Large-scale identification of genes implicated in kidney glomerulus development and function

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To advance our understanding of development, function and diseases in the kidney glomerulus, we have established and large-scale sequenced cDNA libraries from mouse glomeruli at different stages of development, resulting in a catalogue of 6053 different genes. The glomerular cDNA clones were arrayed and hybridized against a series of labeled targets from isolated glomeruli, non-glomerular kidney tissue, FACS-sorted podocytes and brain capillaries, which identified over 300 glomerular cell-enriched transcripts, some of which were further sublocalized to podocytes, mesangial cells and juxtaglomerular cells by *in situ* hybridization. For the earliest podocyte marker identified, Foxc2, knockout mice were used to analyze the role of this protein during glomerular development. We show that Foxc2 controls the expression of a distinct set of podocyte genes involved in podocyte differentiation and glomerular basement membrane maturation. The primary podocyte defects also cause abnormal differentiation and organization of the glomerular vascular cells. We surmise that studies on the other novel glomerulus-enriched transcripts identified in this study will provide new insight into glomerular development and pathomechanisms of disease.
The EMBO Journal (2006) **25,** 1160–1174. doi:10.1038/sj.emboj.7601014; Published online 23 February 2006

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Introduction

The kidney glomerulus is a specialized filtration unit capable of filtering large volumes of plasma into primary urine (Deen, 2004). The glomerulus is made up of three cell types—endothelial cells, mesangial cells and podocytes—and the filter consists of three layers of the glomerular capillary wall: the fenestrated endothelium, the glomerular basement membrane (GBM) and the slit diaphragm (SD) located between the interdigitating podocyte foot processes.

Filter failure results in proteinuria, which may lead to a pathologic chain reaction with end-stage renal disease (ESRD) as a final outcome. About two-thirds of ESRD cases result from primary glomerular insults caused by common systemic diseases (diabetes, hypertension, lupus, infections) and drug-induced toxicity. Although we currently lack knowledge about the molecular pathogenesis of the common glomerular disorders, recent studies of the genetic basis of rare hereditary glomerulopathies have identified several components of the glomerular filter, or the podocytes, as targets of pathogenic pathways (Barker *et al*, 1990; Mochizuki *et al*, 1994; Dreyer *et al*, 1998; Kestilä *et al*, 1998; Klamt *et al*, 1998; Boute *et al*, 2000; Somlo and Mundel, 2000; Zenker *et al*, 2004). For example, defects in GBM-specific proteins, such as type IV collagen and laminin, lead to Alport's syndrome and Pierson's congenital nephrotic syndrome, respectively, emphasizing the role of the GBM in the renal filter. In recent years, the podocytes, and in particular their foot processes and the SD located between them, have been shown to play a central role in glomerular disease. The SD is an orderly structured zipper-like filter with pores smaller than albumin, thus constituting a size-selective molecular sieve (Rodewald and Karnowsky, 1974; Wartiovaara *et al*, 2004). Malfunction or absence of the SD protein nephrin leads to lethal congenital nephrotic syndrome in humans and mice (Kestilä *et al*, 1998; Putaala *et al*, 2001), and absence of Neph1 or FAT-1, which are also believed to be components of the SD, leads to lethal proteinuria in mice (Inoue *et al*, 2001; Liu *et al*, 2003). Defects or absence of the foot process proteins podocin (Boute *et al*, 2000; Tsukaguchi *et al*, 2002), α-actinin-4 (Kaplan *et al*, 2000; Kos *et al*, 2003) and CD2AP cause glomerulopathy (Shih *et al*, 1999; Kim *et al*, 2003). Additionally, mutations in podocyte transcription factors LMX1B and WT1 have been associated with the glomerular disorders in nail-patella, Denys–Drash and Frasier's syndromes (Chen *et al*, 1998; Dreyer *et al*, 1998; Klamt *et al*, 1998).

The recognition of specific glomerulus-associated proteins and the podocytes as central players in developmental biology as well as in the pathogenesis of rare glomerular diseases calls for more comprehensive studies on the glomerular transcriptome and proteome. Such efforts could help to elucidate basic mechanisms of glomerular development and function, but also identify additional causes of rare monogenic diseases, and importantly also pathomechanisms of the common glomerular disorders of systemic origin. The fact that glomeruli constitute less than 10% and podocytes only about 1% of the cells in the kidney complicates glomerulus research, however. Most known podocyte-specific transcripts are not detected by whole kidney molecular profiling methods. To bypass this problem we have generated and large-scale sequenced high-complexity cDNA libraries from glomeruli isolated at different developmental stages. We also constructed a glomerulus cDNA microarray and produced a series of global and glomerulus cell-type-specific transcript profiles. This effort resulted in a large number of novel candidates for genes of importance for glomerulus development and function. As an example, we identified the transcription factor Foxc2 as an early marker for podocytes and used mutant mice to elucidate a specific role for Foxc2 in podocyte differentiation and glomerulus maturation.

Results

Construction and large-scale sequencing of mouse glomerulus cDNA libraries

Using a magnetic bead perfusion method (Takemoto *et al*, 2002), highly purified preparations of kidney glomeruli were isolated from approximately 100 adult and 400 newborn mice in order to generate sufficient quantities of total RNA for the synthesis of high-complexity cDNA libraries. Two standard oligo-dT-primed cDNA libraries were generated from the newborn and adult glomerular RNA. In order to facilitate the identification of rare transcripts, two normalized libraries were also generated from the standard adult library. In the normalized libraries, high-abundance RNA transcripts were suppressed to different degrees (Figure 1A). Test sequencing of 96 random clones from each library indicated that the glomerular libraries were of high complexity (data not shown). The test sequencing also provided an estimate of the number of sequences required to approach saturation in a large-scale sequencing effort. Based on these estimates, we attempted a total of 15 627 sequence reads from the four libraries, which provided 14 171 sequences of 500–800 bp length (91% readability). After vector trimming, a total of 13 368 cDNA sequences longer than 100 bp remained (data for the individual libraries are shown in Supplementary Table S1; gene sequences can be accessed at www.ebi.ac.uk/arrayexpress/, accession number A-MEXP-288). BLAST searches against the ENSEMBL mouse gene predictions resulted in 12 309 high-quality hits (*e*-value $<$1e$-$30, alignment identity $>$85%). A total of 941 sequences did not match ENSEMBL annotated genes, but matched the mouse genome and may therefore represent putative novel gene transcripts. To evaluate the quality of the cDNA library normalization procedure, we studied the distribution of a number of housekeeping genes among the different libraries. As shown in Figure 1B, the relative abundances of housekeeping gene transcripts were decreased in the normalized libraries compared to the standard library.

Composition of the glomerular transcript database (GlomBase)

Comparison with the ENSEMBL mouse gene predictions revealed that out of the total number of 28 055 hitherto annotated genes, 6053 (21.6%) were present in GlomBase. Out of the 25 383 coding genes and 2672 pseudogenes predicted in the mouse genome, 6012 coding genes (23.7%) and 41 pseudogenes were found in GlomBase. We compared the GlomBase content with published literature on gene and protein expression patterns in the glomerulus, demonstrated with cellular resolution by either *in situ* hybridization (ISH) or immunohistochemistry. Out of 170 such genes or proteins, we found 140 (82.4%) in GlomBase (Supplementary Table S2). Although this suggests that a major proportion of the glomerular transcriptome is represented in GlomBase, the 2:1 ratio between sequences (13 368) and annotated genes (6053) also indicates that the libraries were not sequenced to saturation, and hence that part of the glomerular transcriptome is missing in GlomBase. A more comprehensive report on GlomBase will be provided elsewhere (He *et al*, manuscript in preparation).

Identification of candidate glomerulus-specific genes by GlomChip analysis

The cDNA clones of GlomBase were amplified and printed on glass slides (GlomChip), which were used in a series of hybridization experiments aimed at the identification of genes with glomerulus-enriched or glomerulus-specific expression (details on GlomChip design, construction, target preparation and chip hybridization are described in Supplementary Figure S1; GlomChip microarray data can be accessed at www.ebi.ac.uk/arrayexpress/, accession number E-MEXP-492). We first compared glomeruli isolated from 5-day-old mice to non-glomerular kidney tissue. The 357 different genes and 63 ESTs that were significantly upregulated more than two-fold in glomeruli (Figure 1C, gene category 1) encompass 48 genes known to be expressed in the glomerulus (Supplementary Table S2) and included most known podocyte-specific markers, such as nephrin (Nphs1), podocin (Nphs2), podocalyxin (Podxl), synaptopodin (Synpo), protein-tyrosine phosphatase receptor o (Ptpn15, Ptpro, GLEPP1) and Wilm's tumor protein (Wt1) as well as markers for vascular endothelial cells (Supplementary Tables S3 and S4). The concentration of vascular transcripts in category 1 was expected, as vascular cells (endothelial and mesangial cells) together constitute about 85% of the glomerulus, but only a minority of the cells in the remaining kidney tissue. To subtract general vascular markers, we profiled isolated brain capillary fragments. Thereby, category 1 genes were split into category 3 (143 genes and 34 ESTs upregulated in glomeruli; Supplementary Table S5), category 4 (34 genes and one EST upregulated in brain capillaries; Supplementary Table S6) and category 5 (180 genes and 28 ESTs, which were not significantly differentially expressed more than two-fold; Supplementary Table S7). As expected, most known podocyte markers fell into category 3, whereas known endothelial markers collected in categories 4 and 5.

