**Foxc2 is essential for podocyte function**

Daniel Nilsson, Mikael Heglind, Zahra Arani & Sven Enerbäck

Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

**Keywords**

Foxc2, Nrp1, podocyte, proteinuria.

**Correspondence**

Sven Enerbäck, Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, PO Box 440, 40530 Göteborg, Sweden.
Tel: +46 (0)31 786 33 34
Fax: +46 (0)31 41 61 08
E-mail: sven.enerback@medgen.gu.se

**Abstract**

Foxc2 is one of the earliest podocyte markers during glomerular development. To circumvent embryonic lethal effects of global deletion of Foxc2, and to specifically investigate the role of Foxc2 in podocytes, we generated mice with a podocyte-specific Foxc2 deletion. Mice carrying the homozygous deletion developed early proteinuria which progressed rapidly into end stage kidney failure and death around postnatal day 10. Conditional loss of Foxc2 in podocytes caused typical characteristics of podocyte injury, such as podocyte foot process effacement and podocyte microvillus transformation, probably caused by disruption of the slit diaphragm. These effects were accompanied by a redistribution of several proteins known to be necessary for correct podocyte structure. One target gene that showed reduced glomerular expression was Nrp1, the gene encoding neuropilin 1, a protein that has been linked to diabetic nephropathy and proteinuria. We could show that NRP1 was regulated by Foxc2 in vitro, but podocyte-specific ablation of Nrp1 in mice did not generate any phenotype in terms of proteinuria, suggesting that the gene might have more important roles in endothelial cells than in podocytes. Taken together, this study highlights a critical role for Foxc2 as an important gene for podocyte function.

**Introduction**

The forkhead gene Foxc2 is a transcription factor with many vital functions during development, including skeletogenesis and patterning of the aortic branch (Iida et al. 1997). Foxc2 expression has also been identified in podocytes (Dagenais et al. 2004; Takemoto et al. 2006; Brunskill et al. 2011) and it appears as one of the earliest podocyte markers required for correct glomerular development (Takemoto et al. 2006). Using a global knockout mouse model, it has been shown that loss of Foxc2 results in downregulation of slit diaphragm-associated NPHS2 (podocin) as well as collagen IV subunits a3 and a4 (COL4A3 and COL4A4) (Morello et al. 2001; Takemoto et al. 2006), two important components of the glomerular basement membrane (Miner and Sanes 1996; Korstanje et al. 2014). Additionally, the Foxc2 global knockout had reduced glomerular levels of important factors such as rhophilin 1 (Rhpn1) and PODXL (Takemoto et al. 2006). In humans, mutations in FOXC2 have been shown to cause lymphedema-distichiasis syndrome, which in rare cases also result in renal disease (Fang et al. 2000; Erickson et al. 2001; Brice et al. 2002; Yildirim-Toruner et al. 2004). Only heterozygosity of nonsense human FOXC2 mutations have been reported pointing to a requirement of functional FOXC2 for proper development, a hypothesis strengthened by the lethal effects of complete Foxc2 deletion in mice (Iida et al. 1997; Winnier et al. 1997).

Although global knockout mouse models are useful in studying gene functions, developmental or systemic effects in these models could mask a potential later role in differentiated cells and cell-specific requirements cannot be
addressed. The latter problem is highlighted in the case of Lmx1b, another transcription factor found in podocytes (Chen et al. 1998; Dreyer et al. 1998). Similar to Foxc2, global knockout of Lmx1b in mice causes glomerular downregulation of NPHS2, COL4A3, and COL4A4 (Miner et al. 2002). However, these effects on gene expression could not be confirmed when Lmx1b was specifically deleted in podocytes even though the kidney phenotype was profound (Suleiman et al. 2007; Burghardt et al. 2013). This emphasizes the caution one should take when interpreting data from global knockout mouse models.

Conditional deletion of genes, using the Cre-lox system (Hoess et al. 1984), has proven to be an efficient way to circumvent postnatal lethality and developmental issues. Efforts have previously been made to conditionally delete Foxc2 in the kidney, using either Pax or Nephrin promoter to drive Cre expression (Motojima et al. 2016a, 2017). However, Pax2 is predominantly expressed in undifferentiated podocytes (Barienti et al. 2006), leaving the specific role of Foxc2 in differentiated podocytes in vivo unknown, whereas Nephrin promoter has been found to be active not only in podocytes but also in brain and pancreas (Moeller et al. 2000; Putaala et al. 2001).

To be able to study the specific role of Foxc2 in podocytes, we decided to generate mice with conditional Foxc2 knockout using Podocin-Cre transgenic mice (Moeller et al. 2003), a widely used model for podocyte-specific genome modifications.

**Methods**

**Mice**

Generation of Foxc2∆incZ/+ mice was described earlier (Cederberg et al. 2009). Transgenic Pod-Cre mice (alasles Nphs2-Cre or 2.5P-Cre) were a kind gift from L. Holzner (Moeller et al. 2003). Rosa26 Cre reporter strain (+3474) (Soriano 1999), and floxed Nephrin 1 (Nrp1) were genotyped as previously described (Soriano et al. 1999; Gu et al. 2003; Moeller et al. 2003; Cederberg et al. 2009). Floxed Foxc2 allele was genotyped by PCR using primers located on each side of the inserted 5’ loxP site (sense primer 5’- AACTCGCTTTGAGCCAGAA-3’ and antisense primer 5’- CCGTCCGAGTGC-3’). Expected products were 180 bp from the wild-type allele and 220 bp from the floxed Foxc2 allele. Genotyping of the null allele of Nrp1 was performed by PCR, using sense primer 5’- AAGGAGTGGCACAGCATCCTT-3’ and antisense primer 5’- TGGGTGAACT-CAGCCCATTT-3’. Amplification from the Nrp1 null allele was estimated to generate a 350 bp PCR product, whereas wild-type allele should generate a 739 bp band.

**Histology and morphology**

Embryos and kidneys for X-gal staining and standard histology were harvested and immersed in ice-cold 4% (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Medicago) for 1 h. Fixed specimens for cryosectioning were immersed in ice-cold 4% (w/v) sucrose in PBS until they sunk, embedded in OCT Cryomount (Histolab) and cryosectioned at 10 μm thickness.

For detection of β-galactosidase expression, fixed whole embryos or kidneys were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution (5 mmol/L K4Fe(CN)6 (ICN), 5 mmol/L K3Fe(CN)6 (ICN), 5 mmol/L EGTA (Sigma-Aldrich), 0.01% (w/v) deoxycholate (Sigma-Aldrich), 0.02% (v/v) NP-40 (BDH), 2 mmol/L MgCl2 (Scharlau), and 1 mg/mL of X-Gal (Sigma-Aldrich) in PBS) overnight at 37°C. Here, β-galactosidase-expressing tissues were located and photographed under a dissecting microscope.
Stained embryos were postfixed in 10% (v/v) formalin and embedded in a mixture of 25% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and 0.4% (w/v) gelatin in PBS. The solution was solidified by addition of glutaraldehyde (Sigma-Aldrich) to a final concentration of 2.5% (w/v), and the resulting tissue blocks were serial sectioned on a vibratome at 30 μm. Sections were mounted on SuperFrost microscope slides and counterstained for 5 min in 0.1% (w/v) cresyl violet acetate (Sigma-Aldrich) in distilled H₂O₂ and washed for 15 min in 96% ethanol (Solvaco) and for 5 min in PBS.

