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ORIGINAL PAPER

Renal phenotype in heterozygous *Lmx1b* knockout mice (*Lmx1b*$^{+/-}$) after unilateral nephrectomy

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Abstract The nail-patella syndrome (NPS) is a rare autosomal-dominant disorder which is caused by loss-of-function mutations in the transcription factor LMX1B. NPS is characterized by dysplastic nails, absent or hypoplastic patellae, minor skeletal abnormalities and nephropathy (in 20–40% of the cases), which is the most severe aspect of the disorder. The current data suggest that genetic modifiers in the outbred human genetic background are responsible for this variable phenotype. Preliminary work on the function of Lmx1b in the kidney has been performed using *Lmx1b* knockout mice (*Lmx1b*$^{-/-}$). Although *Lmx1b*$^{-/-}$ mice die within 24 h after birth, they exhibit the characteristic NPS features including the renal abnormalities. But in contrast to the situation in human, no phenotype could so far be detected in heterozygous *Lmx1b*$^{+/-}$ mice. This indicates that our understanding of the pathomechanism underlying the nephropathy is still very limited. In an attempt to further evaluate these mechanisms, we tried to induce a renal phenotype in *Lmx1b*$^{+/-}$ mice, and thus model the human (NPS) situation. We applied unilateral nephrectomy as a model to induce nephron loss and detected a significant ($p = 0.02$) reduction in compensatory renal growth in heterozygous knockout animals (*Lmx1b*$^{+/-}$) compared to *Lmx1b*$^{+/+}$ animals, which was correlated with a significantly lower increase in glomerular volume (V_G) ($p = 0.0034$) and an increase in glomerulosclerosis ($p = 0.085$). Thus, Lmx1b deficiency in heterozygous *Lmx1b* (*Lmx1b*$^{+/-}$) knockout mice profoundly affects the compensatory response to nephron loss. Moreover, this is the first report of a phenotype in heterozygous *Lmx1b* (*Lmx1b*$^{+/-}$) knockout animals.

Keywords LMX1B · Uninephrectomy · Compensatory renal growth · Genetic modifiers

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Introduction

The nail-patella syndrome (NPS) is a rare autosomal-dominant disorder characterized by dysplastic nails, absent or hypoplastic patellae, discrete skeletal defects and, in some cases, nephropathy. The disorder is caused by heterozygous loss of function mutations in the *LMX1B* gene, encoding a transcription factor of the LIM homeodomain protein family (Dreyer et al. 1998; McIntosh et al. 1998). Nephropathy is the most serious aspect of NPS. Early signs are proteinuria and less frequently hematuria which, in ~15% of cases, develops towards end-stage renal disease. In the kidney, *LMX1B* is expressed in podocytes during embryonic development and during postnatal life (Morello et al. 2001). Work on *Lmx1b*$^{-/-}$ mice revealed that LMX1B regulates the expression of proteins critical for glomerular function, like the α3 and α4 chains of type IV collagen, podocin, and CD2AP (Morello et al. 2001; Miner et al. 2002; Rohr et al. 2002). It has therefore been proposed that the dysregulation of these genes result in the specific histological changes of the GBM (glomerular basement membrane), which can be found in biopsies from NPS patients (i.e. thickening and lamination of the GBM), and finally in the development of nephropathy. Nevertheless, the typical histological changes in NPS patients do not correlate with the occurence of proteinuria and hematuria (Reviewed in: Meyrier et al. 1990; Bongers et al. 2002). Moreover, using immunohistochemical studies in biopsies from NPS patients with glomerular disease that progressed to end-stage renal disease, Heidet et al. (2003) were not able to detect any changes in the expression of α3 and α4 chains of type IV collagen, podocin and CD2AP at endstage kidney disease. Although early changes in the kidney have not been investigated, so far, the present data may suggest that other, yet unknown pathomechanisms may contribute to the nephropathy in NPS.

