 (encoding the first WW domain) was replaced by the Venus fluorescent protein via Cre-mediated recombination (Figure 1A). Because of the replacement of exon 6, the mutant protein is expected to lose all of the WW domain and most of the PDZ domain. The genomic structure of the disrupted allele of the *Magi2* gene locus was confirmed by Southern blot analysis (Figure 1B) and by PCR amplification (Figure 1C). Furthermore, Northern blot analysis demonstrated a significant reduction of *Magi2* transcripts in the heterozygous mutant, but no transcripts in *Magi2*^{-/-} mice (Figure 1D). In addition, the results of the Northern blot analysis were consistent with those of semiquantitative

Figure 1 Generation of *Magi2*^{-/-} mice. **A:** Wild-type, targeted, and knockout *Magi2* loci, and gene targeting constructs. Exons and *Magi2* cDNA are indicated by white boxes. The reporter cassette encoding the Venus protein was inserted into exon 6 of the *Magi2* gene. As a result, the 5'-portion of exon 6 (25 bp) was connected to the Venus reporter and SV40 polyadenylation sequences. LoxP (gray triangles) and FRT (white ellipses) are also shown, along with positions of restriction enzyme sites and the probes used for Southern blot analyses. **B:** Southern blot analysis of genomic DNA of littermate progeny from *Magi2*^{+/-} crosses. HindIII-digested tail DNA was hybridized with the radiolabeled probes shown in **A**. **C:** PCR analysis of genomic DNA from mice offspring of *Magi2*^{+/-} crosses for the knockout (730 bp) and wild-type (550 bp) alleles. **D:** Northern blot analysis of poly(A)⁺ RNA (2 µg per lane) from the brain of *Magi2*^{+/+}, *Magi2*^{+/-}, and *Magi2*^{-/-} mice (**D**). The blot was sequentially hybridized with *Magi2* and *Gapdh* cDNA probes. RT-PCR analysis of cDNA from the brain of *Magi2*^{+/+}, *Magi2*^{+/-}, and *Magi2*^{-/-} mice (**E**). *Arbp* was used as internal control. E, exon; F, forward primer; H, HindIII; I, intron; KO, knockout; MC1, MC1 promoter; Neo, neomycin-resistance gene; R, reverse primer; TK, thymidine kinase gene; WT, wild-type; 3× pA, 3× Simian virus 40 late polyadenylation signal.

RT-PCR analysis (Figure 1E). From these observations, we concluded that the mutant *Magi2* allele that we had constructed causes the functional loss of MAGI-2 protein.

Magi2^{-/-} Mice Are Neonatal Lethal

The genotypes of offspring from heterozygous mouse crosses at postnatal day 0 (P0) completely followed Mendelian rules of inheritance, with a 63:128:67 ratio of *Magi2*^{+/+}, *Magi2*^{+/-}, and *Magi2*^{-/-} offspring (Table 1). The *Magi2*^{-/-} offspring showed no obvious abnormalities in gross appearance at P0 (Supplemental Figure S1A), nor did their body weight differ significantly from that of heterozygous and wild-type mice. Furthermore, the weight of

several major organs of *Magi2*^{-/-} offspring did not exhibit any abnormality, relative to heterozygous and wild-type mice (Supplemental Figure S1, B–F). However, no *Magi2*^{-/-} mice survived to the day of weaning (3 weeks after birth) (Table 1). We therefore concluded that *Magi2*^{-/-} mice are neonatal lethal.

Magi2^{-/-} Mice Exhibit Anuria and Increased Plasma Creatinine

MAGI-2 protein is highly expressed in certain organs such as brain, kidney, and testis.⁶ In the present study, we examined renal function in *Magi2*^{-/-} mice. In P0 mice, we measured plasma creatinine (which is a breakdown product of creatine phosphate in muscle) as a biomarker of renal function. Most creatinine is filtered out from the blood by the kidney via glomerular filtration and proximal tubular secretion. If filtering by the kidney is deficient or ineffective, the level of creatinine in the blood becomes abnormally high. Plasma creatinine levels of *Magi2*^{-/-} mice were significantly higher (fourfold) than in *Magi2*^{+/+} mice (Figure 2A), suggesting that renal function was impaired in *Magi2*^{-/-} mice. In other types of knockout mice, functional abnormalities of the slit diaphragm frequently result in proteinuria.^{12–16} We therefore tried to collect the urine of *Magi2*^{-/-} mice, but could not detect any in the bladder (Figure 2B), indicating severe impairment of renal function. To address kidney abnormalities at the histological level, we examined kidney tissues under a light microscope after hematoxylin and eosin staining and periodic acid–Schiff staining. We did not observe any histological abnormalities of glomeruli in *Magi2*^{-/-} mice (Supplemental Figure S2).