Standard hematoxylin and eosin staining for histology was performed on cryosections using Mayer’s HTX (Histolab) and eosin Y with phloxine (Sigma-Aldrich). Histochromically stained sections were mounted in Canada Balsam (Sigma-Aldrich), and then examined and photographed using an Eclipse E800 microscope (Nikon).

Kidneys for electron microscopy were fixed overnight in modified Karnovsky’s fixative (2.5% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde, 0.05 mol/L sodium cacodylate (Sigma-Aldrich), 0.02% (w/v) sodium azide (Sigma-Aldrich), pH 7.2). For transmission electron microscopy, kidneys were rinsed in 0.15 mol/L sodium cacodylate and postfixed in 1% (w/v) osmium tetroxide (OsO₄) (Sigma-Aldrich), and 1% (w/v) potassium ferrocyanide (K₃Fe (CN)₆) for 2 h at 4°C. Contrast was enhanced by incubation in 0.5% (w/v) uranyl acetate for 1 h in darkness at room temperature. After dehydration, the filters with attached cells were embedded in Agar 100 resin (Agar Scientific LTD). Ultrathin transverse sections (60–70 nm) of the filters with attached cells were contrasted with uranyl acetate and lead citrate before examination using a Zeiss LEO 912 Omega transmission electron microscope (Zeiss). Digital image files were captured with a MegaView III camera (Soft Imaging Systems).

For scanning electron microscopy, 60 μm vibratome-sections of kidneys were treated by modified osmium-thiocarbohydrazide-osmium method, that is, 2 h incubation in 1% (w/v) OsO₄ at 4°C, 10 min in 1% (w/v) thiocarbohydrazide (Sigma-Aldrich) followed by 1 h incubation in 1% (w/v) OsO₄ at 4°C. Sections were dehydrated in ethanol followed by immersion in hexamethyldisilazane (Sigma-Aldrich) and evaporation in a fume hood. The dried specimens were mounted on stubs and coated with palladium and then examined in a Zeiss 982 Gemini field emission scanning electron microscope equipped with an in-lens secondary electron detector (Zeiss).

Immunohistochemistry

Kidneys were dissected and embedded in OCT Cryomount (Histolab). Cryosections at 10 μm were fixed and permeabilized in ice-cold acetone (Fisher Scientific) for 10 min or fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature and permeabilized in 0.2% (v/v) Triton X-100 in PBS for 15 min at room temperature. Sections were then blocked in 10% (v/v) fetal bovine serum in DMEM medium (Gibco) (blocking solution) for 1 h at room temperature and incubated with primary antibody diluted in blocking solution at 4°C overnight. After washing in PBS, the sections were incubated with secondary antibody and TO-PRO®-3 nuclear stain (Thermo Fisher Scientific) or ProLong® Gold Antifade Mountant (Thermo Fisher Scientific) and photographed using an LSM 510 Meta confocal microscope (Zeiss). Antibodies used are listed in Table 1.

TUNEL staining was performed using Click-iT Plus TUNEL assay with Alexa488 (Thermo Fisher Scientific, C10617) according to manufacturer’s protocol.

Image analysis

WT1 positive cells (according to labeling with WT1 primary antibody) were identified in glomeruli from either Foxc2fl/fl or Foxc2fl/fl; Pod-Cre mice and automatically counted using ImageJ software and the built-in function of “Analyze particles”.

Urine analysis

Spot urine was collected and 2 μL of each urine sample, and BSA standards at 0, 1, 3, and 10 μg, were separated by SDS-PAGE on a NuPAGE Novex 4–12% Bis-Tris gel (Thermo Fisher Scientific). After electrophoresis, the gel was immersed in fixing solution (50% (v/v) methanol (Fisher Scientific), 10% (v/v) acetic acid (Acros)) for 1 h, incubated for 20 min with agitation in staining solution (0.1% (w/v) Comassie brilliant blue R-250 (Bio-Rad), 50% (v/v) methanol, 10% (v/v) acetic acid), and finally incubated several times in destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid) until destained.

Urinary albumin content in spot urine was assessed using Albuwell M indirect ELISA (Exocell) and urinary creatinine content was measured, using Creatinine Companion kit (Exocell). Both measurements were performed according to manufacturer’s instructions. Albumin:creatinine ratio was calculated by dividing μg albumin/mg creatinine/mL urine with mg creatinine/mL urine, getting the ratio μg albumin/mg creatinine.

Cell culture

An immortalized podocyte cell line E11 (Cell Lines Service GmbH) (Schiwek et al. 2004) was maintained at...
33°C RPMI 1640 Medium, GlutaMAX™ Supplement (Gibco) supplemented with 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific) and 10 U/mL IFNγ (Millipore). For differentiation, IFNγ was excluded from the medium and cells were incubated at 37°C until harvesting.

For knockdown experiments, cells cultured at 33°C were transfected with either Foxc2 ON-TARGETplus siRNA pool (GE Dharmacon), or non-targeting ON-TARGETplus siRNA control (GE Dharmacon), using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer’s protocol. After transfection, cells were transferred to 37°C, in medium without IFNγ, to induce differentiation until harvesting after 48 h.

Overexpression of mouse Foxc2 was achieved by retroviral transduction of undifferentiated E11 cells. Full-length coding sequence of mouse Foxc2 (NM_013519.2) were PCR-amplified from genomic DNA (C57Bl/6) (primers used: sense 5’- AAAGGATCCTCTGGGACGCAGTGCAGG-3’ and antisense 5’- AAAGATATCTCAGTATTTGGTGCAGTCGTAAGA-3’) and the PCR product was cloned in retroviral pBabe-puro vector (gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764)) (Morgenstern and Land 1990). Replication-incompetent retroviruses were produced in 293T cells by co-transfection of retroviral vectors together with pVPack-GP and pVPack-Eco packaging vectors (Agilent) and E11 cells was transduced essentially as described (Gerin et al. 2009).

