Rapid Isolation of Glomeruli Coupled with Gene Expression Profiling Identifies Downstream Targets in Pod1 Knockout Mice

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Mouse mutations have provided tremendous insights into the molecular basis of renal and glomerular development. However, genes often play important roles during multiple stages of nephrogenesis, making it difficult to determine the role of a gene in a specific cell lineage such as the podocyte. Conditional gene targeting and chimeric analysis are two possible approaches to dissect the function of genes in specific cell populations. However, these are labor-intensive and costly and require the generation, validation, and analysis of additional transgenic lines. For overcoming these shortcomings and, specifically, for studying the role of gene function in developing glomeruli, a technique to isolate and purify glomeruli from murine embryos was developed. Combined with gene expression profiling, this method was used to identify differentially expressed genes in glomeruli from Pod1 knockout (KO) mice that die in the perinatal period with multiple renal defects. Glomeruli from early developing stages (late S-shape/early capillary loop) onward can be isolated successfully from wild-type and KO kidneys at 18.5 d postcoitus, and RNA can readily be obtained and used for genome-wide microarray analysis. With this approach, 3986 genes that are differently expressed between glomeruli from Pod1 KO and wild-type mice were identified, including a four-fold reduction of α 8 integrin mRNA in glomeruli from Pod1 KO mice that was confirmed by immunostaining. This procedure may be adapted to any transgenic strain, providing a rapid and efficient method to dissect the function of specific genes in glomerular development.

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Over the past decade, gene-targeting studies have identified many genes that play essential roles in glomerular development, including Wilms tumor suppressor gene, LIM homeobox transcription factor 1β, Pod1, Kreisler, vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor, PDGFB/PDGFR-β, endothelial cell tyrosine kinase receptor, angiopoietin-1, and hepatocyte growth factor (reviewed in 1,2). Although knockout (KO) studies have provided definitive proof that these genes are important in renal development, elucidation of the specific role for each of these genes in glomerulogenesis has been difficult because many of them play multiple roles in renal development and in other organs. In our laboratory, we identified the basic helix-loop-helix transcription factor Pod1 (also called Tcf21/capsulin/epicardin) to be expressed in developing and mature podocytes from the S-shape stage of glomerular development onward. However, Pod1 is also expressed in condensing metanephric mesenchymal cells that give rise to stromal cell lineages, to pericytes, to peritubular interstitial cells, and in the mesenchyme of many other organs (3). Pod1 KO mice die in the perinatal period as a result of major lung and cardiac defects. Furthermore, Pod1 is required for sex determination and gonad and spleen development (4–6). Analysis of chimeric mice showed that Pod1 is required in the developing stroma for glomerular development (3); however, the role of Pod1 within podocytes is still unclear. One approach to answer this question is to generate a conditional KO that deletes the Pod1 gene only from podocytes. However, this approach is not yet possible as no Cre driver mouse line is available to excise genes specifically from S-shape stage podocytes when Pod1 is first expressed. Because of this, we sought to develop an alternative method to identify biologic effectors of Pod1 function within the glomerulus.

In this article, we describe an adaptation of the glomerular isolation method developed by Takemoto *et al.* (7) that permits us to isolate glomeruli from embryonic-stage (18.5 d postcoitus [dpc]) kidneys. This is critical in the case of Pod1 KO mice because they die within minutes after birth as a result of respiratory distress. Using this method, we were able to isolate large quantities of glomeruli (approximately 2500) from early developing stages (late S-shape/early capillary loop) onward. Furthermore, we generated RNA from glomerular pools that were

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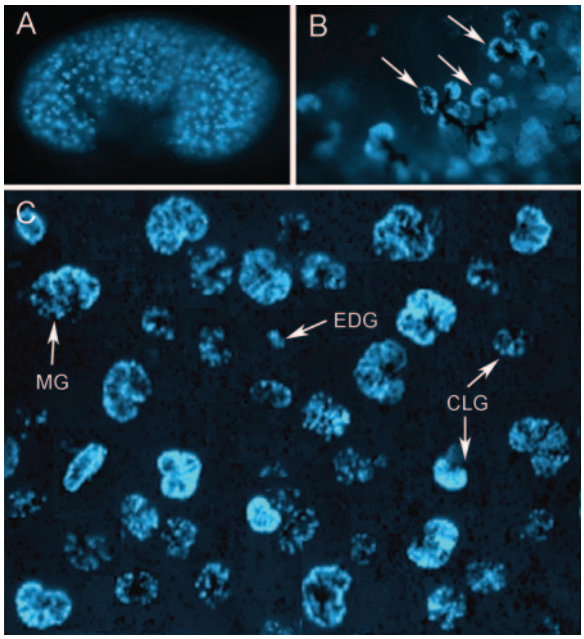

Figure 1. Glomerular isolation under the dissecting microscope. (A) Glomeruli in kidneys from Nephrin–cyan fluorescent protein (CFP) mice at 18.5 d post coitum (dpc) fluoresce under a CFP filter before infusion of Dynabeads. (B) Dynabeads that were injected into the aorta are seen in afferent arterioles (dark outlines) entering glomeruli (arrows). (C) Different developmental stages of glomeruli can be seen after isolation with the magnetic concentrator. EDG, early developing glomeruli (late S-shape/early capillary loop); CLG, capillary loop glomeruli; MG, mature glomeruli.

isolated from Pod1 KO or wild-type mice and used it to generate gene expression profiles. Using this method, we identified a number of genes whose expression is disrupted in Pod1 KO kidneys, including $\alpha 8$ integrin, and confirmed that it is reduced in glomeruli from Pod1 KO mice.

