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# The ECM protein nephronectin promotes kidney development via integrin α8β1-mediated stimulation of *Gdnf* expression

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Development of the metanephric kidney crucially depends on proper interactions between cells and the surrounding extracellular matrix. For example, we showed previously that in the absence of α8β1 integrin, invasion by the ureteric bud into the metanephric mesenchyme is inhibited, resulting in renal agenesis. Here we present genetic evidence that the extracellular matrix protein nephronectin is an essential ligand that engages α8β1 integrin during early kidney development. We show that embryos lacking a functional nephronectin gene frequently display kidney agenesis or hypoplasia, which can be traced to a delay in the invasion of the metanephric mesenchyme by the ureteric bud at an early stage of kidney development. Significantly, we detected no defects in extracellular matrix organization in the nascent kidneys of the nephronectin mutants. Instead, we found that *Gdnf* expression was dramatically reduced in both nephronectin- and α8 integrin-null mutants specifically in the metanephric mesenchyme at the time of ureteric bud invasion. We show that this reduction is sufficient to explain the agenesis and hypoplasia observed in both mutants. Interestingly, the reduction in *Gdnf* expression is transient, and its resumption presumably enables the nephronectin-deficient ureteric buds to invade the metanephric mesenchyme and begin branching. Our results thus place nephronectin and α8β1 integrin in a pathway that regulates *Gdnf* expression and is essential for kidney development.

**KEY WORDS: Kidney, Integrin, Extracellular matrix, GDNF, Mouse, Ureteric bud**

## INTRODUCTION

The metanephric kidney arises through instructive signaling between two cell populations, a mesenchymal population known as the nephric cord and an epithelial population termed the nephric duct (ND). Both of these populations arise from the intermediate mesoderm on approximately embryonic day (E) 9.0 in the mouse. Within the nephric cord, in a region adjacent to the posterior hindlimb bud, there develops a special group of cells known as the metanephric mesenchyme (MM). At ~E11, a signal(s) from the MM elicits the formation of the ureteric bud (UB) from the ND. Once formed, the UB extends toward and then invades the MM, and by ~E11.5 the UB begins branching, thus giving rise to a tubular network that will eventually mature into the collecting ducts of the adult kidney. As the UB invades and branches, it expresses genes that induce the MM to condense and differentiate into various cell types that comprise the nephron. In the absence of invasion by the UB into the MM, the metanephric kidney does not develop (reviewed in Saxen, 1987).

Glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β (TGFβ) superfamily, is a key signal in the initiation of UB formation and subsequent branching (Costantini and Shakya, 2006). GDNF is expressed in the MM and signals to the UB through a receptor complex consisting of the receptor tyrosine kinase RET and a coreceptor, GDNF family receptor alpha1 (GFRα1). GDNF has been shown to induce ectopic UB formation in culture, and mice lacking either the *Gdnf*, *Ret* or *Gfra1* genes display renal agenesis at high penetrance (Cacalano et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Sanchez et al., 1996; Schuchardt et al., 1994). *Gdnf* expression in the MM is regulated by a highly conserved network of transcription factors, and can be activated or inhibited by extracellular signaling molecules such as WNT11, GDF11 and SLIT2 at discrete stages of development (Brodbeck and Englert, 2004; Esquela and Lee, 2003; Grieshammer et al., 2004; Majumdar et al., 2003). It is also known that GDNF signaling is dependent on heparan sulphate glycosaminoglycans, which interact with or are constituents of the extracellular matrix (ECM) (Barnett et al., 2002).

Integrins are cell adhesion receptors that serve as a link between the ECM and the cytoskeleton. Integrins are heterodimers, consisting of two single-pass transmembrane proteins designated as the α and β subunits. Integrins are thought to occupy either an inactive or active state depending on cues received from the extracelluar environment in the form of an appropriate ECM ligand or growth factor signal. They communicate information from the ECM to the cell interior through recruitment of cytoskeletal proteins and kinases. In addition, they are able to communicate information from the cell interior to the ECM, resulting in its proper deposition and remodeling (Delon and Brown, 2007; ffrench-Constant and Colognato, 2004; Giancotti and Ruoslahti, 1999; Hynes, 2002).

Previously, we showed that the integrin subunit α8 (*Itga8*) is expressed throughout the nephric cord, including the MM (Muller et al., 1997). Furthermore, we demonstrated that loss of *Itga8* function invariably results in a delay of invasion of the MM by the UB, which in turn results in a high frequency of kidney agenesis. The molecular mechanism by which *Itga8* function in the MM influences the UB has yet to be determined. Several ECM constituents are known ligands for α8β1 integrin, including fibronectin, osteopontin, tenascin C and vitronectin (Denda et al., 1998a; Denda et al., 1998b; Varnum-Finney et al., 1995). However, data from expression and loss-of-function analyses indicated that

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these ligands are unlikely to be mediators of α8β1 integrin function in the developing kidney. Using an expression cloning strategy we identified a gene that encodes a novel ECM molecule, nephronectin (*Npnt*), that is expressed by the UB and the epithelia of several developing organs (Brandenberger et al., 2001). We demonstrated that α8β1 integrin recognizes nephronectin in binding assays and associates with nephronectin in vivo. In addition, we found that the localization of nephronectin in the kidney is consistent with it mediating α8β1 integrin function during development.

Here we report that mice lacking nephronectin frequently display kidney agenesis. We show that the phenotype arises during the early stages of metanephric development, when the UB is beginning to invade the MM, similar to the phenotype of mice lacking *Itga8* (and therefore α8β1 integrin function). Thus, nephronectin is an ECM protein expressed by the UB that is required for α8β1 integrin function during early stages of UB invasion and branching. Significantly, we demonstrate that *Gdnf* expression is reduced in both *Npnt* and *Itga8* mutants at the time when the invasion of the MM by the UB is delayed. Finally, we present genetic data indicating that *Gdnf* dosage as well as signaling from the receptor tyrosine kinase, RET, impact the penetrance of the *Itga8* mutant phenotype. Taken together, our results suggest that the observed reduction of *Gdnf* expression in the MM is sufficient to explain the phenotypes observed in mice lacking either nephronectin or the α8 integrin subunit.

## MATERIALS AND METHODS

### Targeting of the *Npnt* locus

A probe derived from the 5′ region of the *Npnt* locus was used to screen a mouse 129Sv/J BAC library, RPCI-22 (Roswell Park Cancer Institute). One of 9 clones isolated was used to generate the floxneo targeting construct (Fig. 1B-D) by recombineering (Zhang et al., 1998). Clones harboring BACs that were correctly modified were identified by Southern blot hybridization, which revealed the presence of both the 5′ and 3′ arms. One clone, designated 273P10 NN-1, was electroporated into E14 embryonic stem (ES) cells.

Because of the large size of the targeting construct (300 kb), we used TaqMan (Applied Biosystems) real-time quantitative PCR to identify ES cells in which there was a reduction in copy number of a region that was targeted for insertion (Valenzuela et al., 2003). This involved the use of specific PCRs that amplify the wild-type allele, but fail to amplify the mutated allele because of the increase in sequence length. Candidate ES cell clones that were identified by sequential PCR screens were assessed for reduced wild-type copy number by quantitative Southern hybridization using densitometry. Two clones isolated from the screen were injected into C57BL/6 blastocysts (Transgenic/Targeted Mutagenesis Core, University of California, San Francisco, CA), and both were incorporated into the germ line.