### Table 1. Antibodies used for detection of proteins. (n/a = not applicable, Thermo FS = Thermo Fisher Scientific). Higher dilution used for immunoblotting, lower for immunofluorescence.

| Antibody                  | Protein symbol | Host    | Catalogue # | Company          | Dilution |
|---------------------------|----------------|---------|-------------|------------------|---------|
| Wilm’s tumor protein      | Wt1            | Rabbit  | ab15249     | Abcam            | 1:200   |
| Klf7                      | Mki67          | Rabbit  | ab15580     | Abcam            | 1:200   |
| Actin, α-Smooth Muscle    | Acta2          | Mouse   | F3777       | Sigma-Aldrich    | 1:200   |
| Cd31                      | Cd31           | Rat     | ab7388      | Abcam            | 1:200   |
| Nephrin                   | Nphs1          | Rabbit  | ab58968     | Abcam            | 1:100   |
| Podocin                   | Nphs2          | Rabbit  | P0372       | Sigma-Aldrich    | 1:400   |
| ZO-1/TJP1                 | Tjp1           | Rabbit  | 40-2200     | Thermo FS        | 1:100   |
| Podocalyxin               | Podxl          | Goat    | AF1556      | R&D Systems      | 1:400   |
| Alpha-actinin 4           | Actn4          | Rabbit  | 0042-5      | Immunoglobulin   | 1:200   |
| Nestin                    | Nes            | Rabbit  | ab24692     | Abcam            | 1:200   |
| CD2-associated protein    | Cd2ap          | Rabbit  | ab32741     | Abcam            | 1:400   |
| Integrin beta-1           | Itgb1          | Rat     | MA81997     | Millipore        | 1:200; 1:1000 |
| Integrin linked kinase     | Ilk            | Rabbit  | ab52480     | Abcam            | 1:200   |
| CCN1                      | Cyr61          | Rabbit  | ab24448     | Abcam            | 1:100   |
| Collagen IV subunit a3    | Col4a3         | Rat     | 7076        | Chondrex         | 1:100   |
| Collagen IV subunit a4    | Col4a4         | Rat     | 7073        | Chondrex         | 1:100   |
| Neuropilin 1              | Nrp1           | Goat    | AF566       | R&D Systems      | 1:200; 1:1000 |
| Rabbit IgG Alexa 488      | n/a            | Donkey  | A21206      | Thermo FS        | 1:500   |
| Rabbit IgG Alexa 568      | n/a            | Donkey  | A10042      | Thermo FS        | 1:500   |
| Goat IgG Alexa 488        | n/a            | Donkey  | A11055      | Thermo FS        | 1:500   |
| Rat IgG Alexa 488         | n/a            | Donkey  | A-21208     | Thermo FS        | 1:500   |
| Goat IgG HRP              | n/a            | Rabbit  | P0449       | Dako             | 1:2000   |
| Rat IgG HRP               | n/a            | Goat    | NA935       | GE Lifescience   | 1:20,000 |

Isolation of glomeruli

Glomeruli were isolated using the magnetic bead isolation procedure essentially as described (Takemoto et al. 2002). Briefly, mice were anesthetized followed by perfusion (through the heart) with 2 × 10⁷ Dynabeads (Thermo Fisher Scientific) diluted in 10 mL of PBS. The kidneys were removed, minced, and digested in collagenase (1 mg/mL collagenase A (Roche), 100 U/mL deoxyribonuclease I (QIAGEN) in PBS) at 37°C for 15 min with gentle agitation. The collagenase-digested tissue was gently pressed through a 100-μm cell strainer, washed with PBS and filtered through a new cell strainer without pressing, and the cell strainer was washed with PBS. The cell suspension was then centrifuged at 200g for 5 min and the cell pellet was resuspended in 2 mL of PBS. Finally, glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and washed for at least three times with PBS.
Gene expression analysis

Total RNA from glomeruli and E11 cells was isolated using RNeasy mini or plus micro kits (QIAGEN) according to the manufacturer’s instructions. Reverse transcription of 1 µg of total RNA was carried out using Transcriptor First-Strand cDNA synthesis kit (Roche Life Science), according to the manufacturer’s instructions using random primers. Expression levels of specific miRNAs were quantified by real-time PCR, using Power SYBR green PCR Master Mix (Thermo Fisher Scientific) and normalized to the level of Rplp0. All samples were analyzed in quadruplicate, and mean values were calculated. Primers were designed using Primer-BLAST software (Ye et al. 2012). The sequence of specific primers can be found in Table 2.

Western blot analysis

Cells were lysed in RIPA buffer (50 mmol/L Tris-HCl (MP Biochemicals), pH 8.0, 1% (v/v) EDTA (Sigma-Aldrich), 1% (v/v) Triton X-100 (Sigma-Aldrich), 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich), 0.1% (w/v) SDS (Serva), 150 mmol/L NaCl (Scharlau)), supplemented with 1% (v/v) Tris-buffered saline (TBS)). Specific proteins were detected with indicated primary antibodies (see Table 1). Horseradish peroxidase-coupled secondary antibody was visualized with West Dura Chemiluminescent Substrates (Thermo Fisher Scientific) on a LAS-4000 Luminescent Image analyzer (Fujifilm).

Results

Foxc2 is widely expressed

Previous studies of the function of FOXC2 in mice are mainly based on global knockouts (Iida et al. 1997; Win- nier et al. 1997; Takemoto et al. 2006), and due to embryonic lethality in these models, information on FOXC2 requirement beyond embryonic development is lacking. To identify cells expressing Foxc2, we have previously generated a knocked-in mouse model where the single exon of Foxc2 is replaced by the lacZ gene, encoding β-galactosidase, resulting in lacZ expression under control of the endogenous Foxc2 promoter (Cederberg et al. 2009). By performing X-gal staining on Foxc2lacZ/+ embryos at day 12 after conception (Fig. 1A), we could confirm previous reports showing that Foxc2 is widely expressed during embryonic development (Miura et al. 1993; Kaestner et al. 1996). At embryonic day 12.5, β-galactosidase activity was detected in the developing bone and vertebrae as well as in nephrons and the heart, indicating that the Foxc2 promoter is active in these tissues (Fig. 1B).

Podocyte-specific deletion of Foxc2 results in postnatal lethality and proteinuria

Further investigation of the kidney showed that, based on X-gal staining in adult Foxc2lacZ/+ mice, Foxc2 is expressed in several structures, including glomeruli, vessels, and ureter (Fig. 1C). This expression pattern makes cell-specific roles of Foxc2 in the kidney difficult to dissect, using the global knockout model. We were interested in studying the requirement of Foxc2 specifically in the podocytes, so we sought to develop a different model. A well-characterized model for podocyte-specific deletions is the Pod-Cre transgenic mouse (Moeller et al. 2003). By crossing this mouse with a Rosa26 Cre-reporter strain (Soriano 1999) and performing X-gal staining, we could...
confirm that Cre activity was specific for glomeruli, whereas other structures are unstained (Fig. 1C).

To be able to study the role of Foxc2 specifically in podocytes, we generated a mouse with loxP sequences flanking the single exon of Foxc2 and crossed it with Pod-Cre+/- mice to achieve podocyte-specific deletion of Foxc2. The Foxc2fl/+; Pod-Cre+/- and Foxc2fl/fl control mice were viable, fertile and showed no obvious phenotype, indicating that neither the Cre-transgene nor the presence of loxP sequences in potential regulatory regions of Foxc2 were harmful to the mice. Mating for homozygous Foxc2fl/fl; Pod-Cre+/- mice resulted in litters that, at the time of weaning (3–4 weeks of age), completely lacked the Foxc2fl/fl; Pod-Cre+/- genotype (Fig. 2A), indicating embryonic or early postnatal lethality. Examination of pups from the late embryonic stage revealed that the predicted genotypes were present at normal ratio (Fig. 2B) confirming that the genetic modification is not causing embryonic lethality. Instead, the homozygous deletion of Foxc2 in podocytes causes complete penetrance of postnatal lethality, since animals with this genotype die around postnatal day 10.