The category 3 transcripts represent candidate glomerular markers. To assign these genes further to individual glomer-
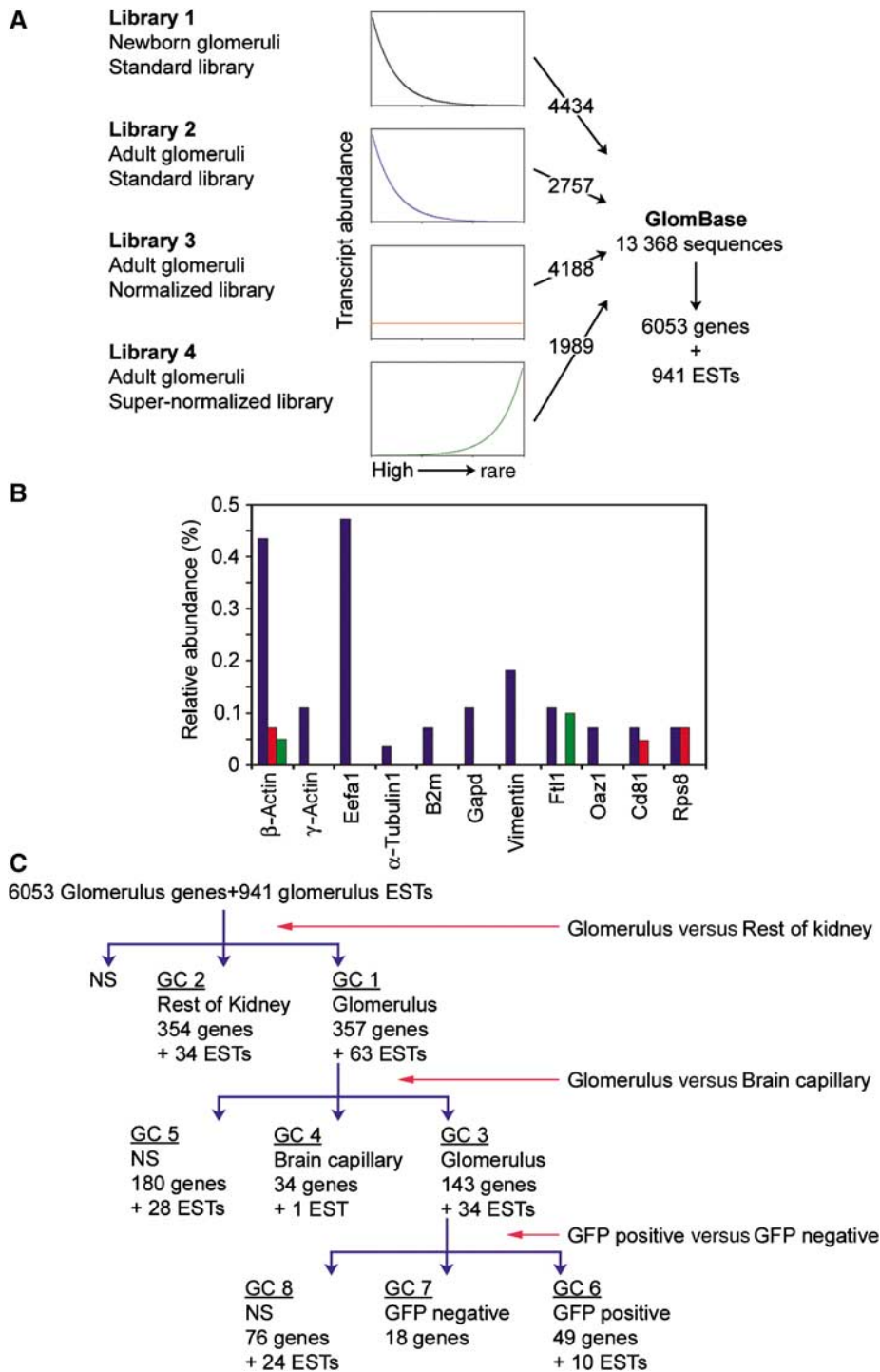
Figure 1 Generation of GlomBase and GlomChip and identification of glomerulus-expressed and -specific genes. (**A**) The four glomerular cDNA libraries, their theoretical distribution of cDNA clones relative to original transcript abundance, the number of sequenced clones from each library and the total number of sequences, annotated genes and ESTs. (**B**) Relative abundance of different housekeeping genes in the adult standard (blue bars), adult normalized (red bars) and adult supernormalized (green bars) libraries. Eef1a1, elongation factor 1 α1; B2 m, β2 microglobulin; Gapd, Glyceraldehyde 3-phosphate dehydrogenase; Ftl1, Ferritin light chain 1; Oaz1, Ornithine decarboxylase antizyme; Rps8, 40S ribosomal protein S8. (**C**) Flow chart of GlomChip hybridizations. GlomChips were hybridized against labeled targets from different tissues: isolated glomeruli, non-glomerular kidney tissue (rest of kidney), brain capillary fragments, GFP-positive glomerular cells and GFP-negative glomerular cells. Stepwise comparisons between pairs of tissues provided lists of significantly upregulated ($>$2-fold) genes in each tissue category, or not significantly different (NS). GC: gene category.

ular cell types, we FACS-sorted GFP-positive podocytes from mice in which GFP expression was activated from the Z/EG transgene by Cre-recombinase expressed under the control of the podocin (Nphs2) promoter (Belteki *et al*, 2005) (Figure 2).

Glomeruli were isolated by Dynabead perfusion from 8-day-old podocin-Cre;Z/EG mice (Figure 3B), and enzymatically digested into single-cell suspensions (Figure 3C). RNA was extracted from 15 000 GFP-positive cells obtained from three
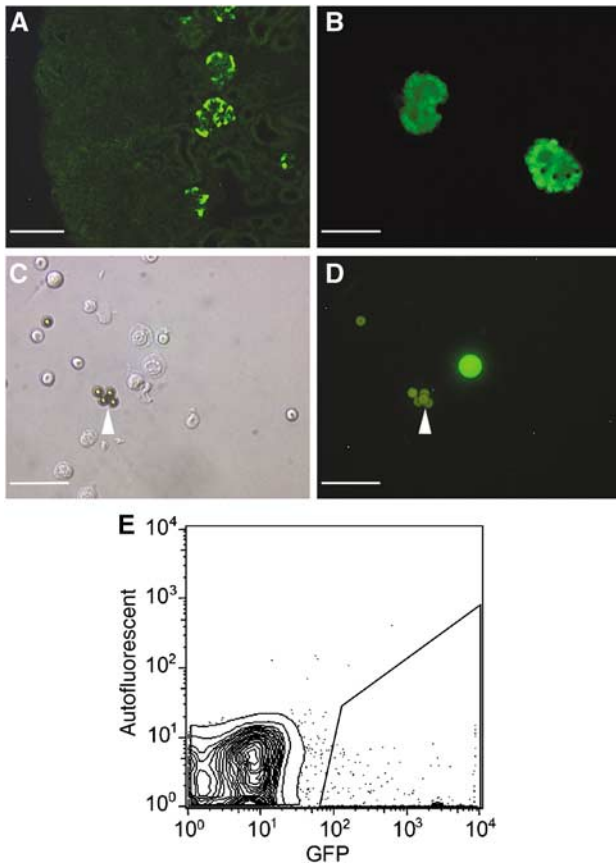
Figure 2 Isolation of podocytes from Podocin-Cre × Z/EG mice. (**A**) Post-natal day 1 kidneys from Podocin-Cre × Z/EG mice examined by fluorescence microscopy. Note the crescent of GFP-positive podocytes in each glomerulus. (**B**) Dynabead-isolated glomeruli from Podocin-Cre × Z/EG mice. (**C, D**) Single-cell suspensions were prepared from isolated glomeruli and evaluated under the microscope with or without fluorescence. Arrowheads indicate Dynabeads. (**E**) Glomerular cells sorted by GFP fluorescence (quadrangle).

mice, and from the same number of GFP-negative cells, and used for GlomChip analysis. By this procedure, we further subdivided category 3 genes into category 6 (49 genes and 10 ESTs upregulated in GFP-positive podocytes; Table I), category 7 (18 genes upregulated in non-podocyte glomerular cells; Table II) and category 8 genes (76 genes and 24 ESTs, which were not significantly differentially expressed more than two-fold) (Supplementary Table S8).

Category 6 genes represent candidate podocyte-specific transcripts. Indeed, most known podocyte-specific transcripts fell among the top 20 genes in this category, as did several other genes known to be highly expressed in podocytes (e.g. Cdnk1c and tau). Category 7 genes instead include several known mesangial cell and juxtaglomerular markers, such as renin1 (Ren1), integrin α8 (Itga8), protease nexin I (Serpine2, PN-1) and mesoderm-specific transcript (Mest). Category 7 genes therefore represent a list of candidate mesangial cell markers. Category 7 genes may also include markers that are specific to glomerular endothelial cells in comparison with other types of endothelium.