Magi2^{-/-} Mice Have Abnormal Podocyte Foot Process Morphology

To address the pathological mechanisms of abnormal renal function in *Magi2*^{-/-} mice, we performed morphological analysis of the glomeruli at the ultrastructure level by scanning electron microscopy, using the glomeruli of wild-type mice as the control. In wild-type mice, the normal interdigitating network of the foot processes was observed in the glomeruli (Figure 3, A and B). However, *Magi2*^{-/-} mice exhibited abnormal morphology of foot processes (Figure 3, C and D): the foot processes were shortened, and the interdigitating network was not well organized.

Table 1 Mendelian Inheritance of Pups Produced by Breeding Pairs of *Magi2*^{+/-} Mice and Neonatal Lethality of *Magi2*^{-/-} Pups

Age	n	Genotype			Ratio
		+/+	+/-	-/-	
Newborn (P0)	258	63	128	67	1.00:2.03:1.06
Weaning (3 wk)	424	138	286	0	1.00:2.07:0.00

The genotypes of newborn pups and weaned pups were analyzed separately.

Figure 2 *Magi2*^{-/-} mice exhibit increased plasma creatinine and anuria. **A:** The plasma creatinine level of *Magi2*^{-/-} mice (black bars) was approximately fourfold higher than that of *Magi2*^{+/+} (white bars) or *Magi2*^{+/-} (gray bars) mice. **B:** *Magi2*^{-/-} mice exhibited a lack of urine in the bladder, in contrast to *Magi2*^{+/+} and *Magi2*^{+/-} mice. Data are expressed as means $\pm$ SEM. $n = 3$ (+/+), $n = 6$ (-/-); $n = 8$ (+/+), $n = 17$ (+/-), $n = 24$ (+/-). * $P < 0.05$; *** $P < 0.001$, Student's *t*-test.

To further address the glomerular abnormalities of *Magi2*^{-/-} mice, we examined the ultrastructure of glomeruli with transmission electron microscopy. The arrangement and morphology of podocyte cell bodies looked normal in both *Magi2*^{+/+} and *Magi2*^{-/-} mice (Figure 3, F and I). Slit diaphragms (Figure 3, G and H) are ordinarily observed as

gaps between foot processes, which normally exit at the basal side in *Magi2*^{+/+} mice. Surprisingly, we could not observe clear slit diaphragms in glomeruli of *Magi2*^{-/-} mice, because fusion and/or adhesion of the foot processes had occurred (Figure 3, J and K). For quantitative evaluation, we determined the gap space between foot processes in *Magi2*^{+/+} and *Magi2*^{-/-} mice (Figure 3E). The gap space in *Magi2*^{-/-} mice was significantly narrowed. From these results, we concluded that the slit diaphragms in *Magi2*^{-/-} mice are impaired.

Nephrin and Dendrin Expression in the Glomeruli of *Magi2*^{-/-} Mice Is Dramatically Reduced

The slit diaphragm is composed of a complex of several proteins. One of these, nephrin, is a transmembrane protein that is a main structural component of the slit diaphragm.¹⁷ MAGI-2 is reported to interact with slit diaphragm components, including nephrin.¹⁸ Interestingly, loss of function of components in the slit diaphragm sometimes affects expression of other components. For example, Yaddanapudi et al¹⁹ reported that loss of function of CD2AP induces a dramatic reduction of dendrin, which is another component of the slit diaphragm.

