The C3H/HeJ inbred mouse is a model of vesico-ureteric reflux with a susceptibility locus on chromosome 12

Inga J. Murawski[1,2], Rita W. Maina[1], Danielle Malo[2], Lisa M. Guay-Woodford[3], Philippe Gros[4], Mary Fujiwara[2], Kenneth Morgan[2] and Indra R. Gupta[1,2]

[1]Department of Pediatrics, McGill University, Research Institute of the McGill University Health Center, Montreal, Quebec, Canada; [2]Department of Human Genetics, McGill University, Research Institute of the McGill University Health Center, Montreal, Quebec, Canada; [3]Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA and [4]Department of Biochemistry, McGill University, Research Institute of the McGill University Health Center, Montreal, Quebec, Canada

Vesico-ureteric reflux is the most common congenital anomaly of the urinary tract, characterized by a defective uretero-vesical junction with retrograde urine flow from the bladder toward the kidneys. Because there is strong evidence for a genetic basis for some cases of vesico-ureteric reflux, we screened 11 inbred mouse strains for reflux and kidney size and identified one strain, C3H/HeJ, that has a 100 percent incidence of vesico-ureteric reflux with otherwise normal kidneys at birth. These mice are predisposed to reflux as a result of a defective uretero-vesical junction characterized by a short intravesical ureter. This defect results from a delay in urinary tract development initially manifested by a ureteric bud arising from a more caudal location along the mesonephric duct. In contrast, C57BL/6J mice (resistant to reflux at birth) have long intravesical ureters, normally positioned ureteric buds, and no delay in urinary tract development. Genome-wide and additional fine mapping of backcross mice, derived from C3H/HeJ and C57BL/6J crosses, identified a significant reflux susceptibility locus, *Vurm1*, on chromosome 12 (peak logarithm of the odds = 7.39). The C3H/HeJ mouse is a model of vesico-ureteric reflux without renal malformation, and further characterization of this model will allow for the identification of a pathway important for urinary tract development, a finding that will serve as a model for the human disorder.

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Correspondence: *Indra R. Gupta, Department of Pediatrics, McGill University, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Quebec, Canada H3H 1P3. E-mail: indra.gupta@muhc.mcgill.ca*

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Vesico-ureteric reflux (VUR) is a congenital urinary tract defect of the uretero-vesical junction in which urine flows retrogradely from the bladder to the ureters and the kidney.[1–3] It affects up to 1% of the population and is associated with recurrent urinary tract infections, renal malformations, hypertension, and end-stage renal disease.[4–8] The uretero-vesical junction is composed of the intravesical ureter and the surrounding bladder musculature, and is compressed during bladder filling to prevent the reflux of urine.[9–11] Specific defects in the uretero-vesical junction have been observed in patients with VUR, including a short intravesical ureter, an abnormal ureteric orifice, and a poorly muscularized ureter and/or bladder.[9,12,13] However, it is unclear whether these defects are primary or secondary manifestations of an abnormal uretero-vesical junction.

Treatment of VUR includes antibiotic prophylaxis to prevent recurrent urinary tract infections that may lead to renal damage and/or surgical treatment to remodel the uretero-vesical junction.[14–16] In spite of these therapies, the incidence of end-stage renal disease secondary to VUR, so-called reflux nephropathy, has not decreased over the past 40 years.[14,15] This strongly suggests that the pathophysiology of VUR and its associated complications need to be re-examined to understand which patients may benefit from treatment.

VUR can have a genetic basis and the discovery of genetic factors associated with this disorder may be the best approach to characterize the phenotypic variability, to improve treatment, and to assess the outcome in patients with VUR. Twin and family studies have shown that VUR is highly heritable.[17–20] Genetic analysis of patients with VUR has shown that the condition is heterogeneous, such that a number of different genes and loci are associated with VUR.[18–23] However, the association of specific genes with VUR has not been consistently shown, and a few of the loci have been replicated in independent study populations. This suggests that a variety of strategies will be required to identify the underlying genes associated with VUR, and

to understand the relationship between these genes and disease progression.

We previously identified two mouse models of VUR with kidney malformations, the *Pax2*$^{1Neu+/-}$ and *Hoxb7/Ret*$^{+/-}$ mice.[24,25] From these models, we have shown that the position of the ureteric bud, the primordial structure that develops into both the kidney and the ureter, is abnormally positioned along the mesonephric duct, and this is associated with malformed kidneys and short intravesical ureters that reflux.[24,25] These models illustrate the relevance of using the mouse to study VUR, as mutations in the associated genes, *PAX2* and *RET*, have been found in humans affected with VUR.[26–28]

Most children with VUR have normal kidneys.[29,30] Therefore, in the present study, our objective was to identify mouse models of VUR without a renal malformation. Eleven inbred mouse strains were screened for VUR and kidney morphology, and from this we identified the C3H/HeJ (C3H) mouse as a model of VUR without a kidney malformation. Urinary tract development and formation of the ureterovesical junction were characterized in C3H mice that are susceptible to VUR and compared with C57BL/6J (B6) mice that are resistant to VUR. Linkage analysis was performed on backcross and intercross mice derived from a C3H and B6 cross, and this helped identify a reflux susceptibility locus, the *Vurm1* locus, so named because it is the first VUR susceptibility locus that was identified in the mouse.

RESULTS

The C3H/HeJ mouse is a model of VUR without a renal malformation

To identify mouse models with VUR, we screened 11 inbred mouse strains for the presence of VUR. We also assessed their kidney size because in humans, VUR can be subdivided on the basis of the presence or absence of a renal malformation.[31] Four strains showed susceptibility to VUR: AKR/J, DBA/2J, CBA/J, and C3H/HeJ, whereas the remaining seven strains were resistant (Figure 1a–c). Kidney size was assessed by measuring whole-mount planar surface area and kidney weight[32] (Figure 1d). Two strains, C3H and B6, showed the most contrasting phenotypes and were analyzed in depth for the remainder of the studies (Figure 1b and c). B6 mice had no VUR and larger kidneys (measured as planar surface area adjusted for body weight ± s.e.: $4.18 \pm 0.08\,mm^2/g$) when compared with C3H mice, which had a 100% incidence of VUR and significantly smaller kidneys ($3.61 \pm 0.06\,mm^2/g$; *t*-test, $P = 2 \times 10^{-7}$). Similar results were obtained when kidney weights were compared (data not shown). C3H mice exhibited both bilateral and unilateral VUR (Table 1), and in the case of unilateral VUR there was no difference in right- versus left-sided VUR. In the VUR-affected mice, the pressure at which reflux occurred in each ureter was noted (30, 60, 90, 120, or 150 cm). If no VUR was observed by the highest pressure, the mice were considered to be unaffected. Among the C3H and B6 litters, equal numbers of males and females were tested and there was no gender difference in reflux among C3H mice (data not shown). C3H and B6 mice were also tested for VUR at 8 weeks of age to determine whether VUR had resolved.[33–35] C3H mice continued to reflux (incidence: 92% (11/12)), whereas B6 mice continued to be resistant, with only one mouse showing a mild unilateral reflux that did not reach the renal pelvis (incidence: 8% (1/12); Table 1).