Validation by in situ hybridization
We employed ISH to establish the cellular expression of some of the candidates for novel podocyte- and non-podocyte-

specific glomerular transcripts. Figure 3A shows by non-radioactive ISH the overall distribution in E18.5 kidney of five novel podocyte transcripts—Semaphorin sem2 (Sem2), Rhophilin 1 (Rhpn1), Cbp/p300-interacting transactivator 2 (Cited 2), Protease inhibitor 15 (Pi15) and Gene X—in comparison with three known podocyte markers, Nphs2 (Boute *et al*, 2000), Podxl (Kershaw *et al*, 1997) and Forkhead box c2 (Foxc2) (Miura *et al*, 1993). Figure 3B shows the overall distribution in E18-5 kidney of three novel mesangial markers: secreted frizzled-related protein 2 (Sfrp2), Aldo-keto reductase family 1 member B7 (Akr1b7) and Lim domain only protein 7 (Lmo7) in comparison with known mesangial, juxtaglomerular and endothelial transcripts insulin-like growth factor binding protein 5 (Igfbp5), Ren1 and endomucin (Emcn), respectively. Commentaries and references to the eight novel glomerular markers are provided in Supplementary Table S9.

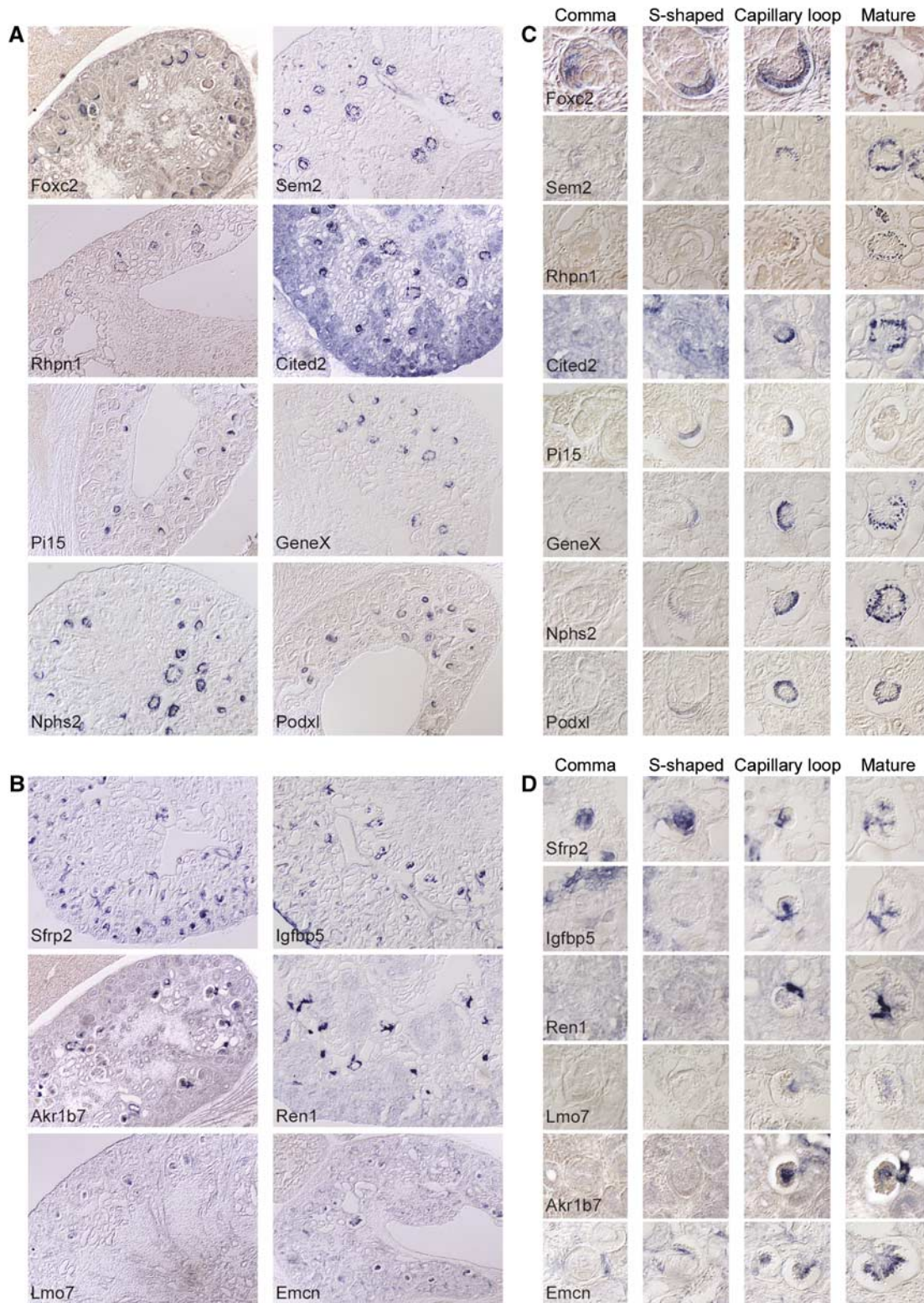### Temporal expression patterns of glomerular cell markers during nephron development
Of the podocyte markers studied, only Foxc2 was expressed during the comma-shaped stage of nephron development, that is, before morphological distinctions can be made between prospective podocytes and tubular epithelium (Figure 3C). Morphologically distinguishable podocytes appear during the S-shaped stage of nephron development at which stage the known podocyte markers Nphs2 and Podxl appeared in developing podocytes, together with the novel markers Sem2, Pi15, Rhpn1 and gene X (Figure 3C). During the capillary loop and mature stages, all podocyte markers were expressed. Foxc2 and Pi15 were the only markers showing a distinct transient peak of expression during S-shaped and capillary loop stages.

Both the known and novel mesangial markers were expressed first during the capillary loop and mature stages, with the exception of Sfrp2. Sfrp2 was first expressed in the epithelium of the comma- and S-shaped nephron stages, and subsequently switched to the mesangium during the capillary loop and mature stages of glomerular development (Figure 3D). In addition to the mesangial cells, expression of all these markers was also noticed in smooth muscle cells of the glomerular afferent and efferent arterioles (Figure 3D and data not shown).

Abnormal glomerular development in foxc2−/− mice
To begin examining the potential developmental role of the glomerulus-specific genes we have identified in this study, we focused on Foxc2. As expression of Foxc2 mRNA and protein in developing podocytes preceded other more definitive podocyte markers (Figures 3C and 4), we studied glomerular development in late-stage *foxc2*−/− embryos (Iida *et al*, 1997). Embryonic day 18.5 *foxc2*−/− embryos had smaller kidneys and reduced numbers of glomeruli compared to +/+ littermates (Figure 4). Mutant glomeruli were also abnormally shaped and contained fewer than normal capillary loops, which were dilated and blood-filled (Figure 5A).

Dilated glomerular capillary loops are characteristic of mesangial cell-deficient glomeruli observed in platelet-derived growth factor-B (*pdgfb*) and PDGF receptor-beta (*pdgfrb*) mutant mice (Levéen *et al*, 1994; Soriano, 1994;

Figure 3 Glomerular gene expression patterns determined by ISH in E18.5 kidneys. (A, B) Overview of the renal expression of glomerulus-expressed genes by non-radioactive ISH. (**A**) Podocyte-expressed genes Foxc2, Sem2, Rhpn1, Cited2, Pi15, GeneX, Nphs2 and Podxl. (**B**) Mesangial/juxtaglomerular-expressed genes Sfrp2, Igfbp5, Akr1b7, Ren1 and Lmo7, and endothelium-expressed gene Emcn (endomucin). (C, D) Temporal expression of podocyte (**C**) and mesangial/juxtaglomerular (**D**) markers during nephron development by non-radioactive ISH. 'Comma' and 'S-shaped' refer to morphologically distinct early stages of nephron development, whereas 'capillary loop' and 'mature' refer to morphologically distinct late stages of glomerulus development. Note that only Foxc2 and Sfrp2 are expressed at the earliest stage of nephron development, whereas all other markers appear in the podocyte and mesangial/juxtaglomerular apparatus cells during S-shaped (podocyte markers) and capillary loop stages (mesangial markers).

