Insight into Podocyte Differentiation from the Study of Human Genetic Disease: Nail-Patella Syndrome and Transcriptional Regulation in Podocytes

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ABSTRACT

In recent years, our understanding of the molecular basis of kidney development has benefited from the study of rare genetic diseases affecting renal function. This has especially been the case with the differentiation of the highly specialized podocyte in the pathogenesis of human disorders and mouse phenotypes affecting the renal filtration barrier. This filtration barrier represents the end product of a complex series of signaling events that produce a tripartite structure consisting of interdigitating podocyte foot processes with intervening slit diaphragms, the glomerular basement membrane, and the fenestrated endothelial cell. Dysregulation of unique cytoskeletal and extracellular matrix proteins in genetic forms of nephrotic syndrome has shown how specific structural proteins contribute to podocyte function and differentiation. However, much less is known about the transcriptional determinants that both specify and maintain this differentiated cell. Our studies of a skeletal malformation syndrome, nail-patella syndrome, have shown how the LIM homeodomain transcription factor, *Lmx1b*, contributes to transcriptional regulation of glomerular basement membrane collagen expression by podocytes. Moreover, they raise intriguing questions about more global transcriptional regulation of podocyte morphogenesis. (*Pediatr Res* 51: 551–558, 2002)

Abbreviations:
Lmx1b, LIM homeodomain transcription factor
NPS, nail-patella syndrome
GBM, glomerular basement membrane

Until recently, much of the study of kidney development has focused on the early inductive events specifying formation of the metanephric kidney. In vertebrate embryogenesis, a primitive nonfunctional kidney rudiment, the pronephros, and a transiently functional mesonephros precede the formation of the permanent metanephric kidney in the fifth week of human development and at E10.5–11 in mouse development. The cells of the excretory and collecting systems derive from mesoderm and undergo a characteristic mesenchymal to epithelial transition superimposed on a process of branching morphogenesis. Multiple molecules, including transcription factors, growth factors, and growth factor receptors, have been identified that regulate the reciprocal inductive interaction between the ureteric bud and metanephric mesenchyme. Several excellent reviews summarize these studies (1–5). These interactions specify the processes of ureteric budding from the mesonephric duct, aggregation of metanephric mesenchyme, its epithelial transformation into glomerular and tubular epithelium, the differentiation into mesangial and stromal cells, and the branching of the ureteric bud to form the collecting duct system. The differentiation of metanephric mesenchyme at the tip of each ureteric bud into the nephron unit traverses morphologically from the renal vesicle, to comma-shaped bodies, to the S-shaped bodies, and, finally, to the epithelialized bodies that become vascularized during glomerular maturation. The end result of these processes is the mature vertebrate kidney with approximately 1.5 million nephron units in humans and 1000–2000 nephron units in mouse (6).

One primary function of the mature vertebrate kidney is filtration of high-molecular-weight proteins present in the blood (not allowing molecules bigger than albumin at 68 kD to cross into urine) (7). This highly specialized function resides in the glomerular filtration unit, composed of 1) fenestrated endothelial cells of the capillary tuft; 2) an intervening GBM, rich in specialized collagens, laminins, and heparin sulfate proteoglycans; and 3) the podocyte, a terminally differentiated visceral glomerular epithelial cell with specialized major cell

processes and smaller foot processes that extend to interdigitate with adjacent foot processes, forming an intervening slit diaphragm (Fig. 1). Disruption of this unit by environmental, immune, or genetic factors results in loss of the filtration function and, ultimately, some or all features of clinical nephrotic syndrome (8).