We generated a null allele for nephronectin by crossing animals heterozygous for the $Npnt^{floxneo}$ allele with animals expressing CRE recombinase under the β-actin promoter (Lewandoski et al., 1997). This cross produced mice carrying the $Npnt^{\Delta ex1}$ allele in which the first exon of *Npnt* was excised without deletion of the neomycin-resistance gene expression cassette (Fig. 1D,E). All the analysis described here was performed on mice carrying this allele on a mixed genetic background (129Sv/J; C57BL/6; FVB/N).

### In situ hybridization

To stage embryos, noon of the day on which a vaginal plug was detected was considered E0.5. Embryos were collected at various stages and the region containing the hindlimb buds was fixed in 4.0% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) overnight at 4°C and cryosectioned at 14 μm. Analysis of gene expression using in situ hybridization with RNA probes was performed according to standard protocols. Data using the following probes are presented: *Gdnf* (Srinivas et al., 1999), *Eya1* (Xu et al., 1999), *Six2* (Xu et al., 2003), *Pax2* (Dressler et al., 1990).

### Histology and immunofluorescence

Embryos at various stages and kidneys from newborn animals were fixed in 4.0% PFA/PBS overnight at 4°C. Tissues were cryosectioned and stained with hematoxylin and eosin according to standard protocols. Sections were stained with the following antibodies: anti-nephronectin (1:100) (Brandenberger et al., 2001), anti-EHS laminin (1:500) (Sigma L9393), anti-fibronectin (1:300) (Sigma F-6140), anti-calbindin D28K (1:600) (Swant CB-38a), anti-collagen type IV (1:500) (LB-1403; Cosmo Bio., Tokyo, Japan), and anti-pax2 (1:100) (PRB-276P; Covance, Princeton, NJ). Confocal imaging was performed on a Zeiss LSM 5 Pascal.

## RESULTS

### Mice lacking nephronectin display renal agenesis at birth

We generated a line of mice carrying, $Npnt^{\Delta ex1}$, which lacks the first exon of nephronectin (see Materials and methods; Fig. 1). Animals homozygous for $Npnt^{\Delta ex1}$ express neither nephronectin protein nor *Npnt* RNA (see Fig. 1G,H), demonstrating that $Npnt^{\Delta ex1}$ is a null allele. $Npnt^{\Delta ex1}$ homozygotes, hereafter referred to as *Npnt* mutants, were born at the expected Mendelian frequency (52 out of 219 mice; 24%). At birth they appeared to be of normal size without any obvious external defects. However, upon dissection, we found that *Npnt* mutant animals ($n$=52) frequently lacked one (23%) or both (46%) kidneys (Fig. 2A-C). The remaining *Npnt* mutants had two kidneys (31%), on average resulting in 58% agenesis (see legend to Fig. 2F). By contrast, 2 of 109 $Npnt^{\Delta ex1}$ heterozygotes displayed bilateral kidney agenesis and none displayed unilateral agenesis (2% agenesis). No agenesis was observed in their wild-type littermates. In most cases the *Npnt* mutant kidneys were smaller than those of their wild-type littermates (Fig. 2A,B). Histological analysis revealed variability in development, with most *Npnt* mutant kidneys containing essentially normal nephrogenic regions (Fig. 2D,E), and a few displaying cystic and dysplastic elements (not shown). Significantly, *Npnt* mutants lacking kidneys did not show evidence of partial ureter development or survival of MM, suggesting that development is perturbed at an early stage, when the UB has yet to invade the MM.

### Renal agenesis in nephronectin-null embryos results from a developmental delay

To determine the developmental origin of the kidney agenesis observed at birth, we examined *Npnt* mutants and their wild-type littermates at early stages of metanephric development. At E11.0, the UB had formed and appeared similar in *Npnt* mutants ($n$=2) and their wild-type littermates (Fig. 3A,B). However, by E11.5, we found a significant difference: whereas in all wild-type embryos the UB had invaded the MM and branched, none of the mutant embryos examined, in wholemount or serial section ($n$=12), showed any evidence of UB invasion of the MM or branching, on either side of the embryo (Fig. 3C,D). By E12.5, the UB had invaded the MM and undergone some branching in all *Npnt* mutants examined ($n$=4). However, the extent of branching was variable, ranging from a single branching event to two rounds of branching, and therefore fewer than the three rounds in their wild-type littermates (Fig. 3E-G).

To determine whether the UB branching observed was sufficient to promote nephrogenesis, we analyzed kidney development at E13.5. In two of four *Npnt* mutants examined, we found that kidneys were developing on both sides in one embryo and on one side in the other. In all cases, the mutant kidneys were markedly smaller than normal. In the other two of four *Npnt* mutants, no kidney development was detected on either side and the MM was beginning to atrophy (five out of eight kidneys expected did not form), whereas