As C3H mice had slightly smaller kidneys (~15%) at birth compared with B6 mice, we examined kidney morphology at birth and at adulthood (8 weeks). Histological analysis of the cortex and medulla showed that there were no gross abnormalities between the two strains (Supplementary Figure S2A–D). These observations are consistent with previous reports showing that C3H mice have normal kidneys, normal blood pressure, and normal renal function.[36–39] The C3H mouse is therefore a model of VUR without an overt kidney malformation, and thus it reproduces the phenotype seen in most children with VUR who have normal kidneys.[29,31]

C3H/HeJ mice have short intravesical ureters and caudal ureteric buds

The intravesical ureter must be of sufficient length to prevent the backflow of urine from the bladder into the ureters.[9,12,13,40] Intravesical ureter lengths were measured at birth and C3H mice had significantly shorter intravesical ureters than B6 mice, for both the left and the right ureters (Figure 2a–c). The length of the left intravesical ureter (mean ± s.e.) was 0.203 ± 0.012 mm, $n = 6$, in C3H mice, and 0.344 ± 0.043 mm, $n = 8$, in B6 mice (*t*-test, $P = 5.5 \times 10^{-3}$). The length of the right intravesical ureter (mean ± s.e.) was 0.215 ± 0.016 mm, $n = 8$, in C3H mice, and 0.314 ± 0.003 mm, $n = 8$, in B6 mice (*t*-test, $P = 1.7 \times 10^{-3}$).

The position from which the ureteric bud emerges along the mesonephric duct has been shown to be important for normal kidney and urinary tract development, and was assessed at embryonic day (E) 11.[24,25,41] C3H embryos had ureteric buds that developed more caudally, closer to the end of the mesonephric duct, whereas B6 embryos had ureteric buds that developed more cranially, further from the end of the mesonephric duct (Figure 2d–f). The position of the ureteric bud relative to the end of the mesonephric duct on the left was (mean ± s.e.) 0.074 ± 0.008 mm, $n = 7$, in C3H embryos and 0.164 ± 0.016 mm, $n = 11$, in B6 embryos (*t*-test, $P = 1.8 \times 10^{-4}$). The position of the ureteric bud relative to the end of the mesonephric duct on the right was (mean ± s.e.) 0.088 ± 0.012 mm, $n = 6$, in C3H embryos and 0.150 ± 0.016 mm, $n = 11$, in B6 embryos (*t*-test, $P = 8.5 \times 10^{-3}$). These data suggest that short intravesical ureters and caudal ureteric buds are phenotypic traits that are associated with VUR.

C3H/HeJ mice exhibit a delay in urinary tract development

We have previously shown that a caudal ureteric bud is associated with a delay in urinary tract development and VUR; therefore, we speculated that the caudal ureteric bud in

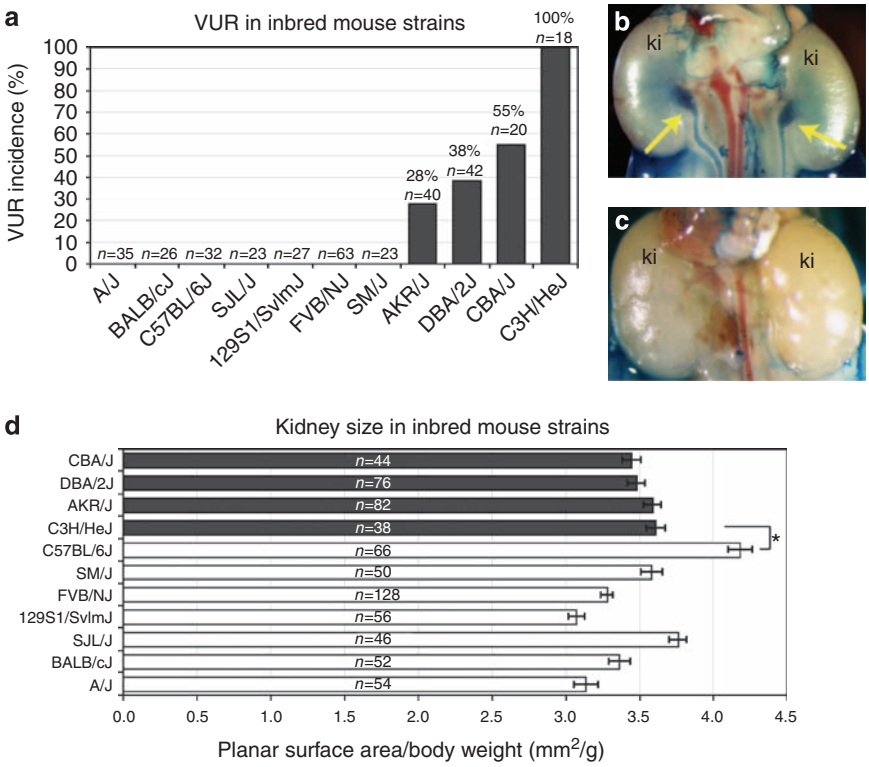

Figure 1 | C3H/HeJ mice have a 100% incidence of vesico-ureteric reflux (VUR) at birth. (**a**) Eleven inbred mouse strains were screened for VUR at birth. The majority of the strains were resistant to VUR. Four strains were susceptible and showed varying incidences of VUR: AKR/J, DBA/2J, CBA/J, and C3H/HeJ (n = number of mice with either unilateral or bilateral VUR). (**b**) VUR is defined by the retrograde passage of dye from the bladder into the ureters and pelvis of the kidneys. The C3H/HeJ mouse shown has bilateral VUR (arrows). (**c**) The C57BL/6J strain is resistant to VUR. (**d**) Kidney planar surface areas adjusted for body weight are shown for the 11 strains at birth (mean ± s.e.). C3H/HeJ and C57BL/6J mice have contrasting kidney sizes (t-test, $^*P = 2 \times 10^{-7}$). The C3H/HeJ strain is the most susceptible to VUR and has smaller kidneys (ki_s) at birth when compared with the C57BL/6J strain. Solid bars indicate strains susceptible to VUR; open bars indicate strains resistant to VUR.