The endothelial cell fenestration likely does not contribute greatly to filtration, but the endothelial cell is required for maintenance of the intact unit. From the study of the *cloche* mutant in zebrafish, it appears that podocyte differentiation can occur independent of endothelial cell differentiation (9). These mutant fish are avascular and, interestingly, podocytes differentiate in the absence of endothelial cells. However, they soon undergo effacement, *i.e.* fusion of podocyte foot processes with eventual loss of GBM attachment, suggesting that maintenance of normal podocyte morphology and function requires endothelial cell function. The GBM derives initially from fusion of two independent basement membranes, that of developing endothelial cells with that of visceral glomerular epithelial cells. As the visceral glomerular epithelial cell differentiates into the mature podocyte, it continues to add newly synthesized GBM, whereas the contribution of the endothelial cell is thought to cease. Importantly, a developmental switch in the global pattern of GBM matrix expression occurs during glomerulogenesis (10, 11). Initially, heterotrimeric type IV collagen containing ubiquitously distributed $\alpha1(IV)$ and $\alpha2(IV)$ chains are laid down in GBM. Gradually, during embryogenesis and in postnatal life, it is replaced with heterotrimeric type IV collagen composed of tissue-specific (predominantly in glomeruli) $\alpha3(IV)$, $\alpha4(IV)$, and $\alpha5(IV)$ chains. Paralleling this is a developmental switch from heterotrimeric laminin 1, containing $\alpha1/\beta1/\gamma1$ chains, to heterotrimeric laminin 11, containing $\alpha5/\beta2/\gamma1$, during embryogenesis and postnatal life (12). The importance of these GBM-specific extracellular matrix components for maintenance of an intact filtration unit has been demonstrated by the study of human and mouse models of renal disease. Patients with recessive or X-linked Alport syndrome develop progressive proteinuria due to mutation in either the autosomal 3(IV) and 4(IV) collagen genes (*COL4A3* and *COLA4*), or the X-linked 5(IV) collagen gene (*COL4A5*) (13, 14). Similarly, mouse mutants null for either *Col4a3* alone or for both *Col4a3* and *Col4a4* also exhibit phenotypes similar to Alport syndrome in humans, albeit with an earlier onset in the double mutant animals (15–17). Although no human genetic defect of laminin 11 has been reported, mouse mutants null for the laminin $\beta2$ gene develop nephrosis, in spite of compensation by embryonic laminin $\beta1$ chain in a morphologically normal GBM (18). Interestingly, in the collagen mutants, laminin isoforms were normally expressed, and, similarly in the laminin mutant, collagen isoforms were normally expressed. Importantly, podocytes differentiated normally in both but showed signs of injury, *i.e.* fusion of podocyte foot processes. These studies show that these GBM-specific matrix components are required for maintenance of normal podocyte morphology and filtration function, but are not required for podocyte differentiation and foot process formation.

MOLECULAR DETERMINANTS OF PODOCYTE STRUCTURE AND FUNCTION

The molecular basis for podocyte differentiation has been less well understood compared with the early inductive events of nephrogenesis. However, a better understanding has now been gained from the study of both human and mouse models of renal disease affecting the filtration unit (Table 1) (8, 19, 20). Podocytes are highly specialized cells with large arborization of cell processes that make up the unique foot processes covering the GBM. Their function is to control the turnover of the GBM and to regulate ultrafiltration of urine. An electron-dense slit diaphragm approximately 40 nm in size extends from foot processes of adjacent cells to form a specialized cell-cell junction (7, 21–23). It follows that cytoskeletal components contribute to its differentiation and function. Detailed morphologic studies show that the microtubular cytoskeleton is found in major cell processes whereas actin filaments form the cytoskeleton of the secondary foot processes that extend from the major processes. Alterations in genes that regulate cytoskeletal organization would therefore be expected to affect podocyte differentiation and/or maintenance and hence result in disruption of the filtration barrier. Both congenital and chronic renal diseases resulting from processes such as diabetes and aging are associated with alteration of podocyte integrity often involving effacement of podocyte foot processes, separation from GBM, and/or proliferation of mesangial cells and parietal epithelium (24, 25).

