Title

Nephronectin and alpha8beta1 integrin: novel roles for adhesion and signaling in the developing metanephric kidney

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Nephronectin and α8β1 integrin: novel roles for adhesion and signaling in the developing metanephric kidney

by

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Preface

I have received guidance, kindness and friendship from a number of people while working on this and I would like to acknowledge them here.

The work presented in this thesis was carried out under the guidance of Louis Reichardt. Lou always offered his advice, critiques and praise more as a colleague then as a figure of authority. He gave me the correct advice during critical times and I want to thank him for that. I feel fortunate to have done my doctoral work in his lab.

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I owe a great deal to Susan, my wife. She has been a source of strength emotionally and intellectually. She will always have my deepest admiration and affection.

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They reared me to find my own way. This work is dedicated them.

Abstract

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

Development of the metanephric kidney crucially depends on proper interactions between cells and the surrounding extracellular matrix. For example, it has previously been shown 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 evidence that the extracellular matrix protein nephronectin is a ligand for α8β1 integrin and is essential during early kidney development. We show that mice 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 α8β1 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.

Chair 

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Chapter 1

Introduction

Introduction

The vertebrate kidney is one of the wonders of the living world. It is an adaptation that is necessary for the survival of large, terrestrial, multi-cellular organisms. It provided evolution with a substrate that would allow for increasing complexity in vertebrate design. In its development, the kidney recapitulates this history in a beautifully simple, elegant process.

All vertebrate kidneys develop from the interactions of two tissues that originate from mesoderm. These interactions are characterized by mutually inductive processes, which, on a fine scale, involve cell proliferation, migration, death, and survival. The coordination of these fundamental cell biological processes for an enormous number of cells requires that cells signal through and adhere to a common medium. This medium is known as the extracellular matrix, ECM. This thesis provides new insight into how the ECM coordinates the organogenesis of the kidney by affecting a signaling pathway that is essential to its development. In order to put in context the results presented herein, what follows is a brief overview of the key morphogenetic and molecular pathways involved in initiating the development of the metanephric kidney.

I. Morphogenetic events in early metanephric development

The mammalian embryonic kidney consists of two components, an epithelial component and a mesenchymal component, both of which are derived from the intermediate mesoderm (IM), the mesoderm located between the paraxial and lateral plate mesoderm. During the very first events of kidney development, the epithelial structure,

the nephric duct (ND) forms from the IM and begins a caudal elongation (Grote et al., 2006; Obara-Ishihara et al., 1999). Elongation of the ND ends in a fusion with the cloaca, an endodermal structure that will contribute to the bladder. When the duct first emerges it is thought to fuse with epithelial tubules that have arisen from the adjacent mesenchymal component, the nephric cord (NC). This fusion results in the formation of the first stage embryonic kidney, the pronephros (Bouchard et al., 2002; Saxen, 1987). The pronephros is a rudimentary structure consisting of the previously mentioned tubules and simple glomeruli that filter the dorsal aorta. While the pronephros is the end stage kidney in fish, it is a vestige in mammals and is not thought to have a function during mammalian development (Saxen, 1987; Torrey, 1965). As the ND elongates, the second stage embryonic kidney, the mesonephros, begins to emerge. This structure is known to come about via the induction of the mesenchyme in the NC by the elongating ND (Boyden, 1927; Sainio et al., 1997; Sainio and Raatikainen-Ahokas, 1999). This induction leads to a mesenchymal to epithelial (MET), transition and the formation of tubules. The mesonephros is similar to the pronephros in that it is made up of tubules and glomeruli that filter the dorsal aorta but differs from the pronephros in that it is considerably longer and contains multiple rudimentary nephrons (Schiller and Tiedemann, 1981). The mesonephros is a functional kidney in reptiles and birds, serving as the adult kidney in these classes. In mammals, the mesonephros is a vestigial kidney (Waddington, 1938). As the mesonephros develops, the pronephros begins to degenerate and, as the ND continues its caudal elongation towards the cloaca, the anterior mesonephros will also begin to degenerate while its posterior aspect will remain to form the efferent tubules and vasa deferentia of the male reproductive system (Tiedemann,

1976). Shortly thereafter, the ND reaches the cloaca and fuses with this structure. It is at this time that the third stage embryonic kidney begins to emerge, the penultimate event in mammalian kidney development, the formation of the metanephros.

In the mouse, metanephric development begins at embryonic day 9.0 - 10.5 with a swelling from the ND that is adjacent to the hind limb. This swelling becomes refined into an epithelial protrusion, know as the ureteric bud (UB) (Saxen et al., 1986). At E10.75, the UB begins to extend into a special population of cells within the NC, known as the metanephric mesenchyme (MM) (Sariola et al., 1982; Saxen et al., 1988). By E11.0, the UB has fully invaded the MM in a dorsal/cranial manner. As invasion proceeds, the UB begins to branch, with the first event happening at E11.5. With the first branching event, the mesenchyme begins to respond by condensing around the branching tips. This condensation signals the beginning of a MET that will eventually give rise to the various epithelial populations that make up a mature nephron (Grobstein, 1953; Saxen et al., 1988). This interaction of the branching UB and the condensing and differentiating MM is known as branching morphogenesis, the process that drives metanephric development (Grobstein, 1955; Pohl et al., 2000; Saxen, 1987; Saxen and Sariola, 1987).

The differentiation of the MM is characterized by the appearance of a pretubular aggregate around the branching tips that exhibit a high degree of mitotic activity. These condensates then mature into renal vesicles. The differentiating cells within these renal vesicles begin to diversify into an asymmetric grouping of epithelial cells known as comma shaped bodies (Ekblom, 1992; Saxen, 1987). These structures then undergo a morphogenetic transformation forming two distinct cavities on opposite sides of the comma shaped mass. These cavities are the very beginnings of what will become the slit

diaphragm in the mature glomerulus. As the formation of the cavities takes place the structures take on a distinctly different appearance and become known as S-shaped bodies (Bertram et al., 2000; Jokelainen, 1963; Mori et al., 2003; Saxen, 1984). Both comma and S-shaped bodies are very apparent by E13.5 in the developing mouse metanephros. S-shaped structures fuse with the branching duct, creating a continuous lumen, and begin to undergo the complex morphogenetic program of nephrogenesis, which is the process that gives rise to the nephron (Ekblom, 1981; Sariola, 2002a; Sariola, 2002b). It is at this time that a third cell linage appears, the endothelial population. These cells migrate into the clefts of the s-shaped bodies and will eventual make up the arterioles of the nascent glomeruli (Holthofer, 1987). In this way the MM is responsible for the cell types that make up the nephron while the UB give rise to the drainage duct or renal pelvis in the mature kidney. Additionally, the MM also is the source of the renal stroma(Cullen-McEwen et al., 2005). This population of cells makes up the interstial region that supports the mature renal pelvis and the associated nephron complex.

The formation of the mature metanephric kidney from its beginnings as a rudimentary epithelia bud and a small population of loose mesenchyme, is an impressive example of the morphogenetic events that are found in all ductile organogenesis. Yet more impressive still, is the cellular and molecular basis of this morphogenesis that is now coming to light. The research in this thesis focuses exclusively on the early events of metanephric development, from the formation of the UB up until several rounds of branching at E13.5. Below is a brief overview of the molecular components involved in these early events.

II. Molecular components of early metanephric development

The morphogenetic and anatomical description of kidney development outlined above was completed by the middle of the twentieth century. In the later part of that century and continuing on into the current one, kidney development has been reassessed under the paradigm of molecular biology. Immunological and, in particular, loss of functional analysis using gene targeting technologies have identified many of the genes involved in this process (Dressler, 2006; Vainio and Lin, 2002; Vainio and Muller, 1997; Yu et al., 2004). This thesis reveals a new role for adhesion in the regulation of gene expression during kidney development. In order to appreciate how this novel pathway fits into the larger picture of the genetic regulation of metanephric development, it is necessary to take a brief overview of what is known about the specific molecular aspects of early metanephric kidney development.

Formation of the metanephric mesenchyme

Metanephric development, like pronephric and mesonephric development, depends on the proper formation, advancement and fusion of the ND with the cloaca. However, the morphological aspects that are specific to metanephric development involve UB formation and the development of the MM from the NC mesenchyme. The MM appears as a distinct population from the NC around E10.0 -10.5 in the mouse. Recently it has been shown that in the absence of the protein, odd-skipped related 1, OSR1, a transcription factor containing a zinc finger motif, the MM does not form (James et al., 2006). Right now, *Osr1* is the only gene reported to be essential for the formation of the

MM from the NC. Some of the first molecular markers of the MM include Wilms tumor homolog 1, WT1 and eyes absent homolog 1, EYA1. WT1 is a marker for the intermediate mesoderm before the origin of the MM. Mice that do not express *Wt1* demonstrate a high degree of kidney agenesis at birth in addition to lacking gonads, adrenals and spleen (Kreidberg et al., 1993). WT1 is thought to be involved in transcription but this factor may play other roles in the regulation of transcription as well (Hohenstein and Hastie, 2006). A number of studies have identified target genes for WT1. Included among these is *Itga8* , α8 integrin subunit, a subject of this thesis (Hosono et al., 1999). However, the role of WT1 in the development of the urogenital system is still unclear. Along with OSR1, EYA1 has also been shown to have a role in MM determination (Sajithlal et al., 2005); however, *Osr1* mutant embryos do not express *Eya1* suggesting that this gene is downstream of *Osr1*. EYA1 has a role in the expression of glial cell line derived neurotrophic factor (GDNF) in the MM, and *Gdnf* expression is the defining functional characteristic of the MM (see below). So, the expression of *Osr1* may be directly linked to that of *Gdnf* via the *Eya1* expression. However, at this time it is unclear whether OSR1 directly recognizes the regulatory sequence of *Eya1*. Based on the above it can be concluded that both OSR1 and EYA1 are important in the transcriptional regulation of MM determination. Recently, a surprising result has been reported for signaling from the fibroblast growth factor family, FGF, in the formation of the MM (Poladia et al., 2006). These authors claim that deletion in the MM of both *fgfr1* and *fgfr2*, the genes that encode the receptors for all the FGF members expressed in the developing kidney, results in lack of a morphologically recognizable MM. However the genetic markers *Eya1* and *Six1*, a *sine oculis* ortholog, are still expressed in the adjacent

NC mesenchyme. Additionally, these mice demonstrate some kidney development. This, along with expression of two key markers of the MM, is hard to reconcile with the claim that the MM is gone. Nevertheless, FGF signaling seems to be important for the development of a normal MM. As of now these are the only *in vivo* results that reveal a direct impact on the development of the MM and there remains much to be uncovered about the regulation involved in the origin of this enigmatic group of cells.

GDNF is a factor expressed by the MM that initiates UB formation

The proper positioning of UB formation adjacent to the MM, the target of UB invasion, is critical in metanephric development and one of the longstanding questions that revolved around the MM was whether it had a role in positioning the formation of the UB from the ND. The advent of functional studies in mice has revealed that a number of mesenchymal factors are involved in the initiation and refinement of the UB. Of these the most critical is GDNF (Saarma, 2000). GDNF is a member of the TGFβ superfamily of growth factors and was originally described to have a role in the maintenance of dopaminergic neurons in the central nervous system. Three groups, working independently, reported that newborn mice, homozygous for a null allele of *Gdnf*, demonstrate bi-lateral, renal agenesis at high penetrance and that the agenesis results from an absence of robust UB formation (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). A few years earlier it had been shown that the loss of expression of the gene for the receptor tyrosine kinase, *Ret*, which is expressed in the UB, also resulted in kidney agenesis, albeit at a reduced frequency (Schuchardt et al., 1994). Considered together, these results suggested that RET was the receptor that was responsible for

GDNF signaling in the ND and UB epithelium. The differences between the penetrance of the phenotypes was later explained when the gene for Gdnf family receptor alpha 1, *Gfra1*, a gene that encodes for a glycophatidylinositol-linked membrane protein expressed in the UB and the MM, was targeted for deletion. Mice that do not express *Gfra1* demonstrate kidney agenesis at the same frequency as mice that do not express *Gdnf* (Cacalano et al., 1998). Again, all of these phenotypes arise due to a failure of robust UB formation or responsiveness. This genetic data along with biochemistry showing that GFRa1 could recognize GDNF demonstrated that GDNF was recognized by the receptor complex made up of the RET9 isoform and GFRα1 (Sanicola et al., 1997; Srinivas et al., 1999). Significantly, it has subsequently been shown that loss of *Gfra1* expression from the MM does not recapitulate the budding phenotype, proving that the co-receptor function is solely within the UB (Enomoto et al., 2004). Because *Gdnf* is expressed in the metanephric mesenchyme, it became apparent that the MM provided this factor to the nephric duct to initiate budding. In this regard, GDNF provision to the ND is the most defining feature of the MM, because it is the key to initiating metanephric kidney development.

Regulation of GDNF signaling in the nephric duct insures proper ureteric bud formation and branching morphogenesis

The formation of the UB from the ND involves the binding of GDNF as a dimer to the co-receptor GFRα1 which then complexes with RET, resulting in auto phosphorylation via receptor dimerization. This phosphorylation recruits signaling factors that are responsible for the mitogenic signal driving cell proliferation within the

ND that results in the swelling observed at E10.0-10.5 (Costantini and Shakya, 2006; Pepicelli et al., 1997). As the UB becomes a distinct structure, invades the MM and begins to branch, expression analysis and genetic mosaic analysis has revealed that *Ret* expression becomes localized to the cells in the budding tip (Shakya et al., 2005; Srinivas et al., 1999). Current data suggests that the signaling pathways involved in UB formation, elongation and branching may be distinct. For example, when an inhibitor of MAP kinase kinase (MEKK), PD98059, is applied to kidney cultures the result is a differential growth of the UB stalk in comparison to its budding tips, while the application to culture of the PI3 kinase inhibitor, LY294002, results in a more profound affect on outgrowth and branching (Fisher et al., 2001; Tang et al., 2002). These results, together with the expression data for *Ret*, point to a role for RET mediated activation of MAP kinase signaling in the cells of the budding tip and broader roles for PI3K signaling in epithelial cells of the UB stalks and branches.

While branching morphogenesis is driven in part by mesenchymal GDNF, positive and negative feedback loops ensure proper branching morphogenesis in response to RET signaling in the UB epithelium. For example, the wingless homolog *Wnt11*, is expressed by the UB in response to RET signaling and is thought to enhance the expression of *Gdnf* in the adjacent MM (Majumdar et al., 2003). Therefore, it insures the continual expression of *Gdnf* by the MM directly opposed to the budding tips where *Ret* is highly expressed. This positive feedback loop is regulated by an antagonist of RET signaling, the *sprouty* ortholog, SPRY1. The Sprouty family was first described in Drosophila as antagonist of FGF receptor signaling. Their mechanism of function is not entirely clear. Data has been presented to show that they can inhibit the small GTPase, RAS and

regulate endosomal trafficking of activated receptor tyrosine kinase (Kim and Bar-Sagi, 2004; Kim et al., 2007; Mason et al., 2006). In the UB, SPRY1 has been shown to antagonize RET. Subsequently, *Spry*1 mutant mice demonstrate ectopic ureteric bud formation at E11.5 and multiplex kidneys at birth (Basson et al., 2005). Like *Wnt11*, *Spry1* expression is activated by RET signaling. Therefore the kinetics of branching morphogenesis is partly regulated by the relative expression of *Wnt11* and *Spry1* within the UB.

Strong *in vivo* genetic evidence supports GDNF being the primary factor involved in UB formation, invasion and branching. However, data from both *in vivo*, loss of function, experiments and *in vitro* kidney culture reveal that other mesenchymal factors have roles in both the initiation and refinement of the UB. These other factors include members of the TGFβ superfamily, cytokines and FGF members. Results using kidney culture have implicated many such factors in both the promotion and inhibition of the budding epithelium(Bush et al., 2004; Qiao et al., 2001). However, functions for few of these factors have been confirmed *in vivo*. A particularly interesting exception is a loss of function study in the kidney for bone morphogenetic protein 4 (BMP4). Mice lacking *Bmp4 expression* develop multiple ureteric buds and demonstrate a lack of robust branching after invasion of the MM (Miyazaki et al., 2000). These observations, and results from culture experiments that were published along with them, support dual roles for BMP4. One is inhibitory, signaling through the receptors Alk3/Alk6, expressed in the ND, mesenchymal BMP4 restricts GDNF responsiveness in the ND to the appropriate area of UB formation. The second role promotes elongation of the stalk once the UB has invaded the MM. In confirmation of this, a gene encoding an inhibitor of BMP action,

know as Gremlin, *Grem1*, has been targeted for deletion in mice with the result being a high frequency of kidney agenesis found in neonates homozygous for the mutated allele (Michos et al., 2004). The agenesis results from a lack of robust UB formation, a phenotype most simply explained in terms of a gain of function for BMP4. In addition to BMP4, roles in UB elongation for the FGF group of signaling factors have recently been confirmed *in vivo*. Conditional deletion from either the UB or the MM of the genes *fgfr1* and *fgfr2*, which encode receptor tryrosine kinases that signal FGF responsiveness, have now been reported with unexpected results (Poladia et al., 2006; Zhao et al., 2004). Importantly in both mutants, the UB is able to form properly from the ND. However these studies have revealed a role for FGFR2 in UB branching when both receptor genes are deleted from the UB with CRE driven under the *Hoxb7* promoter. In the MM both FGFR1 and FGFR2 are found to be involved in the proper formation of the MM. This results in a lack of elongation and branching of the UB. Neither of these phenotypes is as severe as that displayed by mice lacking *Gdnf* expression or *Gfrα1* expression. Therefore FGF members facilitate GDNF driven UB formation.

Regulation of *Gdnf* expression in the MM.

A complex and incompletely understood transcriptional network controls the expression of *Gdnf* in the MM. Within this network is a transcriptional axis similar to that described in the development of the compound eye of Drosophila (Brodbeck and Englert, 2004; Epstein and Neel, 2003; Hanson, 2001). At the top of this axis in the fly are the paired transcription factors twin of eyeless, *toy*, and eyeless, *ey*. Both *ey* and *toy* are master regulatory genes that are responsible for the expression of other transcription

factors including *sine oculis*, *so*, a transcription factor containing a homeodomain. In the development of the fly eye, *so* is paired with a co-activator to form a transcription complex. This co-activator is eyes absent, *eya*, of which *Eya1*, mentioned above, is one of four orthologs found in mammals. In the developing fly eye, this co-activator of transcription is also downstream of the paired transcription factors toy and ey (Silver et al., 2003). This family of transcriptional co-activators is particularly noteworthy because its members have been shown to have innate phosphatase activity, currently the only know example in nature of a dual function transcription factor/phosphatase (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). In the fly eye, *eya* has been shown to be a substrate for Erk, and *in vivo* genetic evidence suggest that its function in transcription is activated by phosphorylation of serine residues (Hsiao et al., 2001). The eya/so complex has been shown to move from the cytoplasm to the nucleus to activate transcription. This has also been shown for the mammalian orthologs (Ohto et al., 1999). As of now, it is unclear whether phosphorylation of EYA1 is necessary for the translocation, but translocation to the nucleus suggests that the complex is able to initiate transcription and, therefore, is in an activated state.

As mentioned above, loss of *Eya1* gene expression results in a defect in MM determination and subsequent kidney agenesis (Xu et al., 1999). Loss of *Eya1* expression also results in the complete absence of *Gdnf* expression within the residual mesenchyme. It is known that EYA1 and the mammalian *sine oculis* ortholog, SIX1, form a complex that is able to activate *Gdnf* expression by recognizing the promoter for this gene (Li et al., 2003). However, in *Six1* mutants, *Gdnf* expression, while reduced, is still on at E10.5, suggesting another transcription factor(s) has a role in the expression of *Gdnf* at

that time (Xu et al., 2003). Importantly, *Gdnf* expression has not been assessed in *Six1* mutant embryos at E11.5, the time at which the first branching event occurs. In contrast to the transcriptional axis in the fly eye, a paired type transcription factor has not been described to regulate the expression of *Eya1* or *Six1* in the developing kidney. Rather, the expression of the gene for the paired transcription factor, PAX2, seems to be regulated in the MM by the EYA1/SIX1 complex because there is loss of *Pax2* expression in the MM of *Six1* deficient animals (Li et al., 2003). Like the EYA1/SIX1 complex, PAX2 has been shown to directly bind the promoter of *Gdnf* and initiate transcription (Brophy et al., 2001). So the exact relationship between EYA1/SIX1 complex, PAX2 and *Gdnf* expression is still unclear. However it would not be surprising if there existed multiple redundant pathways involved in the expression of GDNF because it is such a key factor in kidney development. A potential example of this is a target of the EYA1/SIX complex, a gene known as *Sall1,* an ortholog of Drosophila *spalt*, a homeobox containing transcription factor. *Sall1* is expressed in the MM and some null embryos have a budding phenotype similar to *Gdnf* mutant embryos (Nishinakamura, 2003). However the *Sall1* mutant phenotype is less penetrant than that of *Gdnf* mutants, with some Sall1 null embryos demonstrating UBs that have invaded and branched at E11.5. Importantly, *Gdnf* expression is reduced in the MM of *Sall1* mutants at E11.5. The authors contend that the budding phenotype results from the lack of a key factor provided by the MM (Nishinakamura, 2003). However, they do not identify this factor. While *Gdnf* expression is still detectable in this mutant at E11.5, it could be that this transcription factor is downstream of the EYA1/SIX1 complex in the transcriptional cascade that insures a robust expression of *Gdnf* in the MM.

Transcription factors that may be upstream of *Eya1* expression include the forkhead/winged helix transcription factor, FOXC1 and three members of the HOX11 paralogous family. FOXC1 is thought to be a negative regulator of *Gdnf* transcription due to an anterior expansion of *Gdnf* mRNA expression in the NC of *FoxC1* mutant embryos (Kume et al., 2000). This anterior expansion of *Gdnf* message leads to an increase in mesonephric tubules as well as multiple, ectopic UBs. Because an anterior expansion of *Eya1* expression in the NC mesenchyme accompanies the loss of *FoxC1* expression, it is thought that FOXC1 may inhibit *Gdnf* expression in the anterior region of the NC by repressing expression of *Eya1* (Kume et al., 2000). As of yet, this has not been proven, and it remains to be seen if FOXC1 regulates *Gdnf* expression via repression of *Eya1* expression.

In the developing kidney, as in all of organogenesis, *Hox* genes are expressed early on and help pattern the anterior/posterior axis and the dorsal/ ventral axis of many body plans (Kmita and Duboule, 2003). Over the last five years, it has emerged that the *Hox11* members, *Hoxa11*, *Hoxc11*, and *Hoxd11* are involved in the expression of genes in distinct compartments of the metanephric mesenchyme (Patterson and Potter, 2003). Loss of function analysis has revealed that any one of the transcription factors is dispensable for proper gene expression; however, loss of two family members, *Hoxa11* and *Hoxd11*, has an effect on overall kidney size at birth (Patterson et al., 2001). More profoundly, loss of all three members results in bi-lateral agenesis that is completely penetrant at birth (Wellik et al., 2002). Developmental analysis of these triple mutants at E11.0 has revealed a lack of UB invasion, a phenotype similar to *Gdnf* null embryos. Gene expression analysis of triple mutants at E11.0 has also revealed reductions in *Gdnf*

and *Six2* expression (Wellik et al., 2002). *Six2* is another *sine oculis* ortholog that has been shown to have a role in the maintenance of the undifferentiated metanephric mesenchyme but not *Gdnf* expression (Self et al., 2006). The authors found that *Pax2* and *Eya1* were expressed at this time point in triple mutants but they did not assess the expression of *Six1*, which has been shown to impact *Gdnf* and *Six2* expression. Of particular interest and relevance to the work presented in this thesis, it has been reported that in embryos that lack *Hoxa11* and *Hoxd11*, α8 integrin subunit, *Itga8*, expression is reduced specifically in the ventral aspect of the developing kidney at E13.5 (Valerius et al., 2002). In a previous publication the authors found that *Gdnf* expression was reduced in this same region (Patterson et al., 2001). Expression of *Itga8* has not been assessed in the triple mutant. This thesis clarifies and further defines the relationship between *Gdnf* and α8β1 integrin in the developing kidney. Therefore, it will be important to revisit this result in light of the data to be presented.

From the above one may conclude, that the proper formation of the MM and its expression of *Gdnf* are the key events in the initiation of metanephric development. While the details of the transcriptional regulation of *Gdnf* during the early events of kidney development are still emerging, the expression of *Gdnf* in the developing kidney at E10.0 and E11.5 seems to be mediated in part, via a complex made up of EYA and SIX1. Subsequent maintenance of expression may require the additional transcription provided by PAX2 and SALL1.