Using immunohistochemistry, we confirmed the localization of MAGI-2 protein at the slit diaphragm in wild-type

Figure 3 *Magi2*^{-/-} mice exhibit abnormal morphology of podocyte foot processes. **A–D:** Scanning electron micrographs of podocyte foot processes in *Magi2*^{+/+} mice (**A** and **B**) and *Magi2*^{-/-} mice (**C** and **D**). *Magi2*^{-/-} mice exhibit abnormal morphology. Boxed regions in **A** and **C** are shown at higher magnification in **B** and **D**, respectively. **E:** The distance between foot processes is dramatically decreased in *Magi2*^{-/-} mice (black bars), compared with *Magi2*^{+/+} (white bars). **F–K:** Microstructure of slit diaphragm in *Magi2*^{+/+} mice (**F–H**) and *Magi2*^{-/-} mice (**I–K**) by transmission electron microscopy. In **H**, the slit diaphragm is indicated by arrowheads. Data are expressed as means $\pm$ SEM. $n = 46$ (+/+); $n = 37$ (-/-). *** $P < 0.001$, Student's *t*-test. Scale bars: 10 μ m (**A**, **C**, **F**, and **I**); 1 μ m (**B**, **D**, **G**, and **J**); 200 nm (**H** and **K**). CL, capillary lumen; En, endothelial cell; FP, foot process; GBM, glomerular basement membrane; Po, podocyte; R, red blood cell.

Figure 4 Nephrin and dendrin are dramatically reduced in glomeruli of *Magi2*^{-/-} mice. **A** and **B**: Immunodetection of MAGI-2 (**A**) and MAGI-2 with nephrin (**B**) in *Magi2*^{+/+} mice. The expression patterns of MAGI-2 and nephrin were almost perfectly overlapping. **C**: MAGI-2 was not detected in *Magi2*^{-/-} mice. **D–I**: Nephrin and dendrin levels were normal in the slit diaphragm of *Magi2*^{+/+} mice (**D–F**), but a remarkable reduction of these molecules was observed in *Magi2*^{-/-} mice (**G–I**). Dendrin translocated into nuclei in *Magi2*^{-/-} mice (**H** and **I**, arrowheads). WT-1 was used as a marker for the podocyte nucleus (**F** and **I**). Scale bar = 20 μm.

mice (Figure 4A), which suggested that it functions in the slit diaphragm in the wild-type condition. To investigate localization of MAGI-2 in the slit diaphragm of *Magi2*^{+/+} mice, we used double staining with antibodies against MAGI-2 and nephrin. The results showed identical localization of MAGI-2 and nephrin (Figure 4, A and B), indicating the potential association of MAGI-2 protein with slit diaphragm—composing proteins, such as nephrin. As expected, MAGI-2 was not detected in *Magi2*^{-/-} mice (Figure 4C). MAGI-2 was not observed in renal tubules (data not shown).

The expression of further components of the slit diaphragm was detected by immunohistochemistry, including dendrin, CD2AP, podocin, synaptopodin, WT-1, and podocalyxin. All of these components were detected at the slit diaphragm or in the nuclei of podocytes in *Magi2*^{+/+} mice (Figure 4, D–F, and Supplemental Figure S3, A–D, I). However, expression of nephrin and dendrin was dramatically reduced in *Magi2*^{-/-} mice, although other components (podocin, synaptopodin, WT-1, CD2AP, and podocalyxin) were present at near-normal levels (Figure 4, G–I, and Supplemental Figure S3, E–H, L). In addition, dendrin was detected in the nuclei in *Magi2*^{-/-} mice (Figure 4, H and I). From these results, we concluded that loss of function of MAGI-2 protein affects the expression level and cellular localization of diaphragm-related proteins, including nephrin and dendrin.

Loss of Function of MAGI-2 Results in Elevated Expression of CatL

MAGI-2-deficient mice exhibited a dramatic reduction in nephrin and dendrin expression, along with nuclear translocation of dendrin. Yaddanapudi et al¹⁹ reported that translocation of dendrin induced elevated expression of the lysosomal cysteine proteinase cathepsin L (CatL) in kidney podocytes. We examined CatL expression in kidney by real-time RT-PCR. *Ctsl* mRNA (*Ctsl* encodes CatL) was measured in total RNA obtained from *Magi2*^{+/+} and *Magi2*^{-/-} mice (Figure 5A) and was found to be significantly higher in *Magi2*^{-/-} mice than in *Magi2*^{+/+} mice. We also evaluated expression of *Nphs1* mRNA (*Nphs1* encodes nephrin) and found almost identical expression in *Magi2*^{+/+} and *Magi2*^{-/-} mice. The expression level of *Nphs1* mRNA measured by RT-PCR (Figure 5A) did not agree with that indicated by immunostaining (Figure 4, D and G), perhaps because of accelerated degradation of nephrin protein in *Magi2*^{-/-} mice.