An important podocyte component of the slit diaphragm, required for function of the filtration unit, was identified as mutated in congenital nephrotic syndrome of the Finnish type (NPHS1) (26, 27). The protein nephrin is a transmembrane adhesion molecule with an extracellular domain containing eight Ig domains and one fibronectin domain. It localizes to the slit diaphragm, which is hypothesized to be formed, in part, by interdigitating nephrin molecules that interact along their Ig domains while extending from opposing foot processes (28, 29). Nephrin null mice exhibit massive proteinuria and neonatal death (30). While podocytes and foot processes form, there are no slit diaphragms and podocyte foot processes quickly undergo effacement. These data show that nephrin is required for slit diaphragm formation and maintenance of podocyte function (31). Studies in mice showed that another protein, the CD2 adaptor protein (*Cd2ap*), interacts with the intracellular C-terminal domain of nephrin and is required for stabilization of the slit diaphragm (32). Loss of its function in mice causes congenital nephrotic syndrome with podocyte effacement, increased extracellular matrix production, and mesangial cell hyperplasia. Like its role in T cell–antigen presentation cell signaling, *Cd2ap* may serve to stabilize specialized cell-cell interactions mediated by the Ig family of transmembrane proteins. However, to date, no human condition has yet been associated with *Cd2ap* mutations. The importance of cytoskeletal organization for podocyte maintenance and foot process formation is further underscored by the finding of mutations in α-actinin-4 (*ACTN4*) in patients with dominantly inherited focal segmental glomerular sclerosis (FSGS1) (33). ACTN4 is an actin filament cross-linking protein that is highly expressed
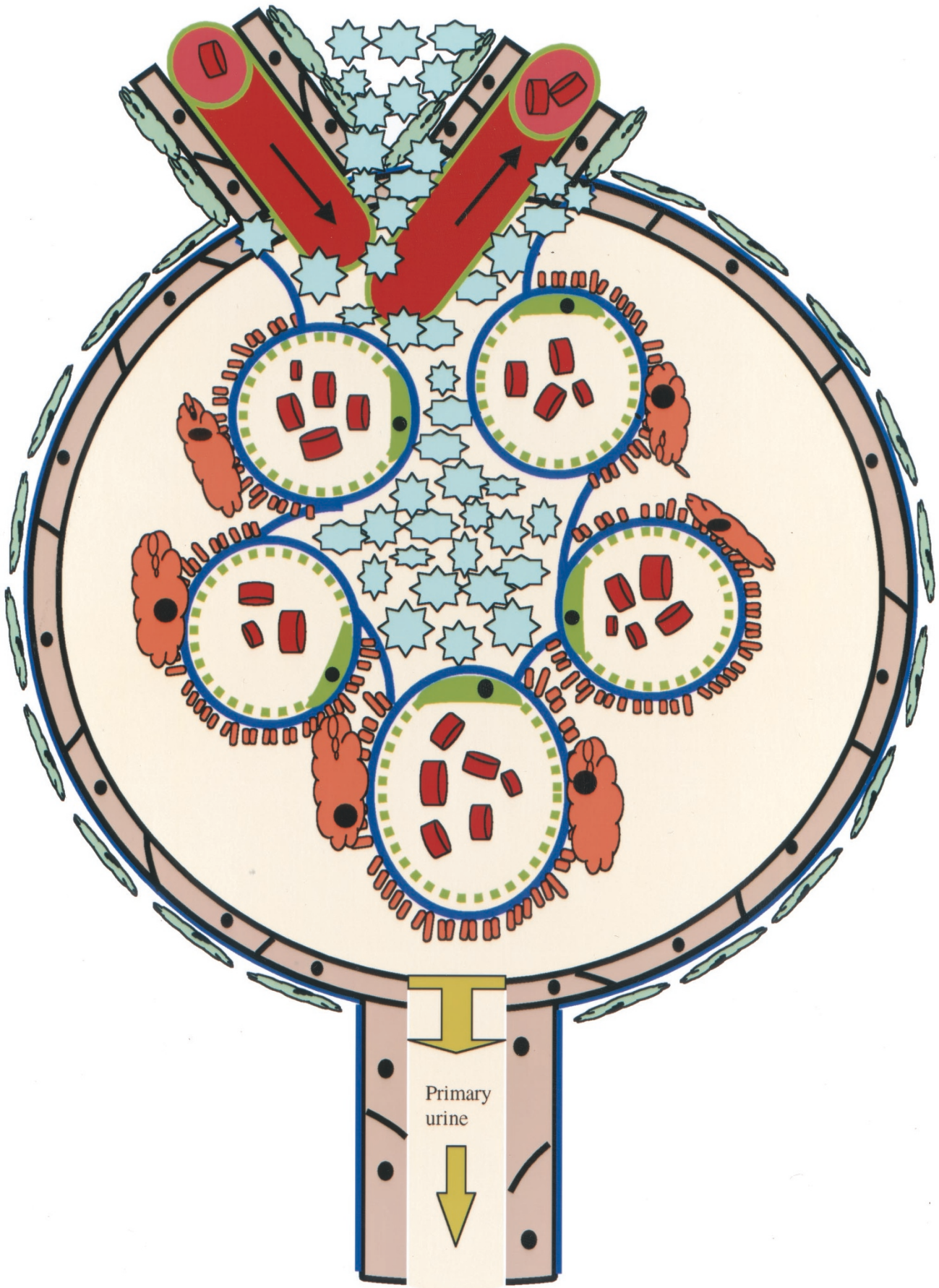
Figure 1. Schematic diagram of the glomerular structure. *Orange,* podocytes; *blue,* basement membrane; *green,* endothelium; *red,* red blood cells; *light blue,* mesangial cells.