Materials and Methods

Experimental Animals

Mice that were used for these studies were maintained at the animal facility of the Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, according to Canadian animal research regulations. ICR (Institute for Cancer Research) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Podocyte-specific Nephrin–cyan fluorescent protein (CFP) mice, expressing a Cyan fluorescent protein in the ICR background, were generated by subcloning the CFP cDNA (gift from A. Nagy and K. Hadjantonakis, Samuel Lunenfeld Research Institute, Toronto, Canada) into the EcoR1 site of the NXPRS nephrin construct (1,8). Pod1 KO and heterozygous (Pod1+/−) mice in the SVJ 129 background were generated as described previously (4,9). VEGF receptor 2–green fluorescence protein (VEGFR-2–GFP) knock-in mice were provided by J. Rossant (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada). In these transgenic mice, a GFP cassette is knocked into the VEGFR-2 locus under regulation of the endogenous promoter; GFP expression in this transgenic line faithfully recapitulates endogenous VEGFR-2 expression (M.E. and J. Rossant, unpublished observations).

Reagents

HBSS was made by the Samuel Lunenfeld Research Institute at Mount Sinai Hospital. Tosylactivated Dynabeads M-450 (product no. 14004) and a magnetic particle concentrator were purchased from Dynal Biotech ASA (Oslo, Norway). Collagenase A was purchased from Roche Applied Science (Mississauga, Ontario, Canada). Deoxyribonuclease I was from Invitrogen Canada Inc. (Burlington, ON, Canada). Cell strainers (0.1 mm diameter) were from Falcon (BD Biosciences, Mississauga, Ontario, Canada). α 8 antibody was provided by Dr. Ulrich Mueller (Scripps Research Institute, La Jolla, CA).

Isolation of Different Stages of Developing Glomeruli

The procedure of isolation of immature and mature glomeruli from embryonic mice is similar to the isolation of glomeruli from adult mice described previously by Takemoto *et al.* (7), with some modifications. As this procedure depends on the circulation of blood and the circulatory pathways are different in embryonic and adult mice, we needed to modify the procedure during Dynabead perfusion. Briefly, embryos at 18.5 dpc were dissected and incubated in PBS on ice. According to the specific pathway of embryonic blood circulation before birth (septal opening between left and right atrium and patent ductus between lung and aortic vessels), the direction of the needle 301/2G (Becton Dickinson and Co., Franklin Lakes, NJ) was adjusted at 70 to 80° to the longitudinal axis of the heart, which allowed almost all perfused Dynabeads to pass through the pathway of the aortic arch leading to the ascending aorta and then to the microvascular circulation of the kidneys (10 ml/4 to 5 min). For decreasing the effect of pressure on the blood vessel wall and for ensuring that enough beads were perfused into the kidneys, the abdominal cavity was opened before microperfusion. The embryo was microperfused with 2×10^7 Dynabeads diluted in 10 ml of HBSS buffer through the beating heart under a dissecting microscope (×6.3). For avoiding the effect of collagenase digestion of the kidney on the glomerular structure, the kidneys first were minced into pieces as small as possible with a scalpel blade after Dynabead perfusion, then collagenase was added. Ten minutes after the digestion procedure was started, the solution was pipetted gently approximately 10 times. After digestion in HBSS with collagenase A (1 mg/ml) and deoxyribonuclease I (100 U/ml) at 37°C for <20 min, the tissue was diluted in 3 ml of HBSS and gently pressed through a cell strainer followed by rinsing with 3 ml of HBSS. Glomeruli that contained Dynabeads were isolated with a magnetic particle concentrator and washed three times with HBSS.

X-Gal Staining, Histology, and Quantification of Isolated Glomeruli

After isolation, glomeruli from Pod1 KO and heterozygous mice were fixed in lacZ fixative for 3 to 4 min at room temperature. After rinsing in the lacZ wash buffer twice, isolated glomeruli were stained with X-gal for 10 min at 37°C, followed by counterstaining with Nuclear Fast Red. These glomeruli were collected with the Dynabead concentrator and further fixed in 10% formalin. Consequently, isolated glomeruli were dropped directly onto glass slides, left to dry at room temperature for 10 min and mounted with GVA mounting solution (cat. no. 00-8000; ZYMED Laboratories Inc., Burlington, Ontario, Canada). Finally, isolated glomeruli were examined and counted under the light microscope. Different stages of glomerular development were identified by the number and shape of isolated structures that contained blue cells (podocytes) on the slide. A Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA) was used for counting glomeruli from 12 kidneys. A total of 14,000 glomeruli were counted. For transmission electron microscopic and scanning electron microscopic (SEM)