Rather than the direct regulation of *Gdnf* transcription, the research presented in this thesis involves the contribution of an extracellular stimuli on the expression of *Gdnf* in the developing kidney. Therefore, before introducing the subject matter of this thesis

it is important to briefly consider the contribution made by the extracellular matrix (ECM) in the development of the kidney.

III. The role of the extracellular matrix in early metanephric development

The organization of cells into complex tissues that make an organ like the kidney requires coordination of migration and gene expression on an enormous scale. This coordination is more refined than can be brought about by the simple diffusion of signaling factors and contact that is mediated cell to cell. In order for this complexity to arise there must exist a substratum for the cells to position in and signal through. A network of proteins known as the extracellular matrix (ECM), provides this substratum.

Many ECM proteins are expressed in the developing metanephros (Kanwar et al., 2004; Lelongt and Ronco, 2003). These include interstitial ECM proteins and ECM proteins which form a special structure, the basement membrane. Of the many interstitial ECM proteins expressed in the developing kidney, there are several with special relevance to the subject matter of this thesis including, fibronectin, the tenascin family, and the osteopontin related proteins. Fibronectin is expressed by the metanephric mesenchyme(Kanwar et al., 2004). At first it is distributed diffusely through the metanephric mesenchyme, after invasion of the UB, fibronectin becomes concentrated in the matrix that surrounds that structure. The role of fibronectin in the developing kidney is still unclear. A null mutation of fibronectin has been generated in mice and embryos homozygous for this allele die early from defects arising from improper mesodermal migration (George et al., 1993). As of yet a conditional deletion of fibronectin from the developing kidney has not been reported. However, using RNAi methodology it has

been reported that knockdown of fibronectin message results in a mild inhibition of UB branching (Sakai et al., 2003). It should be noted that the authors did not verify the loss of protein from these cultures. Additionally, while fibronectin may have a role in branching, using kidney culture it is not possible to determine if it has a role in the initial invasion of the UB into the MM. The tenascins, C, R, X, W ,and Y are a group of ECM proteins made up of various numbers of heptad repeats, EGF repeats, fibronectin type III repeats and fibrinogen type domains (Chiquet-Ehrismann, 2004). Both tenascin-C and Tenascin-W have been reported to be expressed in the kidney (Saga et al., 1992; Scherberich et al., 2004). While the expression of tenascin-W in the developing kidney has not been characterized, the differentiating MM has been reported to expresses tenascin-C (Aufderheide et al., 1987; Kanwar et al., 2004). However, mice homozygous for a null mutation of the gene for tenascin-C do not show a phenotype in the developing kidney. A loss of function analysis for tenascin W has not been reported, so it is not yet known if it has a role in the developing kidney. Additionally, roles for the tenascin family in branching morphogenesis have not been reported in culture (Jones and Jones, 2000). Osteopontin is a member of a loosely defined group of ECM proteins, the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family. This group is characterized by a high amount of glycosylation, proline rich regions, the tripeptide sequence arg-gly-asp, RGD, which is recognized by integrins and casein kinase II target sites. Osteopontin is a protein of ~300 amino acids that, in addition to the above qualities, contains sites for binding calcium and heparin. This protein has been reported to be expressed by the UB and the differentiating MM (Kanwar et al., 2004). While antibodies to osteopontin have been shown to inhibit branching in culture, mice lacking

osteopontin do not display a kidney phenotype (Liaw et al., 1998; Rogers et al., 1997). However, a role in early kidney development has been reported for another member of the SIBLING group protein, Dentin sialophosphoprotein (DSPP). *Dspp* null mice demonstrate a mild branching phenotype and an inability of the MM to undergo proper differentiation (Alvares et al., 2006). No phenotype was reported involving bud formation or invasion.

As in all developing tissues, there are many interstitial ECM proteins expressed in the developing kidney. Some of those expressed in a manner that suggest they could have a role in the early events of metanephric development discussed above. With the exception of fibronectin, whose function has not been determined in the kidney, all of these ECM proteins have been shown not to have essential roles in the early events of bud formation or invasion. This is not the case for some of the constituents of the basement membrane.

The basement membrane is a complex of ECM proteins that is found surrounding epithelial structures and consists of the following components, collagen IV (α1 chain and α2 chain) , laminins, (α1, α3, α5, β1 and γ1 subunits), perlecan and nidogen 1 and 2 (Kreis and Vale, 1999). During metanephric development, the ureteric epithelium contributes all of the components to its own basement membrane. Remarkably, loss of function of most of these components does not disrupt early kidney development (Lelongt and Ronco, 2003). The exceptions include the laminin γ1 subunit (*Lamc1*) and the laminin α5 subunit (*Lama5*). In mice that express a laminin γ1 subunit that is unable to bind nidogens, 90% of newborn mice demonstrate bi-lateral kidney agenesis (Willem et al., 2002). The phenotype involves an apparent inability of the ND to complete its migration and the subsequent lack of budding from the ND. The severity of the

phenotype has revealed an essential role for a nidogen/laminin interaction in the developing kidney. This is revealing because mice lacking nidogen 1 do not display a renal phenotype, suggesting that nidogen 2 can compensate for the loss of nidogen 1 during development. Mice deficient in the laminin α5 subunit, which is a member of the laminins 10 and 11, also demonstrate kidney agenesis at birth but to a much lesser extent (Miner and Li, 2000). At birth 20% of these animals demonstrate either bi-lateral or uni-lateral agenesis. This seems to be the result of a delay or slowing of UB extension. Taken together, these data suggest that the laminins have a role in the proper elongation of the nephric duct and the formation of the ureteric bud from the nephric duct. More profoundly, mice with a mutation in the gene for heparan sulfate 2-O-sulfotransferase, *Hs2st1*, an enzyme that adds a sulfur group to glycosaminoglycan side chains of proteoglycans, demonstrate a highly penetrant phenotype of kidney agenesis at birth (Bullock et al., 1998). The phenotype arises from an apparent defect in MM responsiveness to the UB and a lack of robust branching by the UB once it has invaded the MM. Proteoglycans, (PG), are associated with all basal lamina and the absence or improper expression has adverse effects on development and tissue homeostasis (Lander and Selleck, 2000). This includes the finding that the biological activity of WNT family members depends on the proper expression and distribution of proteoglycans in the developing kidney (Vainio and Uusitalo, 2000). For example, WNT11 function, a key factor involved in the maintenance of GDNF in the MM, is dependant on the proper expression of PG (Kispert et al., 1996; Majumdar et al., 2003). More recently, WNT9B has been shown to be the factor contributed by the UB that induces the MM to condense and begin the differentiation cascade(Carroll et al., 2005). Indeed, the phenotype of mice

deficient for heparan sulfate 2-O-sulfotransferase is most likely explained by disruption of WNT signaling. Heparan sulfate is a known co-receptor for a number of growth factors including GDNF and members of the FGF family (Barnett et al., 2002; Heath et al., 1991). It is likely that the near total penetrance of kidney agenesis demonstrated by *Hs2st* null mice results from the fact that the absence of this enzyme disrupts not only the key signaling of WNT family members but also that of GDNF.

Taken together, the above results demonstrate two fundamental roles for the ECM in the early events of metanephric development: One, the ECM acts as a substrate for the elongation of the ND and the formation, elongation and first branching events of the UB. This role has been revealed by the phenotypes associated with mice that have a mutation in *Dspp*, and more profoundly in mice that have mutations in the genes *Lamc1* and *Lama5*. Two, the ECM acts as a medium through which signaling factors are presented and their effective concentrations are modulated. This is clearly demonstrated in mice with null mutations of *Wnt11* and *Hs2st1*, in which result there is an inability of the MM to respond to the invading UB.

Integrins

Cells respond to the ECM through various receptors expressed on their surfaces. However, the most important class of receptor for the ECM is the integrin family (Bokel and Brown, 2002; ffrench-Constant and Colognato, 2004; Hynes, 2002). Integrins are cell adhesion receptors that are made up of two subunits designated as α and β, both of which are single pass transmembrane proteins. The heterodimer binds its ligand through an interface with both subunits. The structure, both bound and unbound to ligand, of the

extracellular region of integrin has been solved (Xiong et al., 2001; Xiong et al., 2002). The structure reveals a protein complex that undergoes a dramatic conformational change upon activation (Liddington and Ginsberg, 2002). The key to integrin activation is now thought to involve the binding of the protein talin to the cytoplasmic domain of the β integrin tail (Calderwood et al., 2003; Calderwood et al., 2002). This binding, which is mediated by a phosphotyrosine-binding domain (PTB), results in a conformational change in the transmembrane regions of the α and β subunits that is translated into the dramatic extracellular rearrangement that allows for the recognition of ligand (Campbell and Ginsberg, 2004). Integrins are classically known for mediating adhesion, but they have become just as well know for their signaling properties (Giancotti and Ruoslahti, 1999; Martin et al., 2002; Miranti and Brugge, 2002). In this regard, talin serves to nucleate sites of signaling from integrins primarily though interactions with paxillin and focal adhesion kinase (FAK) (Cary and Guan, 1999; Chen et al., 1995; Mitra and Schlaepfer, 2006; Schlaepfer et al., 1999). This non-receptor tyrosine kinase activates through autophosphorylation at tyrosine 397. This recruits other non-receptor tyrosine kinases, such as Src and Fyn that recognize phospho-tyrosine 397 via SH2 domains (Schaller et al., 1994). These kinases phosphorylate other sites that recruit signaling factors such as Grb2 (Schlaepfer et al., 1994). Aside from FAK, integrin signaling is also thought to be mediated through interactions between the transmembrane region of the α subunit and caveolin, which recruits non-receptor tyrosine kinases (Wei et al., 1999). The intricacies of integrin activation and signaling are complex and the reader is referred to a number of reviews on this subject for further details (Danen, 2005; Giancotti and Tarone, 2003; Luo and Springer, 2006). However, it is now appreciated that these

interactions allow integrin activation to feed into multiple signaling pathways. Additionally, it has been shown that integrin adhesion results in specific phosphorylations of tyrosine residues on growth factor receptors (Moro et al., 2002). Conversely, it is known that signaling from both growth factor receptors and G-protein coupled receptors can activate small GTPases that have roles in the recruitment of talin to the cytoplasmic tail of the β subunit of integrin (Han et al., 2006). Such crosstalk is thought to form the basis of robust signal transduction both from the outside in, resulting in cellular migration, proliferation, death and survival, and from the inside-out, with the binding of talin to the β subunit tail resulting in integrin activation and subsequent ECM deposition and remodeling processes.

Currently there are 8 known β subunits and 18 known α subunits, which make up 24 different heterodimers and loss of function analysis of these subunits has revealed roles for integrins in a number of developmental processes (Fassler et al., 1996). In the developing kidney integrins have been shown to have roles in nephrogenesis and the maintenance of ureter formation, but only one has been shown to have an essential role in the early events of metanephric development, integrin α8β1 (De Arcangelis et al., 1999; Kreidberg et al., 1996; Muller et al., 1997).

IV. α8β1 integrin and nephronectin

The identification of a cDNA for integrin α8 subunit (*Itga8*) was first made in the chick from a low stringency hybridization using the *itga5*, α5 subunit, cDNA as a probe (Bossy et al., 1991). These subunits share 46% identity in their primary sequences. Like the α5 subunit, the α8 subunit only forms a heterodimer with the β1 subunit. Expression

analysis of α8 integrin protein revealed it to be highly expressed in the enteric nervous system and the spinal cord of the developing chick at E6 through E11. During E11 in the chick, α8 integrin subunit is expressed in the skin, the enteric nervous system, the retina, auditory system and the mesonephros, the functional kidney in the chick (Bossy et al., 1991). While extensive expression analysis has not been completed in the mouse, the expression of α8 integrin subunit has been examined in the developing kidney (Muller et al., 1997). At E11.5, α8 integrin protein is highly expressed in the nephric cord, adjacent to the nephric duct. It is also expressed by the MM. The expression of α8 protein remains high around the branching UB, especially around the branching tips. As mentioned above, the α8 subunit has an essential role in kidney development. Loss of *Itga8* expression results in bi-lateral kidney agenesis 50% of the time at birth with the other 50% demonstrating variable amounts of kidney development (Muller et al., 1997). Of these less than 2%, develop two kidneys. The phenotype results from a defect in UB invasion and branching. At E11.5 in *itga8* null embryos it is found that the UB has formed and extended towards the metanephric mesenchyme but it has not invaded that field of cells. It is obvious, that in mutants that do form kidneys, the UB must invade and at E13.5 some mutants are found to be developing kidneys; however, the degree of branching morphogenesis is reduced. Subsequently, mutant survivors tend to have smaller kidneys at birth. Additionally, homozygous mutant neonates often display rudimentary kidney development. In this case, a mature ureter is found to be attached to an extremely underdeveloped kidney or an undifferentiated mass of MM. While their kidneys may be smaller, homozygous mutant survivors are completely viable and live a normal life span. Since the description of this phenotype, the exact function of α8β1

integrin in the developing kidney has remained an outstanding question. This thesis will be present data that places α8β1 integrin in a pathway that is critical in the developing kidney, the GDNF/RET pathway.

Another question to emerge from the above studies was the identity of the ligand(s) being recognized by the integrin. This integrin was known to recognize the following ECM proteins: fibronectin, tenascin-C, vitronectin and osteopontin. All of these, with the exception of vitronectin, are expressed in the developing kidney. However, for the reasons discussed above (see introduction part III) none of these was a viable candidate ligand. The one candidate ligand that was expressed in the proper place and time, osteopontin, had been targeted in mice and shown not to have an essential role in kidney development (Liaw et al., 1998). Because of this, the identification of additional ligands that could be mediating α8β1 integrin function was of great interest. A clue to the existence of such a ligand was the ability of a probe, made up of the extracellular domain of the integrin α8β1 fused with alkaline phosphatase, to recognize a protein closely associated with the UB (Muller et al., 1997). To identify this unknown ligand an expression cloning strategy was undertaken that eventually uncovered a novel ECM protein that was recognized by α8β1 integrin and expressed at the right temporal and spatial manner (Brandenberger et al., 2001). This protein was named nephronectin, in anticipation that it was the ligand that mediated α8β1 integrin function in the developing kidney. In this thesis, data from a loss of function analysis of the nephronectin gene, *Npnt*, will show that it is very likely the ligand that mediates α8β1 integrin function during the early event of UB invasion. Additionally, this thesis reports a novel function for this interaction in developing metanephric kidney. We find the α8β1 integrin and

nephronectin are involved in the expression of *Gdnf* by the MM during UB invasion. Interestingly, this is a function that differs from the classic function of ECM proteins as substrates for cellular adhesion.

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Chapter 2

Recognition of nephronectin by integrins

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This work was done in collaboration with Andrea Schmidt, who expressed neph251-561myc in Cos7 cells and collected the data for Fig. 2.3A. Andrea also created the graph for Fig. 2.3A. Ralph Brandenberger contributed the section entitled "Recombinant protein production" for the material and methods. James M. Linton contributed all other data and text presented in this chapter.

Abstract

Nephronectin is an extracellular matrix protein that is expressed in the epithelial components of embryonic kidney. This protein is also expressed in a number of other developing organs including those in which epithelial-mesenchymal interactions are essential. To examine if nephronectin can be recognized by α8β1 integrin when expressed on cells and to examine if it can be recognized by other integrins, we performed adhesion assays using recombinant forms of nephronectin. We found that truncated forms of nephronectin are recognized by α8β1 integrin expressing cells. Additionally, we found that this same form of nephronectin was recognized by a number of other integrins expressed on cells. Rather surprisingly, we find that it is recognized by α4β7 integrin, a non-RGD binding integrin.

Introduction

The extracellular matrix (ECM) provides a substrate for cells to make up the complex tissues found in metazoans (Hutter et al., 2000; Whittaker et al., 2006). Cells recognize ECM components via a number of receptors, including integrins. Integrins are heterodimers consisting of a α subunit and a β subunit that together recognize specific sequences of amino acids within ECM proteins to which they adhere. Integrins can occupy an active or inactive state depending on intracellular signaling, the presence of an appropriate ligand or the presence of certain divalent cations (Humphries et al., 2004; Hynes, 2002).

The integrin α8β1 is expressed in a number of tissues including the mesenchymal compartment of the developing kidney (Muller et al., 1997). Previously, it has been shown that loss of function of α8 integrin subunit in mice results in severe kidney agenesis at birth. The developmental origin of this phenotype was found to involve an invariant lack of ureteric bud (UB) elongation and invasion into the metanephric mesenchyme (MM) at E11.5 in development. Secondarily, in those mutant embryos that overcame the delay of invasion, there was a decrease in UB branching and a decrease in the responsiveness of the MM to the UB (Muller et al., 1997).

Understanding the basis of the phenotype required the identification of an appropriate ligand(s) for the integrin. However, during the early events of UB elongation and invasion, known ligands for α8β1 integrin were ruled out due to either spatial-temporal expression or functional significance (see introduction). Therefore, it was apparent that a novel ligand could have a role in mediating α8β1 integrin function. A subsequent expression cloning strategy using an extracellular portion of α8β1 integrin fused to alkaline phosphatase, α8β1-AP, turned up a previously unknown protein that was subsequently named nephronectin (Brandenberger et al., 2001). This protein was found to contain a number of domains characteristic of ECM proteins, including an n-terminal signal peptide, five EGF like repeats, a highly glycosylated region which contained the tripartite amino acid sequence, arginine, glycine, aspartic acid, RGD and a c-terminal MAM domain (Fig 2.1).

Initial identification of this protein involved asking whether it could be recognized as a ligand by α8β1-AP. While suggestive, the recognition of this protein by α8β1-AP was not a physiologic test of integrin adhesion and it was of interest to know if this protein

could be recognized by α8β1 integrin expressing cells. Additionally, because nephronectin is expressed in a number of other tissues during development, it was of interest to examine the possibility that other integrins could act as receptors for this protein. In order to address these questions, we performed adhesion assays using the cell line, K562. This cell expresses only one endogenous integrin at high levels, α5β1 integrin, and low to undetectable levels of other integrins (Blystone et al., 1994). We obtained lines expressing various integrin heterodimers along with the parental line expressing endogenous α5β1 integrin. We examined how well these lines adhered to a truncated form of nephronectin containing the c-terminal portion of the protein, with or without the RGD site, in the presence of an inhibitory antibody to α5 integrin subunit. Using this assay we find that K562 cells expressing α8β1 integrin recognize this truncated form of nephronectin in a dose dependent manner similar to the way they recognized native, full-length fibronectin. Using several other stable K562 lines expressing other integrin heterodimers, we find that a number of integrins can recognize this form of nephronectin nearly as well as α8β1 integrin, including αvβ3, αvβ5 and αvβ6 integrins. Additionally, the endogenous fibronectin receptor α5β1 is found not to recognize this form of nephronectin while the non-RGD binding integrin, α4β7, recognizes it relatively well.

Materials and Methods

Recombinant protein production

The nephronectin fragments neph251-561 (amino acids 251–561) and neph251-381 (amino acids 251–381) were expressed as NH_2-terminal GST fusion proteins in *E. coli*

(Fig 2.2B,C). Both fragments were generated by PCR and cloned into the pGEX-4T-3 vector (Amersham Pharmacia Biotech). Constructs were verified by sequencing. Recombinant fusion proteins were expressed in *E. coli* BL21 cells. Bacteria were grown at 37°C in LB medium to OD_{600} = 0.8 and were then transferred to 30°C. Cells were induced with 1 mM isopropyl-ß-D-thiogalactopyranoside and grown for an additional 2.5 hr. Cells were collected by centrifugation, resuspended in lysis buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100 containing 1 μg/ml PMSF, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin A), and lysed by sonication. After centrifugation, the supernatant was incubated for 1 h at 4°C with glutathione–Sepharose (Amersham Pharmacia Biotech). Beads were washed with lysis buffer and with PBS and eluted with PBS containing 50 mM reduced glutathione.

For expression of the nephronectin fragment neph251-561myc (amino acids 251–561) in COS7 cells (Fig. 2.2D), a PCR fragment was generated and cloned into the pSecTag2 vector (Invitrogen) containing an Ig chain signal peptide and a c-terminal myc/His_6 tag. COS7 cells were grown in DME (GIBCO BRL) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were transiently transfected with LipofectAmine (GIBCO BRL). After transfection, medium was changed to DME supplemented with Nutridoma HU (Roche Biochemicals) and penicillin/streptomycin. Conditioned medium was collected every 2 d for 8 d.

Cell adhesion assays

For cell adhesion assays, substrate protein was diluted in PBS to the indicated concentrations. Linbro Titertek 96-well plates (Flow Laboratories) were then treated overnight at 4°C with a total volume of 100 μl of substrate solution per well. The wells

were blocked with 10 mg/ml BSA in PBS for 1 hr at 37°C. The cells were harvested with PBS containing 1 mM EDTA, washed once in TBS (24 mM Tris-Cl, pH 7.4, 137 mM NaCl, 2.7 mM KCl), counted, and then resuspended in TBS containing 0.1% BSA, 2 mM glucose, 1 mM $MnCl_2$. The cells were counted once again, and a total of 2.0 x 10^5 cells were plated per well. The cells were incubated for 1.5 hr at 37°C, washed four times with TBS containing 1 mM $MnCl_2$, fixed for 15 min with 2% paraformaldehyde, and stained for 5 min with 2.5% crystal violet in 20% ethanol. Finally, each well was washed four times with water, and adherent cells were lysed with 1% SDS. Absorption values for each well were read at 570 nm using a microtiter plate reader and SOFTmax v2.35 (Molecular Devices). Final absorption values for wells coated with FN or nephronectin GST fusion proteins were determined by calculating the mean absorption value of duplicate or quadruplicate wells and subtracting the mean value from either BSA- or GST-treated control wells run in parallel. For antibody inhibition, cells were preincubated for 15 min on ice with the antibody before plating. Antibody was present throughout the adhesion assay. Antibody BIIG2 (anti- α5) was supplied as an ascites. This ascites blocked adhesion of the parental K562 cells to FN at a dilution of 1:20. K562 adhesion to FN was not affected by a control ascites when used at the same concentration.

Results

Nephronectin is recognized by α8β1 integrin in a dose-dependent manner

K562 cells and K562 cells expressing α8β1 integrin were allowed to adhere to increasing concentrations, (0.06 ug/ml -15.0 ug/ml) of either full-length fibronectin or a truncated form of nephronectin, neph251-561myc (Fig. 2.2D). K562 cells recognized

fibronectin readily as indicated by the plot of OD 570 vs. concentration of protein used to coat the well (Fig 2.3 A open triangles). A total of $2.0X10^5$ cells per well resulted in an adhesion plateau at 3.75 ug/ml. However, the parental line, which expresses $\alpha5\beta1$ integrin almost exclusively, had little or no avidity for neph 251-261 (Fig 2.3A solid triangles). This demonstrates that $\alpha5\beta1$ integrin, which recognizes the RGD sequence in fibronectin, is unable to recognize this form of nephronectin. Like the parental K562 cell line, K562 cells expressing $\alpha8\beta1$ readily bind fibronectin, even in the presence of inhibitory antibody to $\alpha5$ subunit (Fig 2.3A open squares). Here, a total of $2.0X10^5$ cells per well resulted in an adhesion plateau at 7.5 ug/ml. Unlike the parental line, these cells recognize neph251-261 (Fig 2.3A closed squares). Plating the same number of cells as above in wells coated with neph251-561, resulted in an adhesion plateau at 7.50 ug/ml. Therefore, unlike $\alpha5\beta1$ integrin, expression of $\alpha8\beta1$ integrin allows these cells to adhere to neph251-261 with relatively high avidity. This supports the idea that $\alpha8\beta1$ integrin recognizes nephronectin in a physiological meaningful way.

Nephronectin is recognized by multiple integrins

In order to understand if nephronectin could be recognized by other integrins, we performed adhesion assays with a number of K562 clones expressing different combinations of integrin subunits. Cell adhesion assays where carried out using a number of ligands, including neph251-561, which contains the RGD site, and neph251-381, which lacks the RGD site (Fig. 2.2B, C). As positive controls, cell adhesion assays were carried using collagen type III for K562 cells expressing $\alpha1\beta1$ and $\alpha2\beta1$, laminin 5 for cells expressing $\alpha3\beta1$ and fibronectin for all other lines reported here. All lines

adhered to their respective ligands (not shown). All adhesion assays were carried out in the presence of inhibitory antibody to α5 integrin subunit, except in the case of the parental line, K562.