To assess the kidney function, urine from newborn mice was analyzed by SDS-PAGE followed by Coomassie-staining and revealed extensive proteinuria already at 5 days of age in homozygous Foxc2fl/fl; Pod-Cre+/- mice, but not in heterozygous Foxc2fl/+; Pod-Cre+/- mice (Fig. 2C). A more precise measurement of urinary albumin, using ELISA showed that there was no albuminuria in urine from 3-day-old pups, regardless of genotype (Fig. 2D). At postnatal day 5 and 9 however, the albuminuria could be confirmed in Foxc2fl/fl; Pod-Cre+/- mice, whereas albumin levels were normal in the other genotypes tested (Fig. 2D). To compensate for differences in glomerular filtration rate, urinary albumin content is often normalized toward urinary creatinine content,
getting an albumin:creatinine ratio (ACR). However, concerns have been raised that ACR is not suitable in diseases involving severe kidney injury (Waikar et al. 2010). Urinary albumin content was assessed in Foxc2<sup>fl/fl; Pod-Cre<sup>+</sup></sup> 2–9-day-old pups (Fig. 2E) and in parallel normalized toward the urinary creatinine levels (Fig. 2F).
Urinary albumin increased with increasing age (Fig. 2E), whereas ACR makes a sudden drop at 7 days of age after substantial increase at 5 days of age (Fig. 2F). This discrepancy makes ACR an unsuitable method of choice to assess kidney function in this animal model with severe kidney injury.

**Loss of Foxc2 in podocytes causes foot process effacement and microvillus transformation**

Kidneys in the global Foxc2 knockout mice were markedly reduced (Takemoto et al. 2006), but this was not a characteristic shared by mice that had Foxc2 specifically deleted in podocytes. Thus, most likely a phenotype not derived from podocytes. Comparison of kidney weight as % of body weight during the first ten postnatal days did not reveal any differences between the genotypes (Table 3). Although mice lacking Foxc2 in the podocytes display extensive proteinuria already 5 days after birth, no morphological changes of the kidneys could be detected at this stage, using standard hematoxylin-eosin histochemical staining. At 9 days of age, renal lesions, such as enlarged glomeruli and dilated tubuli with proteinaceous content became apparent (Fig. 3A).

Ultrastructural investigation using transmission electron microscopy (TEM) revealed typical signs of podocyte injury. Mice lacking Foxc2 expression in the podocytes exhibited foot process effacement (Fig. 3B). Occasional splitting of the basement membrane was seen at day 5, but could not be detected at later stages. However, at postnatal day 9, apparent folding of the basement membrane between effaced foot processes is observed (Fig. 3B). Other structural changes known to accompany podocyte injury, including thickening of basement membrane or loss of endothelial fenestrae; could not be detected, not even at postnatal day 9 when the mice suffer from end stage kidney failure (Fig. 3B).

TEM images also indicated the presence of microvillus-like structures in the urinary space of the glomeruli of mice with podocyte-specific Foxc2 deletion. Scanning electron microscopy confirmed progression of podocyte microvillus transformation, that is, the presence of microvillus-like structures extending from the cell body of podocytes that lack Foxc2 expression (Fig. 3C). The loss of the typical podocyte structure of interdigitated foot processes was also evident.

**Mesangial expansion but no change in markers for apoptosis or proliferation after Foxc2 deletion in podocytes**

Since Foxc2 has been shown to have major roles in embryonic and podocyte development, knocking out Foxc2 in the podocyte might affect several aspects of podocyte cell biology, such as proliferation, differentiation or even apoptosis; conditions that are known to cause foot process effacement, and proteinuria (Greka and Mundel 2012). Nevertheless, staining kidneys for markers for these features did not show any difference between Foxc2<sup>fl/fl</sup>; Pod-Cre<sup>+/−</sup> and Foxc2<sup>fl/fl</sup> control mice (Fig. 4A). There was no indication of podocyte loss based on number of WT1 positive nuclei or degree of apoptosis detected by TUNEL-assay in Foxc2<sup>fl/fl</sup>; Pod-Cre<sup>+/−</sup> kidneys compared with Foxc2<sup>fl/fl</sup> control kidneys. A more thorough investigation of WT1 positive nuclei confirms the notion that there was no reduction in the number of podocytes (Fig. 4B). By labeling for KI67, a marker for proliferation/dedifferentiation (Scholzen and Gerdes 2000), we could not observe any difference between kidneys from Foxc2<sup>fl/fl</sup>; Pod-Cre<sup>+/−</sup> and Foxc2<sup>fl/fl</sup> control mice. A slight increase in staining intensity for alpha smooth muscle actin (also known as α-SMA or ACTA2), a marker for mesangial cells, was observed, whereas the staining intensity of the endothelial marker CD31 was unaffected by the loss of Foxc2 in podocytes.

**Altered distribution of proteins of the foot processes in podocyte-specific Foxc2 knockout mice**

To explore possible candidates for the phenotype observed in mice lacking Foxc2 in podocytes, we examined a number of proteins that previously have been associated with podocyte damage and/or with a reported link to FOXC2. No obvious difference in expression levels could be detected for any of these (Fig. 5). Especially when labeling for proteins involved in anchoring of podocytes to the basement, like ITGB1, ILK, and CYR61 (cysteine rich angiogenic inducer 61) (Fig. 5A), but also for critical components of the basement membrane, such as COL4A3 and COL4A4 (Fig. 5B), expression appeared normal.

However, although the expression levels appear unaffected, the glomerular distribution of important components of the foot processes, such as NPHS1, NPHS2, TJP1, PODXL, ACTN4, NES (nestin), and CD2AP, was altered (Fig. 5C). Instead of lining the capillary walls of the glomerulus, as seen in healthy Foxc2<sup>fl/fl</sup> mice, these

---

**Table 3.** Kidney weight relative to body weight (b.w.) in newborn mice.

| Genotype         | Kidney weight (% of b.w.) | StdDev |
|------------------|---------------------------|--------|
| Foxc2<sup>fl/+</sup>; Pod-Cre<sup>−/−</sup> | 1.43                      | 0.28   |
| Foxc2<sup>fl/fl</sup>; Pod-Cre<sup>−/−</sup> | 1.37                      | 0.1    |
| Foxc2<sup>fl/fl</sup>                       | 1.30                      | 0.12   |
| Foxc2<sup>fl/fl</sup>                       | 1.25                      | 0.07   |

© 2019 The Authors. Physiological Reports published by Wiley Periodicals, Inc. on behalf of The Physiological Society and the American Physiological Society.
components are mostly limited to the outer rim of the glomeruli and are often detected in a granular pattern in podocytes of Foxc2\(^{fl/fl}\); Pod-Cre\(^{+/-}\) mice.