Table I List of category 6 and 7 genes in FigureC

	ENSEMBL gene ID	Glomerulus cDNA clones ENSEMBL gene name	ENSEMBL Symbol	Ratio 1	Ratio 2	Ratio 3
Gene category 6						
1	ENSMUSG00000026602	Podocin	Nphs2	3.06	6.48	3.63
2	ENSMUSG00000006649	Nephrin	Nphs1	2.97	4.2	3.16
3	ENSMUSG00000030223	Protein tyrosine phosphatase, receptor type, O	Ptpro	2.43	4.55	3.25
4	ENSMUSG00000016458	Wilms' tumor protein homolog	Wt1	2.08	5.47	2.17
5	ENSMUSG00000050010			2.14	4.79	2.67
6	ENSMUSG00000037664	Cyclin-dependent kinase inhibitor 1C	Cdkn1c	2.81	3.51	3.24
7	ENSMUSG00000022580	**Rhophilin 1**	Rhpn1	2.84	3.47	3.12
8	ENSMUSG00000035333	**protease inhibitor 15; cysteine-rich secreted protein**		1.68	3.85	3.49
9	ENSMUSG00000059213			1.84	4.21	2.87
10	ENSMUSG00000015093	Chloride intracellular channel protein 3	Clic3	2.71	2.44	3.73
11	ENSMUSG00000029061	Matrix metalloproteinase 23	Mmp23	2.09	3.55	2.91
12	ENSMUSG00000043079	Synaptopodin	Synpo	2.84	2.57	2.95
13	ENSMUSG00000003411	Ras-related protein Rab-3B	Rab3b	2.25	3.08	3.01
14	ENSMUSG00000046714	Forkhead box protein C2	Foxc2	2.66	3.14	2.54
15	ENSMUSG00000021904	**Semaphorin sem2 (GlomBase annotation)**		3.01	2.68	2.57
16	ENSMUSG00000030513	PACE4A (Fragment)	Pcsk6	1.82	4.23	2.18
17	ENSMUSG00000004105	Angiopoietin-related protein 2 precursor	Angptl2	2.12	2.61	3.04
18	ENSMUSG00000056492			2.51	1.83	2.6
19	ENSMUSG00000025608	Podocalyxin-like protein 1 precursor	Podxl	2.2	2.26	2.41
20	ENSMUSG00000029641			1.6	3.58	1.6
21	ENSMUSG00000032679	CD59A glycoprotein precursor	Cd59a	1.68	3.51	1.53
22	ENSMUSG00000025810	Neuropilin-1 precursor (A5 protein)	Nrp1	2.54	2.72	1.28
23	ENSMUSG00000038831	Ral guanine nucleotide exchange factor RalGPS1A	Ralgps1	1.96	2.16	2.37
24	ENSMUSG00000029887	Maltase-glucoamylase	Mgam	1.28	2.39	2.62
25	ENSMUSG00000020431	Adenylyl cyclase type I	Adcy1	1.53	2.45	2.27
26	ENSMUSG00000005501	Ubiquitin carboxyl-terminal hydrolase 40	Usp40	1.72	1.61	2.87
27	ENSMUSG00000020458	Reticulon 4	Rtn4	1.5	2.25	2.36
28	ENSMUSG00000026817	Adenylate kinase isoenzyme 1	Ak1	1.4	2.41	2.27
29	ENSMUSG00000052516	Roundabout homolog 2 precursor	Robo2	1.9	1.83	2.06
30	ENSMUSG00000026305	Leucine rich repeat (in FLII) interacting protein 1	Lrrfip1	1.18	2.67	1.9
31	ENSMUSG00000024534	Synuclein, alpha interacting protein	Sncaip	1.25	2.45	2.03
32	ENSMUSG00000039910	**Cbp/p300-interacting transactivator 2**	Cited2	1.62	2.46	1.52
33	ENSMUSG00000032625			1.47	1.77	2.29
34	ENSMUSG00000022817	Integrin beta-5 precursor	Itgb5	1.22	1.78	2.45
35	ENSMUSG00000018411	Microtubule-associated protein tau	Mapt	1.93	2.02	1.49
36	ENSMUSG00000018995			1.54	2.09	1.77
37	ENSMUSG00000058056	NIH actin-associated protein palladin (Fragment)	2410003B16Rik	1.24	1.71	2.43
38	ENSMUSG00000017446	C1q and tumor necrosis factor related protein 1	C1qtnf1	2.29	1.42	1.62
39	ENSMUSG00000031217	Ephrin-B1 precursor	Efnb1	1.5	2.2	1.56
40	ENSMUSG00000029638	Glucocorticoid induced transcript 1	Glcci1	1.21	1.11	2.83
41	ENSMUSG00000026584	Ezrin-binding partner PACE-1	Scyl3	1.52	1.41	1.9
42	ENSMUSG00000020520	UDP-N-acetyl-alpha-D-galactosamine,Galnt10	Galnt10	1.74	1.05	2.01
43	ENSMUSG00000027170	Dendritic cell protein GA17	Ga17	1.21	1.93	1.64
44	ENSMUSG00000020044	Metalloproteinase inhibitor 3 precursor	Timp3	1.4	2.15	1.13
45	ENSMUSG00000020601	Tribbles homolog 2	Trib2	1.37	1.52	1.6
46	ENSMUSG00000008348	Ubiquitin	Ubc	1.15	1.45	1.81
47	ENSMUSG00000042675	Yippee-like protein 3	Ypel3	1.21	1.32	1.66
48	ENSMUSG00000027777	Schwannomin interacting protein 1	Schip1	1.12	1.07	1.82
49	ENSMUSG00000000325	Arvcf	Arvcf	1.19	1.01	1.77
1		EST		2.45	3.09	2.77
2		EST		2.49	3.26	2.25
3		EST		1.99	2.99	2.30
4		**EST Gene X (GlomBase annotation)**		2.28	2.79	2.12
5		EST		2.19	2.25	2.72
6		EST		2.24	2.95	1.86
7		EST		2.70	2.01	1.48
8		EST		1.26	1.88	2.64
9		EST		1.55	2.14	1.89
10		EST		1.43	1.33	1.91
Gene category 7						
	ENSEMBL gene ID					
1	ENSMUSG00000026449	Renin 1	Ren1	3.09	6.26	−2.48
2	ENSMUSG00000026185	Insulin-like growth factor binding protein 5	Igfbp5	2.18	3.51	−4
3	ENSMUSG00000024065			3.09	3.09	−3.46
4	ENSMUSG00000026768	Integrin alpha 8	Itga8	1.95	3.01	−3.44
5	ENSMUSG00000027996	**Secreted frizzled-related sequence protein 2**	Sfrp2	1.45	4.05	−2.26
6	ENSMUSG00000033060	**LIM domain only protein 7 (GlomBase annotation)**	Lmo7	1.31	3.2	−2.84
7	ENSMUSG00000054690	Endomucin	Emcn	2.38	1.85	−2.71
8	ENSMUSG00000026249	Protease nexin I	Serpine2	1.88	1.36	−3.68
9	ENSMUSG00000027210	Homeobox protein Meis2	Mrg1	1.3	2.45	−2.54

Table I Continued

	ENSEMBL gene ID	Glomerulus cDNA clones ENSEMBL gene name	ENSEMBL Symbol	Ratio 1	Ratio 2	Ratio 3
10	ENSMUSG00000054435	Immune associated nucleotide 1 isoform b		2.21	1.66	−2.19
11	ENSMUSG00000021759	Lipid phosphate phosphohydrolase 1	Ppap2a	1.77	1.9	−2.09
12	ENSMUSG00000029273	Sulfotransferase family 1D, member 1	Sult1d1	1.3	2.66	−1.78
13	ENSMUSG00000039503			1.51	2.02	−2.1
14	ENSMUSG00000048376	Proteinase activated receptor 1	F2r	2.17	1.58	−1.55
15	ENSMUSG00000028713	Cytochrome P450 4B1	Cyp4b1	1.96	1.61	−1.48
16	ENSMUSG00000011263			1.82	1.49	−1.66
17	ENSMUSG00000051855	Mesoderm specific transcript	Mest	1.21	2.03	−1.64
18	ENSMUSG00000039706	LIM domain binding 2	Ldb2	1.47	1.46	−1.84

Category 6 genes represent podocyte marker candidates and category 7 genes represent mesangial marker candidates. ISH validated novel cell-type-specific transcripts in bold. Clone ID refers to GlomBase cDNA clone. ESTs refer to cDNA clones without unambiguous ENSEMBLE annotation. For some genes (Sem2, GeneX), the given gene name refers to our own (GlomBase) annotation. Ratio 1–3 refer to log 2 ratios for comparison of glomeruli versus rest of kidney (Ratio 1), glomeruli versus brain capillaries (Ratio 2) and podocyte versus non-podocyte glomerular cells (Ratio 3).