Table 1. *List of human genetic syndromes associated with nephrosis and their corresponding genetic loci*

Locus	Gene	Protein	Disease
Xq22.3	*COL4A5*	COL4A5	Alport syndrome, X-linked
2q36–q37	*COL4A3–COL4A4*	COL4A3–COL4A4	Alport syndrome, autosomal recessive
19q13.1	*NPHS1*	Nephrin	Congenital nephrotic syndrome, Finnish type, autosomal recessive
1q25–q31	*NPHS2*	Podocin	Steroid-resistant nephrotic syndrome, autosomal recessive
9q34	*LMX1B*	LMX1B	NPS, autosomal dominant
19q13	*ACTN4*	α-Actinin-4	Focal segmental glomerulosclerosis, autosomal dominant
11p13	*WT1*	WT1	Denys-Drash syndrome, Frasier syndrome, WAGR syndrome

WAGR, Wilms tumor, Aniridia, Genitourinary anomalies, Mental Retardation Syndrome.

in podocytes (34). Interestingly, the FSGS1 mutation caused ACTN4 to bind actin more strongly than wild-type ACTN4, further supporting the critical importance of maintaining normal cytoskeletal homeostasis in podocyte function. Recently, the genetic basis for autosomal recessive steroid-resistant nephrotic syndrome (NPHS2) was identified to be mutations in an integral podocyte membrane protein, podocin (35). Podocin has homology to *Caenorhabditis elegans* Mec-2. Interestingly, Mec-2 has been implicated in mechanosensation linking the cytoskeleton to ion channels (36). This prompts the question of whether podocin might similarly mediate cytoskeletal-cell membrane communication in podocyte foot processes in response to mechanical forces (37).

A host of podocyte cytoskeletal-associated proteins have been described to date (Fig. 2) (38). These include proteins like P-cadherin, which has been also localized to the slit diaphragm. The slit diaphragm can be viewed as a modified adherens junction between podocytes (29, 39). Hence, it is not surprising that zonula occludens protein-1 (ZO1), a multiple-domain membrane-associated signaling protein, has also been localized to the cytoplasmic component of the slit diaphragm (29). GLEPP1 is a single transmembrane protein with an intracellular phosphatase domain and an extracellular domain with eight fibronectin type III repeats. Loss of its function in mice causes decreased GFR and hypertension associated with podocytes with shortened thickened processes (40–42). Synaptopodin is another actin-associated protein found in podocyte foot processes (38). Although no human diseases causing nephrotic syndrome have yet been associated with these molecules, various mouse mutants have highlighted their relevance for podocyte morphogenesis and function. These and other structural components of the podocyte cytoskeleton and slit diaphragm constitute excellent candidates for genetic determinants in the pathogenesis of nephrotic syndrome.
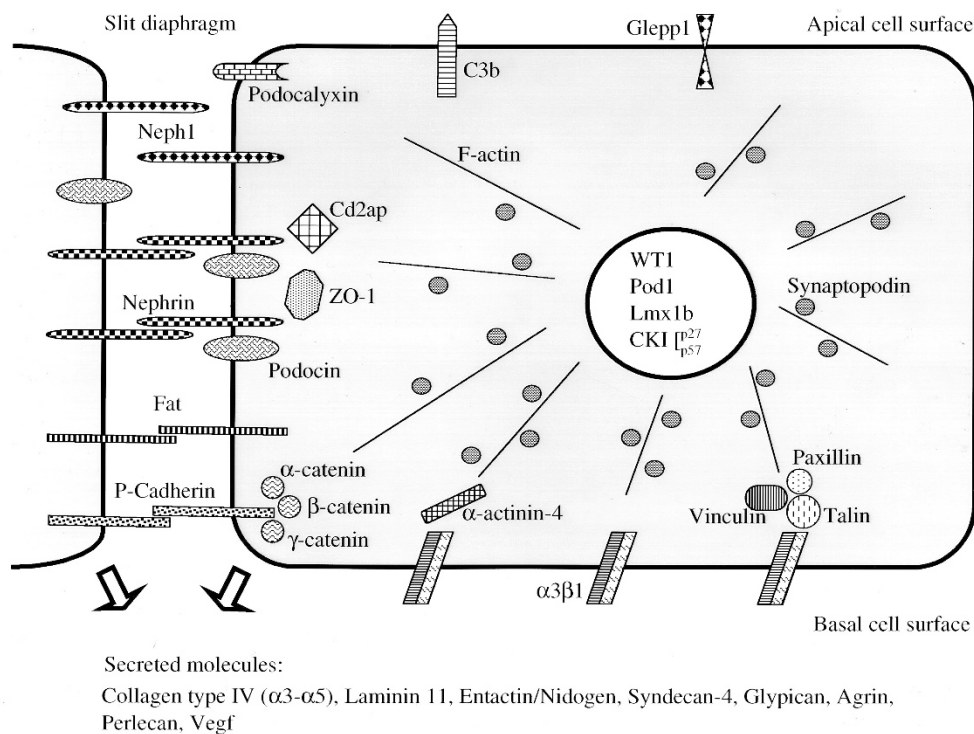