Table 1 | Characterization of VUR in newborn C3H/HeJ, C57BL/6J, F1, N2, and F2 progeny, and adult C3H/HeJ and C57BL/6J mice

	Total mice[a]	VUR[a]	No VUR[a]	VUR 30[b]	VUR 60[b]	VUR 90[b]	VUR 120[b]	VUR 150[b]	Bilateral[a]	Unilateral left[a]	Unilateral right[a]
C3H	18	18	0	1	13	8	3	1	11	2	5
B6	32	0	32	0	0	0	0	0	0	0	0
F1	54	2	52	0	0	1	0	2	1	1	0
F2	116	19	97	0	4	11	4	4	6	8	5
N2	303	140	163	2	27	51	45	41	47	47	46
C3H adult	12	11	1	5	4	5	2	1	6	1	4
B6 adult	12	1	11	0	0	0	0	1	0	1	0

Abbreviation: VUR, vesico-ureteric reflux.
[a]Represents number of mice.
[b]Represents the number of refluxing ureter units at the specified pressure; note that there are missing pressure data for some of the ureters in the C3H, F2, and N2 groups.

C3H embryos might also represent a delay.[24] Kidney and urinary tract development was compared in detail in C3H/HeJ-*Hoxb7/GFP* and C57BL/6J-*Hoxb7/GFP* embryos.[42] Embryos were collected between E10 and E17 and were carefully staged so that only those with similar morphological features and crown-rump lengths were compared. The first morphological difference between the strains became apparent at E11 when the ureteric bud emerged from a more caudal location along the mesonephric duct in C3H/HeJ-*Hoxb7/GFP* embryos compared with C57BL/6J-*Hoxb7/GFP* embryos, confirming our data using *in situ* hybridization to mark the ureteric bud (Figure 2d–f and data not shown). At E13, no gross differences in kidney and urinary tract morphology were noted between the two strains; the ureter had reached the developing bladder and remained attached to the mesonephric duct in both C3H/HeJ-*Hoxb7/GFP* and

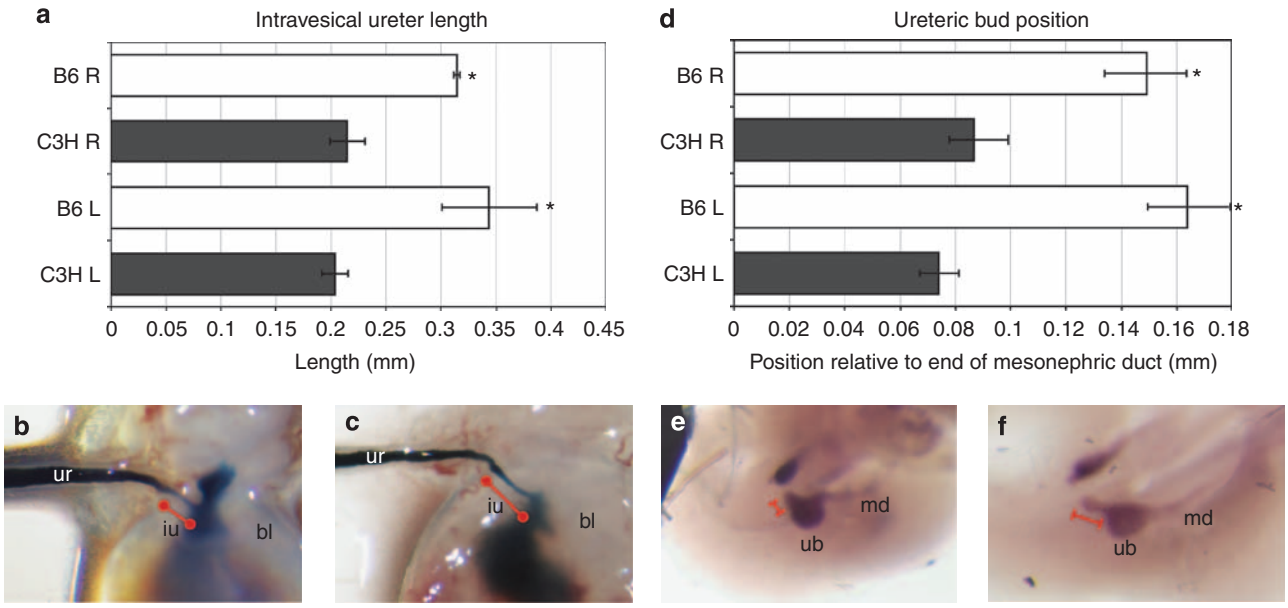

Figure 2 | C3H/HeJ mice have short intravesical ureters and caudal ureteric buds. (**a**) Bar graph representing the length of right (R) and left (L) intravesical ureters in C3H/HeJ (C3H) and C57BL/6J (B6) mice at birth. C3H/HeJ mice have significantly shorter intravesical ureters than C57BL/6J mice on both sides (*t*-test; $*P<0.05$). (**b, c**) Ventral view of newborn bladder and ureter. (**b**) The intravesical ureter (iu) is measured as the length of the ureter (ur) within the bladder (bl) wall (red line). A short right-sided intravesical ureter from a C3H/HeJ mouse is shown. (**c**) A long right-sided intravesical ureter from a C57BL/6J mouse is shown. (**d**) Bar graph representing the position of the ureteric bud relative to the end of the mesonephric duct in E11 embryos. C3H/HeJ embryos have ureteric buds that emerge more caudally than in C57BL/6J embryos on both sides (*t*-test, $*P<0.05$). (**e, f**) Lateral view of E11 embryo showing the mesonephric duct and emerging ureteric bud. (**e**) A ureteric bud (ub) from a C3H/HeJ embryo is seen emerging caudally, closer to the end of the mesonephric duct (md) (red line). (**f**) A ureteric bud from a C57BL/6J embryo is seen emerging more cranially, further from the end of the mesonephric duct.

C57BL/6J-*Hoxb7/GFP* embryos ($n=5$ embryos for each strain, Figure 3a–a′). However, at E14, half (3/6) of the C57BL/6J-*Hoxb7/GFP* embryos showed separation of the ureters from the mesonephric ducts, whereas none (0/5) of the C3H/HeJ-*Hoxb7/GFP* embryos showed this finding ($\chi^2=3.4$, $P=0.06$, Figure 3b–b′). The delay was even more apparent at E15, when all (6/6) of the C57BL/6J-*Hoxb7/GFP* embryos showed separation of the ureters from the mesonephric ducts, whereas only 1/6 of the C3H/HeJ-*Hoxb7/GFP* embryos had complete separation of the ureter from the mesonephric duct ($\chi^2=8.6$, $P=3\times10^{-3}$, Figure 3c–c′ and d–d′). By E17, all C3H/HeJ-*Hoxb7/GFP* ($n=6$) and C57BL/6J-*Hoxb7/GFP* ($n=4$) embryos had complete separation of the ureters from the mesonephric ducts (data not shown). We speculate that the delay in ureter separation disrupts growth of the ureter into the bladder, leading to a short intravesical ureter at birth that refluxes.