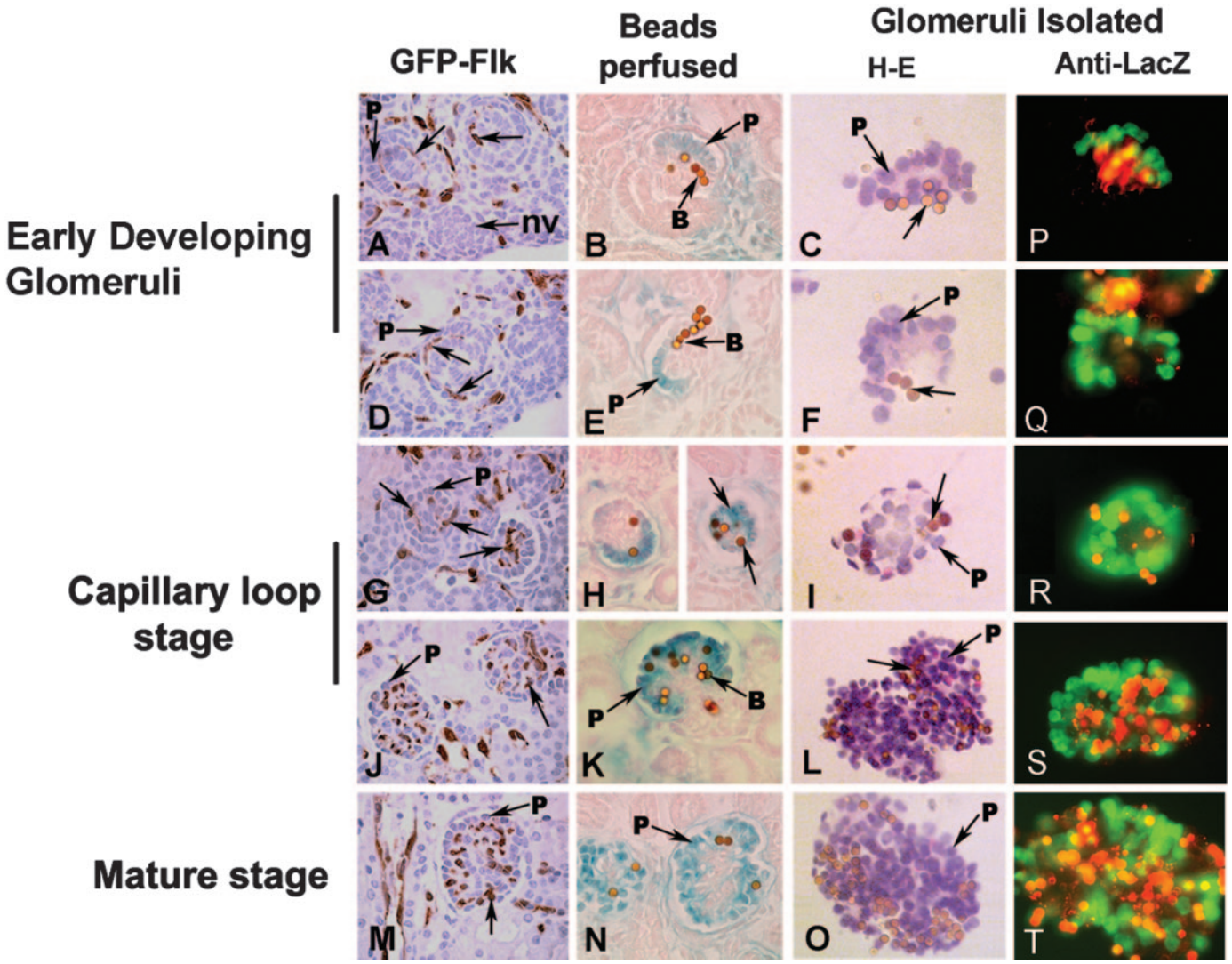

Figure 2. Different stages of glomeruli captured by Dynabeads. Immunostaining to green fluorescent protein (GFP; brown) marks endothelial cells in developing glomeruli isolated from GFP-Flk (VEGFR2) mice (A, D, G, J, and M). LacZ staining (blue) in kidneys from Pod1+/− mice identifies podocytes in developing glomeruli after capture with Dynabeads (gold particles; B, E, H, K, and N); isolated glomeruli are stained with hematoxylin and eosin (H-E) (C, F, I, L, and O) or immunostained with an antibody to LacZ (green) that marks podocytes (P, Q, R, S, and T). Dynabeads appear red in the anti-LacZ column. Early developing glomeruli: Nv, nephrogenic vesicle; unlabeled arrow, vascular cleft; p, podocytes. Capillary loop stage: unlabeled arrows, capillaries, p, podocytes, B, Dynabeads.

examination, isolated glomeruli were fixed with 2% glutaraldehyde in PBS buffer. For SEM, isolated glomeruli were osmicated according to the OTOTO protocol (7,10) and dried using hexamethyldisilazama evaporation (11). Finally, specimens were examined by electron microscopy.

Analysis of RNA Integrity and Microarray Data

Total RNA was purified using the RNeasy Micro Kit (50; Qiagen Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions. The concentration of total RNA was measured by an Eppendorf Biophotometer (AG 22331 CE, Eppendorf, Germany), and the quality was tested by both electrophoreses and electrophogram. Mouse Affymetrix GeneChips 430-2.0 (Santa Clara, CA) were used for RNA microarray analysis, which was performed at the microarray facility in the Center for Applied Genomics (The Hospital for Sick Children, Toronto, ON, Canada). For microarray hybridization, RNA was amplified using the Affymetrix two-cycle kit, and the data analysis was performed using Microsoft Excel program. Four thousand genes whose expression was either up- or downregulated at least two-fold between Pod1 KO and wild-type mice were chosen for further analysis by Affymetrix G-COS, SpotFire, and Array assist programs.

Immunohistochemistry

To demonstrate proof of principle and utility of the microarray screen in Pod1 KO mice, we chose to examine the expression of α 8 integrin, one of the candidate genes related to kidney development, identified in our microarray screen. Because α 8 is expressed in metanephric mesenchymal cells that surround the ureteric bud during early nephrogenesis (12,13) and mesangial cells of mature glomeruli in adult kidneys (14,15), embryonic kidneys were dissected at 13 and 18.5 dpc