Table II List of 25 most downregulated transcripts in *foxc2−/−* glomeruli revealed by GlomChip analysis

	ENSEMBL gene ID	ENSEMBL gene name	ENSEMBL Symbol	Ratio
1	ENSMUSG00000025384			−4.83
2	ENSMUSG00000052957	Growth-arrest-specific protein 1	Gas1	−3.66
3	ENSMUSG00000046714	Forkhead box protein C2	Foxc2	−3.36
4	ENSMUSG00000043984			−3.33
5	ENSMUSG00000053113	Suppressor of cytokine signaling 3	Socs3	−3.32
6	ENSMUSG00000021250	Proto-oncogene protein c-fos	Fos	−3.30
7	ENSMUSG00000026602	Podocin	Nphs2	−3.25
8	ENSMUSG00000035356	IkappaB-zeta	Nfkbiz	−3.24
9	ENSMUSG00000003032	Kruppel-like factor 4	Klf4	−3.19
10	ENSMUSG00000023034	Orphan nuclear receptor NR4A1	Nr4a1	−3.16
11	ENSMUSG00000018899	Interferon regulatory factor 1 (IRF-1)	Irf1	−3.08
12	ENSMUSG00000038418	Early growth response protein 1	Egr1	−3.08
13	ENSMUSG00000043964		BC061259	−2.92
14	ENSMUSG00000029465	Arp2/3 complex subunit p21-Arc	Arpc3	−2.82
15	ENSMUSG00000028024	Glutamyl aminopeptidase	Enpep	−2.73
16	ENSMUSG00000046324	BA207C16.3	D19Wsu12e	−2.70
17	ENSMUSG00000033161	Sodium/potassium-transporting ATPase alpha-1 chain	Atp1a1	−2.70
18	ENSMUSG00000022010	Regulatory protein TSC-22	Tsc22d1	−2.66
19	ENSMUSG00000028195	CYR61 protein	Cyr61	−2.61
20	ENSMUSG00000022580	Rhophilin 1	Rhpn1	−2.60
21	ENSMUSG00000050010			−2.49
22	ENSMUSG00000019970	Serine/threonine-protein kinase Sgk1	Sgk	−2.31
23	EST	Mafb (GlomBase annotation)		−2.29
24	ENSMUSG00000036534			−2.25
25	ENSMUSG00000037664	Cyclin-dependent kinase inhibitor 1C	Cdkn1c	−2.22

Ratio refers to the log 2 ratio between *foxc2+/+* and −/− glomeruli. See also legend to .
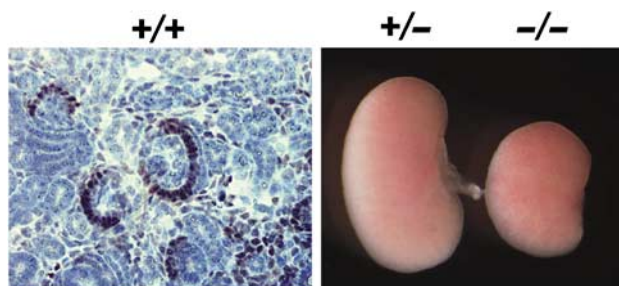
Figure 4 Phenotypic analysis of *foxc2−/−* kidneys. IHC localizes Foxc2 protein mainly to the podocyte crescent of capillary loop stage glomeruli. Note reduced size kidneys in E18.5 *foxc2−/−* compared to +/− mice.

Lindahl *et al*, 1998; Bjarnegård *et al*, 2004). We therefore analyzed the presence and distribution of mesangial cells in the *foxc2−/−* glomeruli. Immunohistochemical staining for the mesangial markers NG2 (cspg4), pdgfrb and desmin in comparison with endothelial (Pecam) and podocyte (Nphs1) markers revealed that mesangial cells were present in *foxc2−/−* glomeruli and had normal marker expression, but their distribution within the glomerulus was abnormal. Instead of forming a branched mesangial core connecting individual capillary loops, the mesangial cells remained localized in a compact cluster at the center of the glomerular tuft (Figure 5A). Additional marker analysis (Ren1, Igfbp5 and Sfrp2) confirmed the normal marker expression but abnormal distribution of mesangial cells in the *foxc2−/−* glomeruli (Figure 5B).

Analysis by transmission electron microscopy (TEM) further confirmed the abnormal mesangial cell distribution in *foxc2−/−* glomeruli (Figure 6A and B). These analyses also showed that the mesangial cells of *foxc2−/−* glomeruli failed to make the normal focal contacts with the GBM.
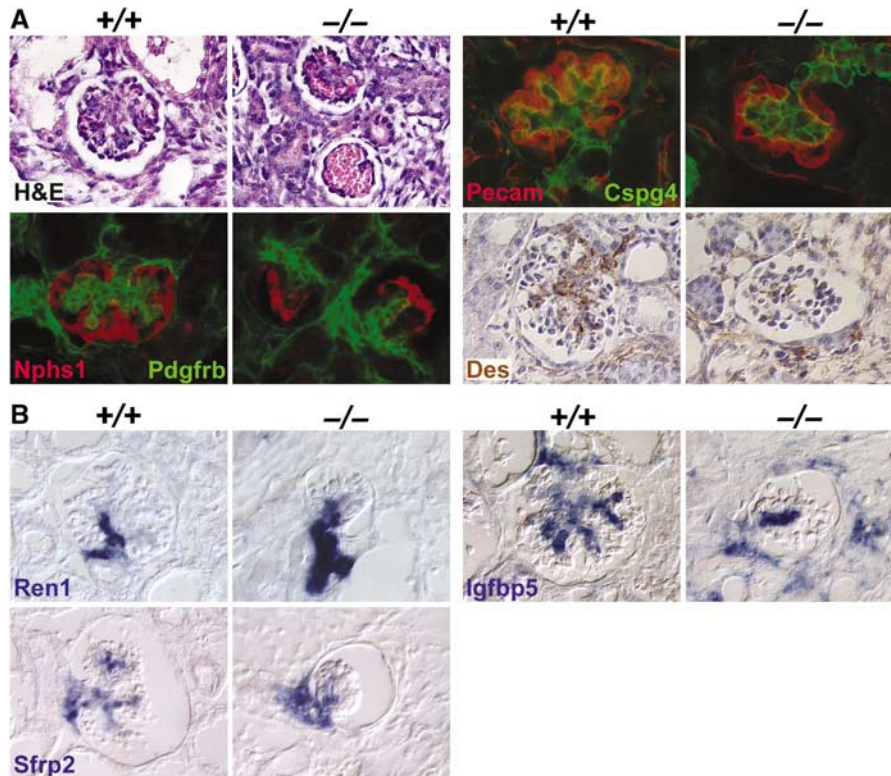
Figure 5 Abnormal organization of mesangial cells in *foxc2*−/− mice. (**A**) Hematoxylin and eosin staining of kidney sections reveals distended capillary loops in *foxc2*−/− glomeruli. Abnormal development of the mesangial core in *foxc2*−/− glomeruli was demonstrated by IHC for the mesangial cell markers Cspg4 (chondroitin sulfate proteoglycan 4, also called NG2), Pdgfrb (platelet-derived growth factor receptor-β) and Des (desmin) in combination with the endothelial marker Pecam (Pecam1/CD31) and Nphs1 (nephrin). (**B**) Non-radioactive ISH for Ren1, Igfbp5 and Sfrp2 confirmed normal marker expression but abnormal organization of the glomerular mesangium in *foxc2*−/− glomeruli, in which the mesangial cells failed to form a tree-like mesangial core.

Podocyte and endothelial differentiation defects in *foxc2*−/− mice

TEM analysis demonstrated that the podocytes lacked foot processes and SD, and were instead connected by structures resembling adherence junctions, characteristics normally present in immature podocytes at the S- and cup-shaped stages of glomerular development (Figure 6C and D). The TEM analysis also showed that the endothelial cells lacked fenestrations (Figure 6C and D), which normally accumulate at the capillary loop stage of glomerular development. The ultrastructural features of the podocytes and the endothelial cells in *foxc2*−/− mice suggest that the differentiation of these two cell types is arrested before the capillary loop stage of glomerular development.

Morphometric analyses of glomerular capillary number, size and fenestration in the *foxc2*−/− glomeruli (Figure 7) confirmed the significant reduction in capillary loop number, the increased capillary size and the almost complete absence of endothelial fenestrations.

GlomChip analysis of *foxc2*−/− glomeruli reveals downstream targets for Foxc2

To gain further insight into the glomerular defects, we transcription-profiled *foxc2*−/− glomeruli by GlomChip analysis. This analysis revealed a total of 501 >2-fold down-regulated and 232 >2-fold upregulated transcripts (Supplementary Table S10), indicating that quite complex transcriptional changes take place as a result of Foxc2 defi-

ciency. Table II shows a partial list of downregulated genes in *foxc2*−/− glomeruli, which include a number of known (Nphs2, Mafb and Cdkn1c) and one novel (Rhpn1) podocyte markers. Several other known (Nphs1, Wt1, Cd2ap) and novel (Sem2, Pi15 and GeneX) podocyte markers were not changed in their expression. Absence of Foxc2 transcripts, as well as the downregulation of Nphs2, Rhpn1, Cdkn1c, Cited2 and Podxl1 transcripts in *foxc2*−/− glomeruli, was also demonstrated by ISH (Figure 8A). IHC revealed significant downregulation of Nphs2 protein, and showed a modest downregulation of Podxl1, whereas Nphs1, Wt-1, Synpo and Cd2ap signals were all comparable to wild type (Figure 8B). Considering that most podocyte markers are turned on simultaneously in glomerular development (Figure 3C), the selective downregulation of some of these markers argues against a general block in the differentiation of podocytes in *foxc2*−/− glomeruli.