Figure 2. Array of genes typically expressed by the mature podocyte. Cell junction and cell junction–associated proteins: nephrin, P-cadherin, Cd2ap, ZO-1; cell adhesion proteins: α3β1 integrin; focal adhesion proteins: talin, vinculin, paxillin; cytoskeletal associated proteins: α-actinin-4, synaptopodin; transmembrane/receptor proteins: podocin, Glepp1, C3b, podocalyxin; transcription factors: WT1, Pod1, Lmx1b; cell cycle–associated proteins: p27, p57; secreted proteins: collagen type IV(α3–α5), laminin 11, entactin/nidogen, syndecan-4, glypican, agrin, perlecan, vascular endothelial growth factor (vegf).
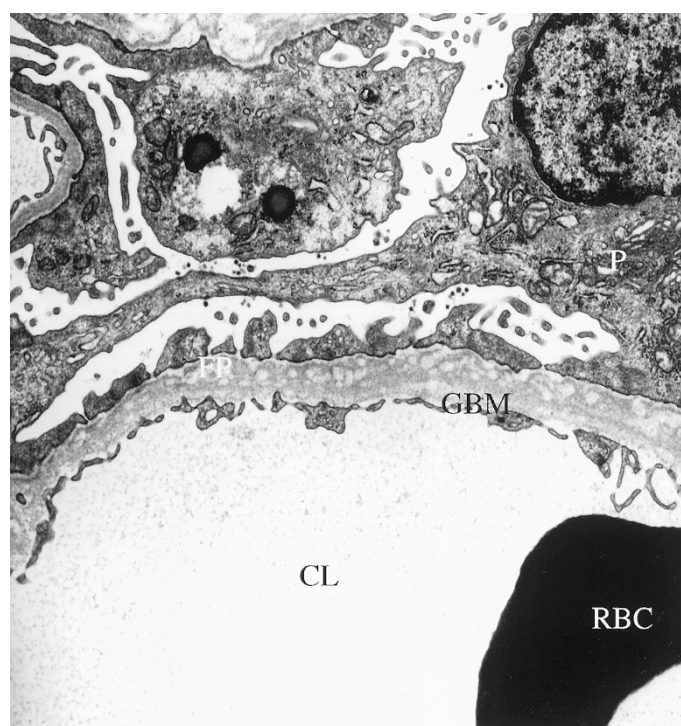
Figure 3. Electron micrograph kidney biopsy from an NPS patient. The patient has a *Lmx1b* mutation (713insA). Thickening of the GBM and fusion of foot processes are observed. *RBC*, red blood cell; *CL*, capillary lumen; *GBM*, glomerular basement membrane; *FP*, foot process; *P*, podocyte.