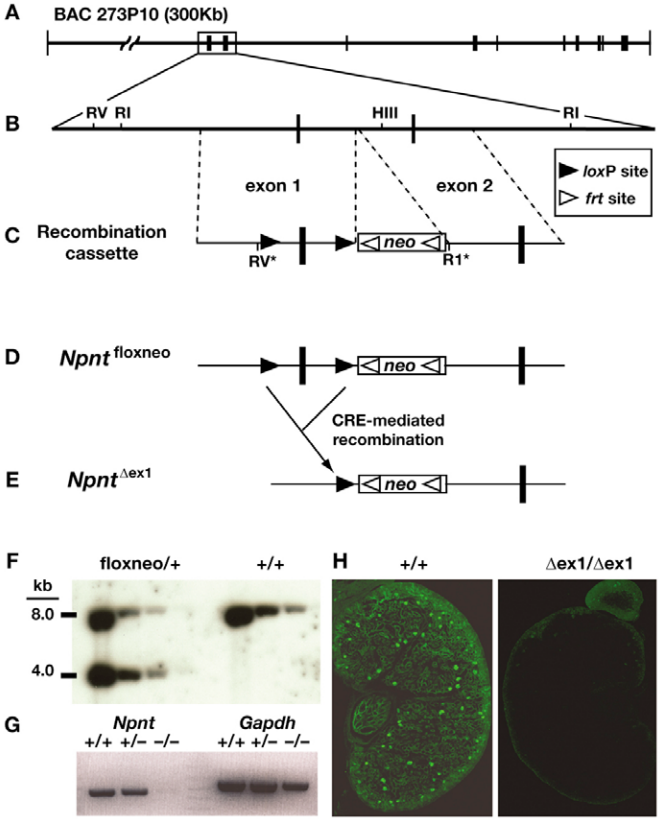


**Fig. 1. Generation of a *Npnt*-null allele.** (**A-F**) Targeting strategy for generating *Npnt* mutant alleles using a BAC containing part of the *Npnt* locus. (A) Representation of the modified BAC (273P10) used for targeting, showing the first 11 exons (vertical bars) of the *Npnt* gene present in this BAC. Boxed region spans exons 1 and 2. (B,C) Illustration of the modifications that were made to the BAC DNA, including insertion of loxP sites in the introns 5′ and 3′ to the first exon, an insertion of a neomycin expression cassette flanked by *frt* sites, and restriction sites (asterisk). (D) Representation of the $Npnt^{floxneo}$ allele, produced following homologous recombination between the modified BAC and the *Npnt* locus in ES cells. (E) Mice carrying $Npnt^{floxneo}$ were crossed to mice expressing CRE recombinase under the β-actin promoter (Lewandoski et al., 1997) to create the $Npnt^{\Delta ex1}$ allele. Note that this allele still contains the neo cassette. (F) Southern blot of DNA from two ES cell clones, one heterozygous for the $Npnt^{floxneo}$ allele and the other wild-type at the *Npnt* locus. An *Eco*RI digest produces an 8 kb wild-type and a 4 kb mutant band. Each clone is represented by a series of three fourfold dilutions (left to right). (**G**) RT-PCR for *Npnt* and *Gapdh* expression in $Npnt^{+/+}$, $Npnt^{+/-}$ and $Npnt^{-/-}$ mice. Total RNA was extracted from spleens of newborn mice using the RNeasy mini kit (Qiagen Inc., Valencia, CA), and reverse transcribed using Superscript II and oligo$(dT)_{12\text{-}18}$ Primer (Invitrogen Corp., Carlsbad, CA). PCR was performed using forward and reverse primers that recognize sequences in *Npnt* exons 4 and 8, respectively, and primers that recognize a sequence in *Gapdh* exon 3. Control reactions without reverse transcriptase were negative for both PCR reactions (not shown). (**H**) Immunostain for nephronectin in kidneys from wild-type and $Npnt^{\Delta ex1}$ homozygous (null) newborn mice using an anti-nephronectin antibody that recognizes sequences in the C-terminal region of the protein (Brandenberger et al., 2001). Primers for wild-type allele, $Npnt^{WT}$, NN-1A: 5′-AGTCCATCCTGATCACTGGCT-3′ and NN-1C: 5′-GCAACCTTCAGCGTCCC-3′, band size 279 bp. Primers for mutant allele, $Npnt^{\Delta ex1}$, NN-1B: 5′-TATGGCTTCTGAGGCGGAA-AGAAC-3′ and NN-1F: 5′-AAGTGGAGCTTCAGGACACAG-3′, band size 509 bp.

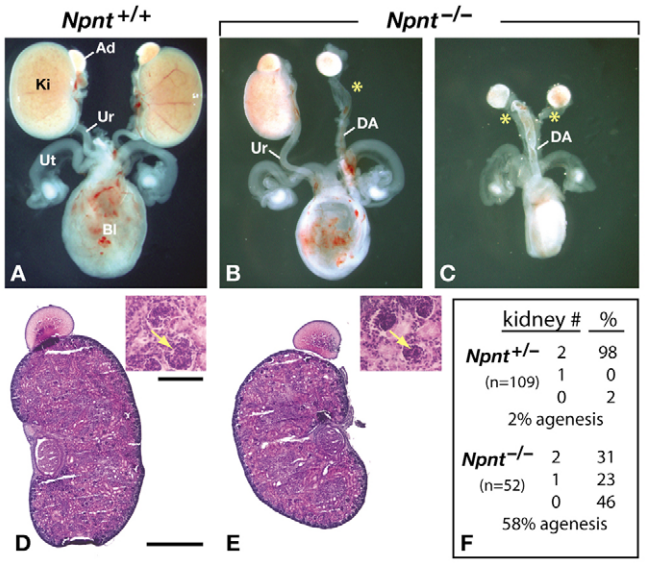


**Fig. 2. Renal agenesis in *Npnt*-null mice.** (**A-C**) Urogenital tracts from newborn female littermates, shown in wholemount. (A) $Npnt^{+/+}$ urogenital tract including kidneys with adrenals, ureters, bladder and uterine horns. (B) $Npnt^{-/-}$ urogenital tract with unilateral kidney agenesis. Note that other than the absence of the right kidney and ureter (asterisk), the urogenital tract appears normal. The adrenal gland on the right is attached to the dorsal mesentery, and part of the dorsal aorta is present. (C) $Npnt^{-/-}$ urogenital tract with bilateral kidney agenesis (asterisks). Again, the rest of the urogenital tract appears normal. (**D**,**E**) Medial sections of $Npnt^{+/+}$ and $Npnt^{-/-}$ newborn kidneys (scale bar, 1 mm). Insets show regions containing glomeruli (arrows) at higher magnification (scale bar, 100 μm), demonstrating that kidney development, including nephron formation, occurs in $Npnt^{-/-}$ kidneys. (**F**) Percentage of *Npnt* heterozygous and homozygous animals with two, one or no kidneys. The percentage agenesis was determined by dividing the number of kidneys [expected (2 per animal) – observed] by the number of kidneys expected. Ad, adrenal gland; Bl, bladder; DA, dorsal arota; Ki, kidney; Ur, ureter; Ut, uterus.

kidneys were developing on both sides in four of four wild-type littermates (no agenesis) (Fig. 3H-K′, and data not shown). It seems likely that the three kidneys that were observed at E13.5 developed in embryos like those in which the UB had undergone several rounds of branching by E12.5 (see Fig. 3F). By contrast, the failure of kidney development at E13.5 may have occurred in embryos like those in which only a single branching event had occurred by E12.5 (see Fig. 3G). From these data it appears that in *Npnt* mutants, UB formation is initially normal, but then the UB fails to invade the MM at E11.5. Subsequently, invasion/branching does occur, but in many cases it appears that the extent of branching is too little to sustain kidney development, resulting in the kidney agenesis observed at birth. Significantly, the lack of invasion of the MM by the UB at E11.5 in the *Npnt* mutants appeared similar to that observed in embryos lacking α8β1 integrin function (Muller et al., 1997).

## The basement membrane is intact in nephronectin-null embryos during kidney development

The ability of a structure such as the UB, a group of polarized epithelial cells, to invade the adjacent mesenchyme depends on proper remodeling of the basement membrane and other matrix constituents that surround the epithelial structures. We therefore were interested in determining whether the basement membranes