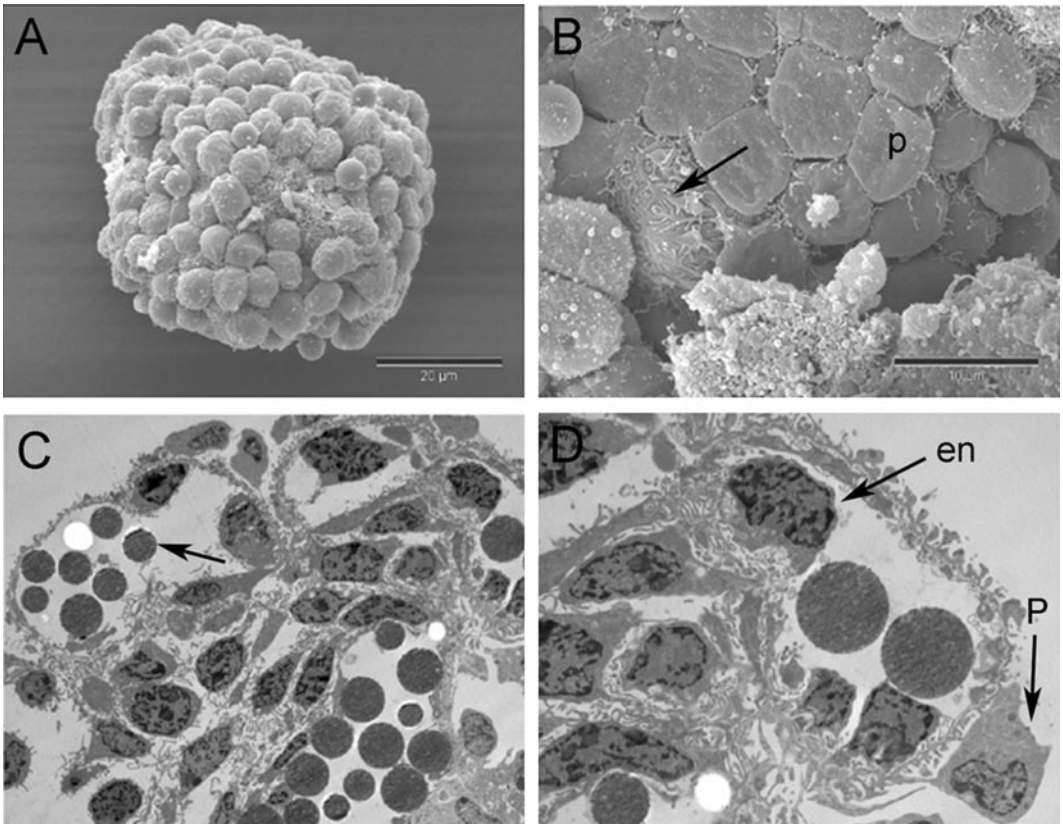

Figure 3. Structural integrity of isolated glomeruli. The quality of isolated glomeruli was examined by electron microscopy. Scanning electron micrographs are shown in A and B, and transmission electron micrographs are shown in C and D. Intact glomerulus with podocytes and multiple foot processes are shown in A and B (unlabeled arrow, foot processes in B). Fenestrated endothelial cells and foot processes of podocytes in contact with the glomerular basement membrane are present in C and D (unlabeled arrow, Dynabeads in C). en, endothelium, p, podocyte.

and fixed in 4% PFA. Ten-micrometer cryosections were prepared as described previously (16). The primary antibodies used were anti–α 8 integrin (1:1500 dilution). Samples were incubated in primary antibody at 4°C overnight. After washing three times for at least 1 h in PBS, samples were incubated with the secondary biotin-related anti-rabbit antibody for 1 h (Vectastain Kit; Vector Laboratories, Burlingame, CA). Samples then were incubated in ABC solution (Vectastain Kit) and further developed with DAB (Peroxidase Substrate Kit DAB, Vector Laboratories) color staining. Samples were counterstained with hematoxylin, dehydrated, mounted, and photographed.

The immunofluorescent staining procedure was described previously (16). The secondary antibodies used were FITC-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA; 1:400) to detect anti–α 8 integrin and Cy3-conjugated donkey anti-mouse IgG (Jackson Laboratories, 1:500) to detect anti-lacZ. Samples were washed three times for 1 h in wash buffer (3% BSA, 0.5% goat serum, and 0.1% Triton X-100 in PBS) and mounted in mounting solution (Sigma, St. Louis, MO) for subsequent microscopic observation.

Results

Isolation of Immature, Maturing, and Mature Glomeruli from Embryonic Mice

To our knowledge, the isolation and characterization of different stages of developing and mature glomeruli from embryonic mice have not previously been reported. To enable us to develop a protocol of glomerular isolation from embryonic kidneys, we first used Nephrin-CFP embryonic mice as glomerular donors, which allowed us to determine the location of Dynabeads in glomeruli under the dissecting microscope immediately following Dynabead perfusion (Figure 1). It also allowed us to examine the morphology and count the number of isolated glomeruli by checking the florescence using a CFP filter (Figure 1). Moreover, it confirmed that this method was successful to isolate large quantities of glomeruli from the embryonic kidneys, because the number of CFP-positive glomeruli and/or cells was very low in the solution discarded after filtration through the cell strainer and collection by the magnetic concentrator. X-gal staining of the kidneys from Pod1+/− embryonic mice perfused with Dynabeads revealed that the beads were distributed in the vessels of all glomerular stages, including early developing (late S-shape/early capillary loop), maturing (capillary loop), and mature glomeruli (Figure 2). However, perfused beads, as expected, were not found in the early S-shaped stages, as no vascular network (Figure 2A) has assembled by this stage. Hematoxylin and eosin staining of glomeruli that were isolated from Nephrin-CFP embryonic mice and immunohistochemistry staining of glomeruli that were isolated from Pod1 heterozygous mice showed that different stages of glomeruli were present on the slides (Figure 2) and demonstrated that this protocol is useful to isolate various

stages of glomeruli from embryonic kidneys. SEM revealed that the morphology of isolated glomeruli was intact (Figure 3A), and primary processes from the main podocyte cell bodies remain wrapped around individual capillary loops in mature isolated glomeruli (Figure 3B). Transmission electron microscopy further demonstrated that the ultrastructure of isolated glomeruli was intact (Figure 3C). The capillary network of isolated glomeruli was seen to consist of fenestrated endothelial cells; in addition, thin diaphragms were sometimes observed extending across these fenestrae. Foot processes of podocytes could be seen in contact with the glomerular basement membrane (Figure 3D). The number of isolated glomeruli that were collected from a single 18.5 dpc embryonic mouse was estimated to be 2164 ± 326, which consisted of 37.6% glomeruli at early developing stage (late S-shaped/early capillary loop), 43.6% at capillary loop stage, and 18.8% at the mature stage (Figure 4).

For ensuring a high quantity and quality of RNA, extraction of total RNA was begun immediately after isolation of the glomeruli. The amount of total RNA that was purified from isolated glomeruli of a single embryonic mouse was estimated to be 438 ng on average. To confirm the integrity of the RNA that was extracted from glomeruli, we performed electrophoretic and electrophogram analysis (Figure 5). Figure 5 shows that the integrity of RNA from glomeruli that were isolated using Dynabead perfusion was comparable to total RNA from snap-frozen whole kidney.