We found that of all the clones K562 cells expressing α8β1 integrin, gave the highest OD 570 value for this assay; therefore, we normalized all the data to this value (Fig. 2.3B). Those clones that recognized nephronectin with the greatest relative avidity were those expressing the integrin heterodimers αvβ6, αvβ3, αvβ5, (Fig. 2.3B). Aside from these, only one other clone tested adhered to neph251-261, the clone expressing α4β7, which adhered with an avidity that was not statistically different from that of clones expressing the integrins αvβ3 and αvβ5, (Fig. 2.3B). The parental line, K562, was found to recognize neph251-5 61 with limited avidity, consistent with the above data. This adhesion is most likely mediated via α5β1 integrin as inhibitory antibody to α5 subunit abolished adhesion (not shown). In contrast, the clone expressing the fibronectin receptor α4β1 integrin did not recognize this form of nephronectin at all. This was also true of clones expressing the collagen and laminin receptors α1β1, α2β1 and α3β1 (Fig. 2.3B). Importantly, none of these clones was found to adhere to neph251-381 (not shown).

Discussion

Nephronectin, an ECM protein that is expressed in a number of developing organs, is a candidate ligand for mediating α8β1 integrin function in the developing kidney (see introduction). Using truncated recombinant protein expressed in COS-7 cells, we found that K562 cells expressing α8β1 integrin adhered to nephronectin in a similar manner as

they did to full-length, native fibronectin, as assessed by plotting a dose response curve (Fig. 2.3 A). In contrast, the parental cell line, K562, adhered well to fibronectin, but poorly to neph251-561, even at coating concentrations of 15 ug/ml (Fig. 2.3A). Therefore, two integrins that are able to recognize a common ligand, fibronectin, differentially recognize this form of nephronectin. This is interesting when considering that α8 and α5 integrin subunits are similar at the primary sequence level (Bossy et al., 1991). Additionally, because α8β1 integrin and α5β1 integrin recognize the RGD sequence in fibronectin (Irie et al., 1995; Mould et al., 1997; Pytela et al., 1985; Schnapp et al., 1995), these results suggest that nephronectin is selectively recognized by α8β1 integrin in a manner dependent on characteristics of the protein other than the RGD site.

Nephronectin is expressed in a number of tissues during development, some of which do not express α8β1 integrin (Brandenberger et al., 2001; Muller et al., 1997). To examine the possibility that this protein is recognized by additional integrins, we performed adhesion assays with a number of K562 clones expressing different combinations of integrin subunits. We found that of all the clones we tested, those expressing α8β1 integrin recognized neph251-561 with the greatest avidity, as assessed by adhesion assays. Other clones that recognized neph251-561 included those expressing αvβ6, αvβ3 and αvβ5 integrin. Because these integrins do not recognize a truncated form of nephronectin not including the RGD site, neph 251-381 (not shown), it is assumed that these integrins are recognizing the RGD sequence in nephronectin. These results were somewhat expected because these are RGD binding integrins and the αv and α8 subunits share a degree of sequence identity at the amino acid level (Hemler, 1999). What was unexpected was the finding that clones expressing the α4β7 integrin

recognized neph 251-561, although less well than the clones expressing the αv integrins (Fig. 2.3B). Sequence gazing reveals that neph251-561 contains the quartet LDDV at amino acids 551-554. As α4β7 integrin is know to recognize the LDVP motif in fibronectin, the IDSP sequence in the N-terminal domain of IgCAM and the LDTS sequence in mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Yang et al., 1998), it seems possible that it could be recognizing the LDDV sequence in neph 251-561. However, α4β1 integrin also recognizes the LDVP sequence of fibronectin (Irie et al., 1997), yet we find that clones expressing α4β1 integrin do not recognize neph 251-561 (Fig. 2.3B). Therefore the exact identification of the sequence recognized by α4β7 integrin in neph251-561 and presumably, full-length nephronection will require more directed studies with deletions of nephronectin.

The above data demonstrate that cells expressing α8β1 integrin can adhere to a truncated form of nephronectin and that this adhesion is comparable to that of fibronectin, a known ligand for this integrin. In this way, these studies lend support to the hypothesis that nephronectin is an *in vivo* ligand for α8β1 integrin in the developing kidney. Additionally, we have shown that other integrins recognize this form of nephronectin supporting the idea that nephronectin is recognized by other integrins *in vivo*. Conclusive evidence for nephronectin as a functional ligand for α8β1 integrin in the developing kidney and, for its importance in other aspects of development will require a functional analysis in mice.

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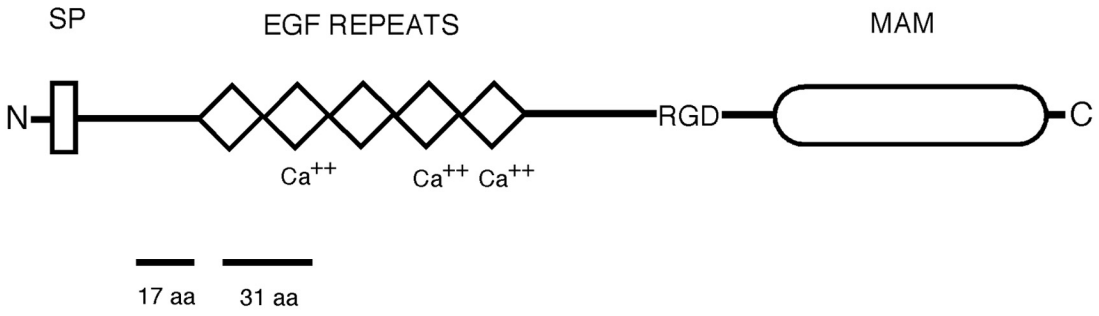

Fig. 2.1. Nephronectin, an extracellular matrix protein. (**A**) The overall domain organization of nephronectin. The protein contains an amino-terminal signal peptide (SP), five EGF like repeats, three of which have putative calcium binding ability. It also contains a proline rich region that is highly glycosylated. This region contains an integrin recognition site, the tripeptide sequence Arg-Gly-Asp (RGD). At its carboxy-terminus is a MAM domain. Two isoforms of nephronectin have been identified a short form of 526 amino acids and a long form, that includes inserts of 17 amino acids and 31 amino acids that flank the first EGF repeat.

Fig. 2.2. Schematic representations of nephronectin fusion proteins. (**A**) Domain structure of nephronectin. (**B**) GST-neph251-561 (neph251-561), contains all the sequence of nephronectin that is C-terminal of the EGF repeats, including the RGD site. (**C**) GST-neph251-381 (neph251-381), contains the all the sequence of nephronectin that is C-terminal of the EGF repeats and ends just before the RGD site. (**D**) sp-neph251-561-myc, (neph251-561myc) contains an n-terminal IgG signal sequence (sp), all the sequence of nephronectin that is C-terminal of the EGF repeats, including the RGD site, and a c-terminal myc tag.

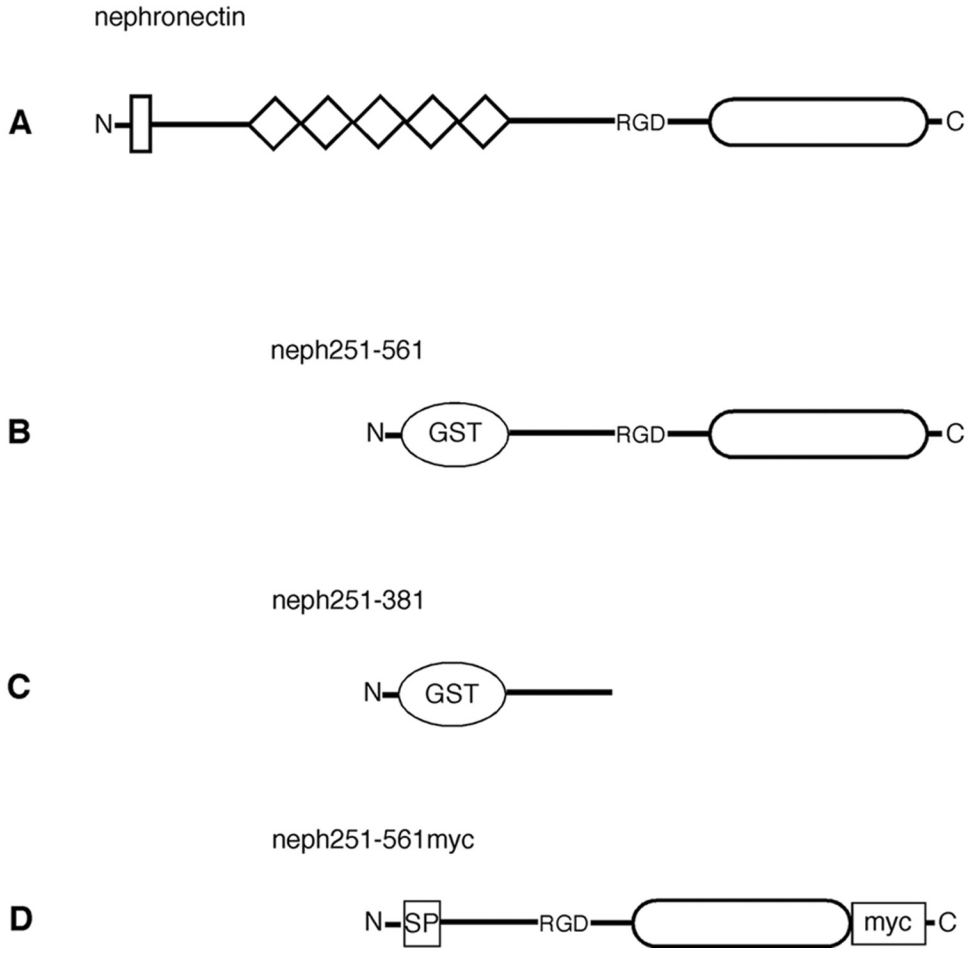
nephronectin
A
N
RGD
C
neph251-561
B
N
GST
RGD
C
neph251-381
C
N
GST
neph251-561myc
D
N
SP
RGD
myc
C

Fig. 2.2

Fig. 2.3. Adhesion of K562 cells, and K562 expressing specific additional integrins, to nephronectin. **(A)** K562 and K562 expressing α8β1 integrin were allowed to adhere to increasing concentrations, 0.06–15.0 μg/ml, of either FN or amino acids 251–561 of nephronectin (NN) purified from CHO cell–conditioned medium. Experiments were carried out in the presence of 1 mM Mn^{2+}. **(B)** Adhesion of K562 cells expressing indicated additional integrin heterodimers to 2 μg/ml of a GST fusion protein containing amino acids 251–561 of nephronectin in the presence of 1 mM Mn^{2+} and the anti-α5 mAb BIIG2 to inhibit α5β1-mediated adhesion. Adhesion of the parental K562 cells that express α5β1 integrin to these two substrates was measured in the absence of the anti-α5 mAb. For all lines, OD values for wells coated with GST alone were subtracted. Adhesion of α8β1 integrin-expressing K562 cells was defined as 100% and adhesion of other cells is expressed as a percentage of that value.

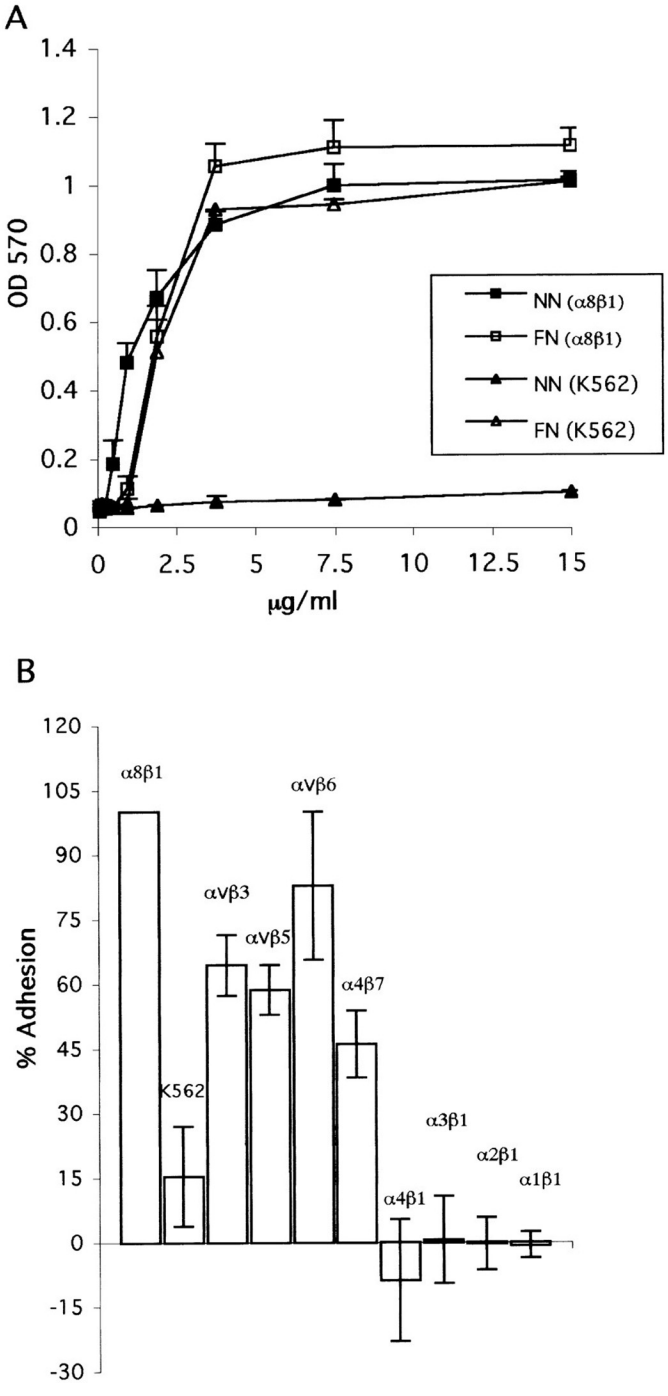
A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 570
0
2.5
5
7.5
10
12.5
15
μg/ml
NN (α8β1)
FN (α8β1)
NN (K562)
FN (K562)
B
120
105
90
75
60
45
30
15
0
-15
-30
% Adhesion
α8β1
K562
αvβ3
αvβ5
αvβ6
α4β7
α4β1
α3β1
α2β1
α1β1

Fig. 2.3

Chapter 3

Loss of function analysis of nephronectin in the developing kidney

The data presented in this chapter were published in the journal Development (Linton et al. Development 2007 134: 2501-2509). The Company of Biologist Ltd 2007

Abstract

Here we present genetic evidence that the extracellular matrix protein nephronectin is an essential ligand for α8β1 integrin during early kidney development. We show that mice 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 nephronectin null mutants specifically in the metanephric mesenchyme at the time of ureteric bud invasion.

Introduction

Of the many factors involved in organogenesis, the extracellular matrix (ECM) plays a central role. It is made up of a diverse array of proteins that impart a variety of functions (Gumbiner, 1996; Werb, 1997). However, in a general sense, it can be thought to serve as an essential medium through which cells communicate and a scaffold to which they adhere, allowing for the organization of cells into tissues (Huang and Ingber, 1999). The developing metanephric kidney provides a particularly relevant model system to investigate the essential qualities of the ECM. For example, it has been shown that in embryos lacking α8β1 integrin (*Itga8*), a cell adhesion molecule that recognizes the ECM, invasion by the ureteric bud (UB) into the metanephric mesenchyme (MM) is inhibited (Muller et al., 1997). This inhibition results in a high frequency of renal

agenesis at birth. A long standing question about α8β1 integrin function has involved the identification of its essential ligands in the developing metanephric kidney (Muller et al., 1997).

Previously, we reported the identification of a gene that encodes a novel ECM protein, nephronectin (*Npnt*) (Brandenberger et al., 2001). Nephronectin contains a number of domains characteristic of ECM proteins, including a signal peptide, five EGF like repeats and a cell adhesion site, the tripeptide sequence Arg-Gly-Asp. Uniquely, it also includes a MAM domain at its c-terminus (see Fig. 2.1). We have demonstrated that α8β1 integrin recognizes nephronectin in binding assays and associates with nephronectin *in vivo* (Brandenberger et al., 2001). Finally, the localization of nephronectin in the kidney was found to be consistent with it mediating α8β1 integrin function during development. However, the question of whether nephronectin has an essential role in the developing kidney has remained outstanding.

Here we report the engineering of mice with a floxed allele of nephronectin. We show that conditional targeting of this allele with CRE recombinase expression driven under the β-actin promoter, results in a null allele. Mice homozygous for this null allele frequently display kidney agenesis at birth. We have found that the phenotype arises during the early events of metanephric development, when the UB is beginning to invade the MM. Similar to *Itga8* mutant embryos, *Npnt* mutant embryos display a lack of UB invasion at E11.5 with complete penetrance. Thus, nephronectin is an ECM protein expressed by the UB that has an essential function in early kidney development. This strongly supports the hypothesis that nephronectin is an essential ligand for α8β1 integrin during the early stages of UB invasion and branching. Significantly, we detected no

defects in ECM organization in the nascent kidneys of *Npnt* mutants. Instead, we found that *Gdnf* expression was dramatically reduced in nephronectin null mutants specifically in the MM at the time of ureteric bud invasion.

Materials and Methods

BAC screen for 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, BAC 273P10, was found to contain the first eleven exons of nephronectin (Fig. 3.1A). This clone was used to generate the floxneo targeting construct (Fig. 3.1B-D) by introducing a targeting cassette through recombineering (Zhang et al., 1998).

Creation of the *Npnt* targeting cassette

Three separate PCR reactions were performed, using ES cell DNA from 129Sv/J embryonic stem (ES) cells, to create a 5' arm, a site of internal homology (floxed region), and a 3' arm. For the 5' arm, forward and reverse primers were used that resulted in a product of 2.86 kb with a Nco1 site at the 5' end of the arm and a BsaA1 site at the 3'end of the arm. For the site of internal homology, forward and reverse primers produced a product of 0.77 kb product containing the first exon of nephronectin. This PCR introduced 5' flanking BsaA1, EcoRV and loxP sites. It also introduced 3' flanking EcoR1 and Not 1 sites. For the 3' arm, a single PCR produced a product of 2.5 kb. This PCR introduced Not1 sites at both the 5' and 3' ends of the arm. To create the complete 5' arm, the internal homology PCR was cut with both Not 1 and BsaA1. That fragment was then ligated with a sub-clone of the 5'arm that had been cut with the same enzymes.

This resulted in a ligation product with the following in 5' to 3' orientation: a Nco1 site, a 2.86 kb arm of homology, a BsaA1 site, a EcoRV site, a lox P site, 452 bp of homology, exon 1 of *Npnt* (182bp), 119 bp of homology, and a EcoR1 site. This ligation product was sub-cloned into pCR4 TOPO, excised with Nco1 and Not1, and blunted with T4 polymerase. The polished restriction fragment was then ligated into the vector pL451 so that the loxP site within the arm and the vector were in the head to tail orientation. This resulted in a construct which consisted of the 5' arm sitting 3' from the neomycin resistance gene, which is flanked with frt sites and driven under the duel Eukaryote/Prokaryote promoters PGK/EM7. The 3' arm fragment was then introduced 5' of the neomycin resistance gene via blunt end ligation. This construct was cut with the restriction enzymes PshA1 and Kpn1 resulting in a 7kb fragment, the targeting cassette (Fig 3.1C).

Recombineering, creation of the *Npnt* floxneo targeting construct

The *E.coli* strain DH10B, containing BAC 273P10, was transformed with plasmid pBAD-ETγ, containing the phage genes *t-recE*, *recT* and *redγ* (Zhang et al., 1998). Transformed bugs were made competent for electroporation in the following manner. Cells were grown in 200 mL Luria broth (LB) until reaching an OD_{600} 0.1. At this point 2 ml 10% L (+) arabinose was added to the culture and incubation proceeded until cells reached an OD_{600} 0.4. Cells were divided into two 100 ml portions and spun at 6000 rpm for 15 min, resuspended in 45 mL ice cold ddH_2O and spun again at 6000 rpm. This was repeated two times more and the cells were finally resuspended in 700 mL of 10% glycerol.

To 50 mL of competent cells, 100 ng of the targeting cassette was added and electroporated using the following conditions: 1.75 kV, 25mF, 200 Ω.
Directly afterward, 1 ml of LB was added and the cells were allowed to incubate for 1 hour at 37 °C. The cells were then plated on LB plates containing 50 mg/mL kanamycin and 100 mg/mL chloramphenicol. Colonies were picked and grown in 5ml LB liquid media contain antibiotics at the above concentrations.
BAC DNA was isolated from each of the cultures in the following manner: Cells were pelleted from 2mL cultures and re-suspended in 250 μL P1 buffer from QIAGEN followed by 250 μL P2 and 350 μL P3. The tubes were then spun at 15,000 rpm for 5' and the supernatant was removed to a fresh 1.5 mL tube. The DNA was precipitated with one volume isopropanol at room temperature for 10 minutes and the pellet was washed once with 100 μL 70% Ethanol.
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 ES cells.

Transfection and ES cell culture

Transfection and ES cell culture was carried out by the Transgenic/Targeted Mutagenesis Core, University of California, San Francisco, CA.

Analysis of neomycin resistant clones for homologous recombination.

To verify homologous recombination we used TaqMan (Applied Biosystems) real-time quantitative PCR to determine loss of native copy number. The strategy was based on (Valenzuela et al., 2003) and involved designing specific PCRs, for the site of insertion for the neomycin resistance gene and the lox P site. These PCRs were designed

to work in the wild-type allele and fail in the mutated allele. By comparing the Ct value for the specific PCRs with the Ct values of the reference PCRs, we were able to determine potential candidate clones based on the ΔCt. Clones which were identified in a first round screen were then subject to a second round of PCRs done in quadruplicate. Those clones that passed the second round were then subjected to a third round of PCRs with a second specific PCR, for the loxP site. Finally, those clones that passed the PCR screen were assessed for loss of copy number by quantitative Southern hybridization, using densitometry.

***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% PFA/PBS overnight at 4° C and cryosectioned at 14 μm. Analysis of gene expression using in situ hybridization with RNA probes was carried out 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).

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) (Brandenbergger et al), 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) (Cosmo Bio. LB-1403), anti-pax2 (1:100) (Covance PRB-276P). Confocal imaging was performed on a Zeiss LSM 5 Pascal.

Results

Generation of a BAC containing a conditional allele for nephronectin

The targeting of the nephronectin locus proved difficult. Use of conventional sized vectors did not result in positive targeting of the locus in ES cells despite the use of two separate targeting vectors, the screening of several thousand clones and the use of four different ES cell lines. For this reason we undertook an unconventional approach to targeting the nephronectin locus. The approach was based on the use of large constructs derived from BAC DNA(Valenzuela et al., 2003). To create a BAC containing a floxed exon of nephronectin, we first cloned a targeting cassette containing at its core a floxed copy of the first exon of the nephronectin gene and a neomycin resistance gene flanked by *frt* sites (see material and methods and Fig. 3.1C). This targeting cassette was introduced into a BAC that contained the first eleven exons of nephronectin (Fig. 3.1A,B). Using this engineered BAC we successfully targeted the nephronectin locus in ES cells, obtaining a number of positive clones (Fig. 3.1F). Two of these clones where injected into C57BL/6 blastocyst to generate chimeric animals. Germline transmission generated mice that contained the $Npnt^{floxneo}$ allele (Fig. 3.1D) By crossing animals heterozygous for this allele with animals expressing CRE recombinase under the control of the β-actin promotor (Lewandoski et al., 1997) we obtained progeny that carried the $Npnt^{\Delta ex1}$ allele, in which the first exon of nephronectin had been excised by CRE-mediated recombination, without deletion of the neomycin resistance gene cassette (Fig. 3.1E). Animals homozygous for $Npnt^{\Delta ex1}$ express neither nephronectin protein nor *Npnt* RNA (Fig. 3.1G, H), demonstrating that $Npnt^{\Delta ex1}$ is a null allele.

Mice lacking nephronectin display renal agenesis at birth

Mice homozygous for the *Npnt*$^{\Delta ex1}$ allele, 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. 3.2A-C). The remaining *Npnt* mutants had two kidneys (31%), on average resulting in 58% agenesis (see legend to Fig. 3.2F). In 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. 3.2A, B). Histological analysis revealed variability in development, with most *Npnt* mutant kidneys containing essentially normal nephrogenic regions (Fig. 3.2D, 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 ureteric bud had formed and appeared similar in *Npnt* mutants (n=2) and their wild-type littermates (Fig. 3.3A,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 whole mount or serial section (n=12), showed any evidence of UB invasion of the MM or branching, on either side of the embryo (Fig. 3.3C,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 3 rounds in their wild-type littermates (Fig. 3.3E-G).