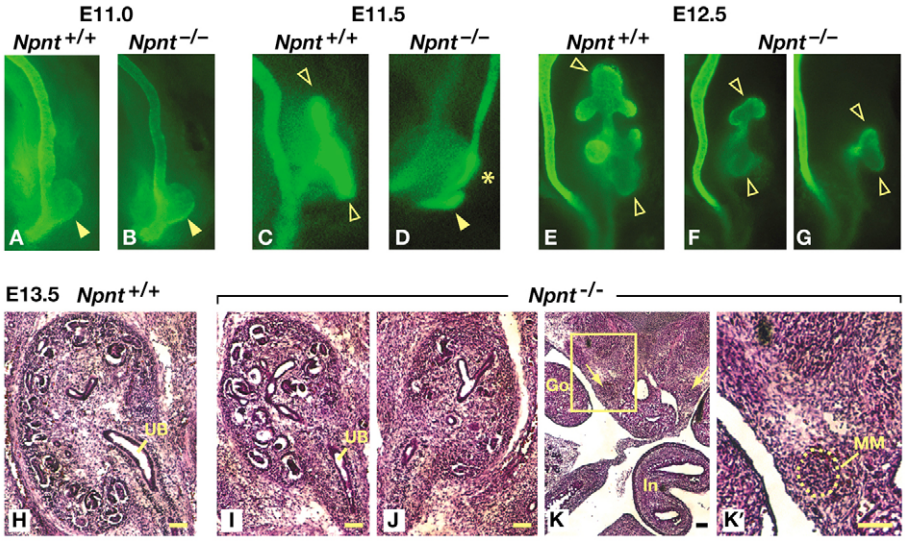


**Fig. 3. Developmental origin of renal agenesis in *Npnt*-null mice.** (**A-G**) Embryos at the stages indicated, immunostained in wholemount for Calbindin. (A) In the *Npnt*$^{+/+}$ embryo at E11.0 the ureteric bud (arrowhead) has invaded the MM. (B) In the *Npnt*$^{-/-}$ embryo the UB (arrowhead) is similar to that in the wild-type embryo. (C) In the *Npnt*$^{+/+}$ embryo at E11.5 the UB has branched (open arrowheads). (D) In the *Npnt*$^{-/-}$ embryo the UB (arrowhead) has not extended into the MM (asterisk) or branched. (E) In the *Npnt*$^{+/+}$ embryo at E12.5 the UB has undergone several rounds of branching (arrowheads). (F,G) *Npnt*$^{-/-}$ embryos, showing the variable extent of branching at E12.5. (**H-J**) Transverse sections through E13.5 *Npnt*$^{+/+}$ and *Npnt*$^{-/-}$ kidneys. (I,J) Left and right kidneys from one embryo. Note that metanephric fields have been invaded by the UB and nephron development is occurring, but the kidneys are smaller than normal in the *Npnt*$^{-/-}$ embryo. Nephrogenesis is occurring, but is less advanced than in the wild-type littermate. (**K**) Transverse section of an E13.5 *Npnt*$^{-/-}$ embryo through the region in which the kidney would normally develop. Note the bilateral kidney agenesis (arrows). (**K′**) Boxed region in K is shown at higher magnification. Broken line demarcates the MM. Scale bars, 100 μm. Go, gonad; In, intestine; MM, metanephric mesenchyme; UB, ureteric bud.

surrounding the ND and the UB were abnormal in *Npnt* mutants. To assess this, we examined two of the core components of the basement membrane, laminin (LN) and collagen IV (COL IV). At E11.5, when the mutant phenotype becomes obvious, staining for either LN or COL IV revealed that the basement membranes surrounding the ND and the UB appeared similar in *Npnt* mutants and their wild-type littermates ($n$=3) (Fig. 4A-F). In a *Npnt* mutant in which the UB had invaded and branched at E13.5, the basement membrane likewise appeared similar to that in the wild-type controls, as assessed with antibodies to LN and COL IV (Fig. 4G-J). To assess the ability of the basement membrane to associate with other ECM proteins, we examined the distribution of fibronectin, an ECM protein that associates with, but is not a core component of, the basement membrane (Hynes, 1986). At E13.5 we found that the localization of this protein was similar in both a *Npnt* mutant and a wild-type littermate (Fig. 4K,L). From these results, we conclude that the basement membrane is comparatively normal in *Npnt* mutants.

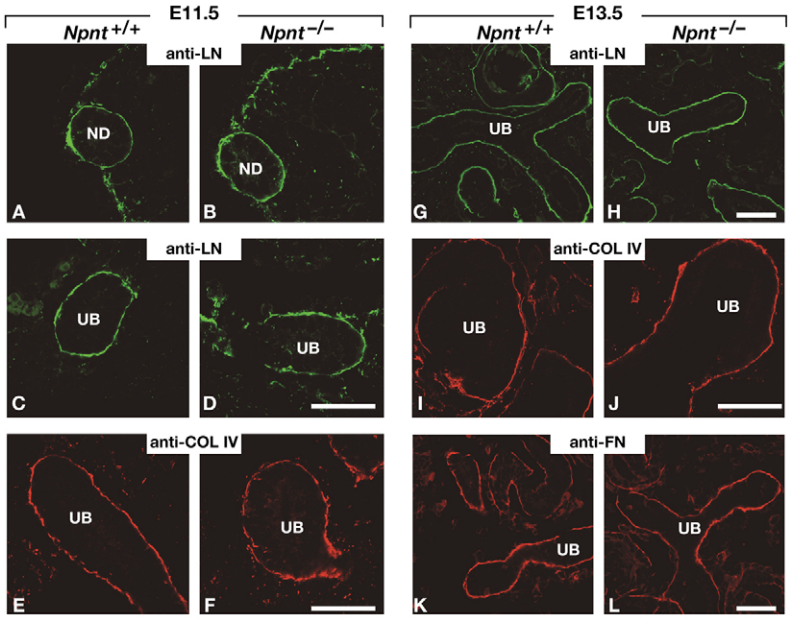


**Fig. 4. The basement membrane is normal in *Npnt*-null embryos during kidney development.** (**A-F**) Transverse sections through E11.5 *Npnt*$^{+/+}$ and *Npnt*$^{-/-}$ embryos stained with antibodies against laminin (LN) or Collagen IV (COL IV). (**G-L**) Transverse sections through E13.5 *Npnt*$^{+/+}$ and *Npnt*$^{-/-}$ embryos stained with antibodies against LN, COL IV or fibronectin (FN). Note the similar staining patterns in mutant and wild-type embryos. Scale bars, 50 μm. ND, nephric duct; UB, ureteric bud.