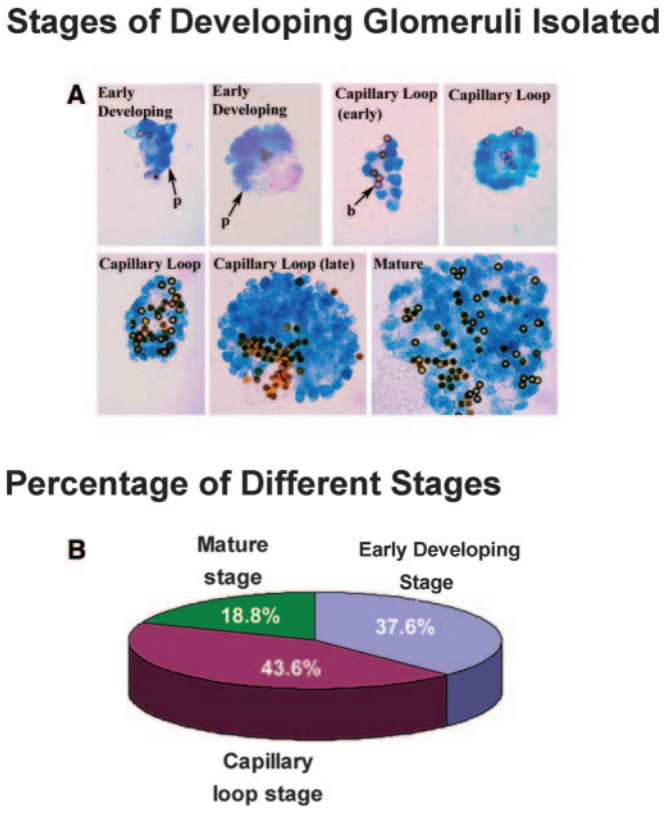

Figure 4. Proportion of glomerular stages isolated by Dynabead perfusion. (A) LacZ staining shows morphology of developmental stages of glomeruli that were isolated from Pod1+/− mice with Dynabeads. (B) The number of each developmental stage isolated as a percentage of total glomeruli.

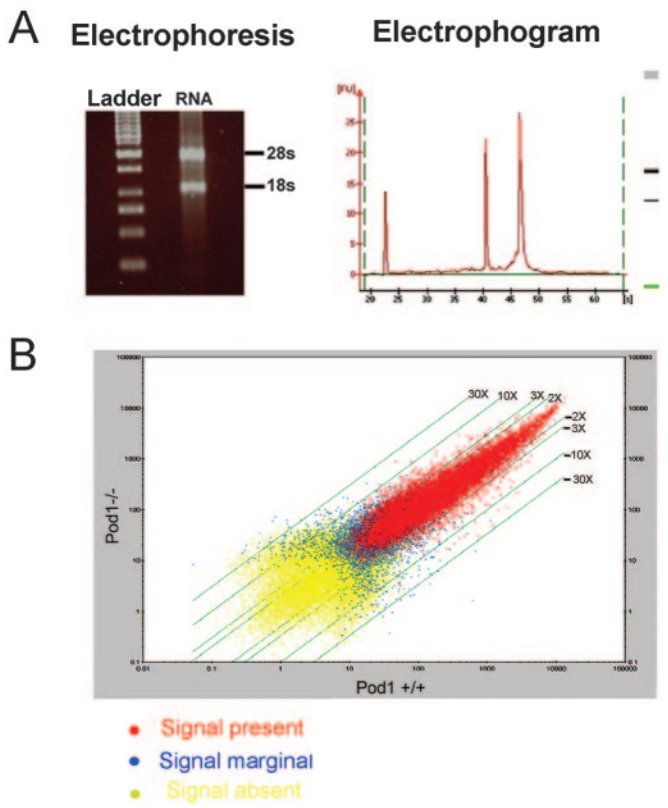

Figure 5. Evaluation of the integrity of RNA isolated for microarray analysis. (A) An ethidium bromide–stained gel of total RNA (left) and electrophogram of RNA after amplification with the Affymetrix two-cycle kit confirm the integrity of RNA purified from isolated glomeruli. (B) Scatter plot of the signal detection of gene expression from isolated glomeruli from Pod1 knockout (KO; −/−) compared with Pod1 wild-type (+/+) mice. Genes represented are from the microarray database.

Characterization of Glomeruli from Wild-Type and Pod1 KO Mice

Pod1 is a transcription factor expressed in many developing organs. We recently found that Pod1 plays an important role in podocytes and endothelial cell differentiation in glomeruli, particularly during remodeling and maturation of the vasculature (unpublished observations). To characterize further the role of Pod1 in the glomerulus, we wanted to isolate mutant glomeruli to profile their gene expression. This is important as the kidneys in Pod1 KO mice exhibit multiple defects, including disruption of ureteric bud branching, arrest of stromal cell development, and marked dysplasia with absence of the renal medulla (3,4). Figure 6 shows that the number of beads deposited was markedly increased in the capillaries of Pod1 KO glomeruli that were microperfused with Dynabeads when compared with wild-type glomeruli. Moreover, this heavy deposition could be observed from the beginning of glomerular formation onward, through early capillary loop stage, and in