It is assumed that NPS is caused by haploinsufficiency (Dreyer et al. 2000) in a dose-dependent manner. This contrasts with the fact that *Lmx1b*$^{+/-}$ mice seem to be phenotypically normal and did not develop any NPS symptoms (Chen et al. 1998; Rohr et al. 2002). Instead, *Lmx1b*$^{-/-}$ animals, although they die within 24 h after birth, develop NPS features including renal insufficiency and the typical renal GBM changes (Chen et al. 1998). This points at a different dose sensitivity in the mouse and therefore questions the use of *Lmx1b*$^{-/-}$ mice for the investigation of the pathomechanisms underlying NPS nephropathy.

Interestingly, only about 20–40% of the NPS patients progress to renal insufficiency despite similar or identical *LMX1B* mutations (Meyrier et al. 1990; Sweeney et al. 2003; Bongers et al. 2005). Based on a reduced penetrance and strong inter- and intrafamilal variable expressivity it is assumed that modifier genes in the mixed human genetic background are causing this phenotypic heterogeneity. The mapping of genetic modifiers influencing the onset and progression of nephropathy is an important issue but, in the case of the fairly rare NPS, can only be addressed by using the mouse as a model system. This would require a strain specific, renal phenotype in heterozygous *Lmx1b*$^{+/-}$ knock out animals. Therefore, the identification of a renal phenotype in *Lmx1b*$^{+/-}$ mice would be extremely useful in order to model the human situation in the mouse and to further investigate the pathomechanisms, as well as the variability of NPS nephropathy.

Here, we performed a thorough investigation of the kidneys of *Lmx1b*$^{+/-}$ mice after induction of nephron loss by unilateral nephrectomy (Unx). Unx in mice is an experimental procedure which leads to compensatory enlargement of the remaining kidney. We have chosen UNX as a model since the response of the remaining nephrons to a reduction of nephron number may play an important role in the development of progressive renal damage (Brenner 1985). On the other hand, UNX causes a mild renal dysfunction, with no direct damage of the remaining kidney and adaptive changes are well investigated (e.g. Nagata and Kriz 1992; Nagata et al. 1992). We detected a significant ($p = 0.02$) reduction in compensatory renal growth (CRG) in heterozygous knockout animals (*Lmx1b*$^{+/-}$) compared to Lmx1b$^{+/+}$ animals, which was correlated with a significantly lower increase in V_G ($p = 0.0034$) and a mild increase in glomerulosclerosis ($p = 0.085$). This is the first report of a phenotype in heterozygous *Lmx1b* knockout animals, and the first report of a connection between CRG and Lmx1b action.

Materials and methods

Animals

About 6–14-week old male *Lmx1b*$^{+/-}$ and *Lmx1b*$^{+/+}$ mice maintained on the C57BL/6 background were

used for the experiments. Initial body weights were 21–30 g. The mice were housed 4–5/cage in a room with a 12:12-h artificial light cycle, a temperature of 21 ± 1°C, and a humidity of 55 ± 5%. The animals had free access to standard chow and high-salt (1% NaCl) water throughout the experiment. Body weight of 6–14-week old mice was recorded, then the left kidney was removed after exposure by a flank incision under anaesthesia with Ketamine/Xylazine. Kidney weight per 100 g body weight was calculated. Animals were allowed to recover for exactly 1 week. After additional 8 weeks (under administration of high-salt water) urine albumin and total protein excretion were measured. Then the animals were killed by dissecting the abdominal artery and bleeding in deep ketamine/xylazine anaesthesia. The right kidney was removed, weighed and the ratio of kidney weight per 100 g body weight was calculated. CRG is expressed as a difference between the relative weight of the left and the right kidney after removal.