To provide further evidence supporting a reduced level of nephrin in the slit diaphragm of *Magi2*^{-/-} mice, we performed

Figure 5 MAGI-2-induced expression of cytosolic CatL is defective. **A**: Quantitative RT-PCR of *Nphs1* and *Ctsl* indicates that *Ctsl* mRNA significantly increases in *Magi2*^{-/-} (black bars) mice, compared with that of *Magi2*^{+/+} mice (white bars), but *Nphs1* mRNA levels in *Magi2*^{-/-} mice are not significantly changed. **B–G**: Immunohistochemical analysis of CatL and synaptopodin. Up-regulation of CatL is observed in the slit diaphragm of *Magi2*^{-/-} mice. Synaptopodin was used as a slit-diaphragm marker. Data are expressed as means ± SEM. ***P* < 0.01, Student's *t*-test. Scale bar = 20 μm. CatL, cathepsin L.

Figure 6 Immunoelectron micrographs of slit diaphragms. **A–D:** MAGI-2 immunological signals (arrowheads) were detected in slit diaphragms of *Magi2*^{+/+} mice (**A** and **B**), but not those of *Magi2*^{-/-} mice (**C** and **D**). **E–H:** Nephrin immunological signals (arrowheads) were detected in slit diaphragms of *Magi2*^{+/+} mice (**E** and **F**) and in *Magi2*^{-/-} mice, in which the signals were decreased and ectopic localization was observed (**G** and **H**, arrowheads). Boxed regions are shown at higher magnification in the adjacent panel with a matching letter. Scale bars: 1 μm (**A**, **C**, **E**, and **G**); 100 nm (**B**, **D**, **F**, and **H**).

immunoelectron microscopy using immunogold-labeled antibody. MAGI-2 and nephrin signals were detected in the slit diaphragms of *Magi2*^{+/+} mice (Figure 6, **A**, **B**, **E**, and **F**). In *Magi2*^{-/-} mice, however, the MAGI-2 signal was not detected, and nephrin expression was both decreased and ectopic (Figure 6, **C**, **D**, **G**, and **H**). To further address the effect of loss of MAGI-2 function, we examined CatL in the kidney of *Magi2*^{+/+} and *Magi2*^{-/-} mice by immunostaining. CatL is normally expressed in glomeruli of *Magi2*^{+/+} mice (Figure 5, **B** and **D**). Interestingly, relatively high expression of CatL was observed in glomeruli of *Magi2*^{-/-} mice (Figure 5, **E** and **G**), compared with compared with the wild type. Synaptopodin was used as a slit-diaphragm marker (Figure 5, **C** and **F**). CatL significantly colocalized with synaptopodin.

MAGI-2 Defect Alters Expression Patterns of Cell Adherence Molecules

We next focused on change in cytosolic CatL, which is reported to induce cleavage of the dynamin GTPase and the actin-associated adapter synaptopodin, resulting in rearrangement of the actin cytoskeleton in the foot process. We suspected that there might be some alteration in the distribution of cytoskeletal-related protein in the foot process. We performed immunostaining of tight junction proteins in the kidney podocyte and detected the localization of claudin-5 and ZO-1 at the slit diaphragm in *Magi2*^{+/+} mice (Figure 7, **A** and **B**, and Supplemental Figure S3J). In *Magi2*^{-/-} mice, interestingly, claudin-5 was detected not only in the slit diaphragm (merged

Figure 7 MAGI-2 defect alters expression patterns of cell adherence molecules. Immunohistochemical analyses of claudin-5 and synaptopodin (**A–D**) and claudin-1 and podocin (**E–H**). In *Magi2*^{-/-} mice, claudin-5 is detected not only in slit diaphragms, but also between the contact surfaces of podocyte cell bodies, as seen at higher magnification (**D**, inset). Fluorescence intensity of claudin-1 in the slit diaphragms of *Magi2*^{-/-} mice is slightly enhanced compared with that in *Magi2*^{+/+} mice (**H**). Synaptopodin and podocin were used as slit-diaphragm markers. Scale bar = 20 μm.