To determine whether the UB branching observed was sufficient to promote nephrogenesis, we analyzed kidney development at E13.5. In 2 of 4 *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 (5 out of 8 kidneys expected did not form), whereas kidneys were developing on both sides in 4 of 4 wild-type littermates (no agenesis) (Fig. 3.3H-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. 3.3F). In 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. 3.3G). 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 basal lamina and other matrix constituents that surround the epithelial structures. We therefore were interested in determining if the basement membranes 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. 3.4A-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. 3.4G-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. 3.4K,L). From these results, we conclude that the basement membrane is comparatively normal in *Npnt* mutants.

Expression of *Gdnf* is reduced in nephronectin null embryos

The above results suggested that, rather than insuring 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. 3.5A-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, the level of *Gdnf* expression in the adjacent limb bud was comparable in the mutant and control embryos (Fig. 3.5A, 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. 3.5C-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. 3.5J-M) and in a mutant embryo in which kidney development was observed at E13.5 (Fig. 3.5N-Q). 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.

Discussion

Nephronectin is a critical 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 a number of other tissues during development, preliminary analysis has shown that all organs except the kidney appear grossly normal in *Npnt* mutants at birth. Consistent with this finding, *Npnt* mutants that survive beyond birth are healthy, 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 also known as EGFL6) (Buchner et al., 2000), which shares 41% oveall 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 if 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). 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 critical 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% vs. 83% agenesis, respectively (Muller et al., 1997). 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. In 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 (unpublished results). 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 clue to the role for nephronectin function in the developing kidney

In order to gain some insight into the molecular basis of the Npnt mutant phenotype, we assessed the expression of a number of genes known to have roles in the developing kidney. At E11.5, the time when Npnt mutant phenotype is completely penetrant, we find that the expression of *Gdnf* is down in *Npnt* mutant embryos compared to wild-type controls. GDNF is a factor with an essential role in the developing kidney. It is the mesenchymal factor that is thought be to the primary inducer of budding from the ND. This result is interesting because GDNF is not expressed in the same cells that express nephronectin, which is expressed in the epithelium of the UB. This means that *Gdnf* expression is being affected by the absence of nephronectin in a cell-nonautonomous fashion. Just as intriguing, we have found that the reduction of *Gdnf* expression in the

MM is transient, being relatively normal at E10.5, down at El1.5, and normal at E13.5 in mutants that have overcome the delay and invaded the MM. Because the data presented above strongly suggest that the early phenotype seen in *Npnt* mutants is the same as that of *Itga8* mutants, we conclude that nephronectin is responsible for mediating integrin α8β1 function. Therefore, the gene expression data point to a possible role in *Gdnf* regulation for nephronectin and α8β1 integrin.

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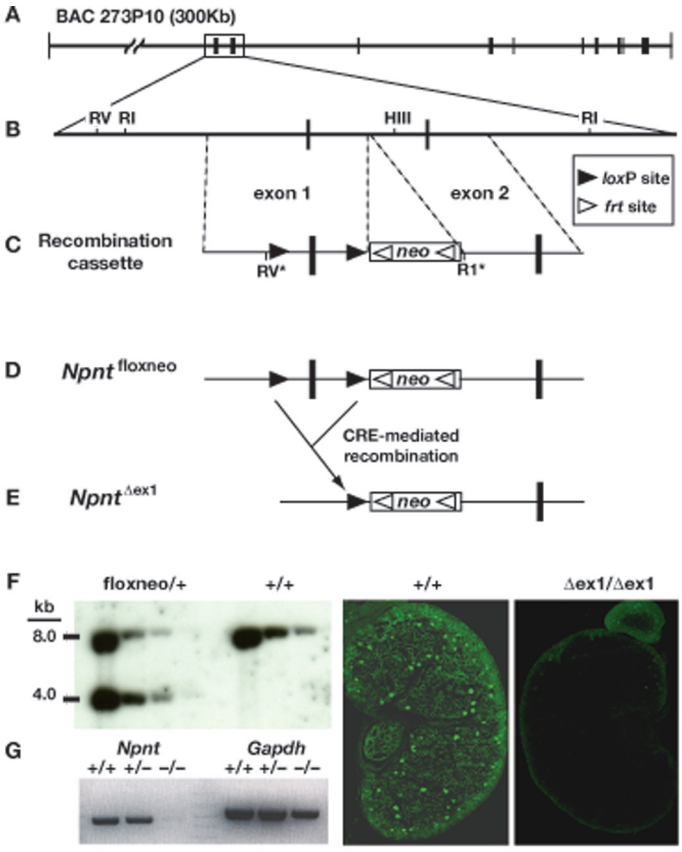
Fig. 3.1 Generation of a *Npnt* null allele

(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 (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 EcoRI digest produces an 8kb wild-type and a 4kb mutant band. Each clone is represented by a series of three, four-fold 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 carried out 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 (RT -) 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).

Fig. 3.2 Renal agenesis in *Npnt* null mice

(A-C) Urogenital tracts from newborn female littermates, shown in whole mount. (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 and 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 kidneys, unilateral or bilateral kidney agenesis. The percentage agenesis was determined by dividing the number of kidneys (expected [2 per animal] – observed) by the number of kidneys expected. Abbrevations: Ad, adrenal gland; Bl, bladder; DA, dorsal arota; Ki, kidney; Ur, ureter; Ut, uterus.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B
RV RI
HIII
RI
exon 1
exon 2
loxP site
frt site
C
Recombination cassette
neo
RV*
R1*
D
Npnt floxneo
neo
CRE-mediated recombination
E
Npnt Δex1
neo
F
floxneo/+
+/+
+/+
Δex1/Δex1
kb
8.0
4.0
G
Npnt
Gapdh
+/+ +/− −/−
+/+ +/− −/−

Fig. 3.1

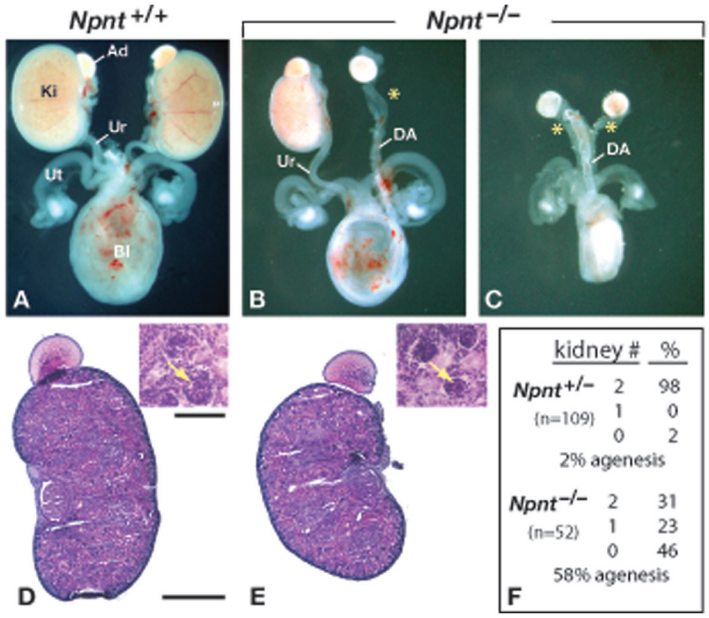
Npnt +/+
Npnt −/−
Ad
Ki
Ur
Ut
Bl
A
DA
Ur
B
DA
C
D
E
kidney #
%
Npnt+/−
(n=109)
2
98
1
0
0
2
2% agenesis
Npnt−/−
(n=52)
2
31
1
23
0
46
58% agenesis
F

Fig. 3.2

Fig. 3.3. Developmental origin of renal agenesis in *Npnt* null mice

(A-G) Embryos at the stages indicated, immunostained in whole mount for Calbindin. (A) In the $Npnt^{+/+}$ embryos, the ureteric bud (arrowhead) has invaded the metanephric mesenchyme. (B) In the $Npnt^{-/-}$ embryo the ureteric bud (arrowhead) is similar to that in the wild-type embryo. (C) In the $Npnt^{+/+}$ embryo the ureteric bud has branched (open arrowheads). (D) In the $Npnt^{-/-}$ embryo the ureteric bud (arrowhead) has not extended into the MM (asterisk) or branched. (E) In the $Npnt^{+/+}$ embryo the ureteric bud has undergone several rounds of branching. (F,G) $Npnt^{-/-}$ embryos, showing the variable extent of branching at E12.5. **(H-J)** Transverse sections through E13.5 $Npnt^{+/+}$ and $Npnt^{-/-}$ kidneys. Panels I and J show the 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 (arrow**s**). **(K')** Boxed region in (K) is shown at higher magnification. Dotted line demarcates the MM. All scale bars, 100 μm.

Abbreviations: Go, gonad; In, intestine; MM, metanephric mesenchyme; UB, ureteric bud.

Fig. 3.4. 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.

Abbreviations: ND, nephric duct; UB, ureteric bud.

Fig. 3.3

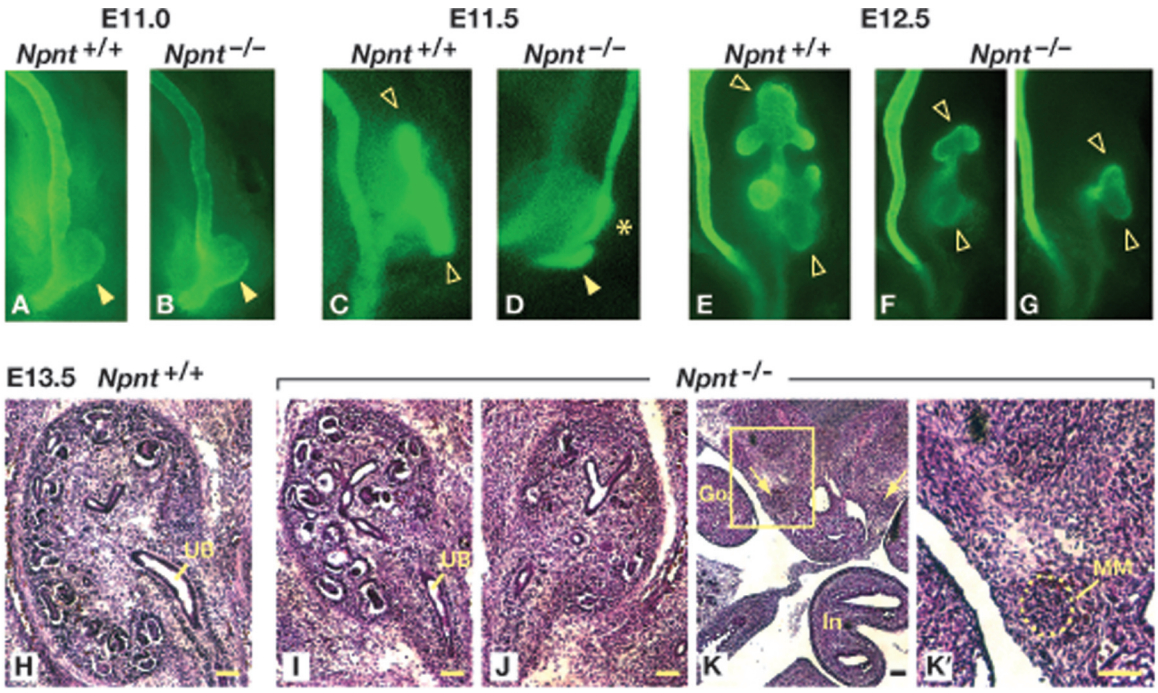
E11.0
E11.5
E12.5
Npnt +/+
Npnt −/−
Npnt +/+
Npnt −/−
Npnt +/+
Npnt −/−
A
B
C
D
E
F
G
E13.5 Npnt +/+
Npnt −/−
UB
UB
Go
In
MM
H
I
J
K
K'

Fig. 3.4

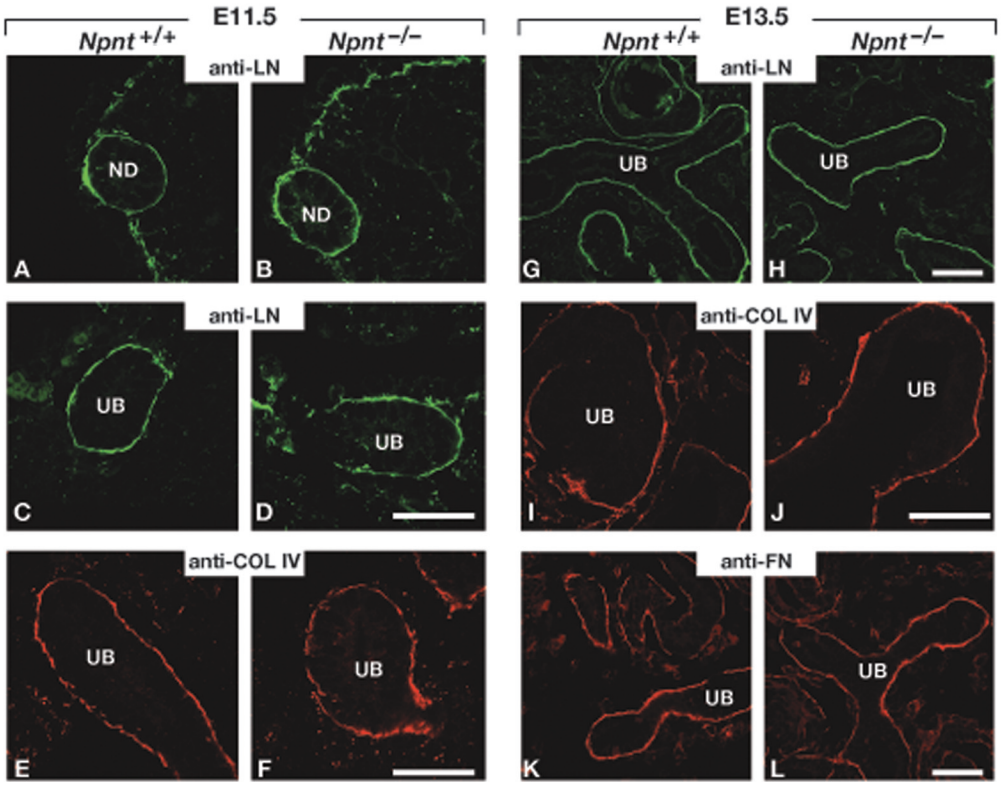
E11.5
E13.5
Npnt +/+
Npnt −/−
Npnt +/+
Npnt −/−
anti-LN
anti-LN
ND
ND
UB
UB
A
B
G
H
anti-LN
anti-COL IV
UB
UB
UB
UB
C
D
I
J
anti-COL IV
anti-FN
UB
UB
UB
UB
E
F
K
L

Fig. 3.5. *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-F) Expression at E11.5 of the genes indicated, as detected 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.

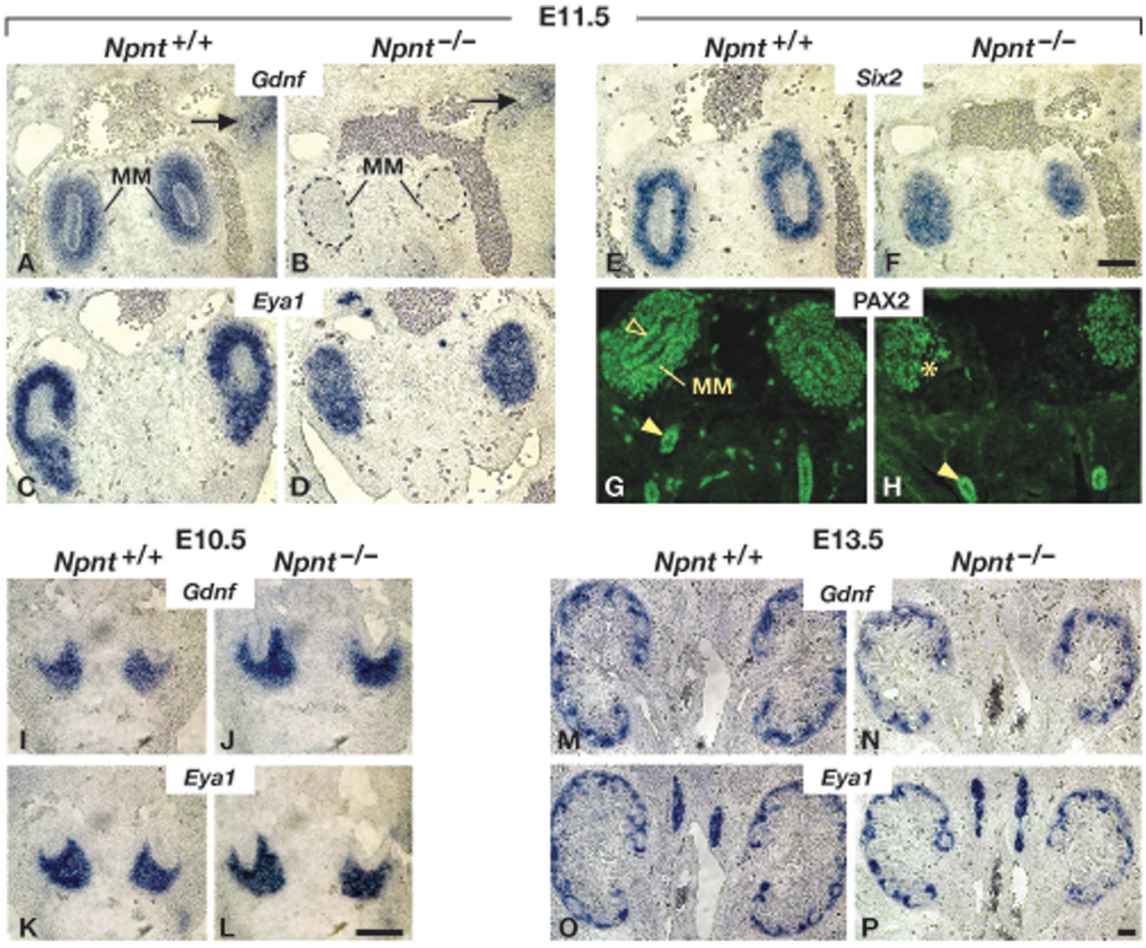
E11.5
Npnt +/+
Npnt −/−
Gdnf
MM
A
B
Six2
E
F
Eya1
C
D
PAX2
G
H
E10.5
I
J
K
L
E13.5
M
N
O
P

Fig. 3.5

Chapter 4

α8β1 integrin stimulates the expression of *Gdnf* in the metanephric kidney

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Abstract

Embryos that do not express α8 integrin subunit (*Itga8*), or its ligand nephronectin (*Npnt*) display an invariant lack of invasion of the ureteric bud into the metanephric mesenchyme during kidney development. Subsequently, both mutant mice demonstrate a high degree of kidney agenesis at birth. The function of α8β1 integrin during the early events of kidney development is unknown. The finding that *Gdnf* expression is reduced in the un-invaded metanephric mesenchyme of *Npnt* mutant mice has provided a clue into a possible role for α8β1 integrin in the developing kidney. Here we show that *Gdnf* expression is also reduced in *Itga8* mice and, like in *Npnt* mutants, the reduction in *Gdnf* expression is transient. Additionally, we provide genetic evidence that this reduction is sufficient to explain the agenesis and hypoplasia observed in both mutants. Our results thus place nephronectin and α8β1 integrin in a pathway that regulates *Gdnf* expression and is essential for kidney development.

Introduction

Organogenesis depends on highly refined communication between cells. Refinement depends on both the temporal and quantitative nature of the signal (Eldar et al., 2004; Holtzer, 1968; Lander, 2007). Examples of this are found in the developing metanephric kidney, where signaling is carried out between two tissues with a common origin from the intermediate mesoderm. In the initiating event in metanephric development, a distinct population of mesenchymal cells, the metanephric mesenchyme (MM), signals to an epithelial structure the nephric duct (ND). This results in the formation of the ureteric

bud (UB). Once the UB has invaded the MM, it in turn signals back to the MM, providing instructive signaling that results in the initiation of a differentiation program within the MM, which will give rise to the secretory epithelium of the nephron (Dressler, 2006; Saxen, 1987). The elicitation of the UB by the MM in now known to be largely mediated by glial cell line derived growth factor, GDNF (Costantini and Shakya, 2006; Sainio et al., 1997). The GDNF signal is transduced in the epithelial cells of the UB through a receptor complex, which results in the expression of genes involved in positive and negative feedback in response to GDNF (see chapter 1 for detailed discussion). The timing, quantity and spatial distribution of GDNF provided to the ND by the MM are critical to the formation of the UB (Grieshammer et al., 2004; Srinivas et al., 1999). While the mechanism of Gdnf expression in the MM is incompletely understood and is currently being worked out, factors expressed by these cells insure the proper temporal, quantitative and spatial expression of *Gdnf* (Brodbeck and Englert, 2004).

Previously, it has been shown that α8 integrin subunit (*Itga8*) is expressed throughout the nephric cord, including the MM (Muller et al., 1997). Additionally, it was demonstrated that loss of α8 integrin subunit function invariably results in a delay of invasion of the MM by the ureteric bud (UB), which in turn results in a high frequency of kidney agenesis. The molecular mechanism by which α8β1 integrin function in the MM influences the UB has yet to be determined. The finding that, at E11.5, *Gdnf* expression is reduced in the MM of embyros lacking a ligand for α8β1 integrin, nephronectin (*Npnt*) provides a clue into this function. This result is particularly insightful considering *Npnt* mutant mice display a similar phenotype to *Itga8* mutant mice (see chapter 3).

Here we demonstrate that, like *Npnt* mutants, *Gdnf* expression is reduced in *Itga8* mutants at the time when the invasion of the MM by the UB is delayed. Additionally, 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

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/phosphate buffer saline (PFA/PBS) overnight at 4° C and cryosectioned at 14 μm. Analysis of gene expression using in situ hybridization with RNA probes was carried out 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 newborns 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.

Penetrance Experiments.

Mice carrying a *Gdnf* null allele, $Gdnf^{lacZ}$ (Moore et al., 1996) were crossed to mice heterozygous for a null allele of *Itga8* (Muller et al., 1997) to create compound heterozygous progeny that were scored for kidney development at birth. Compound heterozygous progeny from this cross were used in subsequent experiments to assess kidney development at birth in *Itga8* homozygous mutant mice with one copy of the $Gdnf^{lacZ}$ allele. Agenesis is reported as a percent difference of the observed number of kidneys to the expected number and was determined using the following equation:

(Expected [2 per animal] – Observed)/Expected

Significance was determined using Student's t-test with n as the number of kidneys and the mean as the percentage of agenesis. For rescue experiments mice hetereozygous for a null allele of *Spry1* (Basson et al., 2005) were crossed to mice heterozygous for the *Itga8* null allele to create compound heterozygous mice. Compound heterozygous mice were then crossed to each other and the offspring were scored for kidney development at birth. Agenesis is reported as above and significance was determined using Student's t-test as above.

Results

***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 other genes expressed in the MM, including the signaling molecule genes *Bmp4*, *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. 4.1A-D and data not shown). Again, *Gdnf* mRNA was not detected in the MM, but *Gdnf* expression appeared comparable in the adjacent limb buds of *Itga8* mutant and control embryos (Fig. 4.1A-B). Similar results were obtained in numerous independent experiments. However, using a radiolabeled *Gdnf* probe we were able to detect a signal at low level (Fig. 4.2). Additionally, in situ hybridization assays revealed no obvious difference between the *Itga8* mutants and controls at E10.5 and E13.5 (Fig. 4.1E-L), indicating that, as in *Npnt* mutants, a8 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 due to 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, $Gdnf^{\Delta lacZ}$ (Moore et al., 1996). To assess kidney development we scored kidney agenesis as described in materials and methods. 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*$^{tacZ/+}$), we observed 53% agenesis (Table 4.1). 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 4.1). The one kidney that was found in the 11 *Itga8*$^{-/-}$;*Gdnf*$^{tacZ/+}$ animals examined was reduced in size and histological analysis revealed it to be highly dysplastic and most likely non-functional (Fig. 4.3). 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.

Reduction *Spry1* Gene Dosage in *Itga*8 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 were 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 *Itga*8 animals resulted in a significant rescue of

kidney development: 25% agenesis in *Itga8*$^{-/-}$;*Spry1*$^{+/-}$ animals (n=20) vs. 71% agenesis in their *Itga8*$^{-/-}$;*Spry1*$^{+/+}$ littermates (n=14). Furthermore, no kidney agenesis was observed in animals homozygous for both the *Itga8* and *Spry1* null alleles (n=8) (Fig. 4.4A). These data provide strong support for the hypothesis that the kidney agenesis that results from the absence of the α8 integrin subunit is due to 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 ureteric bud 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. 4.4B-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 were due to 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 to that in their control (*Itga8*$^{+/-}$; *Spry1*$^{+/-}$) littermates (Fig. 4.2D,E). In contrast, *Eya1* expression was similar in the rescued *Itga8* mutants and their control

littermates (Fig. 4.4F,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. 4.1B), most likely because the presence of the UB in the rescued mutants produces signals that enhance *Gdnf* expression (Majumdar et al., 2003).