As podocytes contribute to the formation of the GBM, we also studied the expression of podocyte-derived extracellular matrix proteins and integrins in *foxc2*−/− glomeruli. We found no difference in the deposition of the early GBM collagen IV chains, col4a1 and col4a2, but noticed significant decreases in the labeling of the mature GBM collagens col4a3, col4a4 and col4a5 (Figure 8B). GBM laminins and proteogly-cans appeared normally expressed, as was the podocyte-restricted integrin α3 chain (Figure 8C). These data suggest that the GBM is deposited normally during early development of *foxc2*−/− glomeruli, but fails to mature during the cup-
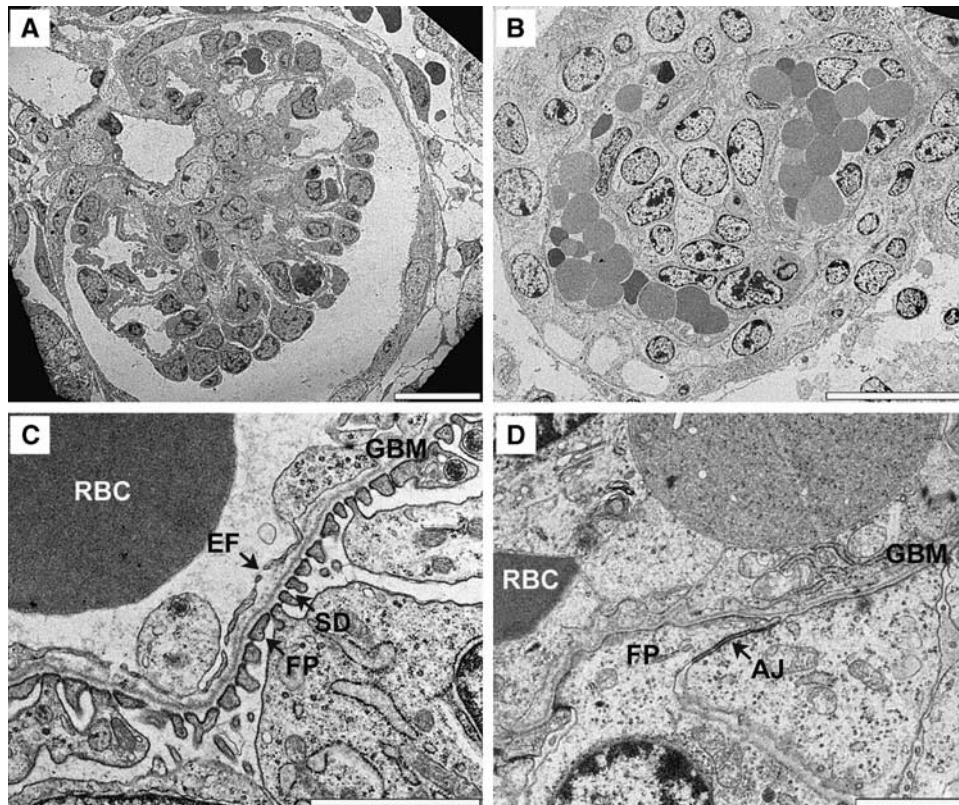
Figure 6 Ultrastructure of *foxc2*−/− glomeruli. TEM analysis of *foxc2*+/+ (**A, C**) and *foxc2*−/− (**B, D**) E18.5 glomeruli. At low magnification (A, B), *foxc2*−/− glomeruli show distended capillaries, which are commonly filled with red blood cells (RBC). High magnification (C, D) reveals aberrant podocyte foot processes (FP) connected by structures resembling adherens junctions (AJ) and thick endothelial cells lacking fenestrae (EF) in *foxc2*−/− glomeruli (D). Bars: 20 μm (A, B), 2 μm (C, D).

shaped and capillary loop stages through the incorporation of col4a3, col4a4 and col4a5 chains.

Discussion

Here we used a platform for glomerulus transcription profiling in the mouse, which combines an efficient method for glomerulus isolation with a tailored array—GlomChip—covering over 6000 glomerulus-expressed genes. Transcription profiling studies on healthy and diseased kidneys, including isolated glomeruli, have previously been reported (Virlon *et al*, 1999; Wada *et al*, 2001; Elalouf *et al*, 2002; Chabardes-Garonne *et al*, 2003; Sarwal *et al*, 2003; Wilson *et al*, 2003; Baelde *et al*, 2004; Higgins *et al*, 2004; Peterson *et al*, 2004; Sadlier *et al*, 2004; Susztak *et al*, 2004). When comparing our study to the earlier reports, it becomes apparent that the detection of glomerulus-specific transcripts, in particular those with podocyte-specific expression, is notoriously problematic. This is likely caused by the scarcity of the relevant cell types, in particular the podocytes, in the analyzed tissue, although different profiling methodologies may also have their limitations. Thus, although global transcript profiling of kidney diseases has already proven useful to categorize certain global renal responses (Sarwal *et al*, 2003), it will likely be of limited value for the glomerular disorders. GlomChip analysis of isolated glomeruli efficiently measured known podocyte-specific transcripts, and has in the present state of our analysis also revealed the existence of five novel podocyte transcripts, as well as three transcripts specific for mesangial and juxtaglomerular cells.

To provide an example of the functional relevance of genes detected by GlomChip analysis, we analyzed the role of the winged helix transcription factor Foxc2 in glomerular development. Critical roles for Foxc2 during the development of several different organ systems have been reported (Iida *et al*, 1997; Petrova *et al*, 2004), including kidney (Kume *et al*, 2000), but its function in podocytes has not previously been studied, although expression has been reported (Miura *et al*, 1993; Rohr *et al*, 2002). Kidney problems have been reported in patients with *FOXC2* haploinsufficiency and lymphedema distichiasis (Yildirim-Toruner *et al*, 2004), but it is not yet known whether these problems reflect a primary kidney defect, or whether they are secondary to other problems in these patients.

We found Foxc2 transcripts in prospective podocytes before the appearance of other podocyte markers, suggesting a role in podocyte differentiation. Indeed, we found that *foxc2*−/− podocytes fail to form foot processes and SD, and instead remain columnar and partially connected by adherence junctions, which are typical features of immature podocytes (Kreidberg, 2003). Other mutants that lack podocyte foot processes *a priori* include knockouts for the podocyte-restricted proteins LIM-homeodomain transcription factors Lmx1b (Chen *et al*, 1998; Morello *et al*, 2001; Miner *et al*, 2002; Rohr *et al*, 2002), the helix–lop–helix transcription factor Pod1 (Cui *et al*, 2003), the transmembrane sialomucin podocalyxin (Doyonnas *et al*, 2001) and α3 integrin (Kreidberg *et al*, 1996). We therefore asked if any of these factors were regulated in their expression by Foxc2. Whereas expression of Pod1, Lmx1b and α3 integrin was normal
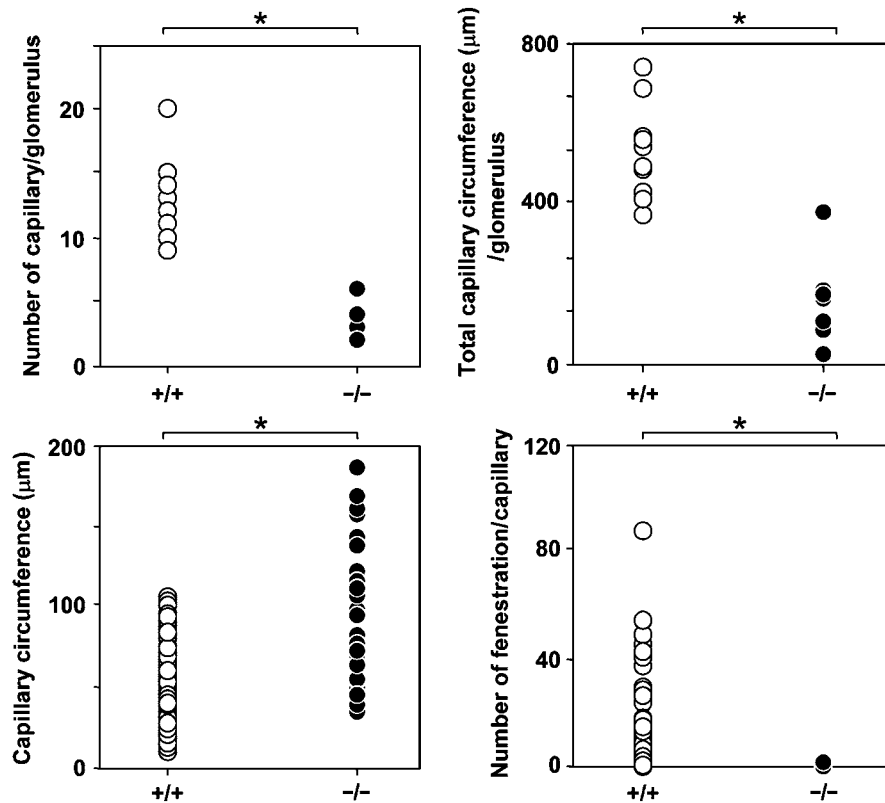
Figure 7 Morphometric analysis of endothelial cell abnormalities in *foxc2−/−* glomeruli. Morphometric analysis of TEM data shows that *foxc2−/−* glomeruli contain fewer capillary lumens, which were dilated; however, total capillary circumferences per glomerulus decreased compared to controls. *Foxc2−/−* glomeruli almost completely lack endothelial fenestrations. A total of 11 glomeruli from two *foxc2+/+* and one *foxc2+/−* kidneys were compared to nine glomeruli from two *foxc2−/−* kidneys. The statistical significance was analyzed using Student's *t*-test. *$P < 0.001$.

in *foxc2−/−* podocytes, as revealed by GlomChip analysis, RT–PCR and IHC, respectively (this study and data not shown), podocalyxin expression was partially downregulated as revealed by both GlomChip analysis and ISH. It is unlikely, however, that the podocalyxin downregulation contributes more than marginally to the glomerular phenotype in *foxc2−/−* mice, given the lack of reported defects in podocalyxin +/− mice (Doyonnas *et al*, 2001).