Figure 8 Schematics for the diaphragm of the glomerulus in *Magi2*^{+/+} and *Magi2*^{-/-} mice. **A:** Structure of the glomerulus. The boxed region is shown in detail in **B** and **C**. **B–E:** In wild-type mice (**B** and **D**), the slit diaphragm, which consists of a gap between podocyte foot processes, has an important role in producing primary urine from blood flow. In the knockout mice (**C** and **E**), the gap is blocked because of stacking and fusion of the foot processes. In **B** and **C**, the transparent plane indicates the cross-sectional area on which the corresponding detailed schematic (**D** and **E**) is based. Schematics are adapted from Ihara et al.⁶

with synaptopodin), but also between the contact surfaces of podocyte cell bodies (Figure 7, C and D). ZO-1 was intact in *Magi2*^{-/-} mice (Supplemental Figure S3M). MAGI-2 was also reported as a component of the adherens junction complex.^{22,23} However, we confirmed that the expression pattern of β -catenin did not change (Supplemental Figure S3, K and N). Instead, in the slit diaphragm of *Magi2*^{-/-} mice we observed low levels of expression of claudin-1, which is usually expressed in the epithelial cells of Bowman's capsule^{24–26} (to prevent urine leaking from the capsule) (Figure 7, E and F). From these results, we concluded that loss of functional MAGI-2 at the slit diaphragm complex results in increased CatL and potentially contributes to disordering of foot processes and to altered expression pattern of adhesion molecules.

Discussion

The potential role of MAGI-2 protein in neuronal tissue is suggested by earlier research, in which knockout of the α form of MAGI-2 gave rise to abnormal spine morphogenesis.³ However, we need to focus on the α , β , and γ splicing variants of MAGI-2, bearing in mind that insertion of the Venus reporter cassette is expected to result in loss of the important

WW and PDZ C-terminal functional domains of the MAGI-2 protein.

Previously, we reported the detailed tissue distribution of MAGI-2 in the Venus knock-in mouse.⁶ Our established Venus knock-in mouse expresses the Venus fusion protein in all splicing forms of MAGI-2, because the Venus expression cassette was inserted into exon 6, which is present in all MAGI-2 splicing forms. Furthermore, the high fluorescence intensity of the Venus protein allows visualization of the detailed distribution of all MAGI-2 forms throughout the organs. MAGI-2 was highly expressed in various types of neurons in brain. We also detected MAGI-2 protein localization in testis spermatids. In addition to the brain and testis, we also reported expression of MAGI-2 in the kidney glomerulus, and its localization in the podocyte.

In the present study, we observed neonatal lethality and anuria in our knockout mice, which reveal the critical function of the MAGI-2 protein in the kidney. We retrieved dead neonates as possible, and analyzed each genotype. Of the dead neonates analyzed, 36/52 (69%) were *Magi2*^{-/-}, whereas the genotype of living neonates at P0 completely followed Mendelian rules of inheritance (the proportion of *Magi2*^{-/-} mice was 26%). Moreover, all deaths of *Magi2*^{-/-}

mice occurred within 48 hours after birth. We therefore concluded that *Magi2*^{-/-} mice exhibit neonatal lethality, unable to survive for more than a few days after birth. Furthermore, we showed by electron microscopy on the kidney of knockout mice that the structure of the slit diaphragm was impaired. Fusion of the podocyte foot processes resulted in loss of the normal slit diaphragm structure. Normally, blood is filtered through the slit diaphragm in the kidney glomerulus to produce primary urine. The anuria phenotype we observed in our knockout mice may be explained by the abnormal morphology of the slit diaphragm. In addition, we confirmed increasing plasma creatinine levels in *Magi2*^{-/-} mice at P0. Taken together, these results indicate that the renal function of excretion of waste matter was entirely lost, and that this caused neonatal lethality of the *Magi2*^{-/-} mice.