In addition to the early phenotype involving a delay in UB invasion at E11.5, *Itga8* mutant embryos that overcome the delay at E13.5 also demonstrate a branching phenotype. *Gdnf* expression appears relatively normal at this stage (Fig. 4.1I,J), so the molecular basis of this late phenotype remains obscure. However, we find that the kidney size of rescued *Itga8* mutant neonates is similar to controls (Fig. 4.5). This suggests that Sprouty1 may have a role in attenuating a receptor tyrosine kinase involved in the molecular basis of the late phenotype.

Discussion

Regulation of *Gdnf* expression via an integrin interaction with the ECM

We have presented data that support a role for α8β1 integrin and its ligand nephronectin in regulating the expression of *Gdnf*, an essential growth factor, in the developing kidney. 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 find that the UB has not invaded the MM. We have demonstrated that *Itga8*;*Gdnf* compound heterozygotes display kidney agenesis at a five-fold 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 the genetic data provide evidence that α8β1 integrin and GDNF are functioning in a common pathway and suggest that α8β1 integrin, via its recognition of nephronectin, plays a role in regulating the expression of *Gdnf*.

A transient requirement for α8β1 integrin and nephronectin in *Gdnf* expression

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.

A specific requirement for α8β1 integrin and nephronectin in *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 to 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.

Other roles for α8β1 integrin in the metanephric kidney

Does α8β1 integrin have essential roles in the early events of metanephric development other than the regulation of *Gdnf* expression in the MM? Integrins are known regulators of ECM remodeling and deposition (ffrench-Constant and Colognato, 2004; Humphries et al., 2004). Given that the *Itga8* mutant phenotype involves the lack of elongation of the UB into the MM, an obvious explanation may involve matrix remodeling or integrity. We have presented data that do not support this idea as an explanation in the *Npnt* mutant (see chapter 3). More significantly, we have looked at laminin distribution around the UB in *Itga8* mutant embryos at E11.5 and do not find any differences in staining patterns between the mutant and controls (unpublished). This result supports the idea that, as in *Npnt* mutant embryos, the basement membrane is intact in *Itga8* mutant embryos at the time when the phenotype is completely penetrant.

Furthermore it does not support the idea that this integrin has a role in matrix integrity, which is independent from its function regulating *Gdnf* expression via recognition of nephronectin, at E11.5.

While we have not assessed the basement membrane in *Itga8* mutants that have overcome the delay at E13.5, the rescue of the *Itga8* phenotype through the reduction of *Spry1* gene dosage results in kidneys that are indistinguishable from controls (Fig.4.5), suggesting this integrin does not have an essential role in the maintenance of matrix integrity during subsequent branching morphogenesis. Indeed, it is challenging to explain the rescue data in terms of any defect in the ECM. This result does not seem to fit with the observations that indicate *Itga8* mutant mice have a branching phenotype not demonstrated by *Npnt* mutant mice. One explanation for the branching phenotype of *Itga8* mice may involve the disruption of communication between the UB and the MM. While *Gdnf* expression is clearly on in *Itga8* mutants that have overcome the delay (Fig. 4.1I,J), robust expression of *Gdn*f may require multiple signaling pathways at this time. It is conceivable that α8β1 integrin may still have a role in its expression via the recognition of ligands other than nephronectin. A testable hypothesis that emerges from these considerations is that a reduced amount of *Gdnf* expression allows for limited branching by the UB, but is insufficient to activate key, GDNF dependent, signaling from the UB to the MM that instruct it to condense and differentiate. Confirmation of this hypothesis will require the quantitative assessment of *Gdnf* expression in *Itga8* mutant embryos that overcome the delay.

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Fig. 4.1. *Gdnf* expression is reduced in the *Itga*8 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-H) Expression at E10.5 and (I-L) at E13.5 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 bar, 100 μm. Abbreviations as in previous figures.

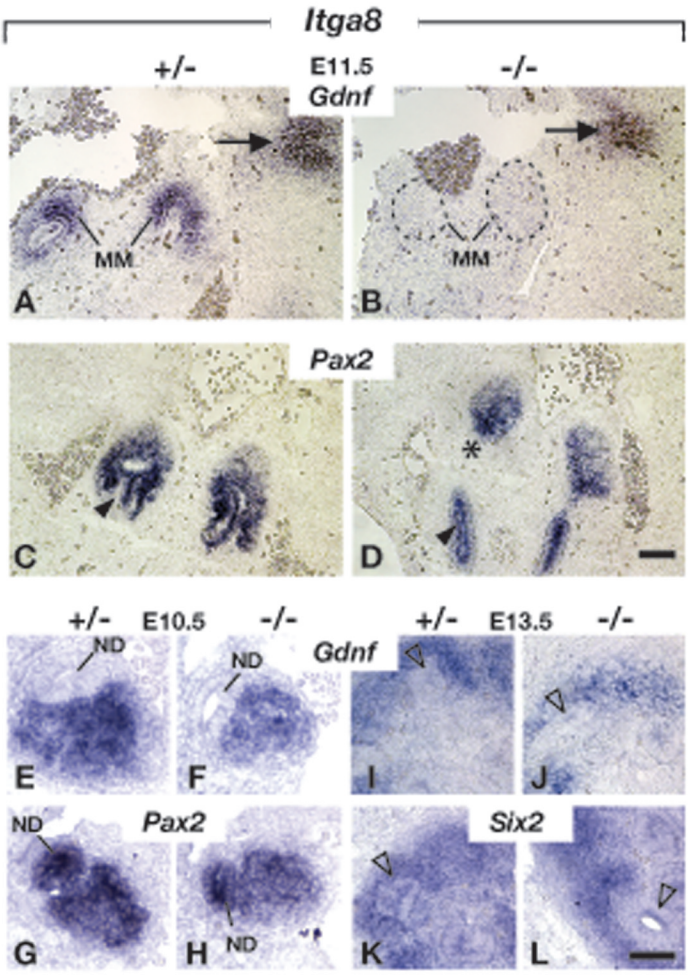
Itga8
+/-
E11.5
-/-
Gdnf
MM
MM
A
B
Pax2
C
D
+/-
E10.5
-/-
+/-
E13.5
-/-
ND
ND
Gdnf
E
F
I
J
ND
Pax2
ND
Six2
G
H
K
L

Fig. 4.1

Fig. 4.2. *Gdnf* expression is not extinguished in the *Itga8* null embryonic kidney at E11.5. (A-F) Transverse sections through *Itga8* $^{+/-}$ and *Itga8* $^{-/-}$ embryos. (A-C) α–[^{35}S]-CTP, UTP *in situ* hybridization at E11.5 for *Gdnf* in *Itga8* $^{+/-}$. Section A is anterior, B is medial and C is posterior. Note expression in hind limb (arrowheads) and metanephric mesenchyme (arrows). (D-F) α–[^{35}S]-CTP, UTP *in situ* hybridization at E11.5 for *Gdnf* in *Itga8* $^{-/-}$. Section D is anterior, E is medial and F is posterior. Note the comparable expression of *Gdnf* in the hind limb (arrowheads) and reduced, but not absent, expression in the metanephric mesenchyme of the *Itga8* null embryonic kidney (arrows).

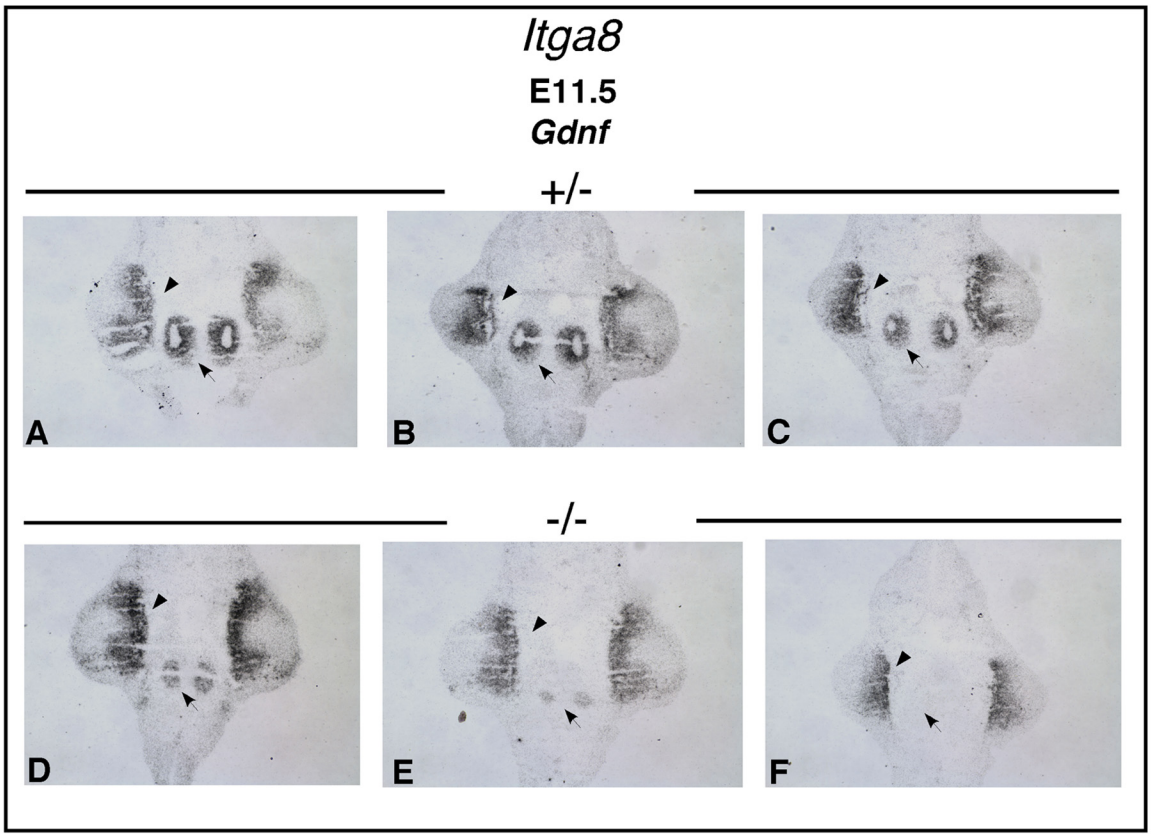

Fig. 4.2

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

n	Genotype	No. 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*$^{+/-}$ x *Gdnf*$^{+/-}$ and BOTTOM: *Itga8*$^{+/-}$ x *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. 3.2F. Significance was determined using Student's t-test with n as the number of kidneys and the mean as the percentage of agenesis.
** $p < 0.005$.

Fig. 4.3. Reduction of *Gdnf* gene dosage in *Itga8* heterozygous and mutant neonates results in renal dysplasia. (A-C) Medial sections from kidneys of newborn mice. (A) Medial section from *Itga8* $^{+/+}$; *Gdnf* $^{+/-}$ newborn kidney. (B) Medial section from *Itga8* $^{+/-}$; *Gdnf* $^{+/-}$ newborn kidney. (C) Medial section from *Itga8* $^{-/-}$; *Gdnf* $^{+/-}$ newborn kidney. Note the trend in (A-C) of the reduction in overall kidney size. (Scale bar, 250 μm) **(A'-C')** Higher magnification of (A-C) showing the nephrogenic region of the developing kidney (indicated with arrows). (A') Nephrogenic region of *Itga8* $^{+/+}$; *Gdnf* $^{+/-}$ newborn kidney. (B') Nephrogenic region of *Itga8* $^{+/-}$; *Gdnf* $^{+/-}$ newborn kidney. Note the mild dysplasia. (C') Equivalent region of *Itga8* $^{-/-}$; *Gdnf* $^{+/-}$ newborn kidney. Note the complete lack of nephrons (arrows) and dramatic increase in dysplasia (arrowhead).

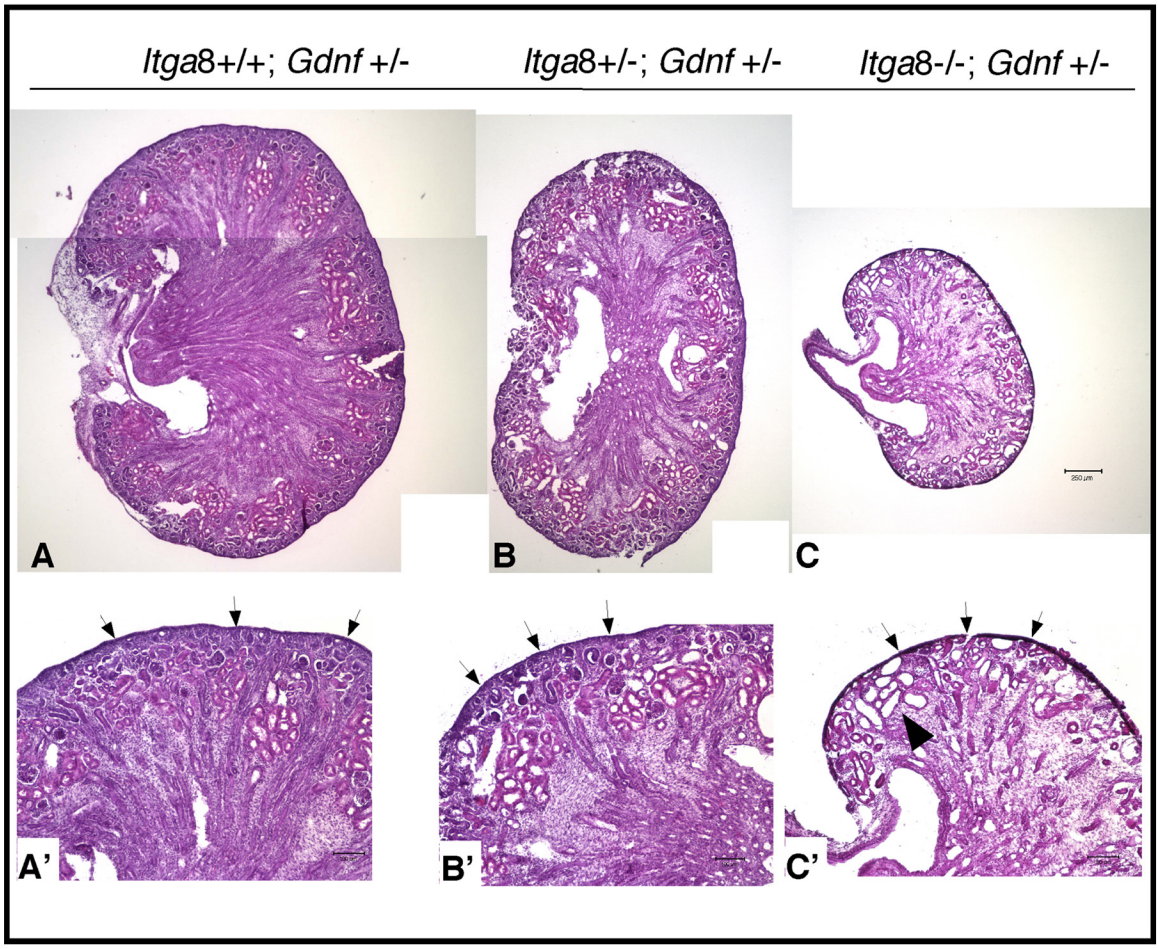

Fig. 4.3

Fig. 4.4. 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*$^{+/-}$ vs. *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 *Itga*8$^{-/-}$; Spry1$^{-/-}$ animals that demonstrated a duplicated ureter phenotype did not appear to differ from the proportion of their Spry1$^{-/-}$ littermates displaying that phenotype. **(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 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.

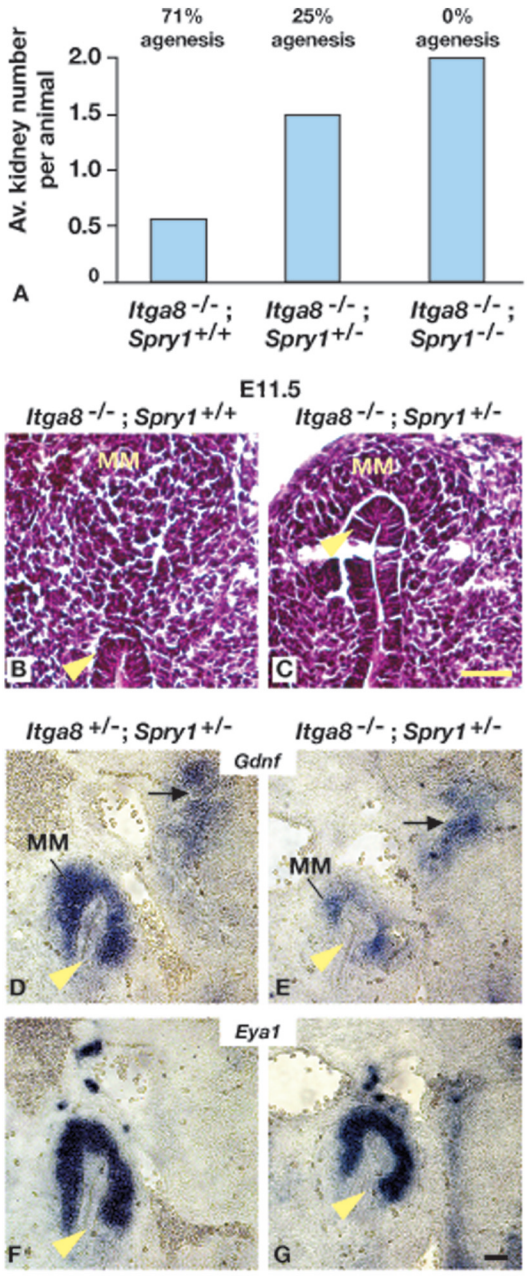
71% agenesis
25% agenesis
0% agenesis
Av. kidney number per animal
2.0
1.5
1.0
0.5
0
A
Itga8 -/- ; Spry1 +/+
Itga8 -/- ; Spry1 +/-
Itga8 -/- ; Spry1 -/-
E11.5
Itga8 -/- ; Spry1 +/+
Itga8 -/- ; Spry1 +/-
MM
B
C
Itga8 +/- ; Spry1 +/-
Itga8 -/- ; Spry1 +/-
Gdnf
MM
D
E
Eya1
F
G

Fig. 4.4

Fig. 4.5. Reduction of *Spry1* gene dosage in *Itga8* mutant mice results in normal kidney size at birth. (A-B) Medial, sagittal sections from kidneys of newborn mice. (A) Medial, sagitttal section from *Itga8* $^{+/-}$; *Spry1* $^{+/-}$ newborn kidney. (B) Medial, sagittal section from *Itga8* $^{-/-}$; *Spry1* $^{+/-}$ newborn kidney. Note the similar histoarchitecture of the control and rescued kidney. **(C-D)** Cross sections of kidneys in (A-B). (C) Cross section from *Itga8* $^{+/-}$; *Spry1* $^{+/-}$ newborn kidney. (D) Cross section from *Itga8* $^{-/-}$; *Spry1*$^{+/-}$ newborn kidney (Scale bar, 250 μm).

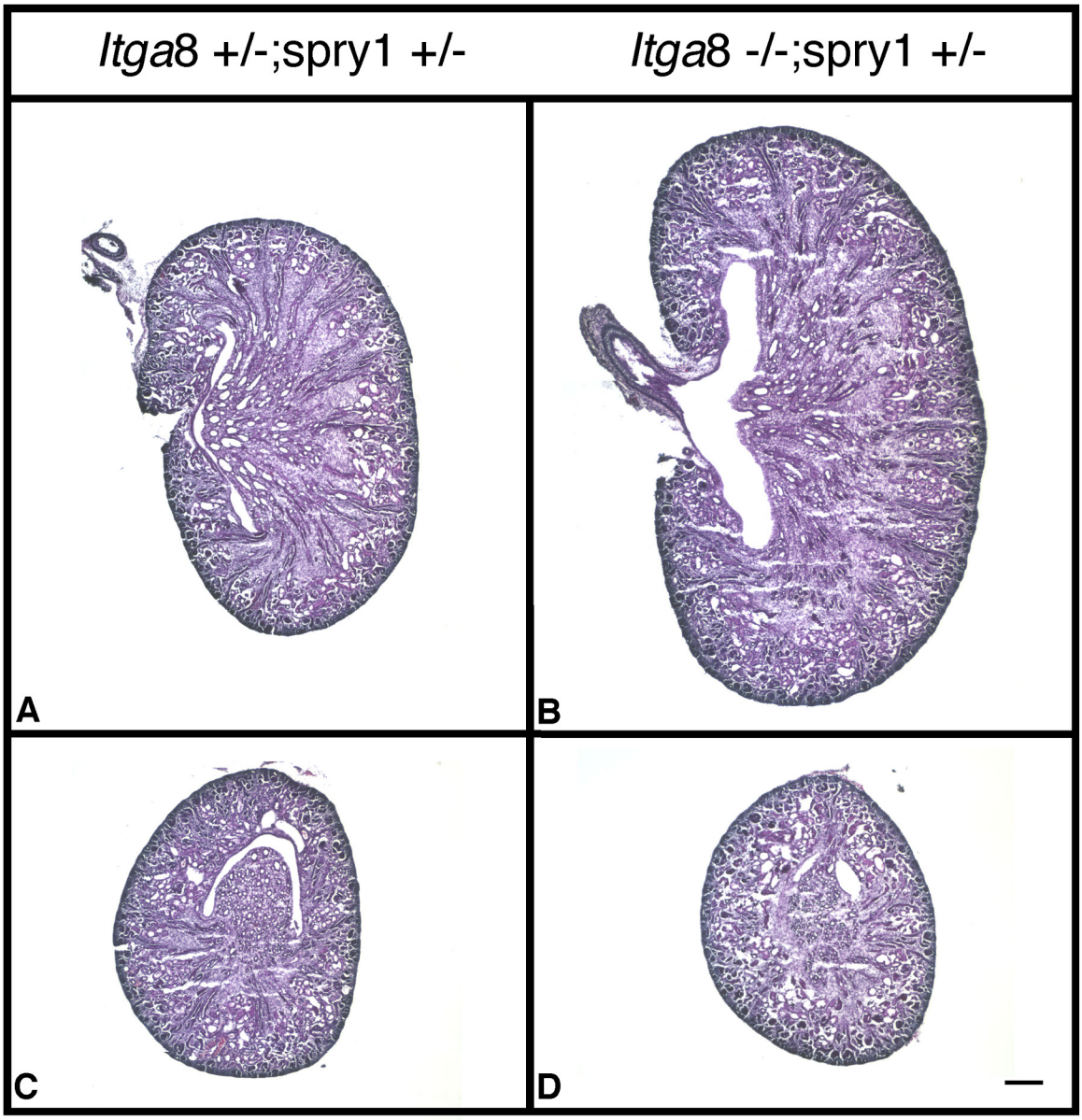

Fig. 4.5

Chapter 5

Preliminary experiments examining the mechanism of α8β1 integrin and nephronectin function

The data presented in this chapter are unpublished.

Abstract

We have found that the extracellular matrix protein nephronectin and its receptor, α8β1 integrin, have essential roles in the early events of metanephric development through the regulation of *Gdnf* expression. Here we present preliminary evidence that α8 integrin, *Itga8*, mutants have decreased levels of activated Erk in the metanephric mesnechyme and that α8β1 integrin can affect a transcriptional regulator of *Gdnf* expression, eyes absent homolog 1, EYA1, a know MAP kinase target. When cells express α8β1 integrin, nuclear localization of a transcriptional complex including EYA1 is found to increase. In addition, we show that the increase in nuclear localization can be abolished with an inhibitor of MAP kinase kinase, MEK. Because nuclear translocation of the transcriptional complex that includes EYA1 is thought to be an indicator of activation, these results hint at the possibility that α8β1 integrin regulates *Gdnf* expression through EYA1 activation.

Introduction

Both α8β1 integrin and its ligand, nephronectin, are essential for proper kidney development (Muller et al., 1997 and chapters 3). Through the recognition of its ligand, nephronectin, α8β1 integrin regulates the expression of glial cell line derived neurotrophic factor, GDNF in the metanephric mesenchyme, MM, of the developing kidney, (see chapter 4). While we have established this on the basis of genetic evidence, we do not have a mechanistic insight on how the regulation is taking place.