Morphological studies

The whole right kidney of uninephrectomized animals were taken, decapsulated and fixed in Methyl-Carnoy, embedded in paraffin, and cut into 4-µm-thick sections. All of the following semiquantitative morphometric investigations were performed in a blinded manner. The glomerulosclerosis and mesangiolysis indices were determined on Periodic acid-Schiff's (PAS)-stained paraffin sections according to a previously described scoring system (scores of 0–4; Kida et al. 1991). For each animal the arithmetic mean of 100 randomly sampled glomeruli was determined using a light microscope under 400× magnification. V_G was estimated using the same slides. Tissue sections were examined on a Olympus light microscope (Olympus CH40, Olympus, Japan) connected to a video camera and a computer-based image analysis system (AnalysisPRO; SIS, Münster, Germany). For each animal 30 glomeruli were randomly selected, systematically digitized using a 40× objective, and examined. Mean value of V_G was than calculated using the formula $V_G = (\beta/k)(A_m)^{3/2}$ where k is 1.1 (size distribution coefficient) and β is 1.38 (shape coefficient for spheres), which is the assumed shape of the glomeruli (Weibel 1979; Remuzzi et al. 1992; Amann et al. 1996). For histological examination of the proximal convoluted tubules, sections stained with PAS were used. For each mouse, the smallest diameter of 100 proximal tubules which were nearly circular, were calculated using the above mentioned computer-based image analysis system. In addition, for each of the proximal tubules the epithelial thickness was measured. Mean diameter and epithelial thickness of the proximal tubules were used for further statistical analysis.

Statistical analyses

Data are given as means with the standard deviation (SD) in brackets. The t test was used to determine intergroup differences. The results were considered significant if the probability of error was $P < 0.05$.

Results

Uninephrectomy (Unx) in mice is an experimental procedure which leads to a loss of approximately half of the nephrons as well as compensatory enlargement of the remaining kidney associated with an increase in V_G and enlargement of the proximal convoluted tubules, resulting in an early reconstitution of the glomerular filtration rate (Kanda et al. 1993; Flyvbjerg et al. 1999). 11 *Lmx1b*$^{+/+}$ and 11 *Lmx1b*$^{+/-}$ mice, both on a C57BL/6 background, underwent Unx and received a high salt diet for 8 weeks in order to increase salt load stress. Renal changes were evaluated following measurement of kidney weights, total protein excretion as well as histological parameters, like V_G, tubular volume, glomerulosclerosis and mensangiolysis index (Table 1). Since the present investigation was designed as a purely comparative study between Lmx1b$^{+/-}$ and Lmx1b$^{+/+}$ mice, no sham-operated animals were included.

Compensatory renal growth (CRG), urine albumin and total protein excretion

Unilateral nephrectomy is followed by CRG of the remaining kidney. We measured CRG by comparing left and right kidney weight in *Lmx1b*$^{+/-}$ and *Lmx1b*$^{+/+}$ animals after uninephrectomy. We

Table 1 Values for right kidney after UNX in *Lmx1b*$^{+/+}$ and *Lmx1b*$^{+/-}$ animals

	Lmx1b$^{+/-}$ (n = 11)	*Lmx1 b*$^{+/+}$ (n = 11)	*t*-test [*p-value*]
Kidney weight gain, mg	74.9 (SD 30.4)	113.6 (SD 40.7)	**0.0200**
Glomerular volume, $10^3\mu m^3$	153.1 (SD18.4)	190.6 (SD 30)	**0.0034**
Diameter of proximal tubules, µm*	35.2 (SD 1.7)	35.1 (SD 1)	0.9100
Epithelial thickness, µm*	11.0 (SD 0.5)	10.7 (SD 0.6)	0.3300
Mesangiolysis index (score 0–4)	0.52 (SD 0.09)	0.52 (SD 0.08)	0.8900
Glomerulosclerosis index (score 0–4)	0.32 (SD 0.11)	0.25 (SD 0.07)	**0.0850**
Uprotein/creatinin	5.9 (SD 6.4)	8 (SD 6.1)	0.4500

Values are means with standard deviation in brackets; n number of kidneys; significant p-values are indicated in bold

* Only n = 6 animals (each) were investigated

observed a significant reduction (p = 0.02) in kidney weight gain in *Lmx1b*$^{+/-}$ animals, with a mean CRG of only 65.9% compared to that in *Lmx1b*$^{+/+}$ animals (Table 1, Fig. 1). Measurement of total protein excretion (Table 1), as well as serum creatinine and serum urea (data not shown) revealed no significant difference between the two groups of animals. Thus, the difference in CRG is not correlated with proteinuria.