The strongly reduced expression of *Col4a3* and *Col4a4* in *foxc2−/−* mice is interesting, as mutations in these genes cause Alport's syndrome. However, it cannot explain the podocyte differentiation defects of *foxc2−/−* mice, as individuals with Alport's syndrome and mice with inactivating mutations in *Col4a3* or *Col4a4* have morphologically normal glomeruli at young age (Miner and Sanes, 1996; Lu *et al*, 1999). Likewise, the loss of podocin in *foxc2−/−* mice cannot fully explain the severe podocyte defects but may be a contributing factor, as lack of podocin leads to a milder foot process defect with partial resemblance to that caused by Foxc2 deficiency (Roselli *et al*, 2004). Additional strongly downregulated genes included the basic domain leucin zipper transcription factor Mafb/Kreisler and the cyclin-dependent kinase inhibitor Cdkn1c/p57. Mice lacking functional Mafb develop nephrotic syndrome and show absence or fusion of podocyte foot processes, but glomerular development proceeds through the capillary loop stage in these mice resulting in histologically normal glomeruli (Sadl *et al*, 2002). Thus, Mafb downregulation may contribute to the foot process defect in *foxc2−/−* podocytes, but cannot explain the glomerular devel-

opmental arrest before the capillary loop stage. Mafb expression is reduced also in *pod1−/−* mice (Sadl *et al*, 2002), suggesting that Foxc2 and Pod1 operate in parallel pathways to regulate Mafb expression. Mice deficient of Cdkn1c/p57 develop podocyte foot processes and SD (Tomari *et al*, 2002) and hence the downregulation of Cdkn1c/p57 unlikely contributes to the *foxc2−/−* podocyte differentiation defect.

GlomChip analysis and ISH also revealed that the expression of two of the newly discovered podocyte markers, Rhophilin 1 (Rhpn1) and Cited2, was strongly diminished in *foxc2−/−* glomeruli. Rphn1 was originally identified as a Rho GTPase binding protein in a yeast two-hybrid screen (Watanabe *et al*, 1996). As a putative Rho interactor, one may speculate that Rhophilin 1 is involved in the cytoskeletal rearrangements required for foot process formation in podocytes. Cited2 has been proposed to act as a negative regulator of hypoxia-inducible factor (HIF)-1 through competitive binding to CBP/p300, and *cited2−/−* mice die at late gestation from defects that are similar to those caused by the HIF-1 target gene VEGF-A (Yin *et al*, 2002). We were not able to document VEGF-A overexpression in *cited 2−/−* glomeruli, which also appeared morphologically normal with the exception that the capillary loops were slightly dilated (M Takemoto, unpublished observations). It is therefore unlikely that the Cited2 downregulation contributes significantly to the phenotypic abnormalities of *foxc2−/−* glomeruli.

As Foxc2 mRNA and protein expression within the glomerulus is restricted to the podocytes, the endothelial and mesangial defects seen in *foxc2−/−* mice are likely second-
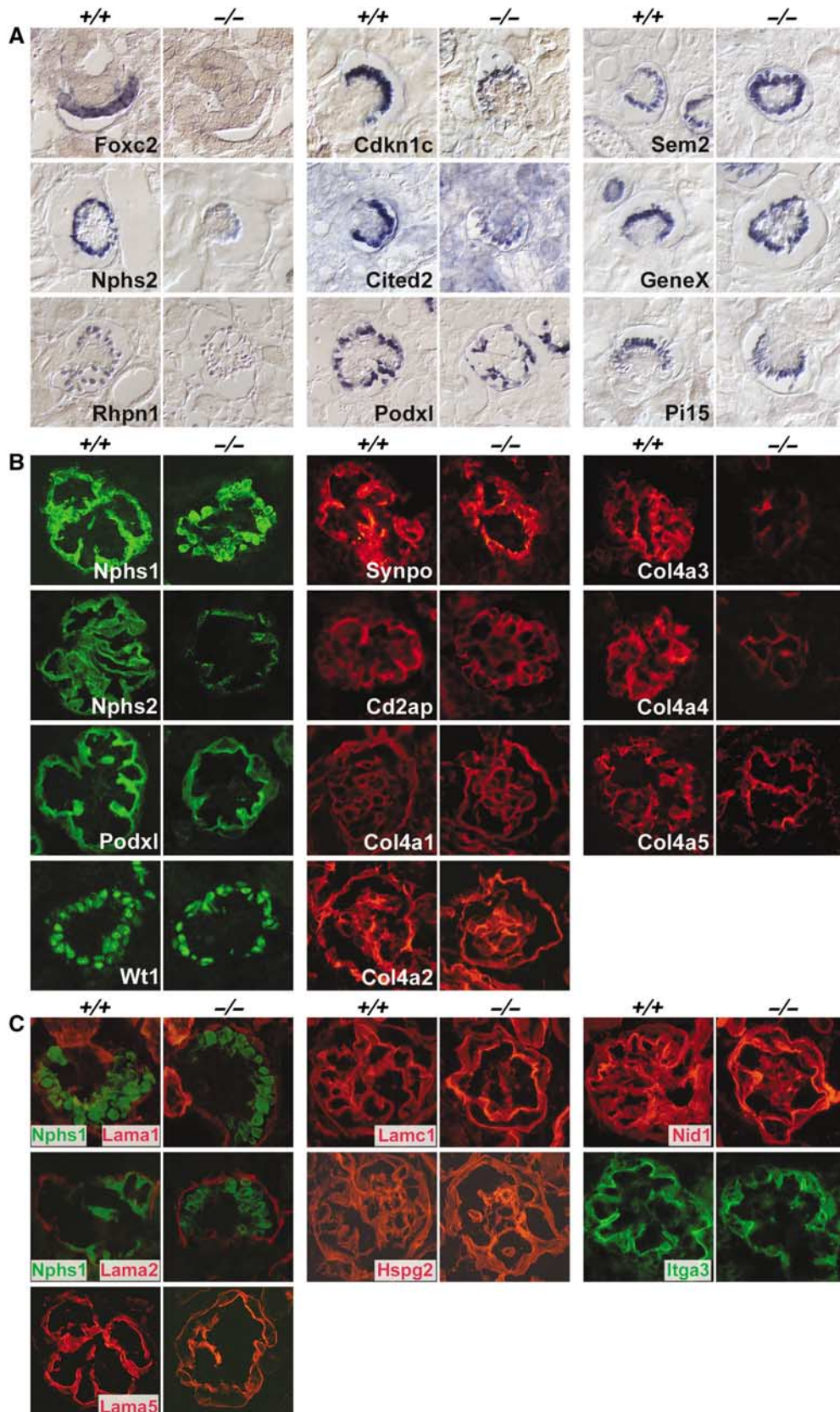
Figure 8 Expression of podocyte genes and proteins and GBM components in *foxc2*−/−glomeruli. The genes/proteins tested were mainly selected to validate from the gene profiling of *foxc2*−/− and *foxc2*+/+ glomeruli (see Table II). (**A**) Non-radioactive ISH. (**B**) Fluorescent IHC. These analyses confirmed the specific and strong downregulation in *foxc2*−/− glomeruli of Nphs2, Rhpn1, Col4a3, Col4a4 and Col4a5, as well as a modest downregulation of Cdkn1c, Cited2 and Podxl, whereas other tested transcripts and proteins were not significantly altered. (**C**) The following GBM and extracellular matrix components were normally expressed in *foxc2*−/− glomeruli as judged by fluorescent IHC analysis: Lama1, Laminin alpha-1 chain; Lama2, Laminin alpha-2 chain; Lama5, Laminin alpha-5 chain; Lamc1, Laminin gamma-1 chain; Hspg2, Heparan sulfate proteoglycan core protein/Perlecan; Nid1, Nidogen/Entactin; Itga3, Integrin alpha-3.