How might an integrin and its ECM ligand regulate *Gdnf* expression? Integrins are classically known as adhesion receptors, which have been shown to have 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).

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 developing kidney, α8β1 integrin activates the MAPK cascade in the MM. 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.

Recent data from genetic studies have identified some of the factors involved in such a transcriptional network (Brodbeck and Englert, 2004). One member of this network that has been shown to have a dramatic impact on *Gdnf* expression is the transcriptional co-activator, eyes absent (*eya*) ortholog 1, EYA1. Mice deficient for *Eya1* do not form a UB due to the absence of *Gdnf* expression and demonstrate bi-lateral kidney agenesis at nearly complete penetrance (Xu et al., 1999). While EYA1 does not have the capacity to bind DNA itself it has been shown to form a complex and translocate to the cell nucleus with SIX proteins that contain homeodomains and whose genes are *sine oculis* orthologs such as *Six1* and *Six2* (Ohto et al., 1999). Significantly, the EYA1/SIX1 complex has been shown to recognize a Gdnf regulatory region (Li et al., 2003). Additionally, *eya*

has been shown to be a target of the MAPK pathway. In the fly, in vivo genetic evidence demonstrates that *eya* activation is dependent on phosphorylation by Erk (Hsiao et al., 2001). From the above a hypothesis can be proposed that places α8β1 integrin upstream of the activation of EYA1 in the MM. Below we present preliminary data from experiments designed to test this hypothesis.

Material and Methods

Expression constructs

For the EYA1V5-His fusion protein, a 1.7 kb PCR product consisting of the *Eya1* open reading frame, ORF, and derived with the primers (below) from IMAGE clone ID 6848408.

'5-CCCGTCGACGCCACCATGGAAATGCAGGATCTAACCAG-3'

'5-CAGGTACTCTAATTCCAAGGCATGATG-3'

This product was cloned into pEF6/V5-His-TOPO (Invitrogen, Carlsbad, CA). The entire insert was verified by sequencing (Fig. 5.2A).

For the SIX1V5-His fusion protein, a 0.858 kb PCR product consisting of the *Six1* ORF and derived with the primers (below) from IMAGE clone ID 4188451.

'5-GCCAGCCATGCTGATGCTGCCGTCGTTTG-3'

5'-GGAACCCAAGTCCACCAAACTGGAGGTGAG-3'

This product was cloned into pEF6/V5-His-TOPO (Invitrogen, Carlsbad, CA). The entire insert was verified by sequencing (Fig. 5.2B).

For the untagged SIX1 protein, a 0.850 kb PCR product consisting of the *Six1* ORF in frame with the stop codon UUA and derived with the primers (below) from IMAGE clone ID 4188451.

‘5-GCCAGCCATGCTGATGCTGCCGTCGTTTG-3’

5’-TTAGGAACCCAAGTCCACCAAACTGGAGGTGAG-3’

The product was cloned into pEF6/V5-His-TOPO (Invitrogen, Carlsbad, CA). The entire insert was verified by sequencing (Fig. 5.2C).

Coating of glass coverslips

Fisher brand microscope cover slips (18mm CIR cat. S175223) were treated with nitric acid overnight, wash extensively with ddH_2O and baked at 270°C overnight. Coverlips were then coated with 0.05% Poly-L-lysine (Sigma, St. Louis, MO) at room temp for 1hr. Coverslips were washed 4 times with ddH_2O and then coated with one of the following protein substrates at 10 ug/ml in PBS, neph251-561 (Fig. 2.2B), neph251-381 (Fig. 2.2C), GST, or Fibronectin (Human full-length) (Invitrogen, Carlsbad, CA). Proteins were allowed to absorb to the glass overnight at 4°C.

Cell culture and transfection

K562 cells expressing α8β1integrin (KA8) and parental K562 cells where plated onto 10cm dishes in RPMI without serum. Cells at 90% confluency where transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with expression constructs; pEF6*Eya1*V5/His, pEF6*Eya1*V5/His, pEF6*Six1*stop. Cells were allowed to recover overnight. Transfected cells were then plated onto glass coverslips, coated as described,

above, in 12 well plate ($7.0X10^5$ – $1.0X10^6$ cells per well). Cells were allowed to adhere overnight, ~16 hours. RPMI without serum was then replaced with RPMI with 10% fetal bovine serum and cells were incubated for another 15 min at 37° C and %5.0 CO_2. Cells were then fixed and stained as described below.

Immunocytochemistry

Cells were fixed on coverslips with 4.0% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15' at room temperature. Coverslips were then washed with PBS and the fixed cells permeablized with 0.2% triton X for 5 min. at room temperature. Cells were incubated with blocking solution (1.0% BSA, 1.0% goat serum in PBS) for 1 hr. at room temperature. Cells were stained with anti-V5 mAb 1:200 (Invitrogen, Carlsbad, CA) in blocking solution overnight at 4 C. Cells were washed twice with PBS for 5 min. at room temperature. Secondary goat anti-mouse conjugated 488 (Molecular Probes, Portland OR) was applied at 1:300 in 1.0% BSA in PBS for 1' at room temperature. Cells were washed twice with PBS for 5 min. at room temperature and stained with DAPI (1:5000 in PBS). Coverslips were mounted with Prolong Antifade Gold (Invitrogen, Carlsbad, CA).

Cell counting

To score cells, seven spots were chosen on each coverslip and fields of cells were imaged using epi-fluorescene under a 20X objective. Fluorescent cells were scored as either demonstrating cytoplasmic and nuclear staining or exclusively nuclear staining (Fig. 5.3A-C). Nuclear localization was determined by co-localization of the fluorescent

signal from the conjugated secondary antibody with a DAPI stain. Coverslips were counted three times, including a blind count. Three separate coverslips were counted for each condition.

Immunohistochemistry

Tissue was post-fixed for 10 min. in 100% methanol (MeOH) at -20°C. Endogenous peroxidases were quenched by treating tissue for 20 min. with the following (10% MeOH, 3.0% H2O2, in TBS (20mM Tris-Cl, pH 7.5, 150 mM NaCl)). Tissue was washed in TBS for 5 min. Tissue was blocked for 1-2 hr. with the following (0.4% Triton, 1.0% glycine, 2.0% BSA, 10% goat serum in TBS). Sections were then treated with primary antibody anti-phospho-p44/42 (1:100) (Cell Signaling, Danvers, MA). Sections were washed with block and detected with the ABC kit (Vector Laboratories) using developing buffer (0.05% DAB;0.03% H_2O_2 in 100 mM Tris-Hcl pH 7.5). The developed sections were counter stained with nuclear fast red.

Western blot analysis

Protein was extracted from cells using 100 µl/coverslip of the following buffer (50 mM Tris-Hcl pH 7.4; 150 mM NaCL;1.0% NP-40; 025% Na-deoxycholate; 1mM EDTA; 1mM PMSF; 1µg/ml aprotinin, leupeptin, pepstatin; 1 mM NaVO and 1mM NaF) for 15 min. at 4 °C. Lysates were passed through 21 gauge needle and spun at 15'000 rpm for 20 min. The lysates were precleared with Protein A/G agarose (Santa Cruz, Santa Cruz, CA) for 1hr at 4 °C. The cleared lysate was then incubated with A/G agarsoe and 2µg of anti-V5 mAb overnight at 4°C. Agarose beads were washed wit h the following buffer (

50 mM Tris-Hcl pH 7.4; 150 mM NaCl;1mM EDTA; 1mM PMSF; 1μg/ml aprotinin, leupeptin, pepstatin; 1 mM NaVO and 1mM NaF) and then boiled in SDS sample buffer, separated on a 12% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked with blocking buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 3% milk powder) at room temperature. For western blots, the nitrocellulose was incubated with antibodies diluted in blocking buffer for 1 h at room temperature, washed three times for 5 min each with wash buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and incubated with secondary antibody (goat anti–mouse IgG, HRP conjugated; Sigma-Aldrich) for 1 h at room temperature. This was followed by three washes of 5 min each with PBS. Nitrocellulose membranes were then developed with ECL (Perkin Elmer, Boston MA)

Results

Decrease in activated MAP kinase, Erk1 and Erk2, in the metanephric mesenchyme of *Itga8* mutant embryos

The extracellular regulated kinases, Erk 1 and Erk 2 are downstream of many cell stimuli including adhesion (Lloyd, 2006). To investigate whether these kinases are involved in the mechanism of α8β1 integrin regulation of *Gdnf* expression in the MM, we sought to assess their active state by using an antibody that recognizes phospho-theronine 202 on p42 (Erk1) and phospho-Tyrosine 204 on p44 (Erk2). We stained *Itga8* heterozygous and *Itga8* mutant embryos at E11.5, the time point at which the phenotype is completely penetrant. Staining of both medial and posterior sections from heterozygous embryos revealed a number of cells in the MM that were positive for

phospho-MAP kinase (Fig. 5.1A,C). However, almost a complete absence of phospho-Map kinase was observed in *Itga8* mutant MM (Fig. 5.1B,D). A number of controls, using *Itga8* heterozygous tissue, were performed to insure the specificity of this signal. These included the staining of a serum only control, which was negative for the signal in both the MM and UB, and a specimen that had been treated with calf intestine alkaline phosphatase, CIP. In this control, the signal in both the kidney and the limb bud was extinguished, suggesting that the signal is real (Fig. 5.1E).

Nuclear translocation of EYA1 is increased in cells by co-expression of α8β1 integrin

The inactivation of MAP kinase in the MM of *Itga8* mutant embryonic kidney focused our attention on downstream targets of MAP kinases within the MM. Of all the potential candidates the eyes absent homolog, EYA1, was of particular interest. EYA1 is attractive because it is both a MAP kinase substrate and a transcriptional activator that has been shown to directly impact *Gdnf* expression (Li et al., 2003; Xu et al., 1999). EYA1 is cytoplasmic and moves to the nucleus as a heterodimeric complex with Six transcription factors (Ohto et al., 1999). Because this is a transcriptional complex, nuclear translocation serves as an indicator of EYA1 activated state. We sought to determine if EYA1 is a physiological target of integrin signaling by examining its activation state, as assessed by nuclear translocation, in cells expressing α8β1 integrin and adhering to various substrates including fibronectin and recombinant nephronectin. Using the cell line K562, we expressed tagged EYA1 (EYA1V5), tagged SIX1 (SIX1V5) and tagged EYA1 with untagged SIX1 (Fig. 5.2). We then allowed the cells to adhere to

fibronectin. The cells were then fixed and stained to assess localization of tagged proteins, using DAPI as a marker for the nucleus. We found that nearly all cells expressing a tagged version of EYA1 contained this protein in both the cytoplasm and the nucleus, while those expressing a tagged version of Six 1 contained that protein almost exclusively in the nucleus (Fig. 5.3A,B). However, when a tagged version of EYA1was co-transfected into cells with an untagged version of Six 1, we found a mixed population, some cells having both nuclear and cytoplasmic localization and some demonstrating complete nuclear localization (Fig. 5.3C). We proceeded with these experiments using K562 cells expressing α8β1 integrin, hereafter referred to as KA8 cells, and plated transfected cells on either poly-L-lysine or fibronectin. Assessing nuclear localization, again using DAPI as a maker for the nucleus, we found that few cells transfected with only EYA1 demonstrated complete nuclear localization of this protein, regardless of the substrate on which the cells where plated. In contrast, almost all cells expressing SIX1 alone demonstrated complete nuclear localization of this protein, again regardless of substrate (Fig. 5.3D). Interestingly, when a tagged version of EYA1 was co-transfected with an untagged version of SIX1 there was a significant difference in the number of cells demonstrating complete nuclear localization of EYA1 when plated on fibronectin (Fig. 5.3D). This suggested that integrin binding of fibronectin could affect the translocation of EYA1 to the nucleus. These results do not distinguish whether this adhesion is specific for α8β1 integrin because α5β1 integrin is able to recognize fibronectin as well or better than α8β1 integrin (see chapter 2). To address this question, we repeated this experiment using recombinant forms of nephronectin protein to which α5β1 integrin does not adhere well (chapter 2). These were truncated forms of

nephronectin, neph251-561, which includes everything c-terminal to the EGF repeats, and neph251-381, which includes the sequence c-terminal of the EGF repeats up to the RGD sequence (see chapter 2). When KA8 cells were plated on these substrates there were significantly more cells demonstrating complete nuclear localization of EYA1V5 when plated on neph251-561 compared to cells plated on neph251-381 (Fig. 5.4A). Additionally, there was a significant difference in complete nuclear localization of EYA1V5 between KA8 cells and the parental line when plated on both substrates. However, we found that a high percentage of KA8 cells plated on poly-L-lysine demonstrated complete nuclear localization of EYA1V5. This percentage was not significantly different from those plated on neph251-561. On poly-L-lysine, KA8 cells still had significantly more nuclear localization of EYA1V5 compared to the parental line. Interestingly, when an inhibitor of MAP kinase kinase (MEK) was added to transfected KA8 cells before adhesion to neph251-561, the number of cells demonstrating complete nuclear localization of EYA1V5 was reduced significantly. The reduction was comparable to the percentage reported for KA8 cells plated on neph251-381 (Fig. 5.4A black bar). This result suggests that MAP kinase has a role in EYA1V5 translocation in these cells. This data fits with the data from the fly showing that eya activity is dependent on Erk phosphorylation (Hsiao et al., 2001).

An EYA1 fusion protein presents distinct banding patterns on SDS PAGE when co-expressed with α8β1 integrin.

Having observed that a relatively high percentage of KA8 cells demonstrate complete nuclear localization of EYA1V5 when plated on neph251-561 and having found that this

percentage can be decreased when these cells are treated with an inhibitor of MEK, we were interested in determining if the phosphorylation state of EYA1 was different in KA8 cells when compared to parental K562 cells. We were also interested in whether the phosphorylated state of the protein changed depending on the whether the cells were plated on neph251-561. We did not have a phosph-specific antibody for EYA1. However, on SDS PAGE an increase in mass due to a phosphate group can change the migration of a given protein. Knowing this protein can be highly phosphorylated, we performed SDS PAGE on immunoprecipitates from K562 and KA8 cells as a first step to assess phophorylation of EYA1. Western analysis on Immunoprecipitates revealed no changes in banding patterns of EYA1V5 when cells were plated on neph 251-561, neph 251-381, or poly-L-lysine (Fig. 5.4B). However, while the fusion protein consistently appeared as a single band of ~ 62 kD from K562 cells, the same protein consistently appeared as a doublet when immunoprecipitated from KA8 cells (Fig. 5.4B inset, arrowheads labeled 1 and 2). This second band, < 62 kD, is a downward shift for EYA1V5. This result indicates that it is probably not due to phosphorylation, which should increase the apparent mass on SDS PAGE. Significantly, this lower band was diminished to near extinction in immunoprecipitates from KA8 cells treated with PD98059, an inhibitor of MEK (Fig. 5.4B inset, compare lane 3 with lane 4). This result suggests that, whatever it physical basis may be, the appearance of this second species is the result of MAP kinase activity.

Discussion

Strong genetic evidence points to a role for α8β1 integrin and its ligand, nephronectin, in the regulation of *Gdnf* expression during the invasion of the UB into the MM (chapters 3 and 4). At this time, the exact mechanism of how integrin signaling is regulating this gene expression is unknown. Here we have attempted to shed light on this by examining the state of MAP kinase (Erk1 and Erk2) activation in the MM of *Itga8* mutant mice. In agreement with data from cell culture that demonstrated a role for α8β1 integrin in MAP kinase activation (Farias et al., 2005), we find that in *Itga8* mutant embryos activated MAP kinase is reduced compared to control embryos (Fig. 5.1). This result led us to investigate targets of MAP kinase in the MM with known roles in *Gdnf* expression. One clear candidate is the transcriptional co-activator EYA1. We have presented preliminary data suggesting that, in K562 cells, complete nuclear localization of a complex, consisting of EYA1V5 and SIX1, is increased when the cells co-express α8β1 integirn, regardless of the substrate on which they are plated. This suggests that nuclear localization of the complex is regulated somehow by the adhesion or signaling properties of α8β1 integrin in these cells. Considering our observation that activated MAP kinase levels are reduced in the MM of *Itga8* mutants and a prior report demonstrating a role in MAP kinase activation by this integrin, we asked if MAP kinase had a role in the increase in nuclear translocation of the complex in these cells. We found that the increase in nuclear translocation was abolished in KA8 cells when they are exposed to an inhibitor of MEK, PD98059, providing the first evidence for a role of MAP kinase in the translocation of the complex. While we have reported a significant change in nuclear localization when KA8 cells are plated on neph251-561 compared to neph251-381, we

found no significant difference in the percentage of cells demonstrating complete nuclear localization between KA8 cells plated on neph251-561 and KA8 cells plated on poly-L-lysine, which is not a known ligand for integrins. Currently, we are unable to explain this result. Certainly, the charge characteristics of the surface of wells coated with poly-L-lysine alone are different than those coated with poly-L-lysine and substrates such as recombinant nephronectin or fibronectin. We have not perform control experiments to rule out the possibility that poly-L-lysine is affecting the activated state of α8β1 integrin. However, we note that there is a significant increase in nuclear translocationn in K562 cells when plated on poly-L-lysine compared to neph251-561. So it would seem that this effect of poly-L-lysine on nuclear translocation is not specific to KA8 cells.

Both nuclear localization and phosphorylation of EYA1 have been correlated with its activated state (Hsiao et al., 2001; Ohto et al., 1999). Yet no clear link exist suggesting that phosphorylation affects complex formation with *sine oculis* orthologs or nuclear localization of the transcriptional complex. Having observed nuclear translocation events that seem correlated with α8β1 integrin co-expression in KA8 cells led us to attempt to assess the phosphorylation state of EYA1V5 in these cells. We examined migration of EYA1V5 using western analysis of immunoprecipitates from KA8 and K562 cells. Contrary to our expectations these experiments revealed no change in migration pattern of EYA1V5 on SDS PAGE when this protein was immunopreciptated from cells plated on neph251-561, neph251-381 or poly-L-lysine (Fig. 5.4B). However, we did observe an intriguing change in the banding pattern of EYA1V5 when this protein was immunoprecipited from KA8 as opposed to K562 cells. It was consistently found that EYA1V5, immunoprecipitated from KA8 cells, migrated as two distinct species as

opposed to a single species when immunoprecipitated from K562 cells (Fig. 5.4B). This second species appears as a band of equal weight and migrates faster than the 62 kD band on SDS PAGE. This has been seen in three separate immunoprecipitates. Because this is SDS PAGE, we expected a shift upward due the increase mass from phosphates. A proper control for this would have been to run lysates from transfected KA8 cells treated with phosphatase. Nevertheless, based on the known physical properties of how phospho-proteins migrate on SDS PAGE, we assume this shift is not the result of phoshorylation. Yet, our observation that the lower band nearly disappeared in an immunoprecipitates from KA8 cells treated with an inhibitor of the MAP kinase pathway, PD98059, suggests that the second species results from MAP kinase activity. One explanation may involve a peculiar quality of EYA1, its known phosphataste activity (Rebay et al., 2005). Although it has not been reported to recognize itself as a substrate, the appearance of this second species could be indicative of a loss of phosphate groups. Assuming this, one can speculate that a decrease in activated MAP kinase could result in a decrease in EYA1 phosphatase activity, explaining the diminishment of the second species.

The above results are presented here for the sake of completeness of the record, which is part of the function of a graduate thesis. The reader is reminded that these results are preliminary and are not the basis on which to draw conclusions. Hopefully they may point the way to more refined experiments.

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Fig. 5.1. Phospho-p44/p42 (phospho-MAP Kinase) is reduced in *Itga8* null embryos at E11.5. (A-E) Transverse sections through *Itga8*$^{+/-}$ and *Itga8*$^{-/-}$ embryos at E11.5. (A) Medial section of *Itga8*$^{+/-}$ embryonic kidney. The ureteric bud (white arrowheads) has invaded the metanephric mesenchyme in which there are numerous phospho-Map kinase positive cells (arrowheads). (B) Medial section of *Itga8*$^{-/-}$ embryonic kidney. The ureteric bud (white arrowheads) has not invaded the metanephric mesenchyme in which there is a noticeable reduction of phospho-Map kinase positive cells (arrowhead). (C) Posterior section of *Itga8*$^{+/-}$ embryonic kidney, in which the metanephric mesenchyme exhibits phospho-Map kinase positive cells (arrowheads). (D) Posterior section of *Itga8*$^{-/-}$ embryonic kidney, in which the metanephric mesenchyme demonstrates a near absence of phospho-MAP kinase positive cells (arrowheads). (E) A section, anterior to that of A, of an *Itga8*$^{+/-}$ embryonic kidney treated with 40U of alkaline phosphatase for 1hr prior to staining with anti-phospho p44/42. Note the lack of any signal in the kidney or surrounding tissue when compared with that in A.

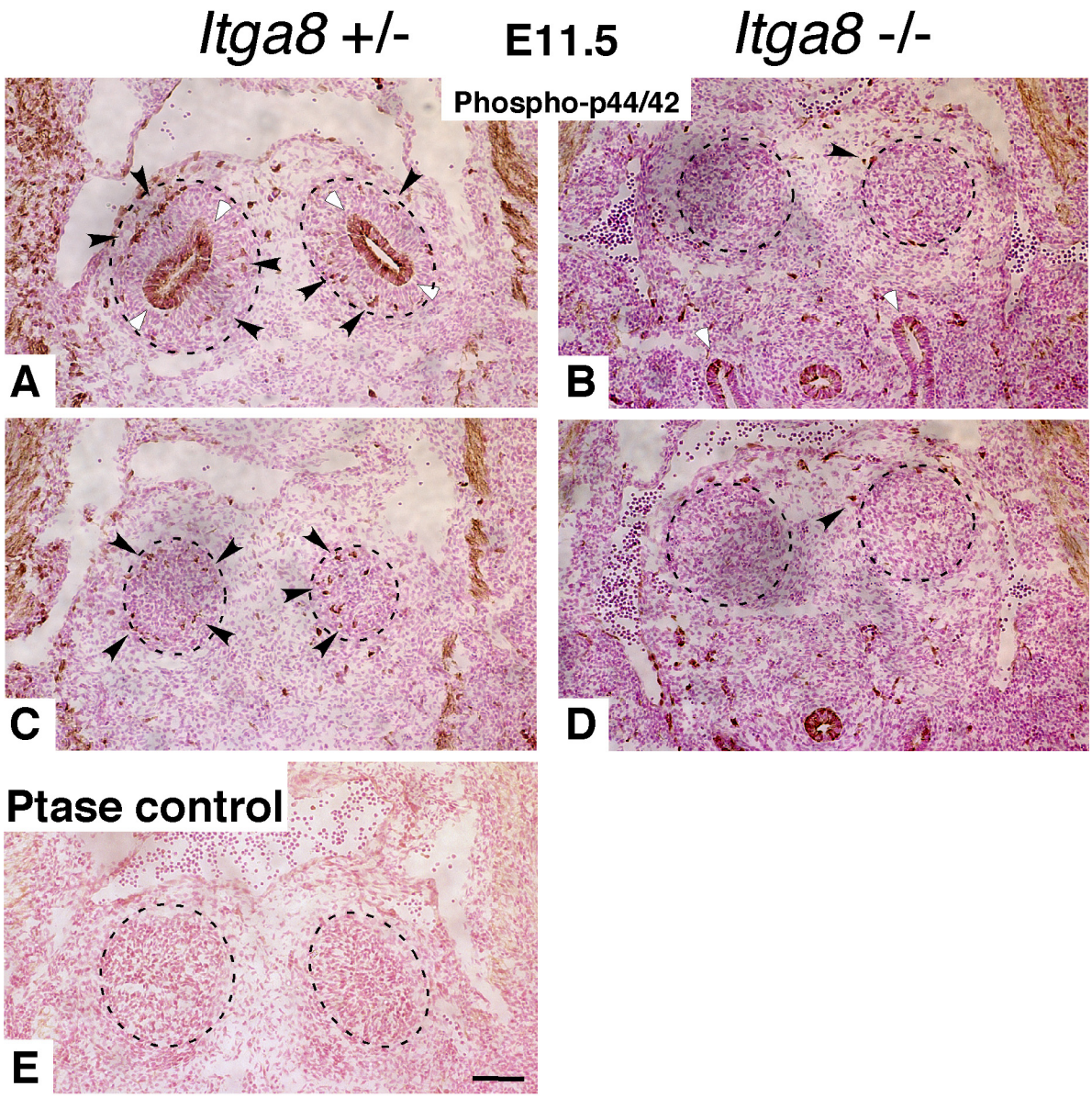

Fig. 5.1

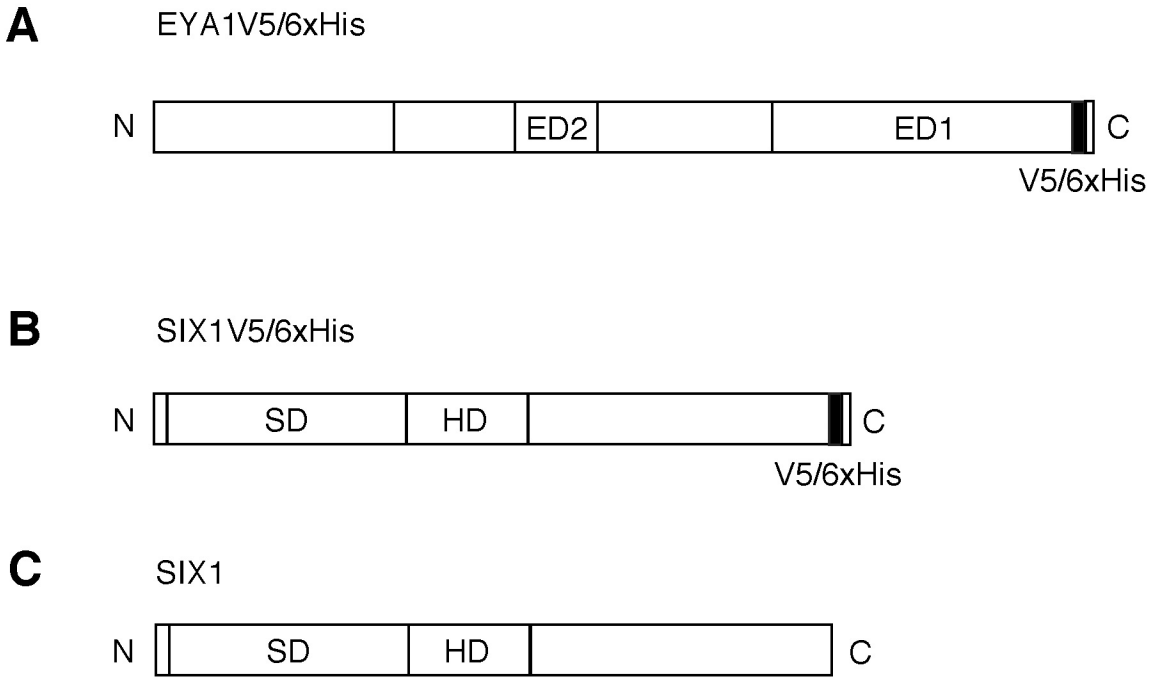

Fig. 5.2. Schematic representations of EYA1 and SIX1 tagged proteins and SIX1 untagged. (**A**). Full-length EYA1 V5/6xHis. (**B**) Full-length SIX1 V5/6xHis (**C**) Full-length untagged SIX1. ED1, eya domain 1; ED2 eya domain 2; SD, six domain; HD, homeodomain.