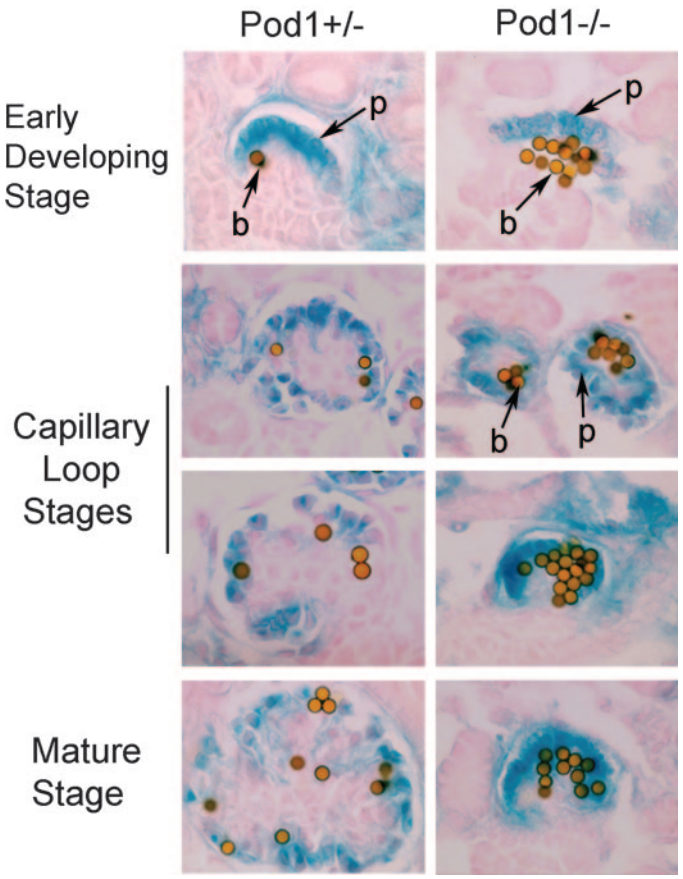

Figure 6. Distribution of perfused Dynabeads in glomeruli from Pod1+/− and Pod1 KO mice at 18.5 dpc. Dynabeads aggregate in greater numbers in all stages of glomeruli from Pod1 KO kidneys compared with Pod1+/− glomeruli, suggesting that the lumen of capillaries are enlarged. Note that glomerular development is arrested at the capillary loop stage in Pod1 KO mice. Blue-stained cells = podocytes.

maturing capillary loop stage when Pod1 KO glomerular development becomes arrested. The results suggest that Pod1 plays an important role in vascular remodeling at the beginning of formation of glomerular architecture during a time when endothelial cells are undergoing differentiation and branching. To identify candidate genes that are related to podocyte and endothelial cell differentiation, we used RNA that was isolated from Pod1 KO glomeruli to screen the mouse Affymetrix GeneChips 430-2.0 compared with RNA from wild-type or heterozygous littermates. RNA was generated from glomeruli that were isolated from a total of 32 mutant and 12 wild-type embryos on two independent occasions. The hybridization results were normalized and analyzed by Affymetrix GCOS, SpotFire, and ArrayAssist programs. When KO samples were compared with control samples, a total of 3986 genes (approximately 8.9% of the 45,000 genes) whose transcript varied by at least 2 SD were identified. Tables 1 and 2 shows a partial list of genes that were most greatly up- or downregulated in glomeruli from Pod1 KO mice compared with wild-type littermates. The complete list of genes that are up- or downregulated at least two-fold is provided in the Appendix (available online only).

As proof of principle and to demonstrate the utility of this approach, we examined the expression of α 8 integrin that was significantly downregulated (four-fold) in glomeruli from Pod1 KO mice. Of note, expression of α 8 integrin has previously been reported in metanephric mesenchymal cells (13,17) and in mesangial cells (14,15) of the glomerulus. Figure 7 shows that the level of α 8 integrin expression is markedly decreased in metanephric mesenchymal cells of Pod1 KO kidneys compared with Pod1+/− kidneys. Furthermore, α 8 integrin is expressed in the mesangial cells of wild-type glomeruli, and this is markedly reduced in glomeruli from Pod1 KO mice. Together, these results demonstrate that this procedure will be valuable to identify downstream biologic effectors in transgenic mice.

Discussion

In this article, we report a technique to isolate large quantities of developing and mature glomeruli from mouse embryos at 18.5 dpc. We show that it is possible to obtain high-quality RNA from glomeruli that are isolated from a single mouse that allows us to perform gene expression profiling from specific renal structures and cell populations. The major advantage of this procedure is that it permits the identification of a large number of differentially expressed genes from relatively homogeneous cell populations. To demonstrate the utility of this procedure in practice, we performed glomerular isolation and gene expression profiling in glomeruli from Pod1 KO mice. Although Pod1 is highly expressed in developing and mature podocytes from the S-shape stage onward, it has not been possible to determine the cell autonomous or non–cell autonomous roles of Pod1 within the glomerulus because of the critical role that Pod1 plays in earlier mesenchymal populations within the kidney. Although conditional gene targeting is a powerful and widely used technique that definitively defines the cell-autonomous role of a gene, it requires that cell-specific Cre-driver lines be available. In the case of Pod1, there are no Cre-driver lines yet available for early stages of podocyte differentiation, *i.e.*, S-shape stage, when Pod1 is first expressed.

Pod1 KO mice die within minutes after birth as a result of lung and cardiac defects. Glomerular development is arrested in Pod1 KO mice at the capillary loop stage. At most, only one capillary loop forms within the mutant Pod1 glomeruli. Despite the major defects in glomerular capillary formation, we show that magnetic beads that were infused into the aorta of Pod1 KO embryos become trapped in the single capillary loop, permitting glomerular isolation. The RNA that was generated from these isolated glomeruli is of high quality and was used to screen the Affymetrix microarray gene chips. A large number of differentially expressed genes were identified in glomeruli from Pod1 mice compared with glomeruli from wild-type littermates; these included NPHS2 (podocin) and Col4α3-2 genes that play important roles in glomerular development and disease.