The VUR phenotype segregates as a recessive trait

To genetically characterize the susceptibility of C3H mice to VUR, crosses were set up between C3H and B6 mice to generate F1, N2, and F2 mice. Analysis of 54 F1 (C3HxB6), 303 N2 (F1xC3H), and 116 F2 (F1xF1) progeny suggested that the VUR phenotype segregates as a recessive trait (Figure 4 and Table 1). The VUR assay that was developed can be scored as a binary trait, by the presence/absence of VUR in at least one ureter, and by the pressure required for VUR to occur in a ureter, as shown in Table 1.[24,25] We recorded pressure because it has been suggested that if a ureter refluxes at a lower pressure, there is a more severe uretero-vesical junction defect.[43,44] The majority (85%) of VURs observed in C3H mice occurred at low pressures, below 90 cm ($\chi^2=25$, $P=6\times10^{-7}$, Table 1), suggesting a severe uretero-vesical junction defect in C3H mice. VUR in the two F1 mice that refluxed occurred at high pressure, suggesting that these mice had a mild form of VUR (Table 1). In the F2 mice, most cases of VUR (68%) were unilateral ($\chi^2=5.2$, $P=0.02$) and the majority (65%) of ureters showed VUR below 90 cm ($\chi^2=4.2$, $P=0.03$, Table 1). In the N2 mice, most (66%) cases were unilateral ($\chi^2=30$, $P=4\times10^{-8}$) and half (48%) of the ureters showed VUR below 90 cm (Table 1). For all of the mice tested, there was no pattern for left- or right-sided predominance of unilateral VUR. Consistent with the results from the parental C3H strain, there was no gender effect: N2 males and females were similarly affected with VUR (females: 49% vs males: 51%).

Given that C3H mice had smaller kidneys than B6 mice in the newborn period, and that kidney size has been shown to be heritable in the mouse,[45] we measured kidney size in the F1, N2, and F2 progeny. Kidney planar surface areas adjusted for body weight were examined and there were no significant differences (mean ± s.e.) when C3H mice ($3.61\pm0.06\,mm^2/g$, $n=38$) were compared with F1 ($3.41\pm0.06\,mm^2/g$, $n=114$) or F2 mice ($3.47\pm0.05\,mm^2/g$, $n=232$; ANOVA, $P=0.3$).

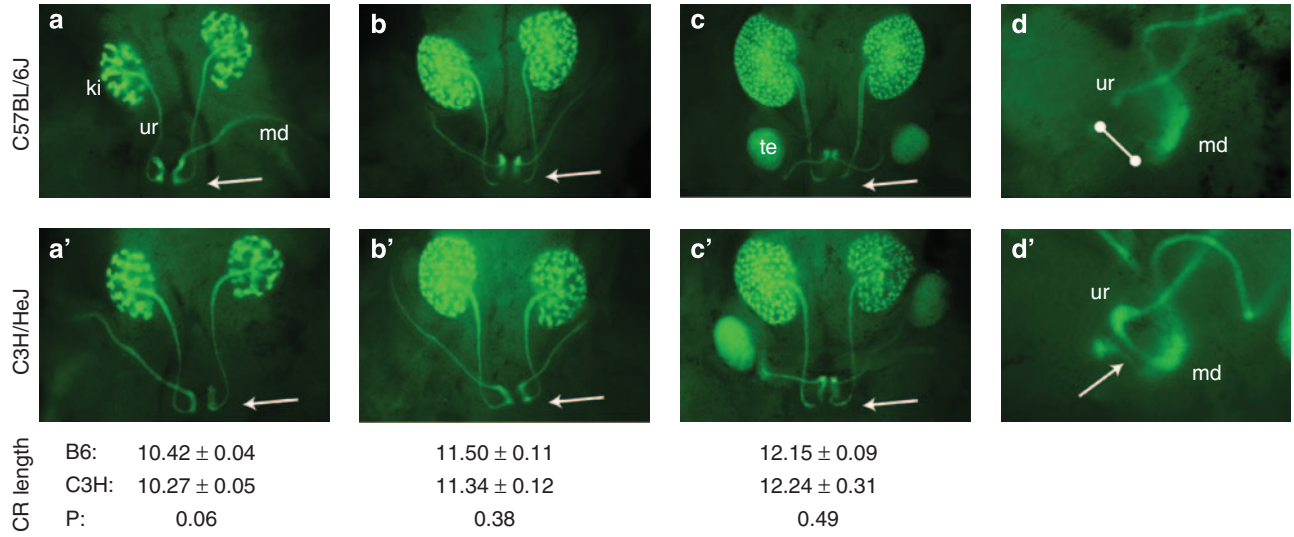

Figure 3 | C3H/HeJ mice exhibit a delay in urinary tract development. The *Hoxb7/GFP* transgene was backcrossed onto a pure C57BL/6J (**a–d**) and C3H/HeJ (**a′–d′**) background, and embryos were dissected at various stages during embryonic development to visualize the developing kidney (ki) and urinary tract. The average crown rump (CR) length (mean ± SE in mm) for each embryonic stage is shown. (**a–a′**) Ventral view of E13 kidneys and urinary tracts. The kidney has undergone several branching events. The mesonephric duct (md) is still attached to the ureter (ur) (arrow) in both C57BL/6J-*Hoxb7/GFP* and C3H/HeJ-*Hoxb7/GFP* embryos. (**b–b′**) Ventral view of E14 kidneys and urinary tracts. (**b**) The kidneys have grown larger, and the ureters in C57BL/6J-*Hoxb7/GFP* embryos have separated from the mesonephric duct and have achieved their own independent insertion into the bladder (arrow). (**b′**) The kidneys have grown in C3H/HeJ-*Hoxb7/GFP* mice as well; however, the ureters remain attached to their respective mesonephric ducts (arrow). (**c–c′**) Ventral view of E15 kidneys and urinary tracts. (**c**) By E15, ureters from a C57BL/6J-*Hoxb7/GFP* embryo have their own independent insertion into the bladder (arrow). A male mouse with testes (te) is shown. (**c′**) In contrast, ureters from a C3H/HeJ-*Hoxb7/GFP* male remain fused to their mesonephric ducts (arrow). (**d**) Lateral view of the left C57BL/6J-*Hoxb7/GFP* ureter shown in **c** that has separated from the mesonephric duct (line). (**d′**) Lateral view of the left C3H/HeJ-*Hoxb7/GFP* ureter shown in **c′** that has not yet separated from its mesonephric duct (arrow).