Fig. 5.3. Nuclear localization of EYA1 and SIX1 tagged proteins in K562 and KA8 cells. (A-C) Expression of tagged proteins in K562 cells. (A) Staining for V5 reveals that tagged EYA1 is located in the cytoplasm and the nucleus. (B) The same staining revealed that most cells transfected with tagged SIX1 demonstrated complete nuclear localization of this protein. (C) When these cells were transfected with tagged EYA1 and untagged SIX1 some demonstrated nuclear and cytoplasmic staining (arrow) and others complete nuclear localization (arrowhead). **(D)** Bar graph representing the percentage of KA8 cells demonstrating complete nuclear localization when transfected with the indicated expression constructs and plated on either fibronectin or poly-L-lysine. Error bars: error is reported as standard error of the mean (SEM).

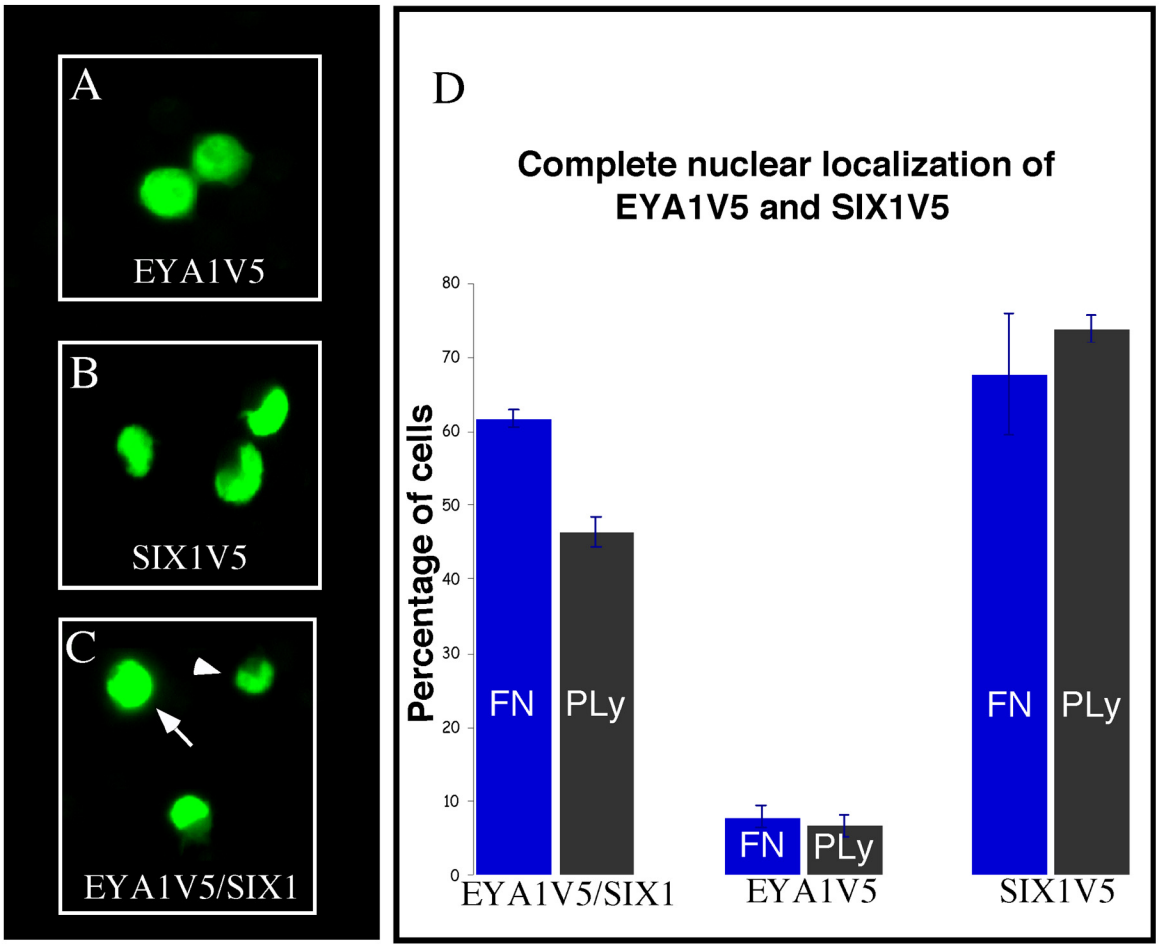
A
EYA1V5
B
SIX1V5
C
EYA1V5/SIX1
D
Complete nuclear localization of
EYA1V5 and SIX1V5
Percentage of cells
80
70
60
50
40
30
20
10
0
FN
PLy
FN
PLy
FN
PLy
EYA1V5/SIX1
EYA1V5
SIX1V5

Fig. 5.3

Fig. 5.4. Analysis of a tagged EYA1/SIX1 complex in K562 and KA8 cells. (A) Bar graph representing the percentage of either K562 cells or KA8 cells demonstrating complete nuclear localization when transfected with tagged EYA1 and untagged SIX1 and plated on the indicated substrates. Note that for all substrates KA8 cells demonstrate significantly more cells with complete nuclear localization. Also, the percentage of KA8 cells, plated on neph251-561, that demonstrate complete nuclear localization is significantly reduced when these cells are treated with an inhibitor of MEK, PD98059. Error bars: error is reported as standard error of the mean (SEM). **(B)** Western analysis of immunoprecipitates (IPs) for the EYA1V5/SIX1 complex from either K562 (lanes 2, 4, 6, 8) or KA8 cells (lanes 1, 3, 5, 7). IP's from cells plated on neph251-561 (lanes 1-4), neph251-381 (lanes 5 and 6), or poly-L-lysine (lanes 7 and 8). IP's from cells plated on neph251-561 and treated with the MEK inhibitor, PD98059, were run in lanes 3 and 4.

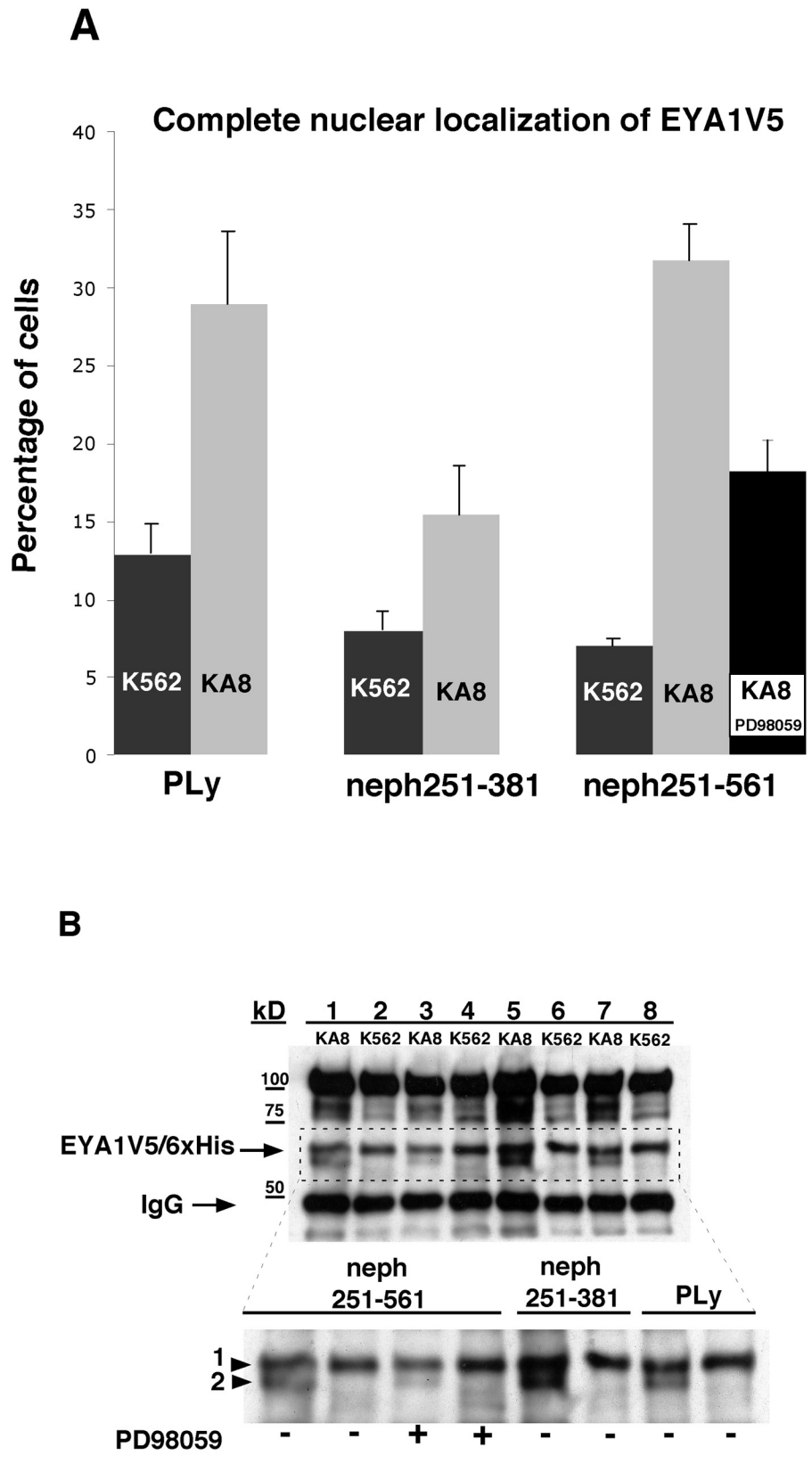
A
Complete nuclear localization of EYA1V5
Percentage of cells
40
35
30
25
20
15
10
5
0
K562
KA8
K562
KA8
K562
KA8
KA8
PD98059
PLy
neph251-381
neph251-561
B
kD
1 2 3 4 5 6 7 8
KA8 K562 KA8 K562 KA8 K562 KA8 K562
100
75
50
EYA1V5/6xHis
IgG
neph
251-561
neph
251-381
PLy
1
2
PD98059
- - + + - - - -

Fig. 5.4

Chapter 6

Summary, Discussion and Future directions

Summary

This thesis reveals two key findings; first, it has presented evidence demonstrating that nephronectin is an essential, early ligand for α8β1 integrin during metanephric kidney development. Second, it has revealed a key insight into α8β1 integrin and nephronectin function by placing them in the GDNF/RET signaling pathway. In addition, it has presented preliminary data that offer the first clues into the molecular mechanism behind the regulation of *Gdnf* expression by α8β1 integrin and nephronectin. What follows are summaries of the key data from each chapter that support the above findings.

Chapter 2 presented experiments that sought to test whether nephronectin can be recognized as a ligand by cells expressing various integrins, including α8β1 integrin. Using the cell line K562, and a number of K562 clones expressing different integrin heterodimers, we carried out adhesion assays using recombinant, truncated forms of nephronectin. These experiments revealed two key findings. One, α8β1 integrin expressed by cells can recognize this form of nephronectin and does so in a dose dependent manner (Fig. 2.3A). Two, a number of other integrins expressed on the same cell type can recognize this form of nephronectin, but cells expressing α8β1 integrin demonstrate the greatest avidity to this form of nephronectin (Fig. 2.3B). These findings support other data, (presented in Brandenberger et al 2001) that suggest nephronectin is a ligand for α8β1 integrin and point to its being the relevant ligand in the developing

kidney. They also open the possibility that nephronectin may have essential roles in other tissues in which α8β1 integrin is not expressed.

Chapter 3 presented the results of a loss of function analysis for nephronectin. The rationale for this experiment was to test the hypothesis that nephronectin was an essential ligand for α8β1 integrin in the kidney and also to uncover essential roles for this protein in other tissues. We generated mice with a floxed allele of nephronectin and targeted this allele for deletion using a mouse that expresses CRE under the β-actin promoter (Fig. 3.1). An analysis of mice homozygous for this allele revealed that mutant mice demonstrate a high degree of kidney agenesis at birth (Fig. 3.2). Analysis of UB formation and early kidney development revealed that while the UB formed in *Npnt* mutant embryos, it was consistently found not to have invaded the MM at E11.5 (Fig. 3.3). Examination of the basement membrane components in nephronectin mutants revealed no outstanding differences when compared to wild-type, littermate controls (Fig. 3.4). Gene expression analysis using *in situ* hybridizaiton revealed that expression of glial cell line derived neurotrophic factor, *Gdnf*, is reduced in the un-invaded metanephric mesenchyme at El1.5 (Fig. 3.5A-H). However, *Gdnf* expression was found to be normal at E10.5, prior to UB formation and at E13.5 in mutants that were developing kidneys (Fig. 3.5I-P). An overall assessment of mutants at birth did not reveal any other gross abnormalities. Mutants that develop kidneys are able to survive well into adulthood and appear healthy. There is some evidence that nephronectin mice may have a defect in cortical development in the brain (see Appendix A).

Chapter 4 presented the results of gene expression analysis of the *Itga8* mutant embryonic kidney and genetic dosage experiments that place the integrin, and its ligand

nephronectin, in a key pathway involved in kidney development. As in the *Npnt* mutant, *Gdnf* expression is reduced in the *Itga8* mutant at E11.5 (Fig. 4.2A-D). Again, as in the *Npnt* mutant, this reduction was found to be transient in *Itga8* mutants, with expression of *Gdnf* being normal at E10.5 and at E13.5 in mutants forming kidneys (Fig. 4.1E-L). Results from gene dosage experiments revealed that reducing the copy number of *Gdnf* in the mutant background dramatically increased the penetrance of the *itga8* phenoytpe (Table 1). To confirm this result and provide further evidence for α8β1 integrin involvement in the GDNF pathway, we performed the converse experiment by attempting to rescue the *itga8* mutant phenotype by enhancing GDNF signaling by reducing the dosage of *Spry1*, an attenuator of GDNF signaling in the UB. These experiments revealed a profound decrease in agenesis in *itga8* mutant neonates with reduced Spry1 expression (Fig. 4.2A). Furthermore, compound mutants demonstrated a complete rescue of agenesis. Inspection of *Itga8* -/-; *Spry1* +/- embryos at E11.5 revealed that the invariant phenotype, lack of UB invasion into the MM, had been rescued (Fig. 4.2B,C). Gene expression analysis of rescued *Itga8* mutants revealed that *Gdnf* expression was not completely rescued (Fig. 4.2D-G). These results demonstrated that the reduction in *spry1* dosage rescued mutants early and that the observed reduction of *Gdnf* in the MM at E11.5 was not a secondary effect due to the absence of the UB from the MM. Taken together, the reduced expression of *Gdnf* in both *Npnt* and *Itga8* mutants, the sensitivity of the penetrance of the *Itga8* mutant phenotype to *Gdnf* dosage and GDNF signaling provide strong evidence for a role of α8β1 integrin and nephronectin in the GDNF/RET pathway.

Chapter 5 presented preliminary data on a possible mechanism involving α8β1 integrin regulation of *Gdnf* expression in the MM of the developing kidney. The

direction of these experiments resulted from an initial observation that activated MAP kinase levels appear down in the *Itga8* mutant MM at E11.5, the time at which we have found the *Itga8* phenotype is completely penetrant (Fig. 5.2). The transcriptional co-activator, EYA1, became an immediate candidate due to its role in the transcription of *Gdnf* and because it is a known target of MAP kinase. Using nuclear translocation as a read out of EYA1 function, we transfected cells expressing the α8β1 integrin, KA8 cells, with constructs expressing a tagged version of EYA1 and untagged version of SIX1 and plated them on various substrates to assess intercellular localization of EYA1 (Fig. 5.3). In a first series of experiments, we found that adhesion of these transfected KA8 cells to fibronectin results in a higher frequency of cells demonstrating complete nuclear translocation of tagged EYA1 compared to these same cells plated on poly-L-lysine (Fig.5.3). In second series of experiments we found that complete nuclear localization of tagged EYA1 was significantly different in KA8 cells when compared to the parental line, K562 (Fig. 5.4A). We also found that significantly more transfected KA8 cells plated on a truncated form of nephronectin that contained the RGD site, demonstrated complete nuclear localization of the EYA1 fusion protein when compared with these same cells plated on a truncated form of nephronectin without the RGD site. The difference in complete nuclear localization could be reduced with an inhibitor of MAP kinase activity, implicating this signaling pathway in the observed translocation. However, unlike our experiments with fibronectin, we found no difference in complete nuclear localization between transfected KA8 cells plated on truncated nephronectin containing the RGD site and these same cells plated on poly-L-lysine. This is a result we are currently unable to explain; however, there are data that show integrins, expressed on

rat sympathetic neurons, can mediate axonal outgrowth on poly-L-lysine in a manner similar to their ability to mediate axonal outgrowth on Laminin. This suggest that poly-L-lysine may be a substrate for integrins (Tomaselli, 1987).

Western analysis of immunoprecipitates from KA8 cells expressing the EYA1 tagged protein revealed that this protein migrated as two distinct bands regardless of the substrate on which these cells were plated (Fig. 5.4B). However, there was a clear absence of a lower molecular weight species from control lysates of the parental cell line, K562. Importantly, the second species was nearly eliminated from immunoprecipitates of transfected KA8 cells treated with PD 98059, suggesting that MAP kinase activity was involved in the appearance of this second species (Fig. 5.4B').

Discussion

The importance of the extracellular matrix in the developing metanephric kidney has been appreciated since it was first used as a model for organogenesis (Grobstein, 1955). However, with the advent of loss of function studies, few ECM proteins or their receptors have been revealed to have specific roles in the early events of metanephric development (Lelongt and Ronco, 2003). One exception is α8β1 integrin, which has previously been shown to be critical for initial UB invasion into the MM and its subsequent branching (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.

Individual integrins usually recognize multiple ligands and the hypothesis that nephronectin should be an essential ligand for α8β1 integrin was weakened by the

likelihood that UB invasion and branching most likely were highly redundant processes. Indeed, we had anticipated the creation of compound mutants lacking other ECM proteins with possible roles in early kidney development, such as osteopontin. For this reason, the similarity in the early phenotypes of *Itga8* mutant mice and *Npnt* mutant mice is striking. It not only demonstrates that the above hypothesis is likely true, at least for the early function of α8β1, but reveals a functional specificity that is unusual. Nephronectin and α8β1 are co-expressed in other tissues including the inner ear where *itga8* mutant mice demonstrate a phenotype (Littlewood Evans and Muller, 2000). While anatomical studies have not been performed, nephronectin null mice do not seem to demonstrate an obvious behavioral phenotype associated with a defect in that tissue. This is also true of any other tissue where it is co-expressed with α8β1 integrin. Therefore, it appears that nephronectin is one of the few ECM components to be specifically essential in the developing kidney.

Differences between the phenotype of *Npnt* null mice and other phenotypes associated with the loss of ECM components.

How does the phenotype of *Npnt* mutant mice differ from the other early kidney phenotypes of mice lacking ECM proteins? As mentioned above, few phenotypes have been described for early budding and branching phenotypes resulting from the loss of an ECM protein. Comparison of the *Npnt* null phenotype with these few phenotypes reveals that it is distinct. For example, the phenotype of mice lacking γ1 laminin subunit (*Lamc1*) involves a lack of invasion of the UB into the MM at E11.5 (Willem et al., 2002). While similar to the *Npnt* mutant phenotype there are some important differences,

including the fact that some *Lamc1* mutants are able to invade the MM at E11.5 in contrast to *Npnt* mutants, in which the UB has never been observed to have invaded the MM at this stage. The invasion phenotype of *Lamc1* mutant mice is most likely a secondary consequence of improper ND elongation, a defect not observed in *Npnt* null embryos. Also, unlike *Npnt* mutant mice, confocal imaging has revealed defects in the basement membrane in these mutants, suggesting that laminin γ1 subunit functions in matrix integrity rather than through the activation of gene expression in the MM. Another laminin subunit shown to have a role in early kidney development is the α5 subunit (*Lama5*) (Miner and Li, 2000). However, the associated phenotype does not involve the initial invasion of the UB into the MM but branching of the UB after invasion as assessed at E13.5. Data from immunofluorescence suggests that, like the γ1 mutant, this branching defect arises from defects in the basement membrane. The penetrance of the *Lama5* phenotype is considerably less than that of *Lamc1* and *Npnt* mutants. Mice lacking heparan sulfate 2-O-sulfotransferase (*Hs2st1*) have a highly penetrant phenotype involving the ability of the MM to respond to the invading UB. However, in the *Hs2st1* mutant the UB has invaded and branched once in the MM at E11.5. Temporally, this gene seems to have an essential role later in development than that of nephronectin. At this time, it seems as if nephronectin is unique among ECM constituents in having an essential role in the initial invasion of the UB into the MM.

