The development of the mammalian kidney from two interacting tissues, the metanephrogenic mesenchyme and the ureteric bud, is ultimately reflected by the presence of two distinct, yet connected systems, the nephrons and the collecting ducts, respectively. The nephrons consist of a filtration unit, the glomerulus, and a subsequent processing portion, the tubule, which empties into the collecting duct. Although it is clearly established that podocytes, like the rest of the nephron, originate from the metanephrogenic mesenchyme, it is far from understood what mechanisms underlie podocyte development, structure, and function. What signal tells parietal cells and podocytes to separate and form Bowman’s space, how does the podocyte orchestrate the formation of primary and foot processes and the interdigitation of the latter, how is the formation of the slit diaphragm regulated, and what molecules mediate the communication between podocytes and glomerular endothelial cells? Fortunately, the identification of genes mutated in patients suffering from hereditary kidney diseases and the increasing data from genetically modified animals have begun to unravel parts of the puzzle.

PATHOLOGIC FEATURES OF PATIENTS WITH NAIL-PATELLA SYNDROME

This chapter will deal with the nail-patella syndrome, a complex of symptoms also known as hereditary osteo-onychodysplasia (HOOD syndrome), Turner-Kieser syndrome, Fong disease, and Osterreicher syndrome. Nail-patella syndrome occurs only rarely; its prevalence has been repeatedly quoted at 1 in 50,000, although this author is not aware of a published epidemiologic study from which this figure has been derived. As already indicated by its name, the syndrome has been christened after the malformed fingernails and toenails and the hypoplastic or even absent kneecaps; however, it is the renal disease that determines the prognosis of individuals with this disorder. Despite being one of the first hereditary diseases for which linkage to a polymorphic marker, in this case the ABO blood group, was established, more than 40 years passed before mutations in the LMX1B gene were found to be responsible for nail-patella syndrome.

The LMX1B gene encodes a transcription factor with two zinc-binding LIM domains at the NH₂-terminus, a DNA-binding homeodomain in the middle, and a putative activation domain at the COOH-terminus. Orthologous genes were first cloned as Lmx1 in the chick\(^5,6\) and as Lmx1.2 in the hamster\(^7\); only later were the human (then called LMX1.2),\(^8\) the murine,\(^9\) and the Xenopus laevis\(^10\) genes identified. The protein is produced in a variety of tissues, and in actuality Lmx1b first gained prominence because it is necessary to specify the dorsoventral axis in the limb.\(^5,6\) A lack of Lmx1b leads to dorsoventral axis defects, which explains the nail and patella symptoms in those suffering from the disorder. Nail changes of variable severity can be noted in 98% of patients, with the thumbnail usually being the most severely or only affected nail.\(^11\) Patellar symptoms have been noticed in 74% of the patients, and again the severity was quite variable.\(^11\) One other pathognomonic sign is the growth of bone spurs on the posterior face of the iliac bone, so-called iliac horns, which was detected in approximately two-thirds of the patients.\(^11\) Nail-patella syndrome can be associated with several other symptoms, of which only one in addition to the renal involvement will be mentioned here.\(^11\) A careful analysis of the expression pattern of the murine Lmx1b gene has demonstrated the existence of the Lmx1b messenger RNA in the anterior segment of the eye.\(^12\) This correlates well with the observation that 10% of patients with nail-patella syndrome present with open-angle glaucoma.\(^11\)

Renal involvement, the first evidence of which was published in 1950,\(^13\) determines the prognosis of patients with nail-patella syndrome. Symptoms may range from light to severe proteinuria, through hematuria, and finally to chronic renal failure. Several studies have addressed this issue, so that the frequency of renal symptoms, which may start shortly after birth or only after several decades, can be estimated at close to 40%.\(^11\) The morphologic changes are most telling on an ultrastructural level and affect the glomerular basement membrane (GBM) and podocytes (Fig. 11-1). In the original reports, a thickened GBM was described that contained both electron-lucent areas\(^14,15\) and fibrillar deposits resembling collagen.\(^14\) Indeed their periodicity of 64 to 66 nm\(^16\) is consistent with that of fibrillar collagen, although in one publication a periodicity of 40 to 60 nm was reported.\(^17\) Furthermore, foot process effacement was observed for some but not all podocytes.\(^14,15\) These first descriptions have been confirmed several times and are important diagnostic criteria.\(^16-21\)