Neuropilin 1 is regulated by Foxc2 in vitro

To further analyze potential candidate FOXC2 targets, quantitative PCR (qPCR) was conducted. The mRNA levels in isolated glomeruli was analyzed by qPCR and, from our list of candidate genes, only neuropilin 1 (Nrp1) had significantly altered expression (approx. 50% lower) in glomeruli from Foxc2\(^{fl/fl}\); Pod-Cre\(^{+/-}\) mice as compared with Foxc2\(^{fl/+}\) mice (Fig. 6A).

Since glomeruli are made up of several cell types, of which podocytes only constitute about 15%, the dependence of Foxc2 in podocytes for expression of these genes...
was further characterized in a podocyte cell line upon knockdown or induction of Foxc2 expression (Schiwek et al. 2004). Transcript analysis showed that the Foxc2 siRNA had a knockdown efficiency of about 75% on Foxc2 mRNA. (Fig. 6B) NRP1 could be confirmed as a potential Foxc2 target in these cells since it was co-
Figure 5. Expression of glomerular proteins. Representative laser confocal images of kidney sections from 9-day old Foxc2<sup>fl/fl</sup>:Pod-Cre<sup>+</sup> and Foxc2<sup>fl/fl</sup> mice using antibodies labeling (A) proteins involved in anchoring of podocytes (ITGB1/b1-integrin, ILK/integrin linked kinase and CYR61/CCN1), (B) basement membrane proteins (COL4A3 and COL4A4), and (C) components of the foot processes (i.e., slit diaphragm proteins (NPHS1/nephrin and NPHS2/podocin), anchoring of slit diaphragm to cytoskeleton (TJP1/ZO-1, ACTN4/α-actinin 4, CD2AP/CD2-associated protein, and NES/nestin), and foot process coating (PODXL/podocalyxin)).
regulated at both the mRNA (Fig. 6B) and protein level (Fig. 6C).

The expression level of NRP1 in podocytes is difficult to assess in vivo, due to strong staining in adjacent endothelial cells. However, co-staining with NPHS2, to label podocytes, reveals loss of co-localization between NRP1 and NPHS2, and a granular distribution of NPHS2, in glomeruli of Foxc2^{fl/fl}; Pod-Cre^{+/-} mice (Fig. 7) emphasizing that NPHS2 has lost its proximity to the glomerular basement membrane.

**Neuropilin 1 is not critical for podocyte function in vivo**

To evaluate the role of NRP1 in podocytes and its potential contribution to the observed phenotype of Foxc2^{fl/fl}; Pod-Cre^{+/-} animals, mice with floxed Nrp1 (Gu et al. 2003), were crossed with Pod-Cre^{+/-} transgenic mice to create podocyte-specific knockout of Nrp1. All Nrp1^{fl/+}; Pod-Cre^{+/-} mice were viable and appeared healthy (Fig. 8A). Not even when provoking the genotype with the addition of a floxed Foxc2 allele, that is, adding a heterozygous deletion of Foxc2 to the homozygous deletion of Nrp1 in podocytes, there were signs of premature death (Fig. 8B). To confirm deletion of floxed Nrp1 in podocytes, genomic DNA from various tissues from Nrp1^{fl/+}; Pod-Cre^{+/-} mice was analyzed by PCR. Only in gDNA from kidney of these mice ~350 bp PCR product could be amplified, using primers located outside of the floxed sequence (Fig. 8C) and given the podocyte-specific expression of Cre recombine it most likely corresponds to deletion in podocytes. Surprisingly, podocyte-specific knockout of Nrp1 did not reduce glomerular mRNA levels of Nrp1 (Fig. 8D).

Although the amount of Nrp1 in glomeruli was not reduced, the levels in podocytes should be eradicated in this
model. To examine whether this causes podocyte injury, urine samples were collected from both young (1 month old) and older mice (14 months old) and analyzed for microalbuminuria, an indicator of podocyte injury, using ELISA. Still, there was no difference in albumin content in the urine of the different genotypes no matter what age or gender analyzed (data not shown), suggesting a minor (if any) contribution of NRP1 to podocyte function.

Discussion

Podocytes have been shown to express the forkhead transcription factor Foxc2 (Dagenais et al. 2004; Takemoto et al. 2006; Brunskill et al. 2011), which appears as one of the earliest markers of podocytes during glomerular development (Takemoto et al. 2006). Characterization of mice with global Foxc2 knockout revealed several kidney defects, including reduced kidney size, abnormal glomerular shape, dilated and blood-filled capillary loops, and failure to produce a proper mesangial core of the glomeruli (Takemoto et al. 2006). In addition, podocytes lacked foot processes and slit diaphragms, and endothelial cells lacked fenestrations. In this model, all mice die in utero or around birth, hence precluding functional analysis of the kidney.

Using in situ hybridization and immunohistochemistry, Takemoto et al. (2006) showed that, in embryonic kidney, FOXC2 was mainly expressed in podocytes, suggesting

Figure 7. Laser confocal microscopy of kidney sections from 9-day-old Foxc2<sup>fl/fl</sup>; Pod-Cre<sup>+</sup> and Foxc2<sup>fl/fl</sup> mice. (A) Images of whole glomeruli, with separate images of NRP1/neuropilin 1 (green) and NPHS2/podocin (red) signal and a merged image for visualization of co-localization. Orange indicates merged/co-localized signals. (B) Zoomed in on part of the same glomeruli as in (A) to further visualize the localization of green (NRP1) and red (NPHS2) signals.
that the histological defects seen in the kidney were derived from arrested podocyte development. However, we show here, using the Foxc2\(^{lacZ/+}\) mouse model (Cederberg et al. 2009), that Foxc2 expression in the kidney is not exclusive to podocytes, but is evident also in other structures. The use of a lacZ – marker for endogenous Foxc2 expression is likely to provide a more sensitive detection method than immunohistochemistry and in situ hybridizations. The additional embryonic expression of lacZ also correlates well with previously reported expression pattern of Foxc2 (Miura et al. 1993; Kaestner et al. 1996; Takemoto et al. 2006), which further validates the Foxc2\(^{lacZ/+}\) model.