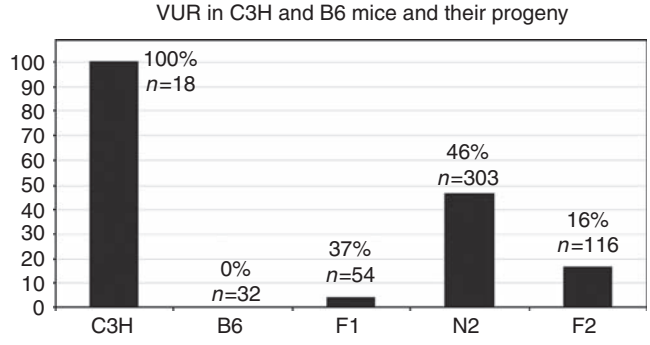

Figure 4 | The vesico-ureteric reflux (VUR) phenotype segregates as a recessive trait. Incidence of VUR in C3H/HeJ, C57BL/6J, F1, N2, and F2 mice at birth. C3H/HeJ and C57BL/6J mice were crossed to create F1 hybrids, N2 backcross progeny ((C3HxB6)F1 × C3H), and F2 intercross progeny ((C3HxB6)F1 × (C3HxB6)F1). The pattern of VUR inheritance is most compatible with an incompletely penetrant recessive trait, as the majority of F1s are unaffected and the number of affected F2 and N2 mice is less than expected for a fully penetrant recessive trait. *N* is the total number of mice tested and % indicates the incidence of VUR.

N2 mice did have significantly smaller kidneys (3.24 ± 0.01 g, $n = 606$) than C3H, B6, F1, and F2 mice ($P < 0.05$ for each comparison) and the same findings were observed when kidney weights were used in the analysis (data not shown). However, refluxing N2 mice did not have significantly smaller kidneys than non-refluxing N2 mice. The kidney planar surface area adjusted for body weight (mean ± s.e.) for N2 mice with VUR was 3.24 ± 0.03 mm^2/g, $n = 140$, and for N2 mice without VUR was 3.24 ± 0.02 mm^2/g, $n = 163$ (*t*-test, $P = 0.9$). These results show that VUR and a smaller kidney size do not segregate together as phenotypes.

A locus on mouse chromosome 12, *Vurm1*, is linked to VUR in the C3H/HeJ mouse

To map VUR-susceptibility genes in C3H mice, a genome-wide scan was performed on a randomly chosen subset of the N2 progeny, consisting of 127 VUR-affected mice and 45 VUR-unaffected mice using 268 single-nucleotide polymorphisms and five microsatellite markers (Supplementary Figure S1). Linkage analysis was performed by scoring VUR as a binary trait. The C3H genotype was over-represented at the proximal end of chromosome 12 in refluxing mice with a peak logarithm of the odds (LOD) score at microsatellite marker D12Mit170 (LOD = 3.97, genome-wide significance $P = 0.004$; Figure 5a). This genomic region is hereafter called the *Vurm1* locus. The 1.5-LOD support interval for *Vurm1* suggests that the locus encompasses 22 cM between markers D12Mit37 and rs6225272.[46] When the pressure at which VUR occurred was included as a covariate in the analysis, the location and value of the peak LOD score were unchanged (genome-wide significance, $P = 0.004$).

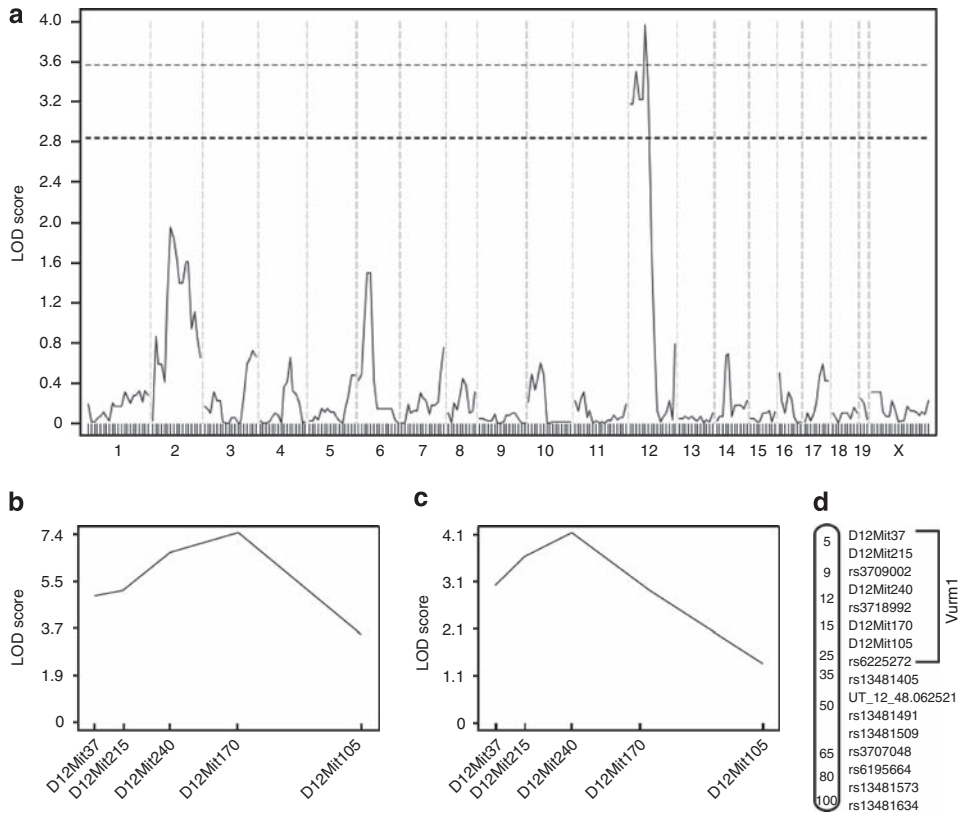

Figure 5 | The *Vurm1* locus on chromosome 12 is associated with vesico-ureteric reflux (VUR) in C3H/HeJ mice. (**a**) Linkage analysis of a genome-wide scan for loci affecting VUR in backcross progeny from a (C3HxB6)F1 × C3H cross. The genome-wide scan was performed on 172 N2 mice (127 VUR-affected and 45 VUR-unaffected). The *x* axis displays the cM location of 268 single-nucleotide polymorphisms (SNPs) and five microsatellite markers on the chromosomes. The *y* axis displays the logarithm of the odds (LOD) score. The two dashed horizontal lines indicate the genome-wide significance thresholds estimated by permutation tests: $a = 0.05$ (lower line), $a = 0.01$ (upper line). The peak LOD score of 3.97 (genome-wide significance $P = 0.004$) was obtained on chromosome 12. This genomic region is named *Vurm1* and encompasses 22 cM between markers D12Mit37 and rs6225272. (**b**, **c**) To confirm the association between chromosome 12 and *Vurm1*, additional genotyping was performed on a larger sample of N2 ($n = 303$) and F2 ($n = 116$) progeny using five microsatellite markers that span the *Vurm1* locus. The *x* axis displays the location of the five microsatellite markers within *Vurm1*. The *y* axis displays the LOD score. (**b**) For the N2 progeny, a peak LOD score of 7.39 was observed at marker D12Mit170 ($P < 0.0001$). (**c**) For the F2 progeny, a peak LOD score of 4.13 was observed at marker D12Mit240 ($P < 0.0001$). (**d**) Name and approximate megabase location of SNPs and microsatellite markers used for mapping on chromosome 12. The location of *Vurm1* is shown.