As proof of principle, we show immunostaining in mutant and wild-type glomeruli for one of the identified genes: α 8 integrin. On microarray analysis, α 8 integrin was reduced four-fold in glomeruli from Pod1 KO mice. Expression of α 8 integrin in mesangial cells of the glomerulus and renal vascular

Table 1. Genes downregulated in glomeruli isolated from Pod1 KO compared to wild-type mice

Affy Probe ID	Gene Title	Gene Symbol	UniGene ID	Fold Difference
1424567_at	RIKEN cDNA 6330415F13 gene	6330415F13Rik	Mm.27469	−27.02
1419717_at	semaphorin 3E	Sema3e	Mm.134093	−20.86
1418093_a_at	epidermal growth factor	Egf	Mm.254772	−18.98
1424719_a_at	microtubule-associated protein tau	Mapt	Mm.1287	−14.24
1415824_at	stearoyl-Coenzyme A desaturase 2	Scd2	Mm.193096	−12.12
1433707_at	gamma-aminobutyric acid (GABA-A) re	Gabra4	Mm.248731	−10.21
1455028_at	microtubule-associated protein tau	Mapt	Mm.1287	−9.46
1441389_at	Mus musculus transcribed sequences	—	Mm.183689	−9.38
1427126_at	heat shock protein 1A	Hspa1a	Mm.275405	−9.23
1452907_at	galactosylceramidase	Galc	Mm.5120	−9.14
1452351_at	RIKEN cDNA C030027K23 gene	C030027K23Rik	Mm.291434	−8.57
1452388_at	heat shock protein 1A	Hspa1a	Mm.6388	−8.54
1460370_at	RIKEN cDNA 2900052H09 gene	2900052H09Rik	Mm.182401	−8.37
1425181_at	RIKEN cDNA 3110007P09 gene	3110007P09Rik	Mm.238094	−8.24
1422155_at	histone 2, H2aa1	Hist2h2aa1	Mm.261587	−8.16
1427127_x_at	heat shock protein 1A	Hspa1a	Mm.275405	−8.06
1437477_at	leucine rich repeat (in FLII) interacting	Lrrfip1	Mm.45039	−7.92
1452474_a_at	ADP-ribosyltransferase 3	Art3	Mm.133535	−7.35
1450224_at	procollagen, type IV, alpha 3	Col4a3	Mm.8069	−7.34
1439618_at	phosphodiesterase 10A	Pde10a	Mm.87161	−7.22
1424208_at	prostaglandin E receptor 4 (subtype EP)	Ptger4	Mm.18509	−7.08
1443906_at	decay accelerating factor 1	Daf1	Mm.101591	−6.84
1416077_at	adrenomedullin	Adm	Mm.1408	−6.84
1438779_at	procollagen, type IV, alpha 3	Col4a3	Mm.8069	−6.70
1457140_s_at	TEA domain family member 1	Tead1	Mm.62480	−6.65
1458381_at	chloride intracellular channel 5	Clic5	Mm.37666	−6.64
1434109_at	RIKEN cDNA A930014C21 gene	A930014C21Rik	Mm.100125	−6.47
1449957_at	protein tyrosine phosphatase, receptor t	Ptprv	Mm.4450	−6.33
1460297_at	nephrosis 2 homolog, podocin (human)	Nphs2	Mm.289099	−6.23
1433885_at	hypothetical protein A630053O10	A630053O10	Mm.38878	−6.20
1422804_at	serine (or cysteine) proteinase inhibitor	Serpinb6b	Mm.36526	−6.16
1450789_at	rhophilin, Rho GTPase binding protein	1Rhpn1	Mm.57052	−6.13
1418162_at	toll-like receptor 4	Tlr4	Mm.38049	−6.10
1447100_s_at	RIKEN cDNA 5730508B09 gene	5730508B09Rik	Mm.19330	−6.10
1422869_at	c-mer proto-oncogene tyrosine kinase	Mertk	Mm.239655	−5.98
1417623_at	solute carrier family 12, member 2	Slc12a2	Mm.228433	−5.83
1426252_a_at	uromodulin	Umod	Mm.10826	−5.71

smooth muscle cells was reported previously (14,18); here we show that it is expressed in mesangial cells of wild-type glomeruli as expected. Furthermore, we show that α 8 integrin staining is markedly reduced in mesangial cells in Pod1 KO glomeruli. This reduction occurs in glomeruli and also in metanephric mesenchymal populations that also normally express Pod1.

The method reported in this article also permitted us to quantify the number of glomeruli that were isolated within each of the major developmental stages: Late S-shape/early capillary loop, capillary loop, and mature. Two factors permitted us to perform this quantitative analysis. First, when we generated the null Pod1-targeting allele, we knocked in a β-galactosidase reporter gene into the Pod1 locus under regulation of the endogenous promoter. Thus, lacZ expression recapitulates the endogenous expression of Pod1 and begins in podocyte precursors during the S-shape stage onward. As a result, it is very easy to identify cells of the podocyte lineage in developing glomerular structures. When counting glomeruli that are stained with X-gal under the light microscope, it thus was possible to exclude definitively contamination of other renal tissue, even when it was minimal contamination. This is an important point for effective quantitative analysis, especially to count the number of early-stage glomeruli isolated. As can be seen in the figures, early developing glomeruli (late S-shape/early capillary loop) are not globular and may have been counted as contaminants on the basis of morphology alone (*i.e.*, it would be hard to distinguish between S-body stage glomeruli

Table 2. Genes upregulated in glomeruli isolated from Pod1 KO compared to wild-type mice