To study the specific role of FOXC2 in the podocyte, we generated a floxed Foxc2 mouse that was crossed with Pod-Cre transgenic mice, a commonly used Cre-transgenic strain for podocyte-specific deletions (Moeller et al. 2003; El-Aouini et al. 2006; Suleiman et al. 2007; Pozzi et al. 2008). In contrast to the global deletion of Foxc2, the conditional deletion of Foxc2 in podocytes did not cause embryonic lethality, but the animals developed heavy proteinuria soon after birth (detected already in 5-day-old pups), and died around postnatal day 10 due to severe kidney failure. Importantly, there were no indications of kidney dysfunction in terms of albuminuria until postnatal day 5. This finding is in bright contrast to a recent work by Motojima et al. 2017 where Foxc2 was conditionally deleted in podocytes, using Nephrin-Cre. In this model, no kidney phenotype was observed unless challenged with combinatorial deletion of Foxc1. It is surprising that the phenotypes are so different. Especially, since nephrin expression appears earlier than podocin during podocyte differentiation (Putaala et al. 2001; Takemoto et al. 2006) with increased risk of developmental effects. Given that Foxc2 is a single-exon gene, both models are required to target the same exon. However, there appears to be a slight difference in the location of the loxP sites in the two models. Motojima et al. inserted the 3' loxP site within the 3' UTR (untranslated region) of Foxc2 (Motojima et al. 2016b), whereas we intentionally avoided this transcribed region due to the risk of affecting crucial, but yet unknown, regulatory elements. Another,
perhaps more plausible explanation, could be that the penetrance of Cre expression in podocytes is different in the two transgenic strains utilized by the different groups. We could show substantial reduction in Foxc2 mRNA in glomeruli of Foxc2fl/fl; Pod-CreCre/+ mice; but unfortunately no such data were presented by Motojima et al. Hence, it is difficult to compare data from these two models since there might be differences in knockout efficiency.

Ultrastructural analysis of kidneys from Foxc2fl/fl; Pod-CreCre/+ mice confirmed that podocytes lacking Foxc2 develop foot process effacement, a common, but non-specific feature of podocyte injury, as well as extensive podocyte microvillus transformation, another manifestation of podocyte damage (Patek et al. 1999; Roselli et al. 2004; El-Aouni et al. 2006). Interestingly, the podocytes in Foxc2fl/fl; Pod-CreCre/+ mice seem to develop properly since at postnatal day 3, the presence of interdigitated foot processes and normal urinary albumin concentrations could be demonstrated. Other typical signs of glomerular injury, such as thickening of the basement membrane or loss of fenestrated endothelial cells, the latter a feature of the global Foxc2 knockout mouse (Takemoto et al. 2006), could not be detected in Foxc2fl/fl; Pod-CreCre/+ mice; not even at day 9 when the mice suffer from end-stage kidney failure. Additionally, in contrast to findings in the global Foxc2 knockout mice, podocyte-specific Foxc2 deletion did not affect kidney size.

Loss of Foxc2 in podocytes does not affect podocyte number, proliferation/dedifferentiation or apoptosis, conditions known to cause foot process effacement and proteinuria (Greka and Mundel 2012). There are signs of mesangial expansion at the late stage of renal failure in these mice and this might be an indication of progressive glomerular injury (Dalla Vestra et al. 2001). Notably, the mesangial expansion seen in the podocyte-specific Foxc2 knockout mice is the opposite effect of what was observed in the global knockout of Foxc2, where the mesangial core failed to develop properly (Takemoto et al. 2006). This inequality adds to the conclusion that these two models have distinct phenotypes, one a general developmental phenotype and one a specific podocyte function phenotype.

To investigate potential Foxc2 targets, we pursued a candidate gene approach based on either phenotypic similarities and/or a reported link to Foxc2. The importance for proper glomerular function have been reported for NPHS2 (Boute et al. 2000; Roselli et al. 2004), TJP1 (Itoh et al. 2014), PODXL (Doyonnas et al. 2001), COL4A3 (Miner and Sanes 1996), COL4A4 (Korstanje et al. 2014), and RHPN1 (Lal et al. 2015), targets that were all down-regulated in glomeruli of the global Foxc2 knockout mouse (Takemoto et al. 2006). In addition to these genes, we analyzed targets that share phenotypic similarities with Foxc2 and have an established function or expression in podocytes, that is, NPHS1 (Lenkkeri et al. 1999), ACTN4 (Kos et al. 2003), NES (Perry et al. 2007), and CD2AP (Shih et al. 2001).

Unexpectedly, none of these proteins showed detectable changes in expression levels in podocyte-specific Foxc2 knockout mice when examined by immunohistochemistry. This was particularly surprising for those candidates that were affected in the global knockout of Foxc2. However, this finding shows many similarities with studies of another transcription factor, LMX1B, where proposed downstream targets derived from a global knockout model, that is, NPHS2, COL4A3, and COL4A4, could not be confirmed when conditionally deleting Lmx1b in podocytes (Suleiman et al. 2007; Burghardt et al. 2013). This further emphasizes the benefit of studying the in vivo role of genes in cell-specific knockouts rather than global deletions, since effects on the cellular level observed in global knockouts might be derived from accumulated or developmental effects.

One striking feature observed after conditional deletion of Foxc2 in podocytes is the change in distribution of components of the foot process. Such components normally accumulate along the capillary lining, being associated with the basement membrane, but was observed in a cytoplasmic, often granular, and rim-like pattern in the Foxc2-depleted podocytes. A change in distribution of podocyte markers is often observed after foot process effacement (Shirato 2002). Proteins that are involved in the anchoring of the podocyte (ITGB1 and ILK) or constituting components of the basement membrane (COL4A3 and COL4A4) do not share this altered expression pattern. A thorough investigation of other anchoring or basal lamina components, like integrins and laminins, was performed on Foxc2 global knockout mice, without any detectable changes (Takemoto et al. 2006). These findings, together with the ultrastructural analyses, suggest that Foxc2 expression in podocytes is crucial to maintain the architecture of foot processes.

We identified Nrp1, a transmembrane co-receptor that can bind either VEGF or semaphorins (Gu et al. 2003), as a downstream Foxc2 target in vitro. A link between these two genes has already been proposed in endothelial cells (Hayashi and Kume 2008), so we investigated the glomerular expression of NRP1. Strikingly, the co-localization that NRP1 possess with the podocyte-specific NPHS2 in healthy kidneys was completely lost in podocytes that lacked Foxc2. Encouraged by these findings, and the many indications that NRP1 might have an important role in podocytes and the development of proteinuria (Bondeva et al. 2009; Loeffler et al. 2013; Patnaik et al. 2014; Bondeva and Wolf 2015), we pursued this target further. Surprisingly, conditional deletion of Nrp1 in...
podocytes in vivo did not cause any kidney-related effects at all. It did not even reduce glomerular mRNA levels of Nrp1, which suggests that the reduced levels that were observed in the glomeruli from Foxc2 conditional knockouts might be derived from glomerular cells other than podocytes. Increased Nrp1 expression has also been shown to lead to increased leakage of vessels (Roth et al. 2016), which might even indicate that reduced levels of Nrp1 in endothelial cells is a response to decrease the leakage in the glomeruli. A recent report also shows that expression of Nrp1 in mesangial cells is vital for proper glomerular function when deleted using Pdgfrb-Cre (Bartlett et al. 2017), indicating that Nrp1 are more likely to play a role in mesangial cells rather than podocytes.