Markers on two other chromosomes reached suggestive linkage. On chromosome 2, marker rs13476573 reached suggestive linkage (LOD = 1.95, $P = 3.4 \times 10^{-3}$) and the allele-effect plot showed an over-representation of the homozygous C3H genotype suggesting the presence of another susceptibility locus. On chromosome 6, marker rs13478728 approached suggestive linkage (LOD = 1.5, $P = 9.3 \times 10^{-3}$), and the allele-effect plot showed an over-representation of the heterozygous genotype suggesting the presence of a locus that confers resistance to VUR. These minor peaks may harbor other VUR-causing genes or modifier genes for *Vurm1*.

To confirm the linkage between chromosome 12 and *Vurm1*, additional genotyping was performed on a larger sample of N2 ($n = 303$) and F2 ($n = 116$) progeny using five selected microsatellite markers spanning the *Vurm1* locus. For the N2 progeny, a peak LOD score of 7.39 was observed at marker D12Mit170 ($P < 0.0001$, Figure 5b). For the F2 progeny, a peak LOD score of 4.13 was observed at marker D12Mit240 ($P < 0.0001$, Figure 5c).

BXH mice with VUR have short intravesical ureters

To further define the location of the *Vurm1* locus, 10 recombinant inbred BXH lines were screened for VUR and intravesical ureter length. Kidney size was also assessed. Among the ten lines, the incidence of VUR ranged from 0 to 88% (Figure 6a), and there was an inverse correlation between incidence of VUR and length of the intravesical ureter ($R^2 = 0.92$, $P = 1.4 \times 10^{-4}$). Strain incidences for VUR were correlated with mean intravesical ureter lengths as VUR and intravesical ureter measurement could not be obtained in the same mouse due to technical limitations. Intravesical ureter lengths are reported as pooled data (Figure 6b), as there was no significant difference when left and right intravesical ureters were examined separately. When kidney size was assessed, there was no relationship between the incidence of VUR and kidney size ($R^2 = 0.17$, $P = 0.6$), nor between the length of the intravesical ureter and kidney size ($R^2 = 0.02$, $P = 0.9$), similar to the findings in N2 mice. These results suggest that VUR and short intravesical ureters, but not kidney

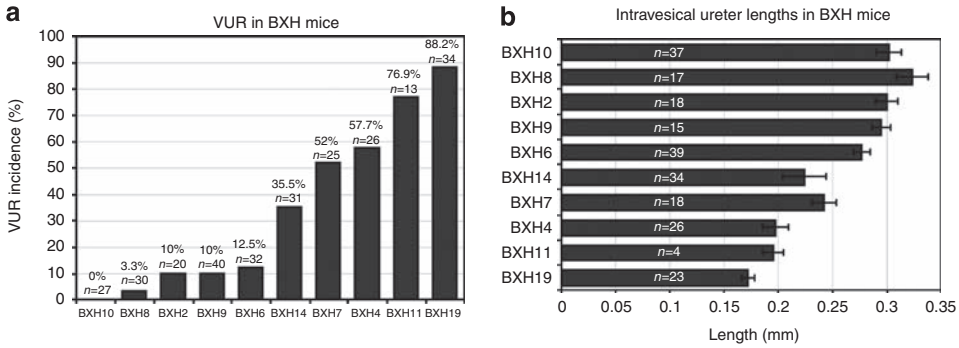

Figure 6 | BXH mice that reflux have short intravesical ureters. (**a**) Ten BXH recombinant inbred lines were tested for vesico-ureteric reflux (VUR) at birth and the incidence of VUR is shown for each line. The BXH lines are shown in ascending order of incidence of VUR. There is a large range in the incidence of VUR from 0 to 88%, suggesting the presence of modifier loci that interact with *Vurm1*. (**b**) Shown in the same order as in **a** are average intravesical ureter lengths for each of the BXH lines. As the incidence of VUR increases among the different BXH lines, the length of the intravesical ureter decreases.

size, segregate together as phenotypes, and that the length of the intravesical ureter is a strong determinant of VUR.

The phenotype data collected for the 10 BXH lines were compared with the genotype data within *Vurm1* for each of the lines. The results from this analysis show a region within *Vurm1* (between rs13481303 and rs6225272) in which BXH lines susceptible to VUR share the C3H genotype (Figure 7). The majority of the BXH lines support the location of *Vurm1* in this region. However, three lines are discordant. The BXH4 line has B6 genotypes within *Vurm1*, but a 57.7% incidence of VUR and short intravesical ureters. The BXH9 and BXH6 lines have C3H genotypes within *Vurm1*, but low incidences of VUR and long intravesical ureters. These results suggest either that a new mutation arose in these lines, or that the susceptibility may be due to yet-to-be identified genes elsewhere in the genome. Taken together, analysis of the BXH lines indicates that there must be modifier loci to explain the variable susceptibility to VUR that ranges from 0 to 88%. These modifier genes may be on chromosomes 2 and/or 6, as inferred on the basis of the initial genome-wide scan.

Comparison of C3H/HeN and C3H/HeJ substrains and *in silico* mapping

To determine whether the VUR phenotype is specific to the C3H/HeJ substrain, or whether the phenotype is the result of an ancestral mutation, we phenotyped C3H/HeN mice for VUR, intravesical ureter length, and ureteric bud position. Consistent with the HeJ substrain, the HeN substrain had a 93% incidence of VUR (28/30), short intravesical ureters (C3H/HeN L: 0.252 ± 0.010 mm, $n = 7$; C3H/HeN R: 0.244 ± 0.008 mm, $n = 7$), and caudal ureteric buds (C3H/HeN L: 0.089 ± 0.007 mm, $n = 7$; C3H/HeN R: 0.077 ± 0.005 mm, $n = 6$), when compared with B6 mice (t-test, $P < 0.005$ for intravesical ureter and ureteric bud position). These results suggest that the mutation associated with VUR is ancestral to the HeJ and HeN substrains.[47] We therefore used an *in silico* approach to prioritize the 198 candidate genes for *Vurm1* (NCBI Build 37.1). By identifying regions where the four VUR-susceptible inbred strains and the susceptible BXH lines shared genotypes that were different from the B6 genotypes, a number of promising regions were identified (Figure 7). From our phenotypic analysis of C3H/HeJ and C3H/HeN mice, we inferred that the candidate gene within *Vurm1* will likely be expressed and function during urinary tract and ureteric bud development. Three interesting candidate genes as per these criteria include retinol dehydrogenase 14 (*Rdh14*), odd skipped related 1 (*Osr1*), and Tribbles homolog 2 (*Trib2*).