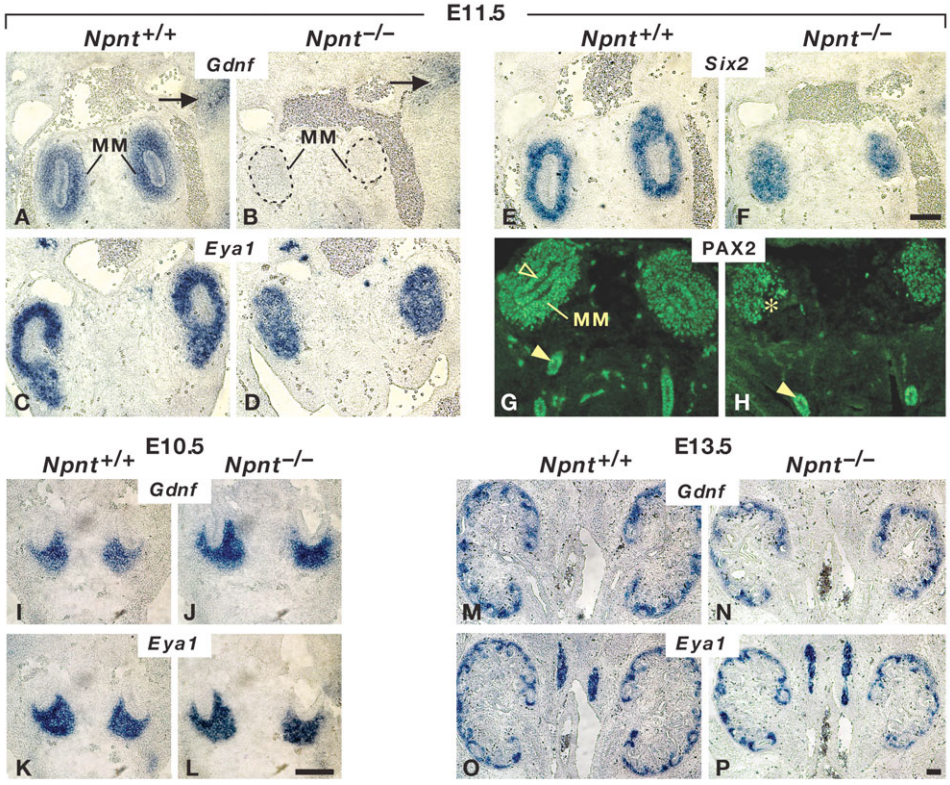


**Fig. 5. *Gdnf* expression is reduced in the *Npnt*-null embryonic kidney at E11.5 but is normal at E10.5 and E13.5.** (**A-P**) Transverse sections through *Npnt*$^{+/+}$ and *Npnt*$^{-/-}$ embryos. (A-H) Expression at E11.5 of the genes indicated, as detected (A-F) by in situ hybridization or (G,H) by immunostaining. (A,B) Note the apparent absence of *Gdnf* RNA in the MM of the mutant (demarcated by dotted circles), whereas the level of *Gdnf* expression appears comparable in the adjacent limb bud (arrows) in mutant and wild-type embryos. (C-F) Note that expression of the *Eya1* and *Six2* transcription factor genes is similar in mutant and wild-type MM. (G,H) PAX2 protein is detected in both the UB (solid arrowhead) and its branches (open arrowhead), as well as in the MM. Note the lack of invasion of the UB into the MM of the *Npnt* mutant (asterisk). (I-L) Expression at E10.5 and (M-P) at E13.5 of the genes indicated, as detected by in situ hybridization. Note that *Gdnf* and *Eya1* expression appears comparable in *Npnt*$^{+/+}$ and *Npnt*$^{-/-}$ embryos at these stages, although the mutant embryonic kidneys are smaller than normal at E13.5. Scale bars, 100 μm. Abbreviations as in previous figures.

## Expression of *Gdnf* is reduced in nephronectin-null embryos

The above results suggested that, rather than ensuring a normal basement membrane, nephronectin recognition by α8β1 integrin may facilitate key signaling events within the MM that promote kidney development. To investigate this possibility, we analyzed the expression, by in situ hybridization and antibody staining, of various genes known to have roles in kidney development. Because a lack of invasion of the MM by the UB was invariably observed in both *Npnt*- and *Itga8*-null mutants at E11.5, we performed our analysis at this stage. We examined the expression of key genes expressed in the ND and UB, including *Pax2*, *Ret*, *Gfra1* and *Emx2* and found that they all were expressed at comparable levels in both *Npnt* mutants and wild-type controls at E11.5 (Fig. 5A-H, and data not shown). The expression of genes that mark the MM was also assessed, including *Gdnf*, *Eya1*, *Six2* and *Pax2*. Interestingly, we did not detect *Gdnf* RNA in the *Npnt* mutant MM. Importantly, however, the level of *Gdnf* expression in the adjacent limb bud was comparable in the mutant and control embryos (Fig. 5A,B). Similar results were obtained in seven separate experiments with seven separate mutant embryos. The expression domains and levels of *Eya1* and *Six2* RNA and PAX2 protein, which are expressed in the MM and are known to have roles in controlling *Gdnf* expression (Brodbeck et al., 2004; Brophy et al., 2001; Xu et al., 1999), were similar in *Npnt* mutants and their wild-type littermates (Fig. 5C-H). Taken together, these results demonstrate that, at E11.5, the MM is present in *Npnt* mutants and displays normal expression of several genes known to be upstream of *Gdnf*, and provide evidence that nephronectin has a role in promoting *Gdnf* expression in the MM at this stage.

To determine the stage-specificity of this effect, we examined expression of *Gdnf* and *Eya1* at E10.5, before the phenotype becomes obvious, and at E13.5, when kidney development is progressing in some *Npnt* mutant embryos. Remarkably, we found that *Gdnf* RNA levels appeared normal in the two mutant embryos examined at E10.5 (Fig. 5I-L) and in a mutant embryo in which kidney development was observed at E13.5 (Fig. 5M-P). We could not determine whether *Gdnf* was also expressed in those mutants in which UB invasion and branching was not detected at E13.5 because the MM had begun to degenerate. These data suggest that nephronectin is transiently required for normal *Gdnf* expression in the MM at E11.5, the time when the phenotype in *Npnt* mutants is first obvious and is invariably observed.

## *Gdnf* expression is reduced in *Itga8*-null embryos at E11.5

In view of the apparent similarity between the kidney phenotypes of *Npnt*- and *Itga8*-null mice, we were interested to examine *Gdnf* expression in *Itga8*-null homozygotes, hereafter referred to as *Itga8* mutants. We compared the expression at E11.5 of *Gdnf* and several

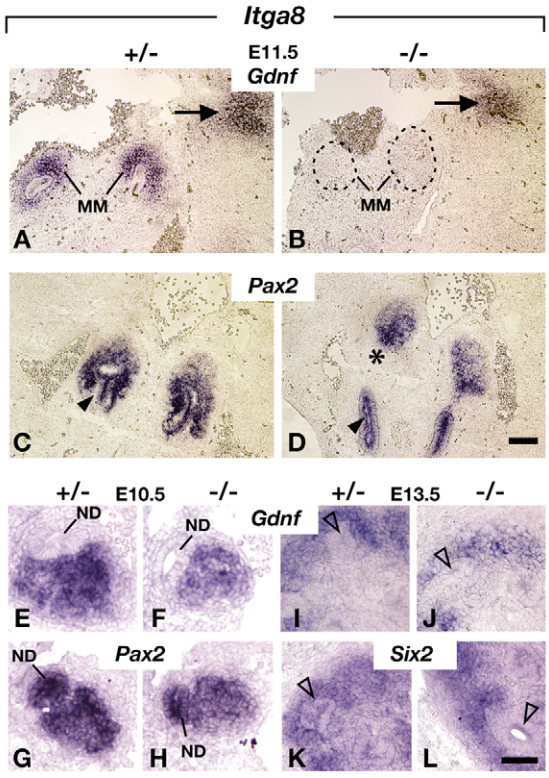


**Fig. 6. *Gdnf* expression is reduced in the *Itga8*-null embryonic kidney at E11.5 but is normal at E10.5 and E13.5.** (**A-L**) Transverse sections through *Itga8*$^{+/-}$ and *Itga8*$^{-/-}$ embryos, showing gene expression as detected by in situ hybridization. (A,B) Note the apparent absence at E11.5 of *Gdnf* RNA in the MM of the *Itga8*$^{-/-}$ mutant (demarcated by dotted circles), whereas the level of *Gdnf* expression appears comparable in the adjacent limb buds (arrows) in *Itga8*$^{+/-}$ and *Itga8*$^{-/-}$ embryos. (C,D) *Pax2* is expressed in both the MM and the UB (arrowhead). Note the lack of UB invasion in the *Itga8*$^{-/-}$ MM (asterisk). (E-L) Expression at E10.5 (E-H) and at E13.5 (I-L) of the genes indicated. Note that expression of *Gdnf*, *Pax2* and *Six2* is similar in *Itga8*$^{+/-}$ and *Itga8*$^{-/-}$ MM at these stages. Open arrowheads point to UB branches in the MM at E13.5. Scale bars: 100 μm. Abbreviations as in previous figures.