GENETIC FINDINGS AND BIOCHEMISTRY OF LMX1B

Back-to-back reports in 1998 established that nail-patella syndrome was caused by mutations in the LMX1B gene on human chromosome 9q34.\(^2,19\) and additional reports in the same year confirmed this initial finding.\(^2,4\) The human LMX1B gene consists of 8 exons and encodes a ≈7-kb messenger RNA, which apparently can be alternatively spliced\(^22\) and therefore gives rise to a 395-amino acid\(^23\) and a 402-amino acid
protein with signature motifs of a transcription factor. It contains a DNA-binding homeodomain extending from position 219 to 278 and two zinc-binding LIM domains at the NH2-terminus, whereas the COOH-terminus is believed to serve as a transcriptional activator domain. LIM domains (consensus motif CysX2-CysX16-18 HisX2-CysX2-CysX16-18 CysX2-His/Cys) were first described in 1990, in contrast to zinc-binding domains of the Cys3His3 and the Cys4 class they do not bind to DNA but rather mediate protein-protein interactions (see review). Indeed, two groups have reported on the interaction between the LIM domains of LMX1B on the one side and the helix-loop-helix protein E47 and the transcriptional adapter protein LDB1 on the other side. E47 is encoded by the E2A gene, which by alternative splicing also gives rise to the E12 protein, and it serves as a strong co-activator of LMX1B. LDB1, however, decreases the transcriptional activation properties of LMX1B. The picture becomes somewhat more complicated when one compares the interaction between LDB1 and the closely related protein LMX1A. In the absence of E47, LDB1 did not influence the transcriptional activity of LMX1A, whereas in its presence it negatively affected the interaction between LMX1A and E47.

It is very surprising that most mutations in the LMX1B gene have been detected in those regions of the gene encoding the LIM domains and the homeodomain, and only very few in the COOH-terminal activation domain. In those cases in which the mutated proteins were subjected to a functional test, mutations in the homeodomain resulted in decreased or even absent binding to DNA, and a mutation in the second LIM domain resulted in the decreased activation of a reporter gene. The modularity of the protein precipitates the question whether a genotype-phenotype correlation can be established for the various mutations, all the more so because E47 preferentially interacts with the second LIM domain of LMX1B (and of LMX1A); moreover, a similar case can be argued for LDB1, which binds more strongly to the second LIM domain of the closely related LMX1A protein. Thus far no such correlation could be demonstrated, but this situation could be due to the limited number of affected families.

One important issue still to be addressed concerns the means by which the mutated gene leads to the disease. Because nail-patella syndrome is inherited in an autosomal dominant fashion, haploinsufficiency, a gain of function, or a dominant-negative effect could represent the underlying pathologic mechanism. The term haploinsufficiency indicates that too little of a protein is present to fulfill its physiologic functions—for example, only 50% if the mutation results in an inactive or absent protein and the nonmutated allele is not upregulated. A gain-of-function mutation leads to the synthesis of a mutant protein with a novel function, for example, a constitutively
active protein due to the absence of an inhibitory domain. Finally, a dominant-negative effect is seen when the mutant protein interferes with the binding of the wild-type protein to a protein “X,” which in turn is essential for the biologic effect of the wild-type protein. For the following reasons it is unlikely that either of the latter two explanations is correct. First, mutations have been described that are predicted to lead to the synthesis of a severely truncated protein without even the first LIM domain,\(^{35}\) thus virtually ruling out a gain-of-function and a dominant-negative effect; second, the analysis of several mutant proteins has failed to demonstrate a dominant-negative effect on the wild-type LMX1B protein.\(^{54}\) Therefore, the current hypothesis is that haploinsufficiency results in nail-patella syndrome, although this contrasts with the failure of mice with only one inactivated Lmx1b allele to show any symptoms.\(^{33}\) One somewhat provocative explanation could be that in addition to the germline mutation, the patients suffer from somatic mutations in the second LMX1B allele; thus, on a cellular level nail-patella syndrome would have to be considered a recessive disease. Such an assumption is not totally speculative, because a similar scenario has already been described for autosomal dominant polycystic kidney disease.\(^{34}\)

### THE Lmx1b-KNOCKOUT MOUSE

The Lmx1b-knockout mouse represents a major tool toward a better understanding of the pathogenesis underlying nail-patella syndrome. It was created by replacing exons 3 to 7 with a selection cassette, thus deleting the region encoding the second LIM domain, the homeodomain, and the COOH-terminal trans-activation domain.\(^{33}\) As already pointed out above, mice with only one inactivated Lmx1b allele show no symptoms even over an extended observation period.\(^{33}\) Homozygous knockout mice die within 24 hours after birth; the cause of death has not yet been determined. In analogy to the human syndrome, the knockout mice lack patellae and present with footpads even on the dorsal side of their limbs because the dorsifacial signal usually provided by Lmx1b is missing.\(^{9}\)