In accord with this idea, we showed that loss of MAGI-2 protein function induces nuclear localization of dendrin and elevated expression of CatL. CatL induced cleavage of the dynamin GTPase and the actin-associated adapter synaptopodin, resulting in rearrangement of the actin cytoskeleton in the podocyte.^{20,21} We expected to find that the anuria was due to changed expression patterns in cell adherence molecules between foot processes. Interaction between MAGI-2 and tight junction or adherens junction proteins in epithelial cells has been reported.^{22,23} We performed immunostaining of claudin-5, ZO-1, and β -catenin in the kidney podocyte. Both ZO-1 and β -catenin were intact in the slit diaphragm of *Magi2*^{-/-} mice (Supplemental Figure S3, M and N); however, claudin-5 was detected not only in the slit diaphragm (merged with synaptopodin), but also between the contact surfaces of podocyte cell bodies (Figure 7, C and D). In addition, we observed low-level expression of claudin-1 in the slit diaphragm of *Magi2*^{-/-} mice (Figure 7, G and H). Alteration in expression patterns of claudin-5 and claudin-1, which are responsible for adhesion of foot processes, is one of the abnormalities that possibly caused fusion of the podocyte foot processes and the abnormal structure of the slit diaphragm, ultimately leading to the anuria phenotype in *Magi2*^{-/-} mice.

Interestingly, the MAGI-2 knockout mice exhibited decreased immunoreactivity of nephrin, compared with the wild type (Figure 4, D and G). In contrast to the immunostaining results, measurement of *Nphs1* mRNA levels showed no difference between *Magi2*^{+/+} and *Magi2*^{-/-} mice (Figure 5A). A reasonable explanation for this discrepancy would be that nephrin is regulated at the protein level. Indeed, nephrin protein is controlled at the protein level by endocytosis.^{27–29} We suggest that the loss of functional MAGI-2 accelerates the degradation of nephrin protein, but does not affect nephrin transcription.

In the present study, we discovered a critical renal function of MAGI-2 protein, using *Magi2* knockout mice. The phenotype obtained quite clearly demonstrates the importance of this gene product in the maintenance of renal function. However, there are still unanswered questions about differences in the phenotype (as, for example, the difference in proteinuria and anuria). In previous studies of slit diaphragm-related proteins, such as

nephrin and dynamin, the knockout mice exhibited proteinuria, but not anuria. Our present data cannot explain the phenotypic differences between proteinuria and anuria at the cellular and molecular level.

We summarize our findings in Figure 8. Primary urine is usually produced from blood flow at the kidney glomerulus (Figure 8A). In wild-type mice (Figure 8, B and D), the slit diaphragm, which composes the gap in the podocyte foot processes, has an important role in kidney function. The morphology of the podocyte foot process was abnormal in *Magi2*^{-/-} mice; the podocyte could not maintain the slit diaphragm structure, because of stacking and fusion of the foot processes (Figure 8, C and E). One possible molecular mechanism for the abnormal podocyte foot processes in *Magi2*^{-/-} mice is abnormal distribution of one or more types of cell adhesion-related molecule. In ongoing work, we are trying to detect expression of E-cadherin and P-cadherin, to explore further evidences of cell adhesion-related abnormalities. Although we do not yet have conclusive data, the expression status of these adhesion-related molecules may reveal the molecular pathogenesis of how functional loss of MAGI-2 affects the podocyte.

Our present study is the first to demonstrate that MAGI-2 has an essential role in kidney function and maintenance of the structure of the slit diaphragm of the glomerulus. In further studies, conditional knockouts could be created by crosses with transgenic mice expressing Cre only within the podocyte.^{30–34} Functional loss of MAGI-2 in these mutants would be specifically induced only within the podocyte; this would contribute to a fuller understanding of the phenotypic differences we observed.

Acknowledgments

We thank Dr. Kan Katayama (Karolinska Institute, Stockholm, Sweden) for anti-nephrin antibody; Prof. Andrey Shaw (Washington University School of Medicine, St. Louis, MO) for anti-CD2AP antibody; Prof. Emiko Isogai (Tohoku University, Sendai, Japan) for technical help in generating anti-MAGI-2 antibody; Terumi Shibata, Kaori Takahashi, Junichi Nakamoto, and Mitsutaka Yoshida (Juntendo University Graduate School of Medicine, Tokyo, Japan) for excellent technical assistance in electron microscopy; and Dr. Kaoru Inoue for technical support in histological analysis of kidney sections.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.06.019>.

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