other genes expressed in the MM, including the signaling molecule genes *Bmp4* and *Gdf11* and the transcription factor genes *Eya1*, *Pax2*, *Six2*, *Wt1* and *Sall1*, in *Itga8* mutants and their control littermates. Consistent with what we observed in *Npnt* mutants at E11.5, we detected no change in expression of any of these genes in *Itga8* mutants at E11.5 except *Gdnf* (Fig. 6A-D and data not shown). Again, *Gdnf* RNA was not detected in the MM, but *Gdnf* expression appeared comparable in the adjacent limb buds of *Itga8* mutant and control embryos (Fig. 6A,B). Similar results were obtained in five separate experiments with five separate mutant embryos. However, using a radiolabeled *Gdnf* probe we were able to detect a signal at low level (data not shown). In addition, in situ hybridization assays revealed no obvious difference between the *Itga8* mutants and controls at E10.5 and E13.5 (Fig. 6E-L), indicating that, as in *Npnt* mutants, α8 integrin is transiently required for normal *Gdnf* expression in the MM at E11.5.

## Genetic interaction between *Itga8* and *Gdnf*

In order to test the hypothesis that the kidney agenesis we observed in *Npnt* and *Itga8* mutants is because of a reduction in *Gdnf* expression, we took a genetic approach. One prediction of this hypothesis is that reducing the dosage of the *Gdnf* gene should increase the penetrance of the mutant kidney phenotype. For these studies we performed crosses to produce *Itga8*-null heterozygotes and homozygotes carrying one copy of a *Gdnf*-null allele, *Gdnf*$^{lacZ}$ (Moore et al., 1996). To assess kidney development we scored kidney agenesis as described in the legend to Fig. 1F. We found that animals heterozygous for either *Itga8*- or *Gdnf*-null alleles displayed 0% and 9% agenesis, respectively. These data are consistent with previously published frequencies of kidney agenesis in these mutant heterozygotes (Moore et al., 1996; Muller et al., 1997). However, in animals heterozygous for both the *Itga8*- and *Gdnf*-null alleles (*Itga8*$^{+/-}$;*Gdnf*$^{lacZ/+}$), we observed 53% agenesis (Table 1). Furthermore, the frequency of kidney agenesis in animals homozygous for the *Itga8*-null allele was increased from 59% to 96% by heterozygosity for the *Gdnf*-null allele (Table 1). The one kidney that was found in the 11 *Itga8*$^{-/-}$;*Gdnf*$^{lacZ/+}$ animals examined was reduced in size and histological analysis revealed it to be highly dysplastic and most likely non-functional (data not shown). These results support the hypothesis that the reduction in *Gdnf* expression that results from a loss of α8β1 integrin function is responsible for the kidney agenesis in *Npnt*- and *Itga8*-null mutants.

**Table 1. Effect of reducing *Gdnf* gene dosage on kidney agenesis in *Itga8*-null mice**

| *n* | Genotype | Number of kidneys observed (percent agenesis) |
|---|---|---|
| 14 | *Itga8*$^{+/-}$ | 28 (0%) |
| 17 | *Gdnf*$^{+/-}$ | 31 (9%) |
| 17 | *Itga8*$^{+/-}$;*Gdnf*$^{+/-}$ | 16 (53%)** |
| 11 | *Itga8*$^{+/-}$ | 22 (0%) |
| 11 | *Itga8*$^{-/-}$ | 9 (59%) |
| 11 | *Itga8*$^{-/-}$;*Gdnf*$^{+/-}$ | 1 (95%)** |

Kidneys were dissected from newborn progeny derived from the following crosses: top, *Itga8*$^{+/-}$ × *Gdnf*$^{+/-}$; bottom, *Itga8*$^{+/-}$ × *Itga8*$^{+/-}$;*Gdnf*$^{+/-}$. Note that the *Gdnf*-null allele used in these crosses was *Gdnf*$^{lacZ}$ (Moore et al., 1996). Here *n* is the number of mice examined. The percentage of agenesis was determined as described in the legend to Fig. 2F. Significance was determined using Student's *t*-test, with *n* as the number of kidneys and the mean as the percentage of agenesis. **$P$<0.005.

## Reduction in *Spry1* gene dosage in *Itga8*-null embryos rescues kidney development

To further test that hypothesis, we sought to rescue kidney development in *Itga8*-null mice by enhancing the GDNF signaling pathway in vivo. To do this, we made use of a null allele of the Sprouty1 (*Spry1*) gene, which has been shown to be involved in antagonizing the function in the UB of RET, the receptor for GDNF (Basson et al., 2005; Basson et al., 2006). *Spry1*-null mutants display ectopic ureteric budding from the ND, resulting in multiple ureters and multiplex kidneys as a consequence of excess GDNF signaling. We reasoned that if the kidney agenesis phenotype in the *Itga8* mutants was due primarily to reduced *Gdnf* expression, it should be possible to overcome this deficiency by reducing *Spry1* gene dosage and thereby increasing sensitivity of the UB to the small amount of GDNF produced in the *Itga8* MM at E11.5. Indeed, we observed that heterozygosity for a *Spry1*-null allele in *Itga8*-null animals resulted in a significant rescue of kidney development: 25% agenesis in *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ animals ($n$=20) versus 71% agenesis in their *Itga8*$^{-/-}$;*Spry1*$^{+/+}$ littermates ($n$=14). In general, *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ kidneys appeared to be of normal size. Furthermore, no kidney agenesis was observed in animals homozygous for both the *Itga8*- and *Spry1*-null alleles ($n$=8) (Fig. 7A). These data provide strong support for the hypothesis that the kidney agenesis that results from the absence of the α8 integrin subunit is because of a reduction in *Gdnf* expression.

Histological analysis revealed that the rescue of the *Itga8*-null phenotype by reduction of *Spry1* gene dosage takes place early in kidney development. Like *Npnt* mutants, all *Itga8*-null embryos displayed a lack of invasion of the MM by the UB at E11.5; however, in all *Itga8*-null embryos that were heterozygous for a *Spry1*-null allele ($n$=5) the UB on one or both sides was found to have invaded the MM at this stage (9/10 UBs invaded) (Fig. 7B,C). This finding provided us with an opportunity to address an important question: is the reduction in *Gdnf* expression that we observed at E11.5 in *Itga8* mutants secondary to the lack of invasion of the MM by the UB at E11.5? The latter is a viable possibility because the UB is known to promote *Gdnf* expression in the MM at later stages by producing signaling molecules such as WNT11 (Majumdar et al., 2003).