Affy Probe ID	Gene Title	Gene Symbol	UniGene ID	Fold Difference
1431225_at	SRY-box containing gene 11	Sox11	Mm.41702	18.17
1417552_at	fibroblast activation protein	Fap	Mm.41816	11.97
1454803_a_at	histone deacetylase 11	Hdac11	Mm.206218	11.36
1448590_at	procollagen, type VI, alpha 1	Col6a1	Mm.2509	8.80
1454159_a_at	insulin-like growth factor binding protein 2	Igfbp2	Mm.141936	7.54
1449368_at	decorin	Dcn	Mm.56769	6.99
1451456_at	Unknown (protein for MGC:66590)	MGC66590	Mm.299908	6.91
1423607_at	lumican	Lum	Mm.18888	6.76
1427038_at	preproenkephalin 1	Penk1	Mm.2899	6.60
1448229_s_at	cyclin D2	Ccnd2	Mm.294136	6.57
1421694_a_at	chondroitin sulfate proteoglycan 2	Cspg2	Mm.158700	6.57
1439427_at	claudin 9	Cldn9	Mm.103738	6.12
1419497_at	cyclin-dependent kinase inhibitor 1B (P27)	Cdkn1b	Mm.272101	5.98
1418450_at	immunoglobulin superfamily containing leucine-	Islr	Mm.38426	5.94
1424367_a_at	homer homolog 2 (Drosophila)	Homer2	Mm.228	5.92
1439757_s_at	Eph receptor A4	Epha4	Mm.3249	5.90
1416006_at	midkine	Mdk	Mm.57181	5.85
1429871_at	hyaluronan mediated motility receptor (RHAMM)	Hmmr	Mm.116997	5.83
1452250_a_at	procollagen, type VI, alpha 2	Col6a2	Mm.1949	5.82
1439556_at	neural cell adhesion molecule 1	Ncam1	Mm.4974	5.77
1448754_at	retinol binding protein 1, cellular	Rbp1	Mm.279741	5.71
1434129_s_at	expressed sequence AI447312	AI447312	Mm.205393	5.55
1439380_x_at	GTL2, imprinted maternally expressed untranslated	Gtl2	Mm.200506	5.40
1429051_s_at	SRY-box containing gene 11	Sox11	Mm.41702	5.39
1416211_a_at	pleiotrophin	Ptn	Mm.3063	5.29
1426658_x_at	3-phosphoglycerate dehydrogenase	Phgdh	Mm.298899	5.28
1417378_at	immunoglobulin superfamily, member 4	Igsf4	Mm.275051	5.24
1421074_at	cytochrome P450, family 7, subfamily b, polype	Cyp7b1	Mm.278588	5.09
1448956_at	START domain containing 10	Stard10	Mm.28896	5.08
1442144_at	Emu1 gene	Emu1	Mm.153507	5.03
1427256_at	chondroitin sulfate proteoglycan 2	Cspg2	Mm.158700	5.02
1434667_at	procollagen, type VIII, alpha 2	Col8a2	Mm.296327	5.01
1450288_at	cadherin 6	Cdh6	Mm.57048	4.98
1427883_a_at	procollagen, type III, alpha 1	Col3a1	Mm.249555	4.93
1452183_a_at	GTL2, imprinted maternally expressed untranslated	Gtl2	Mm.200506	4.93
1416168_at	serine (or cysteine) proteinase inhibitor, clade	Serpinf1	Mm.2044	4.93
1421075_s_at	cytochrome P450, family 7, subfamily b, polype	Cyp7b1	Mm.278588	4.87
1417845_at	claudin 6	Cldn6	Mm.86421	4.82
1416473_a_at	neighbor of Punc E11	Nope	Mm.209041	4.81
1448550_at	lipopolysaccharide binding protein	Lbp	Mm.218846	4.80
1423153_x_at	complement component factor h	Cfh	Mm.8655	4.80

and portions of proximal tubular contaminants). However, that there is intense lacZ staining in these structures confirms that they are glomerular in origin.

Second, we were able to determine which "vascular stage" is required for successful isolation. According to elegant studies by Hyink *et al.* (19), it is known that vascular progenitors migrate into the vascular cleft to produce the glomerular capillary network during the S-shape stage. Using a VEGFR-2–GFP reporter mouse strain (the enhanced GFP is knocked into the VEGFR-2 locus), we were able to observe and confirm VEGFR-2–positive cells in the cleft at the late S-shape/early capillary loop stage. Immediately after perfusion with Dynabeads, the beads could be seen in the lumen of the capillary forming in the cleft of late S-shape/early capillary loop stage glomeruli on tissue sections (Figures 2 and 6). In conclusion, we report a highly efficient method to isolate embryonic glomeruli, provide

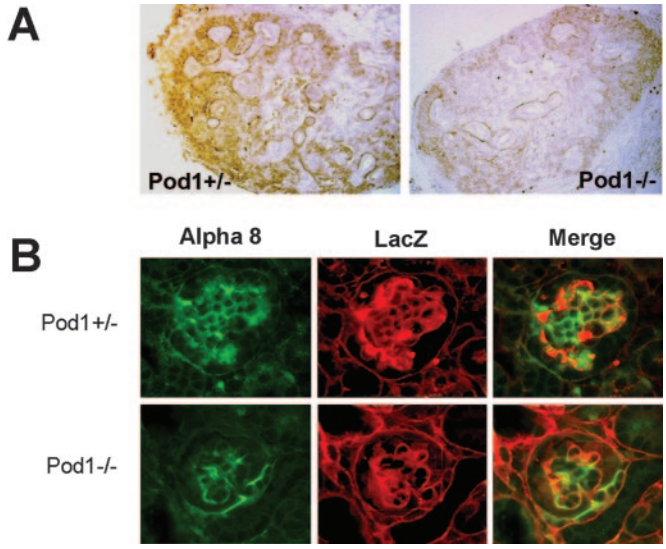

Figure 7. Expression of α 8 Integrin in Pod1 KO and wild-type kidneys. (A) Immunohistochemical staining of kidneys from mice at 13.5 dpc shows expression of α 8 integrin in condensing mesenchyme around ureteric buds of wild-type kidneys. Expression is markedly reduced in kidneys from Pod1 KO mice. (B) Glomeruli are double labeled with an antibody to α 8 integrin (green) and lacZ (red). LacZ is expressed in Pod1-expressing cells (podocytes and peritubular interstitial cells), whereas α 8 integrin is expressed in mesangial cells adjacent to endothelium. Note that α 8 integrin is reduced in glomeruli from Pod1 KO mice.

quantification of stages isolated, and demonstrate the utility and application of this protocol for gene expression profiling. This is a useful method to profile gene expression and identify relevant biologic effectors in glomeruli from KO mice.

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