Morphological indices of renal damage, glomerulus geometry and proximal tubule diameter

A comparative morphological investigation of the right kidney after uninephrectomy was performed. Here, we detected a significant difference in the V_G (p = 0.0034). In *Lmx1b*$^{+/-}$ animals the mean V_G was 153.1 (SD 18.4) compared to 190.6 (SD 30) in *Lmx1b*$^{+/+}$ and thus constitute only 80.3% of that in *Lmx1b*$^{+/+}$ animals (Fig. 2, Table 1). These data correspond well with the differences in CRG (Fig. 1), which is basically caused by an increase in V_G and an enlargement of the proximal tubules (Review: Cingel-Ristic et al. 2004). Interestingly, measurement of the proximal convoluted tubules revealed no difference in diameter as well as epithelial thickness (Fig. 3, Table 1).

In order to investigate the effect of renal hypertrophy and increased salt load on renal damage we measured mesangiolysis and glomerulosclerosis (Fig. 4, Table 1). While the mesangiolysis index was similar in heterozygous knockout and *Lmx1b*$^{+/+}$ animals (p = 0.89), the glomerulosclerosis index was slightly higher in *Lmx1b*$^{+/-}$ mice (p = 0.085) with values of 0.32 (SD 0.11) versus 0.25 (SD 0.07). Of note, no tubulo-interstitial or vascular damage was seen in *Lmx1b*$^{+/+}$ or *Lmx1b*$^{+/-}$ animals after Unx.

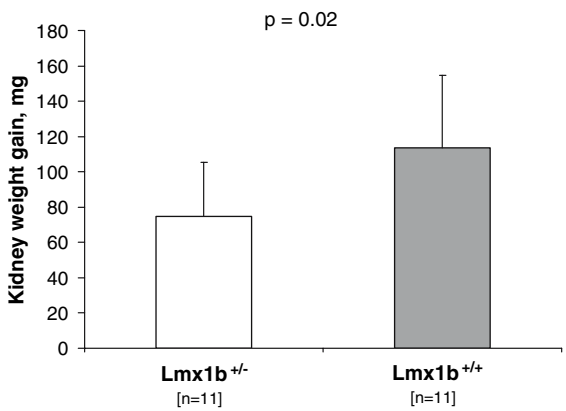

Fig. 1 Effect of uninephrectomy on kidney weight in *Lmx1b*$^{+/-}$ and *Lmx1b*$^{+/+}$ animals. *Error bars* represent 1 SD

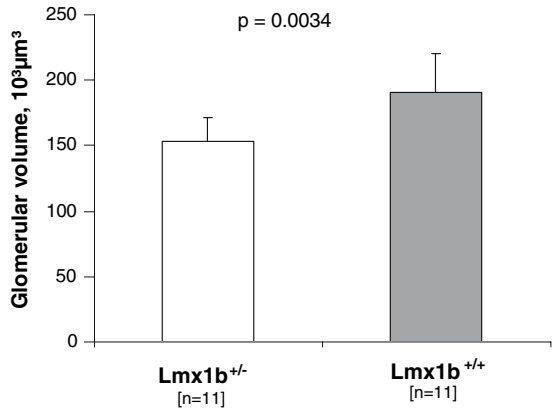

Fig. 2 Effect of uninephrectomy on mean glomerular volume (area). *Error bars* represent 1 SD