Although thus far only a limited expression analysis of the Lmx1b gene has been published,\(^{9,35}\) it is consistent with the kidney phenotype. Renal symptoms in the Lmx1b\(^{-/-}\) mice are already evident from their smaller kidney size; by light microscopy a decreased glomerular tuft area has been observed.\(^{33}\) and proteinaceous material can be found in the tubules.\(^{9}\) More marked changes are noticed on the ultrastructural level. Podocytes in homozygous knockout mice elaborate only rudimentary, if any, foot processes (Fig. 11-2A).\(^{33,36}\) Moreover, the mutant podocytes are not connected by a slit diaphragm but instead by a structure resembling an adherens junction (Fig. 11-2B).\(^{33,36}\) Structural alterations were not, however, restricted to the podocyte layer but also extended to the GBM and the glomerular endothelium. During regular kidney development, the GBM originates from both the podocytes and the endothelial cells and therefore consists of two layers early on, before subsequently the typical structure with a lamina rara externa, a lamina densa, and a lamina rara interna emerges. In the Lmx1b\(^{-/-}\) mice a two-layered GBM is still found in some regions\(^{35}\); it is not clear at this point whether the fusion of the two layers never occurred or whether both layers fused and split again. In contrast to the findings in kidneys from individuals with nail-patella syndrome, we could detect no fibrillar deposits in the GBM but rather found fibrils in Bowman's space.\(^{33}\) However, because of their periodicity of \(?=17\) nm, these fibrils probably represent fibrin and not collagen. In wild-type kidneys, the glomerular tuft is lobulated and the endothelial cells form a fenestrated endothelium, whereas in homozygous Lmx1b-knockout mice the lobulation of the glomerular tuft is greatly reduced and the fenestrae were found at a much reduced frequency.\(^{33}\)

How may these renal alterations be explained on a molecular level? After the positional cloning of the NPHS1 gene,\(^{37}\) the gene mutated in patients with congenital nephrotic syndrome of the Finnish type, a steadily increasing wealth of information has accumulated on the biochemical nature of podocyte-specific structures.\(^{38}\) The NPHS1 gene encodes nephrin, an integral membrane protein with a large extracellular domain, which was later recognized as an essential component of the slit diaphragm.\(^{39,40}\) Soon afterwards two other genes were found to be mutated in individuals with hereditary kidney diseases. Mutations in the NPHS2 gene, which encodes the slit diaphragm-associated protein podocin, lead to steroid-resistant nephrotic syndrome,\(^{41}\) and mutations in the ACTN4 gene, which encodes the actin-bundling protein \(\alpha\)-actinin-4, lead to focal segmental glomerulosclerosis.\(^{42}\) Because podocytes in homozygous Lmx1b-knockout mice lack foot processes and a slit diaphragm, \(\alpha\)-actinin-4, podocin, and nephrin were obvious candidates for proteins whose down-regulation leads to the observed morphologic changes. Surprisingly, however, nephrin is still present in podocytes of Lmx1b\(^{-/-}\) mice,\(^{33,35,36}\) which at the same time suggests that nephrin is not solely responsible for the formation of a slit diaphragm. Podocin, by contrast, is absent both on the messenger RNA and the protein levels\(^{33,36}\) (Fig. 11-2C,D). A discrepancy exists with respect to the synthesis of CD2AP, another slit diaphragm–associated protein.\(^{44}\) Although our group has found only a slight reduction of CD2AP in podocytes of homozygous Lmx1b-knockout mice,\(^{33}\) another group found a reduction of CD2AP protein levels similar to those of podocin.\(^{30}\) Although the reason for these differing results is unclear, they may well be due to the use of different mouse strains.

There are also clues concerning the explanation of the defects in the GBM and in the glomerular endothelium. The main components of the GBM are the \(\alpha3\)-, \(\alpha4\)-, and \(\alpha5\)-chains of collagen IV, which because of their involvement in Alport syndrome and the phenotype of the respective knockout mice\(^{43-45}\) are prime candidates as targets of Lmx1b. Indeed the \(\alpha3\)- and the \(\alpha4\)-chains, but not the \(\alpha5\)-chain, of collagen IV are absent in the GBM of Lmx1b\(^{-/-}\) mice.\(^{33}\) We have observed this in our studies as well. The reduced lobulation of the glomerular tuft and the decreased number of fenestrae in the glomerular endothelium may be due to the reduced synthesis of vascular endothelial growth factor,\(^{33}\) which has repeatedly been shown to act in a critical concentration range.\(^{38}\)