If the observed reduction in *Gdnf* expression was because of the lack of signals from the UB, one would predict that in those *Itga8* mutants in which UB invasion was rescued by reducing *Spry1* gene dosage, *Gdnf* expression would be restored to the level found in wild-type embryos. However, when we assessed *Gdnf* expression in the MM of *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ embryos at E11.5, we found that the level of *Gdnf* RNA was substantially reduced compared with that in their control (*Itga8*$^{+/-}$;*Spry1*$^{+/-}$) littermates (Fig. 7D,E). By contrast, *Eya1* expression was similar in the rescued *Itga8* mutants and their control littermates (Fig. 7F,G). These data demonstrate that *Gdnf* expression in the MM is still reduced by the absence of the α8 integrin subunit in the rescued embryos. However, the signal was stronger than that found in *Itga8*-null embryonic kidneys (Fig. 7B), most likely because the presence of the UB in the rescued mutants produces signals that enhance *Gdnf* expression (Majumdar et al., 2003).

## DISCUSSION

The importance of the ECM in the developing metanephric kidney has been appreciated since the kidney first began to be used as a model for organogenesis (Grobstein, 1955). However, loss-of-function studies have revealed that few ECM proteins or their receptors have specific roles in the early events of metanephric development (Lelongt and Ronco, 2003). One exception is α8β1 integrin, which we have previously shown to be crucial for initial UB invasion into the MM and its subsequent branching, as well as for epithelialization of the MM (Muller et al., 1997). Our finding that *Npnt*-null embryos phenocopy the early phenotype of *Itga8*-null embryos (Muller et al., 1997) strongly suggests that nephronectin is the ligand mediating α8β1 integrin function during the early events of kidney development. In addition, we have found that the expression of *Gdnf*, a key factor in kidney development, is severely reduced in *Npnt* as well as in *Itga8* mutant MM at E11.5, the time at which we invariably observed a lack of invasion of the MM by the UB in both mutants. These data suggested that α8β1 integrin might be involved in a pathway upstream of *Gdnf* expression. Our genetic analysis provides strong support for this hypothesis. It therefore appears that we have uncovered a novel pathway in which an integrin, through recognition of its ECM ligand, is directly involved in the regulation of an essential signaling molecule in the developing kidney.

### Nephronectin is a crucial factor in kidney development

Assessment of kidney development at birth in *Npnt* mutants revealed a high frequency of agenesis, with 46% of the mutants displaying bilateral agenesis and 23% unilateral agenesis. Only 31% of mutant animals had two kidneys, most of which were smaller than normal but otherwise appeared unaffected. Surprisingly, although nephronectin is expressed in several other organ systems during development, preliminary analysis has shown that all organs except the kidney

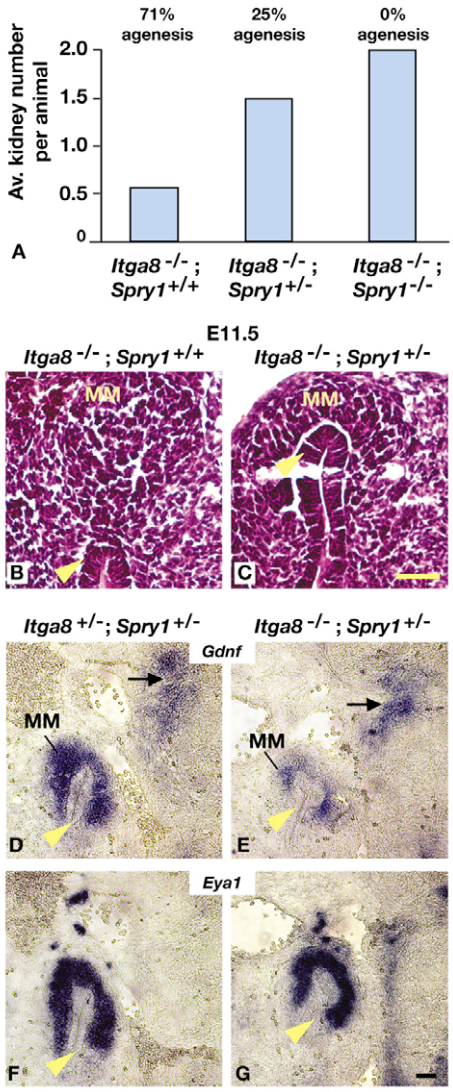


**Fig. 7. Reducing *Spry1* dosage in *Itga8*-null embryos rescues kidney development.** (**A**) Graph illustrating the average number of kidneys per animal at birth in animals of the genotypes indicated. Note that the percent agenesis is significantly reduced in *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ versus *Itga8*$^{-/-}$;*Spry1*$^{+/+}$ animals ($P$<0.005; see legend to Table 1). The rescue of kidney development was complete in *Itga8*$^{-/-}$;*Spry1*$^{-/-}$ animals. However, we found the proportion of *Itga8*$^{-/-}$;*Spry1*$^{-/-}$ animals that demonstrated a duplicated ureter phenotype did not appear to differ from the proportion of their *Spry1*$^{-/-}$ littermates displaying that phenotype (Bason et al., 2005). (**B**,**C**) Transverse sections through embryonic kidneys of the genotypes indicated, stained with hematoxylin and eosin. Note the characteristic lack of invasion of the MM by the UB (arrowhead) at E11.5 in an *Itga8*$^{-/-}$;*Spry1*$^{+/+}$ embryonic kidney. The UB has invaded the MM at E11.5 in an *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ embryonic kidney. (**D-G**) Transverse sections through embryonic kidneys of the genotypes indicated, showing expression at E11.5 of *Gdnf* and *Eya1*, as detected by in situ hybridization. (D,E) Note the substantial reduction in the level of *Gdnf* expression in the MM of the *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ mutant compared with that in the control (*Itga8*$^{+/-}$;*Spry1*$^{+/-}$) embryo, despite invasion of the UB into the MM (arrowhead). Arrows point to *Gdnf* expression in the limb buds, which is similar in both genotypes. (F,G) *Eya1* expression is similar in *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ and control (*Itga8*$^{+/-}$;*Spry1*$^{+/-}$) embryos. Note invasion of the UB into the MM of the *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ and control (*Itga8*$^{+/-}$;*Spry1*$^{+/-}$) embryos (arrowheads). Scale bars: 50 μm.

appear grossly normal in *Npnt* mutants at birth. Consistent with this finding, *Npnt* mutants that survive beyond birth are healthy and fertile, and have an apparently normal life span. This suggests that in tissues other than the developing kidney, the presence of other ECM proteins compensates for the absence of nephronectin. Among the other ECM proteins that could replace nephronectin is Mam domain and EGf domain-containing protein (MAEG) (Buchner et al., 2000), which shares 41% overall amino acid identity with nephronectin and has been shown to be a ligand for α8β1 integrin (Osada et al., 2005). However, at present, little is known about MAEG function and expression, and it remains to be determined whether this ECM protein has roles in organogenesis.