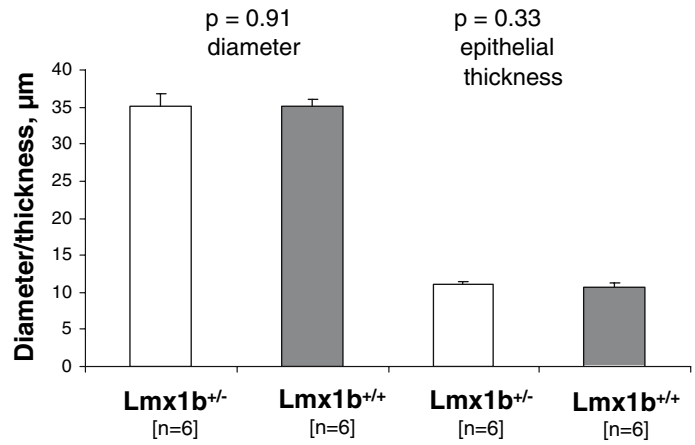

Fig. 3 Effect of uninephrectomy on mean diameter and epithelial thickness of proximal convoluted tubules. Since analysis of the first six animals of each group revealed nearly identical values (see Table 1) we abandoned further measurements. *Error bars* represent 1 SD

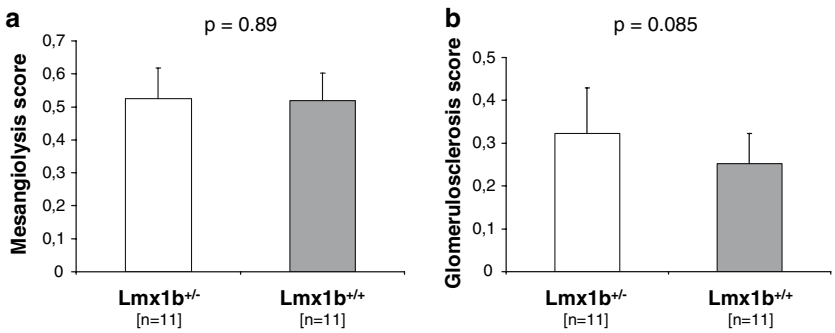

Fig. 4 Effect of uninephrectomy on mean mesangiolysis (**a**) and glomerulosclerosis (**b**) index. *Error bars* represent 1 SD

Discussion

So far, we and others were not able to detect a renal phenotype in heterozygous *Lmx1b*$^{+/-}$ knockout animals in C57BL/6, as well as in JF1, C3H and DBA2 (F1 animals) genetic backgrounds by means of conventional histological/morphological examination (Endele et al. in preparation). In the present study we investigated the kidneys of *Lmx1b*$^{+/-}$ mice after induction of renal damage by Unx. Unx in mice is an experimental model which leads to compensatory enlargement of the remaining kidney associated with an increase in V_G and enlargement of the proximal convoluted tubules. The response of the remaining nephrons to a reduction of nephron number may play an important role in the development of progressive renal damage (Brenner 1985). We were able to detect an effect of Lmx1b haploinsufficiency on CRG after Unx. *Lmx1b*$^{+/-}$ animals showed a mean renal growth of only 65.9% of that in *Lmx1b*$^{+/+}$ animals with a *p*-value of 0.02. This result is supported by the measurement of V_G which was also significantly reduced ($p = 0.0034$) compared to *Lmx1b*$^{+/+}$ animals. It has been demonstrated by numerous reports, that in mice CRG of the remaining kidney after Unx is caused by an increase in V_G and by an enlargement of the proximal convoluted tubules (Flyvbjerg et al. 1999, 2002; Natesan and Reddy 2001; Al Banchaabouchi et al. 2001). While the mechanisms responsible for the glomerular hypertrophy in CRG are largely unknown, at least for proximal-tubule growth a cell-cycle dependent mechanism, which involves TGFβ as well as cdk2/4:cyclinD and leads to cellular hypertrophy (not hyperplasia), has been described (Review: Preisig 1999; Liu and Preisig 2002). In any way, the cellular mechanisms responsible for CRG were found to be influenced by several factors, including endogenous hormones (Flyvbjerg et al. 1999), and growth factors like insulin-like growth factor-1 (IGF-1) (Chang et al. 2002; Flyvbjerg et al. 2002; Cingel-Ristić et al. 2004). Recently, Flyvbjerg et al. (2002) demonstrated that Vascular endothelial growth factor (VEGF) plays a major role in the glomerular compensatory response