Obviously the Col4a3, Col4a4, Nphs2, and possibly also the Cdkap gene are good candidates to be directly regulated by LMX1B. The closely related LMX1A protein, whose DNA-binding homeodomain is 100% identical to that of LMX1B, binds to AT-rich stretches of DNA,\(^{30}\) and it can therefore be assumed that LMX1B recognizes similar motifs. Indeed, it has been demonstrated that LMX1B binds to AT-rich sequences in the first intron of the COL4A4 gene\(^{35}\) and in the promoter region of the NPHS2\(^{33,36}\) and CD2AP\(^{35}\) genes. It is not clear,
however, whether these sequences actually mediate transcriptional activation of the respective target genes by LMX1B. Whereas a reporter construct with six concatemerized LMX1B binding sites of the COL4A4 intron led to a fivefold activation of the reporter gene⁵⁵ and a construct with four concatemerized LMX1B binding sites of the NPHS2 promoter led to an approximately twofold activation of the reporter gene,⁶ we were not able to demonstrate the activation of a reporter construct with 4.4 kb of the NPHS2 promoter by LMX1B.⁵⁴ Furthermore, we could not show the activation of the endogenous NPHS2 gene in HeLa cells by stably transfected LMX1B.⁵³

**Figure 11-2** Glomerular changes in the Lmx1b-knockout mouse. A, No foot processes can be detected in homozygous Lmx1b-knockout mice. B, Neighboring foot processes of homozygous Lmx1b-knockout mice are not connected by a slit diaphragm but by a structure resembling an adherens junction. C and D, By immunohistochemistry podocin can be detected in the podocytes of wild-type but not of mutant mice. [From Rohr C, Prestel J, Heidet L, et al: The LIM-homeodomain transcription factor Lmx1b plays a crucial role in podocytes. J Clin Invest 109:1073–1082, 2002.]

**PERSPECTIVE**

The analysis of the Lmx1b-knockout mouse has yielded remarkable new insights into podocyte differentiation in general and the regulation of podocyte differentiation by Lmx1b in particular. There are, however, several open questions, the most important of which is the identification of LMX1B target genes. Obviously Nphs2, the gene encoding podocin, is one prime candidate, because it is not expressed in Lmx1b-knockout mice. There are, however, several experimental findings that cast doubt onto this assumption. Very importantly, the Nphs2 as well as the Col4a3 and Col4a4 genes were still
active after the podocyte-specific inactivation of Lmx1b in mice. Second, podocin (as well as CD2AP and the α3- and α4-chains of collagen IV) was still detected in glomeruli from patients with nail-patella syndrome, which of course could be due to a species difference; the transcriptional regulation of this gene may differ in mice and humans. Third, we were not able to find an activation of the endogenous NPHS2 gene by transfectected LMX1B in HeLa cells, a human cervical cancer cell line, which would be predicted if LMX1B is solely responsible for the activation of NPHS2. Such a scenario, however, is unlikely, because then NPHS2 should be expressed in those other tissues where Lmx1b also is produced (e.g., the developing limb, eye, skull, and mesencephalon), which has not been observed. Thus, it is likely that additional co-factors are required for, for example, may make a target gene such as NPHS2 accessible to LMX1B only in podocytes. Alternatively, a variable assembly of co-factors may exist in those different tissues where LMX1B is produced such that the tissue-specific expression of LMX1B target genes is achieved. Could LDB1 and E47 be those co-factors? Although LDB1 and E47 are important transcriptional adaptor proteins for LMX1B at least in vitro, it is doubtful whether they can confer podocyte specificity to LMX1B because they are also found in a wide variety of tissues. Furthermore, the podocyte-specific inactivation of Ldb1, but not that of E2a, resulted in a podocyte defect. Another challenge therefore will be to determine whether LDB1 and E47 are necessary for the activity of LMX1B in the podocyte and what other transcriptional adaptor proteins and DNA-binding transcription factors are important for the podocyte-specific expression of genes. For our ultimate goal of deciphering the transcriptional network acting in podocytes, we also must look in the other direction and ask what transcription factors activate the LMX1B gene in podocytes. In Lmx1b-knockout mice, the zinc-finger transcription factor Wt1 is still present, but regrettably the Wt1 knockout leads to renal agenesis, so that it remains unknown whether Lmx1b is expressed in the absence of Wt1. The absence of two other transcription factors, the basic helix-loop-helix protein Pod1 and the leucine zipper protein MaFB, also results in podocyte defects, but again it is not known whether they are directly or indirectly linked to Lmx1b. Recent results obtained from the analysis of chimeric mice suggest that podocytes without a functional Pod1 protein develop normally, and that therefore the podocyte phenotype in Pod1-knockout mice is not cell autonomous in nature but instead is due to a defect in the surrounding stroma. Although it is tempting to speculate that in analogy to the interaction between LMX1B and the helix-loop-helix protein E47, Lmx1b physically interacts with the helix-loop-helix protein Pod1 in podocytes, such an interaction would not have to be considered essential for podocyte function at present. The study of Lmx1b has given us exciting new insight into the biology of the podocyte, an essential component of the renal filtration system. Thus far we have only begun to understand gene regulation in this fascinating cell type, and it is certain that many more important discoveries lie ahead.

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References