ary, pinpointing the critical interdependence of the glomerular cell types during development. The glomerular capillary ballooning observed in *foxc2*−/− glomeruli resembles that seen in mice lacking PDGF-B (Levéen *et al*, 1994) or PDGF-Rβ (Soriano, 1994), but whereas *pdgfb* and *pdgfrb* mutants fail to recruit mesangial cells into the glomerular space, mesangial cells are present in the *foxc2*−/− glomeruli, where they appear to be unable to make the contacts with the GBM that are required for GBM folding. This phenotype is reminiscent of that observed in mice expressing a mutant laminin α5 with its G domain replaced by that of laminin α1, leading to loss of interaction with integrins present on mesangial cells (Kikkawa *et al*, 2003). The *foxc2*−/− phenotype does not appear to involve significant downregulation of PDGF-B, Rβ or laminin α5, however, suggesting that other factors controlled by Foxc2 are involved in the establishment of mesangial cell– GBM contacts. Importantly, we did not find abnormal expression of any of the tested mesangial markers, suggesting a defect in the GBM rather than in the mesangial cells. Irrespective of its cause, we asked whether the deficient mesangial cell–GBM adhesion could be responsible for any of the observed changes in podocyte or endothelial cell differentiation. Podocyte foot processes and SD as well as glomerular endothelial fenestrations develop in the complete lack of mesangial cells (Levéen *et al*, 1994; Soriano, 1994), suggesting that mesangium–GBM contacts or mesangial cell-derived signals are not required for podocyte and glomerular endothelial differentiation. Also, by GlomChip analysis of *pdgfb*−/− and *pdgfrb*−/− glomeruli, we conclude that the lack of mesangial cells does not influence expression of any of the known or novel podocyte markers (M Takemoto *et al*, unpublished observations). Thus, available data suggest that Foxc2 regulates the expression of podocyte genes in a cell-autonomous fashion.

In summary, Foxc2 deficiency leads to glomerular developmental arrest, including primary podocyte defects and secondary endothelial and mesangial cell abnormalities. Foxc2 deficiency causes complex but specific changes in the expression of podocyte genes and proteins, including those mutated in Alport's syndrome and steroid-resistant nephrotic syndrome. It is not possible to explain the *foxc2*−/− podocyte defect by the changes in expression of any individual podocyte protein for which genetic information is available in mice or humans. More likely, the podocyte defects are caused by changes in the expression of a combination of genes that are directly or indirectly regulated by Foxc2. GlomChip analysis demonstrated that these changes are indeed very complex, affecting more than 700 different genes. It will be interesting to compare these results to glomerular transcription profiles of other podocyte transcription factor mutants, particularly Lmx1b and Pod1, which closely resemble *foxc2* mutants in their podocyte phenotypes. A preliminary comparison with *pod1* mutants (Cui *et al*, 2005) suggests a significant overlap in podocyte target genes. It will also be important to study glomerular development in *foxc1* mutants, given that Foxc1 and Foxc2 collaborate in the development of several other organ systems (Kume *et al*, 2001).

Materials and methods

Animals
RNA for cDNA library construction and microarray hybridization was isolated from C57BL/6 and 129/sv strains of mice or hybrids between the two strains. Podocyte isolation experiments were performed using podocin-Cre, Z/EG double transgenic mice, which also contained ICR background (Eremina *et al*, 2002). Foxc2 mutant mice (Iida *et al*, 1997) were bred on mixed C57Bl6J × ICR background (Petrova *et al*, 2004). Mice were housed in accordance with Swedish animal research regulations. Committees for ethics in animal research in Göteborg and Stockholm approved animal experiments.

RNA preparation and cDNA library construction
Glomeruli were isolated from newborn and adult mice using Dynabead perfusion (Takemoto *et al*, 2002). Using RNeasy mini kits (Qiagen), 400 μg of glomerular total RNA was isolated from about two million glomeruli obtained from 100 mice of ages ranging between 3 weeks and 6 months. An additional 350 μg of glomerular total RNA was isolated from approximately 200 000 glomeruli obtained from 400 newborn mice of ages 1–5 days. The RNA was used to produce standard oligo-dT-primed cDNA libraries (Soares *et al*, 1994) (custom synthesis by Incyte), one each from 'adult' and 'newborn' glomerular RNA. In addition, two normalized libraries were generated from the adult standard library using a modification of the technique described by Soares *et al* (1994), in which high-abundance transcripts were suppressed to different degrees.

Sequencing and annotation
Based on test sequencing results, we attempted a total of 15 627 5′ sequence reads from the same number of random cDNA clones (custom sequencing by Incyte and Genome Vision) selected from the four libraries, which provided 14 171 sequences of 500–800 bp length (91% readability). After vector trimming, a total of 13 368 cDNA sequences longer than 100 bp remained (data for the individual libraries are shown in Supplementary Figure S1 and Supplementary Table S1). BLAST searches against the ENSEMBL mouse gene predictions (ENSEMBL mouse release 26.33b.1, 2004-09-03; http://www.ensembl.org/) resulted in 12 309 high-quality hits (*e*-value <1e−30, alignment identity >85%) representing 6053 different genes. A total of 941 sequences did not match ENSEMBL annotated genes, but matched the mouse genome. BLAST searches were also performed against NCBI EST databases and the mouse Unigene cluster database (http://www.ncbi.nlm.nih.gov/UniGene/).

Tissue isolation
Mouse glomeruli and brain capillary fragments were prepared as described (Enge *et al*, 2002; Takemoto *et al*, 2002). Podocytes were separated from isolated glomeruli from 8-day-old Podocin-Cre, Z/EG double transgenic mice. In these mice, GFP expression was activated from the Z/EG transgene by Cre-recombinase expressed under the control of the podocin (Nphs2) promoter (Belteki *et al*, 2005). Glomeruli were isolated by Dynabead perfusion from 8-day-old podocin-Cre;Z/EG mice, and enzymatically digested into single-cell suspensions as follows: isolated glomeruli were incubated with trypsin solution containing 0.2% trypsin–EDTA (Sigma-Aldrich), 100 μg/ml heparin and 100 U/ml DNase I in PBS for 25 min at 37°C, with mixing by pipetting every 5 min. The trypsin was inactivated with soybean trypsin inhibitor (Sigma-Aldrich) and the cell suspension sieved through a 30 μm pore size filter (BD Bioscience). Cells were collected by centrifugation at 200 *g* for 5 min at 4°C and re-suspended in 1 ml PBS supplemented with 0.1% BSA. To separate GFP-expressing (GFP+) and GFP-negative (GFP−) cells, glomerular cells were sorted using a FACSVantage SE (BD Bioscience) operating at a sheath pressure of 22 psi. Autofluorescent cells were excluded by analyzing the emission of orange light (585 nm). Before sorting, the frequency of GFP-positive cells was 2–5%. After sorting, the GFP-negative fraction contained <0.07% GFP-positive cells. RNA was extracted from 15 000 GFP-positive cells obtained from three mice, and from the same number of GFP-negative cells, and used for GlomChip analysis.

GlomChip data analysis
Microarray data were normalized and processed using the bioconductor limma package (Smyth, 2004). For comparison between two samples, each clone was examined for differential expression at the 5% individual significance level. Multiple test correction was made using the false discovery rate method (Benjamini and Hochberg, 1995).

IHC, ISH and TEM

The following commercial antibodies were used: rabbit anti-mouse Pecam-1 (Pharmingen), rabbit anti-rat Cspg4 (Chemicon), rat anti-mouse Pdgfrb (Biosite), mouse anti-human desmin (DAKO), rabbit anti-human Wt 1 (Santa Cruz), mouse monoclonal against Synpo (Progen), rat anti-Nid1 (Chemicon), rat anti-Lamc1 (Chemicon), rabbit anti-Itga3 (Chemicon), rat anti-Hspg2 (Seikagaku Corp), and fluorochrome- and horseradish peroxidase-conjugated secondary antibodies (Molecular Probes and Vector Laboratories). Antibodies already available in author laboratories were rat anti-mouse Foxc2 (NM), rabbit anti-mouse Nphs1 (KT) and rat monoclonal anti-mouse Col4a1, Col4a2, Col4a3, Col4a4 and Col4a5 (YS). Other antibodies obtained from generous colleagues were rabbit anti-mouse Nphs2 (Corinne Antignac, Hôpital Necker-Enfants Malades, Paris, France), rabbit anti-rat Podxl (Hiroshi Kawachi, Niigata University School of Medicine, Niigata, Japan) and rabbit anti-mouse Cd2ap (Eero Lehtonen, University of Helsinki, Helsinki, Finland), rat anti-Lama1, Lama2, Lama5 (Lydia M Sorokin, Lund University, Lund, Sweden). For IHC, kidneys were frozen in OCT compound and sectioned at 8 μm. Sections were air-dried, fixed in ice-cold acetone for 10 min, rinsed in PBT (PBS with 0.1% Tween 20) and blocked in PBS containing 1% BSA for 30 min except when anti-collagen IV antibodies were used (see Gross *et al*, 2004).

Primary antibodies were diluted in 1% BSA in PBS and applied overnight at 4°C. After PBT rinse for several times, fluorophore-conjugated secondary antibodies were applied for 2 h. Sections were rinsed in PBT, mounted in Mowiol/DABCO and viewed with a fluorescence microscope. Non-radioactive ISH and TEM analyses were performed as previously described (Boström *et al*, 1996; Lahdenkari *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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