after uninephrectomy. Interestingly, using RNase protection assay Rohr et al. (2002) showed that Lmx1b-deficient podocytes produce less VEGF-mRNA indicating that *Lmx1b*$^{-/-}$ mice secret less VEGF than do their wild-type counterparts. Assuming a direct dosis relation, it may be suggested that even *Lmx1b*$^{+/-}$ mice produce less VEGF in the podocytes, which may contribute to the reduction in CRG and V_G compared to *Lmx1b*$^{+/+}$ mice. This aspect, however, was not addressed in the present study. Moreover, we cannot exclude a general growth-inhibitory effect in *Lmx1b*$^{+/-}$ animals.

There was no difference in the diameter or epithelial thickness of the proximal convoluted tubules indicating that the observed difference between *Lmx1b*$^{+/-}$ and *Lmx1b*$^{+/+}$ kidneys is restricted to the glomeruli. Since in adult mice *Lmx1b* is exclusively expressed in the podocytes, the data indicate that the effect is linked to podocyte function and requires a critical minimal Lmx1b concentration.

While the mesangiolysis index was similar in *Lmx1b*$^{+/-}$ and *Lmx1b*$^{+/+}$ animals, it is especially remarkable that we found a slight increase in glomerulosclerosis in *Lmx1b*$^{+/-}$ animals. Although the observed *p*-value is only 0.085, we think that this observation is significant, since glomerulosclerosis usually progresses with increasing renal hypertrophy (Nagata et al. 1992; Fogo 2001). Here, the situation is vice versa, with a decrease in glomerular hypertrophy compared to *Lmx1b*$^{+/+}$ animals and an increase in glomerulosclerosis. Although we cannot decide whether there is a functional relationship between CRG and glumerulosclerosis this result may have two reasons: (1) the additional salt load leads to an increased damage of glomeruli in *Lmx1b*$^{+/-}$ mice independent from the reduced CRG, or (2) the inability to undergo complete CRG by Lmx1b haploinsufficiency exerts an additional pathogenic effect on the glomeruli. Currently there are no further data in order to support any one of these possibilities.

In summary, we were able to demonstrate the first specific phenotype in *Lmx1b*$^{+/-}$ mice. The reduced CRG in combination with a lower V_G and a higher glomerulosclerosis in response to subtle renal injury and work load is the result of Lmx1b haploinsufficiency. Renal hypertrophy represents a compensatory mechanism induced by various types of renal damage (Review: Wesson 1989). Although we did not present a direct proof, based on the present study it may at least be speculated that this mechanism is also affected in the kidneys from NPS patients and may thus lead to a different response to renal damage causing the variable nephropathy. This is supported by the fact that changes in the amount of the currently known Lmx1b-induced proteins [podocin, CD2AP, α3(IV)- and α4(IV) collagen] do not play the primary role in NPS nephropathy (Heidet et al. 2003). Therefore, the present results represent a novel aspect for the understanding of the underlying pathomechanisms of NPS nephropathy and will be the subject of further studies. Using the *Lmx1b*$^{+/-}$ mice as a model, we are planning to investigate the observed changes on the ultrastructural level, as well as by comparative expression studies of potential Lmx1b-induced genes. In addition, it will be interesting to investigate the observed effect in *Lmx1b*$^{+/-}$ mice after transfer of the mutation in different genetic backgrounds. Strain specific differences in the CRG reduction in *Lmx1b*$^{+/-}$ animals would possibly allow the genomic mapping of factors modifying Lmx1b action.

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