TRANSCRIPTIONAL REGULATION OF PODOCYTE DIFFERENTIATION

Transcription factors regulating the specification of early reciprocal induction of ureteric bud and metanephric mesenchyme have been well described. These include *Pax2*, *Lim1*, *Eya1*, *n-Myc1*, and *Emx2* (5). In contrast to the study of cytoskeletal and architectural proteins specifying podocyte function, few transcriptional regulators of podocyte morphogenesis have been studied either in humans or in mouse models. The Wilms' tumor gene (*WT1*) is a zinc finger containing transcription factor that acts both early during ureteric bud–metanephric mesenchyme interaction and later during podocyte morphogenesis and maintenance (19, 43). Although loss of its function places patients at risk for development of Wilms' tumor, subtle mutations may produce congenital malformation of the urogenital system observed in Denys-Drash syndrome and Frasier syndrome (44). The former is characterized by diffuse mesangial sclerosis, male-pseudohermaphroditism, and Wilms' tumor. It is thought to arise from dominant negative action. The latter is associated with severe nephrotic syndrome, gonadal dysgenesis, and risk of developing gonadal tumors, especially gonadoblastoma. Renal biopsy in these patients often shows focal segmental glomerular sclerosis and the phenotype is thought to be the result of alteration of the dosage ratio between alternatively spliced *WT1* isoforms. Interestingly, children with diffuse mesangial sclerosis without evidence of Denys-Drash syndrome have been found to harbor mutations in *WT1* (45). Clearly, *WT1* functions early during metanephric kidney development and then later during podo-

cyte morphogenesis. Heterozygous germline loss of function can result in tumorigenesis when associated with somatic mutation of the other allele, whereas the genitourinary phenotype marked by early nephrotic syndrome is associated with dominant mutations with incomplete penetrance. The downstream targets, however, are poorly characterized and the relationship of WT1 to the architectural proteins that specify the podocyte phenotype is still unknown.

NPS AND PODOCYTE FUNCTION

Insight into an important transcriptional regulator of podocyte function has recently been gained from the study of a human skeletal malformation syndrome, NPS. NPS is a dominantly inherited skeletal dysplasia characterized by hypoplasia of nails and patella, joint abnormalities, iliac horns, and nephropathy (46). Although only approximately 30–40% of patients have clinical nephropathy, histopathologic changes in the kidney have been reported even in fetal cases of NPS (47, 48). We and others have shown that NPS is caused by heterozygous mutations in the LIM homeodomain transcription factor *LMX1B* (49–55). LIM domains are zinc finger–containing motifs that have been found in over 40 different proteins. The LIM domain mediates protein-protein interactions and has been implicated in diverse biologic processes (56, 57). LIM homeodomain transcription factors compose a large family with over nine members and have been shown to be of critical importance in cell type differentiation, including pituitary gland differentiation (58). *Lmx1b* is a LIM homeodomain transcription factor originally described in chick as being required for dorsoventral pattern formation during limb development (59). *Lmx1b* expression during mouse limb development initially localizes to dorsal limb bud mesenchyme at E10.5. It continues to be highly expressed in dorsal mesenchyme until E16.5. This expression pattern predicts the consequences of loss of function in humans, *i.e.* dysplasia of dorsal mesenchyme–derived tissues, including nails and patella, and joint anomalies (60, 61). However, the observation of renal dysplasia in NPS pointed to a previously unrecognized function for *Lmx1b* during renal development. *Lmx1b* expression begins early in the S-shaped bodies, and, in contrast to its transient expression in the limb, expression in the kidneys continues postnatally in visceral glomerular epithelium (60, 62).