DISCUSSION

In this study, we identified four inbred mouse strains with a susceptibility to VUR. Of these, the C3H mouse has a 100% incidence of VUR and normal kidneys. Therefore, its phenotype reproduces that observed in the majority of children with VUR who do not have a renal malformation.[29,30] We showed that the C3H mouse has caudally shifted ureteric buds, short intravesical ureters, and a delay in urinary tract development, three phenotypes that have previously been associated with a refluxing urinary tract.[24,25] By crossing the C3H refluxing strain to the B6 non-refluxing strain and analyzing the VUR phenotype in N2 and F2 progeny, the pattern of VUR inheritance was found to be most compatible with a complex recessive trait caused by the interaction of several genes, with a major gene showing incomplete penetrance. Linkage analysis identified a 22-cM susceptibility locus, *Vurm1*, on chromosome 12. This region is orthologous to human chromosome 2p24–25, which was recently identified in a whole-genome linkage scan of patients with primary non-syndromic reflux from the United Kingdom and Slovenia.[23]

Our results in the C3H mouse and in the BXH lines show the importance of the length of the intravesical ureter. The average length of the intravesical ureter appears to be shorter in patients with VUR, although these observations have been made on a limited number of patients of varying ages.[9,12] A comparative analysis of intravesical ureters from different animal species also suggests that differences in a species' propensity to reflux are likely due to differences in the length of the intravesical ureter; however, no causal

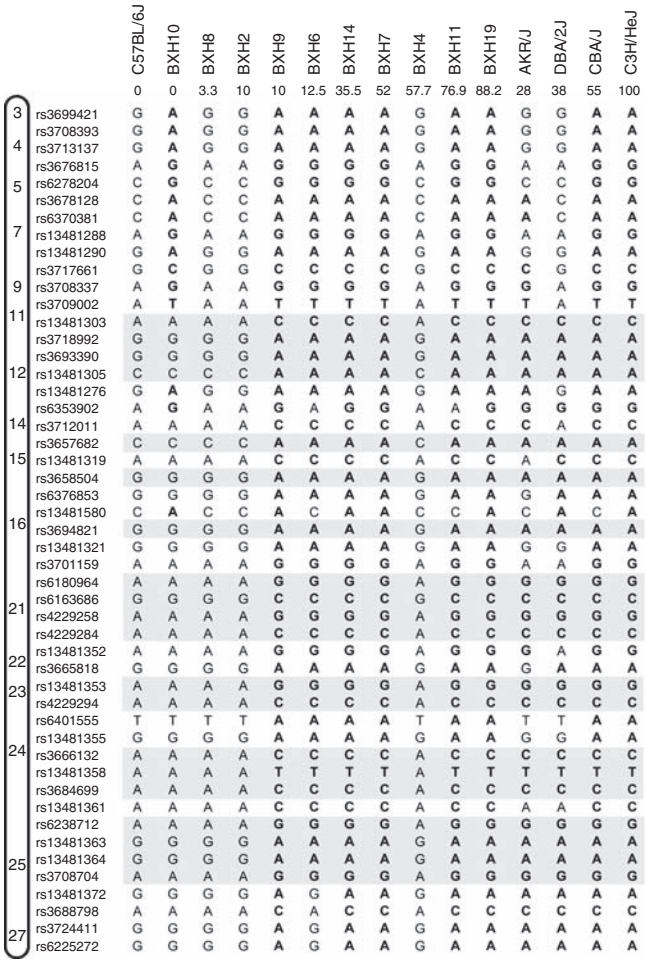

Figure 7 | *In silico* mapping of the *Vurm1* locus. Single-nucleotide polymorphism (SNP) genotype data were obtained for the four inbred mouse strains that were susceptible to vesico-ureteric reflux (VUR; AKR/J, CBA/J, DBA/2J, and C3H/HeJ), the non-refluxing C57BL/6J mouse, and the 10 BXH lines. Selected SNPs within *Vurm1* are shown with their approximate megabase (Mb) positions. Incidence of VUR is indicated at the top of the figure. Analysis of the BXH lines supports the location of *Vurm1* in this region on chromosome 12. Shaded regions indicate priority regions for candidate gene analysis, as the four susceptible inbred strains and the susceptible BXH lines share genotypes that differ from C57BL/6J genotypes. The three SNPs present in the genome-wide scan are shown in bold.

relationships were shown.[48] Our results are consistent with these observations, but stronger in that we have a much larger sample size of animals that were carefully standardized for age and for the measurement itself. The length of the intravesical ureter is therefore an important determinant of the competence of the uretero-vesical junction: a shorter intravesical ureter is associated with a higher incidence of VUR, while a longer intravesical ureter is protective against VUR.

The ureteric bud emerges from a caudal position in C3H embryos, as seen in other mice with VUR, such as the *Pax2*$^{1Neu+/-}$ and *Hoxb7/Ret*$^{+/-}$ mice.[24,25] However, the two latter models are associated with severe kidney malformations, whereas the C3H mouse has a normal kidney phenotype. Although all of these models have abnormally positioned ureteric buds, the gene defects in *Pax2*$^{1Neu+/-}$ and *Hoxb7/Ret*$^{+/-}$ mice also affect the later stages of kidney development, which ultimately leads to a malformed kidney. We speculate that the protein encoded by the *Vurm1* locus exerts an effect during formation of the ureteric bud, and either it does not have a role during the later stages of kidney development, or, alternatively, there are other genes that can replace its function such that no kidney defect arises.

The caudally shifted ureteric bud in C3H embryos is associated with a delay in urinary tract development, a short intravesical ureter, and VUR. However, the mechanism by which a shifted ureteric bud leads to a refluxing urinary tract is unknown. The caudally shifted ureteric bud could interact with a population of mesenchymal cells that are unable to support normal growth and maturation of the ureter. Alternatively, the caudally shifted ureteric bud, which is in closer proximity to the future bladder, could be exposed to a new domain or concentration of signaling factors released from the bladder that perturb the timing of ureter separation. Furthermore, the delay in ureter maturation might disrupt bladder growth such that less bladder surrounds the uretero-vesical junction, and this might prevent the ureter from migrating to its final position within the bladder wall, leading to a shorter intravesical ureter. By identifying the genetic factors that lead to VUR, we will gain a better understanding of their function during these critical steps during the formation of the urinary tract and the uretero-vesical junction.

Within the *Vurm1* locus, there are 198 candidate genes. After analyzing genotypes that are common between the refluxing BXH and inbred mice, we identified three potential gene candidates: *Rdh14*, *Osr1*, and *Trib2*. *Osr1* is the earliest known marker of the intermediate mesoderm, which gives rise to the mesonephric duct and ureteric bud.[49] Knockout *Osr1*$^{-/-}$ mice fail to develop the mesenchyme that surrounds the ureteric bud and have a thin mesonephric duct.[50] *Rdh14* encodes for an enzyme involved in the oxidation of retinol into retinaldehyde, which is a precursor involved in the synthesis of retinoic acid. Although its role during development has not been characterized, the vitamin A pathway and its metabolite, retinoic acid, are critical for kidney and urinary tract development and direct many aspects of morphogenesis.[51,52] Mutations in retinoic acid receptors, *Rarα* and *Rarβ2*, or in *Raldh2*, an enzyme involved in the oxidation of retinaldehyde into retinoic acid, disrupt both kidney and urinary tract development: the kidneys are hypoplastic and the ureters fail to separate from their respective mesonephric ducts.[51,52] *Trib2* is a serine/threonine kinase expressed in the metanephric mesenchyme, and although knockout mice have normal kidneys, their urinary tracts have not been assessed.[53] Owing to their demonstrated or potential roles during formation of the ureteric bud and the urinary tract, all three genes are excellent candidates for *Vurm1*. Preliminary results have not identified any coding mutations in these three genes, nor any expression differences in mRNA obtained from E14 kidneys with the attached ureter.