To conclude, we show for the first time that Foxc2, but not Nrp1, expression is essential for proper podocyte function in vivo. We could clearly demonstrate that podocytes that are deprived from Foxc2 lose their unique architecture and hence their ability to prevent proteins from leaking into the urine. Nevertheless, despite the dramatic phenotype, it is not associated with detectable alterations in levels of important glomerular components, and previously proposed targets, including NPHS2, COL4A3, COL4A4, or Rhpn1. Further studies will therefore be needed to identify genes regulated by Foxc2 in podocytes and how Foxc2 regulates them during normal kidney physiology to maintain proper podocyte structure and function.

Acknowledgment

The authors thank B. R. Johansson and Y. Josefsson for expert technical assistance in electron microscopy and Martin Lidell and Anne Uv for critical reading of the manuscript. We also thank the Karolinska Center for Transgene Technologies (KCTT, Stockholm, Sweden) for technical assistance in generating floxed Foxc2 mice. The Pod-Cre transgenic mice were a kind gift from Lawrence B. Holzman.

Conflict of Interest

None.

References

Bariety, J., C. Mandet, G. S. Hill, and P. Bruneval. 2006. Parietal podocytes in normal human glomeruli. J. Am. Soc. Nephrol. 17:2770–2780.

Bartlett, C. S., R. P. Scott, I. A. Carota, M. L. Wnuk, Y. S. Kanwar, J. H. Miner, et al. 2017. Glomerular mesangial cell recruitment and function require the co-receptor neuropilin-1. American Journal of Physiology-Renal Physiology 313:F1232–F1242.

Bondeva, T., and G. Wolf. 2015. Role of neuropilin-1 in diabetic nephropathy. J. Clin. Med. 4:1293–1311.

Bondeva, T., C. Ruster, S. Franke, E. Hammerschmid, M. Klagsbrun, C. D. Cohen, et al. 2009. Advanced glycation end-products suppress neuropilin-1 expression in podocytes. Kidney Int. 75:605–616.

Boute, N., O. Gribouval, S. Roselli, F. Benessy, H. Lee, A. Fuchshuber, et al. 2000. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat. Genet. 24:349–354.

Brice, G., S. Mansour, R. Bell, J. R. Collin, A. H. Child, A. F. Brady, et al. 2002. Analysis of the phenotypic abnormalities in lymphoedema-distichiasis syndrome in 74 patients with FOXC2 mutations or linkage to 16q24. J. Med. Genet. 39:478–483.

Brunskill, E. W., K. Georgas, B. Rumballe, M. H. Little, and S. S. Potter. 2011. Defining the molecular character of the developing and adult kidney podocyte. PLoS ONE 6:e24640.

Burghardt, T., J. Kastner, H. Suleiman, E. Rivera-Milla, N. Stepanova, C. Lottaz, et al. 2013. LMX1B is essential for the maintenance of differentiated podocytes in adult kidneys. J. Am. Soc. Nephrol. 24:1830–1848.

Cederberg, A., M. Grande, M. Rhedin, X. R. Peng, and S. Enerback. 2009. In vitro differentiated adipocytes from a Foxc2 reporter knock-in mouse as screening tool. Transgenic Res. 18:889–897.

Chen, H., Y. Lun, D. Ovchinnikov, H. Kokubo, K. C. Oberg, C. V. Pepicelli, et al. 1998. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat. Genet. 19:51–55.

Dagenais, S. L., R. L. Hartsough, R. P. Erickson, M. H. Witte, M. G. Butler, and T. W. Glover. 2004. Foxc2 is expressed in developing lymphatic vessels and other tissues associated with lymphedema-distichiasis syndrome. Gene Expr. Patterns 4:611–619.

Dalla Vestra, M., A. Saller, M. Maurer, and P. Fioretto. 2001. Role of mesangial expansion in the pathogenesis of diabetic nephropathy. J. Nephrol. 14(Suppl 4):S51–S57.

Doyonnas, R., D. B. Kershaw, C. Duhme, H. Merkens, S. Chelliah, T. Graf, et al. 2001. Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. J. Exp. Med. 194:13–27.

Dreyer, S. D., G. Zhou, A. Baldini, A. Winterpacht, B. Zabel, W. Cole, et al. 1998. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. Nat. Genet. 19:47–50.

El-Aouni, C., N. Herbach, S. M. Blattner, A. Henger, M. P. Rastaldi, G. Jarad, et al. 2006. Podocyte-specific deletion of integrin-linked kinase results in severe glomerular basement membrane alterations and progressive glomerulosclerosis. J. Am. Soc. Nephrol. 17:1334–1344.

Erickson, R. P., S. L. Dagenais, M. S. Caulder, C. A. Downs, G. Herman, M. C. Jones, et al. 2001. Clinical heterogeneity in lymphoedema-distichiasis with FOXC2 truncating mutations. J. Med. Genet. 38:761–766.
Fang, J., S. L. Dagenais, R. P. Erickson, M. F. Arlt, M. W. Glynn, J. L. Gorski, et al. 2000. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am. J. Hum. Genet. 67:1382–1388.

Gerin, I., G. T. Bommer, M. E. Lidell, A. Cederberg, S. Enerback, and O. A. Macdougald. 2009. On the role of FOX transcription factors in adipocyte differentiation and insulin-stimulated glucose uptake. J. Biol. Chem. 284:10755–10763.

Greka, A., and P. Mundel. 2012. Cell biology and pathology of podocytes. Annu. Rev. Physiol. 74:299–323.

Gu, C., E. R. Rodriguez, D. V. Reimert, T. Shu, B. Fritsch, L. J. Richards, et al. 2003. Neurophin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev. Cell 5:45–57.

Hayashi, H., and T. Kume. 2008. Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. PLoS ONE 3:e2401.

Hoess, R., K. Ahrens, and N. Sternberg. 1984. The nature of the interaction of the P1 recombinase Cre with the recombining site loxP. Cold Spring Harb. Symp. Quant. Biol. 49:761–768.

Iida, K., H. Koseki, H. Kakinuma, N. Kato, Y. Mizutani-Koseki, H. Ohuchi, et al. 1997. Essential roles of the winged helix transcription factor MFH-1 in aortic arch patterning and skeletonogenesis. Development 124:4627–4638.

Itoh, M., K. Nakadate, Y. Horibata, T. Matusaka, J. Xu, W. Hunziker, et al. 2014. The structural and functional organization of the podocyte filtration slits is regulated by Tjp1/ZO-1. PLoS ONE 9:e106621.

Kaestner, K. H., S. C. Bleckmann, A. P. Monaghan, J. Schlondorff, A. Mincheva, P. Lichter, et al. 1996. Clustered arrangement of winged helix genes fkh-6 and MFH-1: possible implications for mesoderm development. Development 122:1751–1758.

Korstanje, R., C. R. Caputo, R. A. Doty, S. A. Cook, R. T. Bronson, M. T. Davison, et al. 2014. A mouse Col4a4 mutation causing Alport glomerulosclerosis with abnormal collagen alpha3alpha4alpha5(IV) trimers. Kidney Int. 85:1461–1468.