NPS patients have characteristic histologic findings on renal biopsy including thickened and split GBM and fusion of podocyte foot processes (Fig. 3). Interestingly, *Lmx1b* heterozygote mice have normal-appearing glomeruli at both the light and ultrastructural levels (data not shown). In contrast, *Lmx1b* null mice die shortly after birth and have severe renal abnormalities, including smaller kidneys and tubules distended with periodic acid-Schiff positive staining material consistent with severe proteinuria (60). Importantly, glomeruli are normally formed and podocytes normally express nephrin. However, glomeruli show GBM alterations similar to that observed in NPS as well as podocytes with ultrastructural features of arrested development (60, 62). The GBM alterations raised the question of whether *Lmx1b* might regulate structural compo-
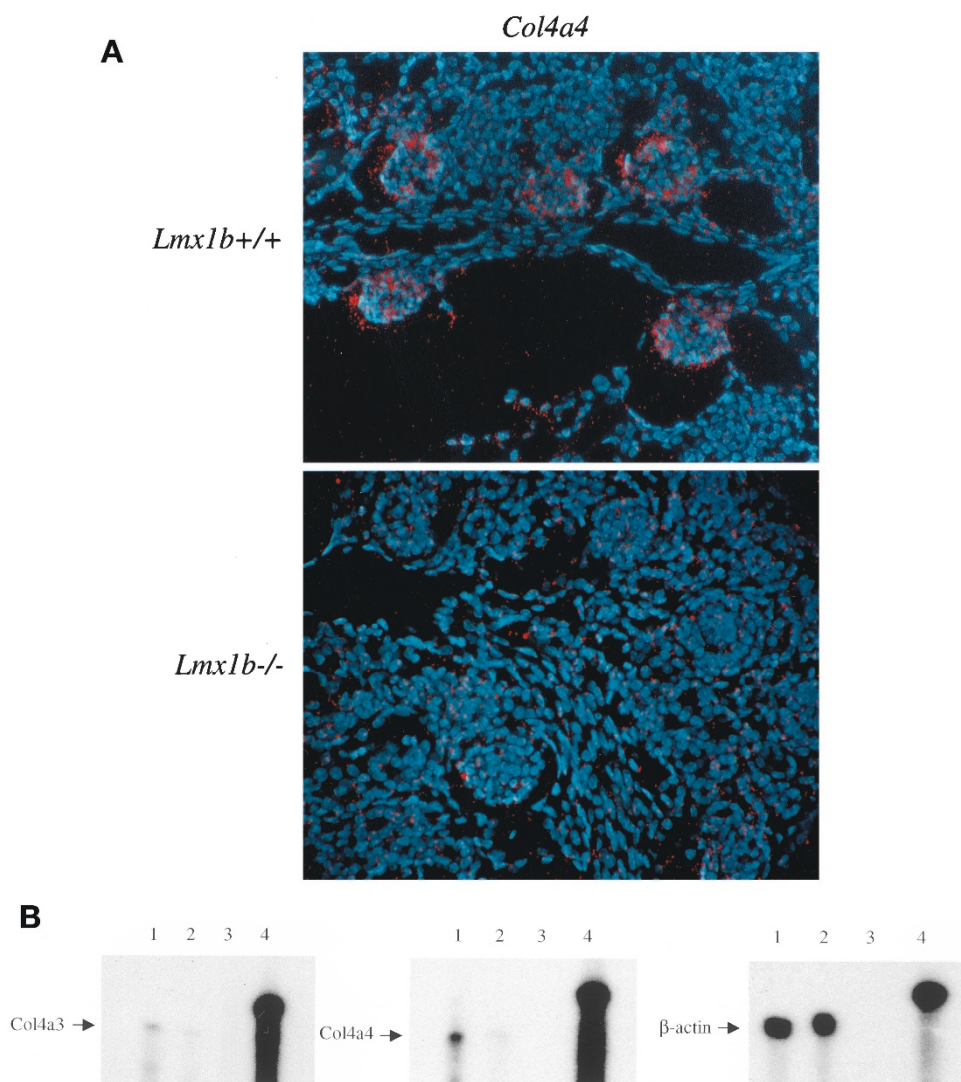
A

Col4a4

Lmx1b+/+

Lmx1b-/-

B

Figure 4. (*B*) *In situ* hybridization assay using a riboprobe specific for *Col4a4* of newborn kidney sections from wild-type and *Lmx1b−/−* mutant mice. Expression of α4(IV) collagen is almost absent in the mutant kidney compared with its wild-type counterpart. (*B*) Ribonuclease protection assay of kidney RNA derived from *Lmx1b* mutant kidneys using *Col4a3* and *Col4a4* probes. *Lane 1:* wild-type kidney total RNA; *lane 2: Lmx1b* mutant kidney total RNA; *lane 3:* yeast RNA; *lane 4:* RNase negative control. *Left panel, center panel,* and *right panel* show experiments with *Col4a3*, *Col4a4* and β-actin-specific probes, respectively. An equal amount of total RNA has been used in lanes 1 and 2. Protected fragments corresponds to detectable level of expression in wild-type kidneys, whereas decrease in the protected fragment corresponds to much decreased expression of *Col4a4* and some decrease in *Col4a3* mRNA in mutant kidneys.