## Nephronectin is an essential ligand for α8β1 integrin during the initial events of kidney development

Our analysis has revealed that although a UB forms in *Npnt* mutants, it consistently fails to invade the MM at E11.5. Significantly, this phenotype very closely resembles the early phenotype of *Itga8* mutants (Muller et al., 1997) (Fig. 6D). Since its identification, nephronectin has been a candidate ligand for α8β1 integrin in the developing kidney and this similarity in phenotype strongly points to nephronectin as an essential ligand for α8β1 integrin during the crucial early process of UB invasion.

Although the *Npnt* and *Itga8* mutant phenotypes appear very similar at E11.5, there are some important differences at later stages. One is that *Npnt* mutants display kidney agenesis at a lower frequency than *Itga8* mutants, 58% versus 83% agenesis, respectively (Muller et al., 1997) (Fig. 2F). A possible explanation for this is that there may be functional redundancy with another ligand(s) expressed by the UB, which can be recognized by α8β1 integrin and can mediate responsiveness of the MM. If so, the expression of this ligand might be responsible for enabling the UB in *Npnt* mutants to undergo the delayed invasion and branching that we observed at E12.5, which in some cases must be sufficient for kidney formation. By contrast, *Itga8* mutants should be unable to respond to any ligand, and therefore display complete agenesis. The finding that kidneys occasionally form in *Itga8* mutants raises the possibility that another integrin may compensate for the absence of α8β1 integrin.

Differences between the *Npnt*- and *Itga8*-null mutants might also reflect differences in the genetic backgrounds of the mice. Although the background of the *Itga8* mutants was largely C57BL/6, with some minor contribution remaining from 129Sv/J, the *Npnt* allele has been maintained on a mixed background with contributions from C57BL/6, 129Sv/J and FvB/N. In support of this explanation, we have observed that *Itga8* mutant survival increases dramatically on an outbred background (J.M.L. and L.F.R., unpublished). Once the *Npnt*-null allele has been bred onto a pure background the penetrance of the homozygous phenotype may more closely resemble that in *Itga8* mutants.

## A role for nephronectin and α8β1 integrin in regulating *Gdnf* expression in the developing kidney

Here we have presented data that support a role for α8β1 integrin and its ligand nephronectin in a pathway that regulates the expression of *Gdnf*, an essential growth factor in kidney development. Using in situ hybridization, we have shown that *Gdnf* expression is severely reduced in *Npnt*- and *Itga8*-null embryos at a time when we invariably found that the UB has not invaded the MM. We have demonstrated that *Itga8*;*Gdnf*-compound-null heterozygotes display kidney agenesis at a fivefold higher frequency than is observed in *Gdnf*-null heterozygotes, and that reducing the level of *Gdnf* gene dosage increases the penetrance of the *Itga8*-null phenotype. Furthermore, we found that by reducing the dosage of a gene that encodes an attenuator of GDNF signaling, Sprouty1, and thus enhancing the sensitivity of *Itga8*-null mutants to GDNF, we decreased the penetrance of the *Itga8*-null phenotype. Taken together, these data provide genetic evidence that α8β1 integrin and GDNF function in a common pathway and suggest that α8β1 integrin and its ECM ligand play a role in regulating the expression of *Gdnf*.

Of special interest, our results show that the severe reduction in *Gdnf* expression in *Npnt* and *Itga8* mutants is transient: in *Npnt* and *Itga8* mutants at E10.5, *Gdnf* RNA levels appeared normal, at E11.5 *Gdnf* RNA was barely detectable, and at E13.5 *Gdnf* RNA was readily detected in those mutants in which sufficient UB branching had occurred such that kidney development proceeded. This transient effect may be indicative of multiple factors working at different times during kidney development to produce the normal pattern of *Gdnf* expression. For example, WNT11, which has been shown to maintain *Gdnf* expression in the MM, seems to be required only after UB invasion (Majumdar et al., 2003). According to this hypothesis, lack of either nephronectin or α8β1 integrin results in a severe decrease in *Gdnf* expression, which causes a delay in UB invasion that is subsequently overcome by the presence of other factors, possibly WNT11, or perhaps members of the fibroblast growth factor (FGF) or TGF-β families, which may have facilitating roles in regulating *Gdnf* expression.

An alternative explanation for the reduction in *Gdnf* expression is that it is a secondary effect of the absence of the UB from the MM in *Npnt* and *Itga8* mutants. We have addressed this possibility by assaying for *Gdnf* expression in $Itga8^{-/-}$;$Spry1^{+/-}$ embryos, in which α8β1 integrin function is lacking but the UB has invaded the MM at E11.5. We found that in these $Itga8^{-/-}$;$Spry1^{+/-}$ embryos the level of *Gdnf* expression at E11.5 was substantially reduced compared with that in their $Itga8^{+/-}$;$Spry1^{+/-}$ littermates. This result demonstrates that loss of α8β1 integrin causes a substantial decrease in *Gdnf* expression in the MM even in the presence of a UB and, therefore, strongly supports our hypothesis that the recognition of nephronectin by α8β1 integrin in the developing kidney is necessary for robust *Gdnf* expression.

## A possible mechanistic explanation of α8β1 integrin-mediated effects on *Gdnf* expression in the developing kidney

How might an integrin and its ECM ligand regulate *Gdnf* expression? Integrins are classically known as adhesion receptors, which have been shown to play roles in organizing the cytoskeleton and activating intercellular signaling pathways (ffrench-Constant and Colognato, 2004; Humphries et al., 2004; Hynes, 2002). There is an extensive literature demonstrating that in cell culture, integrin-mediated cell adhesion together with growth factor signaling can promote mitogenesis, cell viability and gene expression (ffrench-Constant and Colognato, 2004; Giancotti and Ruoslahti, 1999). In mammary gland cultures, β1 integrins have been shown to synergize with prolactin signaling to activate Stat5 and thus to play a role in maintaining the differentiated state of the glandular epithelium and its expression of β-casein (Akhtar and Streuli, 2006; Faraldo et al., 1998; Naylor et al., 2005).

With respect to α8β1 integrin, it has been shown that its recognition of fibronectin activates both the MAPK and PI3K pathways in cell culture systems (Farias et al., 2005). These data raise the possibility that in the kidney, α8β1 integrin activates the MAPK cascade in the

MM. In support of this, we have observed reduced levels of phospho-ERK in the MM of *Itga8* mutants (J.M.L. and L.F.R., unpublished). Therefore, it is conceivable that signaling by α8β1 integrin synergizes with a growth factor signal in the MM to activate the MAPK cascade that then impinges on the transcriptional network involved in regulating *Gdnf* expression.

The signaling properties of integrins have been appreciated for some time, but there is as yet very little in vivo evidence demonstrating roles for integrin signaling in regulating gene expression. The data presented here suggest that this is a key function of integrin in the developing kidney. Further studies will be needed to identify the specific signaling pathway(s) and target genes involved.

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