Kos, C. H., T. C. Le, S. Sinha, J. M. Henderson, S. H. Kim, H. Sugimoto, et al. 2003. Mice deficient in alpha-actinin-4 have severe glomerular disease. J. Clin. Investig. 111:1683–1690.

Lal, M. A., A. C. Andersson, K. Katayama, Z. Xiao, M. Nukui, K. Hultenb, et al. 2015. Rhophilin-1 is a key regulator of the podocyte cytoskeleton and is essential for glomerular filtration. J. Am. Soc. Nephrol. 26:647–662.

Lenkkeri, U., M. Mannikko, P. McCready, J. Lamerdin, O. Gribouval, P. M. Niaudet, et al. 1999. Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. Am. J. Hum. Genet. 64:51–61.

Loeffler, I., C. Ruster, S. Franke, M. Liebisch, and G. Wolf. 2013. Erythropoietin ameliorates podocyte injury in advanced diabetic nephropathy in the db/db mouse. Am. J. Physiol. Renal Physiol. 305:F911–F918.

Miner, J. H., and J. R. Sanes. 1996. Molecular and functional defects in kidneys of mice lacking collagen alpha 3(IV): implications for Alport syndrome. J. Cell Biol. 135:1403–1413.

Miner, J. H., R. Morello, K. L. Andrews, C. Li, C. Antignac, A. S. Shaw, et al. 2002. Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation. J. Clin. Investig. 109:1065–1072.

Moeller, M. J., I. A. Kovari, and L. B. Holzman. 2000. Evaluation of a new tool for exploring podocyte biology: mouse Nphs1 S’ flanking region drives LacZ expression in podocytes. J. Am. Soc. Nephrol. 11:2306–2314.

Moeller, M. J., S. K. Sanden, A. Soofi, R. C. Wiggins, and L. B. Holzman. 2003. Podocyte-specific expression of cre recombinase in transgenic mice. Genesis 35:39–42.

Morello, R., G. Zhou, S. D. Dreyer, S. J. Harvey, Y. Ninomiya, P. S. Thorner, et al. 2001. Regulation of glomerular basement membrane collagen expression by LMX1B contributes to renal disease in nail patella syndrome. Nat. Genet. 27:205–208.

Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18:3587–3596.

Motojima, M., S. Ogihara, T. Matusaka, S. Y. Kim, N. Sagawa, K. Abe, et al. 2016a. Conditional knockout of Foxc2 gene in kidney: efficient generation of conditional alleles of single-exon gene by double-selection system. Mamm. Genome 27:62–69.

Motojima, M., S. Tanimoto, M. Ohtsuka, T. Matusaka, T. Kume, and K. Abe. 2016b. Characterization of kidney and skeleton phenotypes of mice double heterozygous for Foxc1 and Foxc2. Cells Tissues Organs 201:380–389.

Motojima, M., T. Kume, and T. Matusaka. 2017. Foxc1 and Foxc2 are necessary to maintain glomerular podocytes. Exp. Cell Res. 352:265–272.

Patek, C. E., M. H. Little, S. Fleming, C. Miles, J. P. Charliere, A. R. Clarke, et al. 1999. A zinc finger truncation of murine WT1 results in the characteristic urogenital abnormalities of Denys-Drash syndrome. Proc. Natl. Acad. Sci. USA 96:2931–2936.

Patnaik, A., P. M. LoRusso, W. A. Messersmith, K. P. Papadopoulos, L. Gore, M. Beeram, et al. 2014. A Phase Ib study evaluating MNRP1685A, a fully human anti-NRP1 monoclonal antibody, in combination with bevacizumab and paclitaxel in patients with advanced solid tumors. Cancer Chemother. Pharmacol. 73:951–960.
Perry, J., M. Ho, S. Viero, K. Zheng, R. Jacobs, and P. S. Thorner. 2007. The intermediate filament nestin is highly expressed in normal human podocytes and podocytes in glomerular disease. Pediatr. Dev. Pathol. 10:369–382.
Pozzi, A., G. Jarad, G. W. Moeckel, S. Coffa, X. Zhang, L. Gewin, et al. 2008. Beta1 integrin expression by podocytes is required to maintain glomerular structural integrity. Dev. Biol. 316:288–301.
Putaala, H., R. Soininen, P. Kilpelainen, J. Wartiovaara, and K. Tryggvason. 2001. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum. Mol. Genet. 10:1–8.
Roselli, S., L. Heidet, M. Sich, A. Henger, M. Kretzler, M. C. Gubler, et al. 2004. Early glomerular filtration defect and severe renal disease in podocin-deficient mice. Mol. Cell. Biol. 24:550–560.
Roth, L., C. Prahst, T. Ruckdeschel, S. Savant, S. Westrom, A. Fantin, et al. 2016. Neuropilin-1 mediates vascular permeability independently of vascular endothelial growth factor receptor-2 activation. Sci. Signal. 9:ra42.
Schiwek, D., N. Endlich, L. Holzman, H. Holthofer, W. Kriz, and K. Endlich. 2004. Stable expression of nephrin and localization to cell-cell contacts in novel murine podocyte cell lines. Kidney Int. 66:91–101.
Scholzen, T., and J. Gerdes. 2000. The Ki-67 protein: from the known and the unknown. J. Cell. Physiol. 182:311–322.
Shih, N. Y., J. Li, R. Cotran, P. Mundel, J. H. Miner, and A. S. Shaw. 2001. CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am. J. Pathol. 159:2303–2308.
Shirato, I. 2002. Podocyte process effacement in vivo. Microsc. Res. Tech. 57:241–246.
Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21:70–71.
Suleiman, H., D. Heudobler, A. S. Raschta, Y. Zhao, Q. Zhao, I. Hertting, et al. 2007. The podocyte-specific inactivation of Lmx1b, Ldb1 and E2a yields new insight into a transcriptional network in podocytes. Dev. Biol. 304:701–712.
Takemoto, M., N. Asker, H. Gerhardt, A. Lundkvist, B. R. Johansson, Y. Saito, et al. 2002. A new method for large scale isolation of kidney glomeruli from mice. Am. J. Pathol. 161:799–805.
Takemoto, M., L. He, J. Norlin, J. Patrakka, Z. Xiao, T. Petrova, et al. 2006. Large-scale identification of genes implicated in kidney glomerulus development and function. EMBO J. 25:1160–1174.
Waikar, S. S., V. S. Sabbisetti, and J. V. Bonventre. 2010. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int. 78:486–494.
Winnier, G. E., L. Hargett, and B. L. Hogan. 1997. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. Genes Dev. 11:926–940.
Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134.
Yildirim-Toruner, C., K. Subramanian, L. El Manjra, E. Chen, S. Goldstein, and E. Vitale. 2004. A novel frameshift mutation of FOXC2 gene in a family with hereditary lymphedema-distichiasis syndrome associated with renal disease and diabetes mellitus. Am. J. Med. Genet. A 131:281–286.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Strategy for generation of mice with conditional-ready floxed Foxc2 allele.

Figure S2. Analysis of urinary albumin concentration in young and old mice.