nents of GBM on a transcriptional level. We have found that *Col4a1* and *Col4a2* expression was comparable in *Lmx1b* wild-type and null mice (62). In contrast, expression of kidney-specific *Col4a3* and *Col4a4* was dramatically decreased at both protein and mRNA levels (Fig. 4). This suggested that *Lmx1b* is required for expression of *Col4a3* and *Col4a4* and the developmental switch from the ubiquitous basement membrane collagens to the GBM-specific isoforms. The tandem genomic organization of *Col4a3* and *Col4a4* (63) suggested that *Lmx1b* might direct transcriptional regulation of this locus and loss of their expression is not a secondary effect. In fact, within an otherwise poorly conserved *Col4a4* intron 1 sequence, we have identified a conserved LMX1B binding site at its 3' end (62). Together, the data suggest that *Lmx1b* may serve as a transcriptional activator required for coordinate expression of *Col4a3* and

Col4a4, making it a key transcriptional determinant of GBM formation. These data beg the question of whether it might directly regulate the developmental switch in matrix expression. Although *Lmx1b* is required for collagen expression, it is likely not sufficient to specify the switch, inasmuch as we have found that laminin β1 and laminin β2 expression is unchanged in *Lmx1b* null mice kidneys (data not shown). However, the phenotypic severity of the null mice suggests that *Lmx1b* must regulate other genes required for podocyte function. The nephropathy in *Lmx1b* null mice is histologically more severe and of earlier onset than that observed in the *Col4a3* or double *Col4a3/Col4a4* mouse mutants (15, 16). Hence, loss of α3(IV) and α4(IV) collagen chains alone cannot account for the full renal phenotype and it is likely that *Lmx1b* regulates additional genes that contribute to podocyte function and maintenance. Candidate genes in-

clude cytoskeletal and architectural genes such as podocin, synaptopodin, *Cd2ap*, *ZO1*, etc.

The analysis of the expression of these genes in *Lmx1b* null mice and the search for potential LMX1B binding sites in their respective promoters will help to determine whether they are members of the list of *Lmx1b* targets that account for its role in podocyte differentiation. Other critical questions remain about the role of *Lmx1b* regulation in podocyte differentiation: What transcription factors regulate and cooperate with *Lmx1b* function? It is likely that *Lmx1b* functions in different development pathways in a context-dependent and organ-specific fashion and is, in part, dictated by different combinations of proteins that interact with it. For example, the closely related LIM homeodomain transcription factor *Lmx1a* has been described as transactivating the insulin promoter cooperatively with a helix-loop-helix transcription factor *E47* (64). Interestingly, another helix-loop-helix protein, Pod1/capsulin/TCF21, is specifically expressed in S-shaped bodies and continues to be expressed in maturing podocytes, paralleling the expression pattern of *Lmx1b* (65). Loss of its function results in hypoplastic lungs and kidneys that lack mature glomeruli or nephrons (66). It will be important to determine whether *Pod1* might interact with *Lmx1b* to coordinately regulate the collection of architectural genes that define podocyte differentiation and function.

The study of *Lmx1b* in NPS has shown that it is required for basement membrane collagen expression. Moreover, the severity of the mouse phenotype suggests that loss of additional podocyte genes accounts for arrested podocyte differentiation. Candidate targets for *Lmx1b* include multiple cytoskeletal and integral membrane proteins important for podocyte differentiation and maintenance. Elucidating the potential interaction of *Lmx1b* with the few other transcription factors identified in podocyte function, including *WT1* and *Pod1*, will be important in understanding the transcriptional network coordinating podocyte differentiation. Ultimately, this will impact on our understanding of diseases characterized by nephrotic syndrome and on the development of restorative strategies for these disease processes.

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