From clinical studies in humans with VUR, there is clearly a need to re-examine the development of the urinary tract and its relationship with VUR for a better understanding of the pathogenesis of VUR. Genome-wide mapping studies in humans have identified several loci associated with VUR, but the underlying genes are yet to be identified.[18,20–23,54] We have expanded these genetic studies to include inbred mice, and have identified a locus on mouse chromosome 12. By identifying the gene responsible for VUR in the C3H mouse, we will gain new knowledge about the biology of urinary tract defects in humans.

MATERIALS AND METHODS

Animal breeding: inbred mouse strains

The following 11 inbred mouse strains were purchased from Jackson Laboratories between 6–8 weeks of age: A/J, BALB/cJ, C57BL/6J, SJL/J, 129S1/SvImJ, FVB/NJ, SM/J, AKR/J, DBA/2J, CBA/J, and C3H/HeJ. C3H/HeN mice were purchased from Charles River Laboratories, Wilmington, MA, USA. We selected strains from the major genealogical groups that represented priority strains set by the Mouse Phenome Database. F1 hybrids were generated by crossing C3H/HeJ and C57BL/6J inbred strains. Backcross (N2) [(C3HxB6)F1 × C3H] and intercross (F2) [(C3HxB6)F1 × (C3HxB6)F1] progeny were generated following standard breeding schemes. For all animal experiments, multiple litters from each strain or cross were examined. Animal protocols were approved by the McGill University Health Center Animal Care Committee and Ethics Committee and are in accordance with the rules and regulations of the Canadian Council of Animal Care.

Vesico-ureteric reflux and intravesical ureter lengths

Eleven inbred strains were tested for the presence of VUR at postnatal day (P) 1 as previously described.[25] C3H and B6 mice were also tested for VUR at 8 weeks in the same manner. VUR was identified by the retrograde passage of methylene blue into the ureters or renal pelvis. During injection, the syringe was raised vertically from 30 to 150 cm by increments of 30 cm every 10 s. The hydrostatic pressure, represented by the height of the column of methylene blue in relation to the level of the mouse, was recorded for each ureter when VUR was observed. In the case of bilateral VUR, the average pressure of the two ureter units was used for statistical analysis. If VUR was not observed by 150 cm of pressure, the mouse was considered to be unaffected. Body and kidney weights were obtained for all mice. Kidney whole-mount planar surface areas were measured using SPOT (v.3.5.9), as previously described.[24,32] Histological analysis was performed as previously described.[25] The length of the intravesical ureter was measured as previously described.[24] The dye was photographed when moving through the ureters into the bladder and the distance between the bladder periphery and the site of dye exit was defined as the intravesical ureter length and measured using SPOT (v.3.5.9). All measurements were taken by one observer who was blinded to the strain of the mouse.

In situ hybridization

Embryos were generated by performing timed 2-h early-morning matings. The visualization of a vaginal plug was recorded as E0 and embryos were collected at E11 for *in situ* hybridization. Embryos were staged using Theiler's criteria and crown-rump lengths were measured such that embryos with similar morphological features and of similar size were used for analysis. Whole-mount *in situ* hybridization was performed as previously described.[24,55] A digoxygenin-labeled UTP (Roche, Basel, Switzerland) *c-Ret* probe was used to visualize the mesonephric duct and the ureteric bud. The position of the ureteric bud was defined as the distance between the caudal edge of the mesonephric duct and the start of the ureteric bud.[24,25] All measurements were taken by one observer who was blinded to the strain of the embryonic tissue.

Animal breeding: C3H/HeJ-*Hoxb7/GFP* and C57BL/6J-*Hoxb7/GFP* mice

To examine kidney and urinary tract development in C3H/HeJ and C57BL/6J mice, the *Hoxb7/GFP* transgene, which expresses green fluorescent protein throughout the mesonephric duct and its derivatives, was backcrossed for 10 generations onto a pure C57BL/6J and C3H/HeJ background.[42] Embryos from timed matings were collected between E10 and E17. Embryos were staged in the same manner as for the *in situ* experiments. Embryos were dissected using fluorescent microscopy and the developing mesonephric ducts, ureteric buds, ureters, and kidneys were imaged using SPOT (v.3.5.9).

Genotyping and linkage analysis

An initial genome-wide screen was performed on 172 N2 animals using the Illumina mouse low-density linkage panel that includes 268 informative single-nucleotide polymorphisms between the C3H and B6 mouse strains and five microsatellite markers (D12Mit37, D12Mit215, D12Mit240, D12Mit170, and D12Mit105). Linkage analysis was performed with J/Qtl v1.3.0 using marker regression and 10,000 permutations (http://research.jax.org/faculty/churchill/software/Jqtl).[56] Linkage analysis was performed using VUR as a binary trait (present or absent). To confirm the results identified in the genome-wide scan, all of the 303 N2 and 116 F2 progeny were genotyped for five microsatellite markers. PCR conditions were 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 sec, and 72 °C for 1 min, followed by a final incubation at 72 °C for 7 min. PCR products were electrophoresed on 4% agarose gels (Wisent, Quebec, Canada).

BXH recombinant inbred mouse lines and *in silico* mapping

Ten recombinant inbred BXH (B6xC3H) lines were purchased from Jackson Laboratories: BXH2/TyJ, BXH4/TyJ, BXH6/TyJ, BXH7/TyJ, BXH8/TyJ, BXH9/TyJ, BXH10/TyJ, BXH11/TyJ, BXH14/TyJ, and BXH19/TyJ and tested for VUR. Kidney sizes and the length of the intravesical ureters were measured as described above from multiple litters. Single-nucleotide polymorphism genotype data for the 10 BXH lines and the inbred strains were obtained from the Wellcome-CTC Mouse Strain SNP Genotype Set (http://www.well.ox.ac.uk/mouse).

DISCLOSURE

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SUPPLEMENTARY MATERIAL

Figure S1. Supplementary Figure 1: Genetic map of informative SNPs between C3H/HeJ and C57BL/6J.

Figure S2. Supplementary Figure 2: Histological analysis of C3H/HeJ and C57BL/6J kidneys.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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