Nephronectin is dispensable for mediating the late function of α8β1 integrin

While nephronectin mice demonstrate a consistent lack of UB invasion of the MM at E11.5, which is very similar to the phenotype of *itga8* mutants, they do not seem to

demonstrate a branching phenotype like that of *itga8* mutants (Muller et al., 1997). UB branching has not been analyzed extensively in *Npnt* mutants at E13.5, but at birth *Npnt* mutant mice do not display the rudimentary kidney development that is found in some *Itga8* mutants. Such rudiments are presumably the result of delayed invasion and a lack of response by the MM. The most obvious explanation is that α8β1 integrin is recognizing an alternate ligand at this later time. There are a number of candidate ligands, expressed in the correct time and space, to be mediating the branching of the UB and the condensation and differentiation of the MM. One of these is fibronectin (Hynes, 1986). Fibronectin, FN, is expressed diffusely within the MM before invasion (Ekblom, 1981). After invasion, FN becomes incorporated into the basement membrane surrounding the UB. While FN is not expressed in the correct spatial manner to be mediating the initial invasion of the UB, it may play a role in the subsequent branching program. At this time, a conditional deletion of FN gene expression in the MM has not been reported, so the question of whether FN has an essential function in the developing kidney is still unanswered. Osteopontin, OPN, is a known ligand for α8β1 integrin and is expressed in the UB and to some extent in the MM (Denda et al., 1998; Kanwar et al., 2004). While it is expressed in the proper manner to mediate the function of α8β1 integrin during both the initial invasion and subsequent branching, mice lacking OPN do not exhibit a kidney phenotype (Liaw et al., 1998). So, if it has a role in these events, OPN would be playing an accessory role to another ECM, such as FN. Other ligands recognized by α8β1 integrin include tenascin-C, tenascin-W, vitronectin, and the latency associated peptide of TGFβ, LAP. These are either not expressed at the right time or not the right place to mediate α8β1 function in the developing kidney (Aufderheide et al.,

1987; Scherberich et al., 2004; Seiffert et al., 1991; Yang et al., 2007). At this time, it would seem FN is the most likely candidate; yet there exist other intriguing possibilities. The ECM protein, Mam domain And EGF domain containing protein" (MAEG), also known as EGFL6, is very similar to nephronectin, sharing 41% overall amino acid similarity, and can be recognized as a ligand by α8β1 integrin (Buchner et al., 2000; Yeung et al., 1999). However, because there is very little data on the expression of this protein during development, it is unclear whether it is expressed in the developing kidney. The presence of this protein in other tissues during development could explain the lack of phenotypes elsewhere in *Npnt* null mice. The ECM protein, Dentin sialophosphoprotein (DSPP), is a member of the Small Integrin-Binding Ligand N-Linked Glycoproteins, SIBLING family, of ECM proteins (Alvares et al., 2006; Fisher et al., 2004). This is an emerging family, which includes OPN and is grouped not on the basis of genetic homology but on the basis of similar exon-intron structure, the inclusion of the casein kinase recognition site, SSEE, a poly-proline site and an integrin recognition site, RGD. All of the current members are located in a cluster on human chromosome 4. Recently, DSPP has been assigned a role in branching morphogenesis at the exact time branching deficits are observed in *Itga8* null mice (Alvares et al., 2006). DSPP is expressed by the cells of the UB from E13.0 on and could therefore be a ligand for α8β1 integrin. However, this remains to be determined.

Emerging evidence for integrins and their ligands in the regulation of gene expression

The versatility of integrins and the complexity of ECM protein diversity and function is an emerging story and continues to surprise, twenty years after the discovery of integrins. We have presented data that support a role for an integrin and an ECM protein ligand in the regulation of gene expression in the developing kidney. While the idea that gene expression can be affected by integrin signaling is not new, we believe the data presented in this thesis are novel because they were derived from in vivo genetic experiments, which assign an essential function to a particular integrin, α8β1 integrin, and its ligand, nephronectin. As summarized above and in the introduction, there are other ligands for α8β1 integrin in the developing kidney, but none of these can completely substitute for the loss of nephronectin. Additionally, there are other α subunits expressed in the loose MM including α4, α9 and αv that could potentially recognize these ligands (L. Schnapp, personal communication), yet loss of α8 integrin subunit cannot be compensated for in the MM. The profound impact of the loss of this specific interaction, which is required in a narrow window of time during development, seems unique. Our conclusions are based on genetic evidence, but such *in vivo* data on integrin gene regulation is not unprecedented. 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). Here a large class of integrins, all the integrins expressed in the mammary gland that contain the β1 subunit, has an impact on gene expression.

Alternate explanations for the phenotypes

Contrary to a direct role in the expression of *Gdnf*, it is conceivable that integrin α8β1, by binding nephronectin, helps remodel the matrix before budding because it has been shown to be expressed in the mesenchyme adjacent to the ND (Muller et al., 1997). Roles for matrix remodeling have been assigned to the SIBLING family of ECM proteins (Ogbureke and Fisher, 2004). Interestingly, one of the characteristics of this family is their location in a cluster on Human chromosome 4, 4q21.3, very near the locus containing the Human ortholog of nephronectin, 4q24 (Fisher and Fedarko, 2003). Nephronectin shares a number of other similarities to members of this family as well, including proline rich regions, an N-linked glycosylation site, and the integrin recognition sequence RGD. However, there are also key differences. For example, none of the six SIBLING members have EGF repeats. Nephronectin does not contain a phosphorylation site for casein kinase and its exon/intron boundaries differ from that of the SIBLINGS. Even if nephronectin is not a member of the SIBLING family, it is conceivable that nephronectin could perform similar roles in matrix turnover via the activation of matrix metalloproteinases. Contrary to this idea, we consistently observe that the UB has formed and elongated toward the MM at E11.5 in *Npn*t and *Itga8* mutants (chapter 3). While we have not done ultrastructural analysis on the basement membrane in these mutants, we have presented immunofluorescence data that demonstrates the basement membrane around the mutant ND and UB appears intact, suggesting that any matrix remodeling necessary for bud initiation is not perturbed (chapter 3). Indeed, it is challenging to explain the rescue data, presented in chapter 4, in terms of any defect in

the ECM. Therefore, the simplest explanation is that α8β1 integrin functions in the MM by affecting the expression of *Gdnf* .

Apart from the integrity of the matrix, another explanation of the phenotype could involve a role for α8β1 integrin and nephronectin in the distribution of proteoglycans, which allow the matrix to bind and present GDNF in a proper manner (Barnett et al., 2002). However, we find that in Npnt mutants the ND is able to respond to GDNF at E11.0, having already formed a bud that has begun elongation toward the MM. Likewise, we find that mutant escapees respond to GDNF at E13.5. So any presentation or increase in effective concentration of GDNF in the matrix facilitated by integrin α8β1 and nephronectin would be important for only a limited time. While we cannot rule out this possibility, we find this idea hard to reconcile with our data that shows an obvious reduction in *Gdnf* messenger RNA at E11.5 in the MM of both *Npnt* and *Itga8* null embryos. Currently the simplest explanation is that the agenesis in these mutants results from a lack of *Gdnf* expression.

Future directions

The elucidation of mechanism

Having presented data that place α8β1 integrin and nephronectin in a pathway that regulates *Gdnf* expression, we are presented with the question of the underlying mechanism of this regulation. In this thesis, I have presented preliminary data that provides a first attempt at shedding light on this mechanism. The data demonstrating a reduction in activated MAP kinase within the MM at E11.5 may not be surprising

considering the many pathways that impact signaling mediated by these kinases (Lloyd, 2006; Rubinfeld and Seger, 2005). However, with the knowledge that *Gdnf* levels are reduced in these mutants at that same time and that α8β1 integrin can activate MAP kinase, this result provided a lead in to thinking about mechanism. I have presented preliminary results that began to test a hypothesis based on the activation of a possible target of MAP kinase in the MM, EYA1, an activator of *Gdnf* expression (Li et al., 2003; Xu et al., 1999). However, as mentioned at the end of chapter 5 of this thesis, this is only a beginning. In the future it will be important to pursue mechanistic insight on several fronts. First, it will be important to investigate the activation state of a number of key signaling factors within the MM of both *Npnt* and *Itga8* null embryos. These signaling factors include PI3K, which α8β1 integrin has been shown to activate (Farias et al., 2005). The kinase, Akt, a key survival factor in the MM and a signaling factor downstream of RET in the UB (Kurokawa et al., 2003). Additionally, Akt has been shown to have a role in UB outgrowth (Tang et al., 2002).. A role for Rho family guanosine triphosphatases is also worth investigating**.** Streuli and colleagues have demonstrated a role for inetgrin regulation of gene expression in mammary gland cultures. This gene regulation is mediated in part by Rac activation of JNK Map kinase (Akhtar and Streuli, 2006). Although FAK sits atop many signaling pathways, activated FAK levels are worth investigating as well. It has been reported that the phenotype of the inner ear of *Itga8* mutant mice may be due to a lack of FAK co-localization with α8β1 integrin (Littlewood Evans and Muller, 2000). Analysis of activation state of these various signaling proteins will provide an entry point to more thorough analysis. Due to the limited amount of material that can be obtained from the embryonic kidney at E11.5,

these experiments will be most easily accomplished using immunohistology. Such experiments depend on the availability of antibodies that give reproducible results in tissue. Kidney culture provides the ability to obtain data that will refine the general observations derived from immunohistological investigations. Culture allows for a number of approaches including dominant negative analysis, siRNA, viral transfection, and antibody inhibition. The primary obstacle to this type of analysis is the time point of complete penetrance of the *Npnt* and *Itga8* mutants. Robust kidney culture is optimal from E11.5, once the UB has invaded and branched once within the MM (Saxen and Lehtonen, 1987). For analysis of an invasion phenotype, it will be optimal to culture the UB and MM before invasion. Whether this can be done reproducibly is questionable.

Aside from studies directed at signaling within the MM, it will be important to investigate more fully the role of nephronectin and α8β1 integrin in matrix integrity and remodeling. The data presented here revealed no obvious defects in the basement membrane (chapter 3), however ultra-structural analysis will be necessary to confirm this result. It is possible that in addition to their role in *Gdnf* regulation, nephronectin and α8β1 integrin may have other roles during invasion of the UB into the MM that impact the penetrance of the phenotypes displayed by both mutants. This is particularly relevant in regards to the branching phenotype found in *Itga8* mutants. It could be that α8β1 integrin has roles in matrix integrity that are independent of its interaction with nephronectin. This thesis has not ruled out a role in matrix integrity for either nephronectin or α8β1 integrin.

The Identification of the late ligand(s)

Mice that do not express nephronectin, *Npnt* mutants, do not exactly phenocopy mice the *Itga8* mutant phenotype. At birth *Npnt* mutant mice have not been observed to display the rudimentary development that is sometimes exhibited by *Itga8* mutant mice. This may be due to an essential role for α8β1 integrin in branching morphogenesis. Whatever its basis, the difference between the *Npnt* mutant phenotype and the *Itga8* mutant phenotype may be explained the presence of an alternate ligand that can be recognized after invasion and during the initiation of branching morphogenesis, referred to here as a late ligand(s). It is obvious that in order to understand the *Itga8* mutant phenotype completely will require the identification of this ligand(s). Some of the candidates have been mentioned above in the discussion section; here I would like to suggest experimental approaches aimed at the identification of this ligand(s).

As mentioned above, several mice exist with mutations in genes for ECM proteins that are recognized by α8β1 integrin. In particular, it will be informative to cross mice with mutations in these genes with mice carrying the *Npnt* allele. For example *Spp1* (OPN) heterozygote crossed with *Npnt* heterzygous mice could reveal a branching phenotype in progeny heterozygous for both null alleles. These studies could be pursued with production of mice that are compound homozygous for both null alleles. To gauge the amount of redundancy that exits within the ECM in both the budding and branching programs, this approach could be pursued with the various other null alleles for genes of ECM proteins, some of which have been discussed above. For those ECM protein genes that have not been targeted, such as MAEG/EGFL6 or alleles that do not yet exist, such as a conditional fibronectin allele, it will be necessary to turn to culture. Unlike the early

phenotype of Npnt, later branching phenotypes can be analyzed with culture reproducibly; however, there are caveats associated with the use of culture as a means to assign essential roles for genes in kidney development. One outstanding example of this is OPN. Culture experiments using Ab to OPN showed a clear inhibition of branching when applied to culture (Rogers et al., 1997). It is now known that *Spp1* mutants do not demonstrate a phenotype in any aspect of kidney development (Liaw et al., 1998). Fibronectin function has been investigated in culture as well. Using siRNA methodology, the authors of this study claimed to have observed a clear reduction in branching morphogenesis in cultured rudiments (Sakai et al., 2003). No quantitative data was presented for this phenotype and the images presented, while presenting a noticeable reduction in branch number, were not dramatic. More importantly, our attempts to reproduce these results have meet with little success (unpublished results). Based on our experience, genetic experiments will be the most reliable means of assessing redundancy of ECM function in the UB branching program.

Have all the ligands in the developing kidney for α8β1 integrin been identified? It is still possible that another, novel ligand is mediating the late function of α8β1 integrin. The functional screen that identified nephronectin was carried out using an E13.0 heart library(Brandenberger et al., 2001). This means there may be kidney specific ECM proteins that were not uncovered. A first step in addressing this question could involve using the α8β1-AP to probe the developing kidneys, assuming they do develop, of compound mutants, such as *Npnt* --/-; *Spp1* -/-, to provide evidence for the existence of other ligands.

What would the function a late ligand(s) be? One possible function of this ligand(s) would be in matrix remodeling and turnover, a critical process during branching morphogenesis (Pohl et al., 2000). It is now clear that integrins are involved in remodeling process via the activation of various proteases(Larsen et al., 2006). One interesting possibility is that α8β1 integrin is recognizing a SIBLING protein and activating a MMP. However, our observations from the rescue experiments involving the reduction in *Spry1* copy number in the *Itga8* mutant suggest that kidney size in neonates is similar to that of controls (Fig. 4.5). This observation is hard to reconcile with the idea that the *Itga8* mutant branching phenotype arises due to a defect in the ECM. While we have shown that *Itga8* mutant mice which overcome the delay in invasion express *Gdnf*, as assessed by in situ hybridization, we have not quantified the amount. As touched upon in the discussion to chapter 4, it may be the case that additional ligands become necessary for proper *Gdnf* expression as branching morphogenesis gets underway. Experiments that assess *Gdnf* expression in compound mutants, described above, would partly address this possibility. More directly, this possibility should be tested by quantification of the *Gdnf* expression in the *Itga8* mutants that go on to develop kidneys.

Deeper analysis of Npnt null mice; is there an essential function for nephronectin in the brain?

Superficial inspection of *Npnt* mutant mice at birth revealed no phenotypes other than kidney agenesis. However, we have not ruled out defects in other tissues. Based on our previous analysis of nephronectin expression during development, areas of interest include: the lens of the eye, the inner ear, the lung and heart (Brandenberger et al., 2001).

Of these the inner ear is of particular interest. It has been previously reported that *Itga8* mutant mice have defects in stereocilla development within the sensory epithelium of the utricle (Littlewood Evans and Muller, 2000). A number of genes involved in deafness have turned out to be ECM proteins (Kashtan, 1999; Sundstrom et al., 1999). We are therefore interested to see if a similar defect is to be found in *Npnt* mutants. Another area that may harbor a phenotype in the *Npnt* mutant is the brain. It has been reported that nephronectin is expressed in the developing and adult brain(Allen_Institute, 2003; Yamazaki et al., 2004). While, the gross morphology of the brains of *Npnt* mutant mice appears normal, closer inspection has revealed abnormalities of the cortices of newborn *Npnt* mutants that are rather striking (Fig. A1, A2). These abnormalities include large, ectopic clusters of cells within the layers of the cortex, wavy cortical layering and large areas of disorganized cells (Fig. A1B,D,F). At this time all of the mutant neonate brains that we have observed have displayed one or a number of these phenotypes. Additionally, we have observed defects in a 21 day old *Npnt* mutant (Fig. A2). In this one example, we observed a cluster of cells within a cortex, just ventral to the hippocampus and a large a-cellular region in the striatum. Otherwise, this mutant displayed normal cortical layering. We have also observed serious abnormalities in at least one heterozygous neonate. The genotype of this animal was confirmed by PCR several times. We cannot conclude that this is the result of a dominant phenotype because it is the only heterozygote that we have examined. Our numbers need to be increased before we can make a statement about penetrance.

It would also be premature to conclude that these cortical defects are due to the loss of nephronectin protein. One possibility is that loci adjacent to the nephronectin locus

where somehow disrupted in the targeting (see Appendix A). Such a disruption may inhibit the expression of a proximal gene. Due to the high degree of linkage it would be rare to have progeny homozygous for this mutation and not homozygous for mutant allele of nephronectin. However, the one heterozygous animal, mentioned above, may represent this rare example.

It is interesting to note that the group of cells identified as expressing nephronectin during development are the Cajal-Retzius population of transient neurons (Yamazaki et al., 2004). These cells have been ascribed functions in proper cortical layer during development. It is currently thought that they accomplish this through the secretion of the ECM protein reelin (Frotscher, 1998; Soriano and Del Rio, 2005). While distinct from the defects reported for mice null for reelin protein, the defects described above are characteristic of layering defects. It is tempting to speculate that nephronectin may be an essential cue to migrating neuronal progenitors. However, due to the uncertainties and a low observational n, concluding that nephronectin does have a role in the developing brain would be premature. It remains to be seen.

Conclusion

In 2001, I began my first attempts at targeting the nephronectin locus. As outlined in this thesis, it was hypothesized that this ECM protein was an essential ligand in the developing metanephric kidney for $\alpha 8\beta 1$ integrin. While the hypothesis betrays our expectation at that time, we did not expect the phenotype of *Npnt* mice to compliment that of *Itga8* mutant mice in such an extraordinary way. It was a clean and concise answer to a long-standing question. It was also pleasing to assign a somewhat

unexpected role for this integrin and its ligand in the regulation of gene expression during kidney development. Of course, new questions emerge about how this works and so, α8β1 integrin and nephronectin still have something to teach us about how it all comes together.

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Appendix A

Brain histology

Examples of brain malformations observed in *Npnt* mutants are shown here. Abnormalities at birth are consistent with layering defects due to improper migration of neural progenitors. These abnormalities include large clusters of cells located in the cortex, undulations in cortical layering, and defects in hippocampal morphology (Fig. A.1). Currently, we have observed five brains from mutant neonates and all have displayed one or more of these abnormalities. Additionally, we have observed one mutant at three weeks of age (P21) that demonstrated malformations in the cortex and striatum (Fig. A2). At this time, we have only examined one mutant brain at P21. Importantly, we have observed one heterozygous neonate with similar defects. For this reason, and the low number observations, it is uncertain whether these defects are the result of a lack of nephronectin expression.

As mentioned in the discussion section, it is possible that the targeting of the nephronectin locus may have resulted in the disruption of a proximal gene. Examination of the end sequence of the BAC we used to target the *Npnt* locus reveals that it has a 3' end in the *Npnt* locus. Inspection of adjacent, 5' loci on chromosome 3 reveals three putative genes, *Gstcd*, a gene that encodes a glutathione S-transferase domain, *Ints12*, a gene encoding a protein with a zinc finger, PHD-type domain, and an annotated gene identified with a Riken cDNA, 9130221D24Rik, encoding a protein with guanyl-nucleotide exchange factor activity. Sequence for this last gene is not found in the BAC we used for targeting. However, it is proximal and therefore disruption of a regulatory

region could impact its expression. None of these genes have been reported to have roles in the developing brain.

The survey of brain development in the *Npnt* mutant was done in collaboration with Natasha Shinsky-Bjorde. Natasha embedded, sectioned and stained the brains from both P0 mice and P21 mice. Additionally, Natasha contributed to the imaging and analysis of the sections. Brains were dissected from newborn mice (P0) and fixed by submersion in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 16hr at 4 °C. The brains of three week old mice (P21) were harvested after perfusion with 4%PFA in PBS. Brains were embedded in agarose and sectioned with a vibratome. Perfusion and nissel staining were carried out using standard procedures.

Fig. A.1. Abnormalities in the brains of *Npnt* mutants at birth. (A-F) Coronal sections through the brains of a wild-type (A, C, E) and two *Npnt* mutants (B, D, F). (A) Medial, coronal section of wild-type brain demonstrating normal cortical layering (arrow) and histoarchiteture in adjacent striatum (arrowhead). (B) Medial, coronal section of *Npnt* mutant brain. Note the large cellular cluster located in the cortex (arrow), the a-cellular region in the adjacent striatum (arrowhead) and enlarged ventricle (asterisk). (C) Posterior section of wild-type brain demonstrating normal histoarchiteture in the hippocampus (arrow). (D) Posterior section of mutant brain demonstrating highly disorganized histoarchiteture in the hippocampal region (arrow). (E) Anterior section of wild-type brain showing normal layering of the hippocampus and both outer (arrowheads) and inner (arrow) cortical layers. (F) Equivalent section through the brain shown in D demonstrating normal hippocampal development but a large ecoptic cellular cluster (arrow) in the cortex as well as undulating layers (arrowheads).

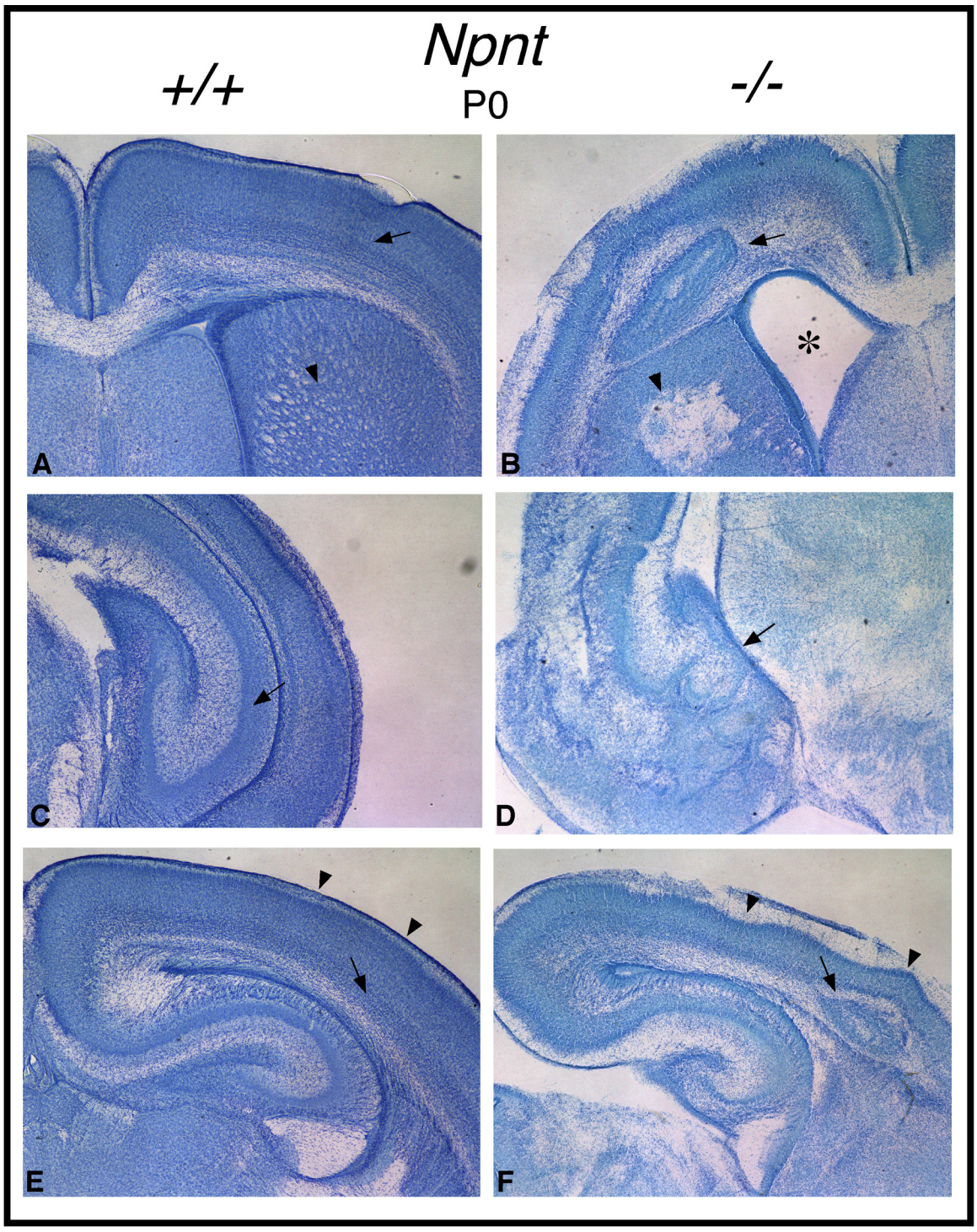

Fig. A.1

Fig. A.2. Abnormalities in the brains of *Npnt* mutants at 3 weeks of age. (A-D) Coronal sections through the brains of a wild-type (A, C) and a *Npnt* mutant (B, D). (A) Medial, coronal section of wild-type brain demonstrating normal cortical layering (arrowhead) and cellular organization (arrow). (B) Medial, coronal section of *Npnt* mutant brain exhibiting a large cellular cluster (arrowhead) located ventral to the hippocampus. This section also contains a highly disorganized area (arrow) just dorsal lateral to the hippocampus. Asterisk indicates an area of damage that may be due to sectioning. (C) Normal striatal histoarchitecture (arrow) in the wild-type is seen in this section that is adjacent to that shown in A. (D) In an adjacent section to B, another highly disorganized region in the striatum is apparent in the brain of the *Npnt* mutant (arrow).

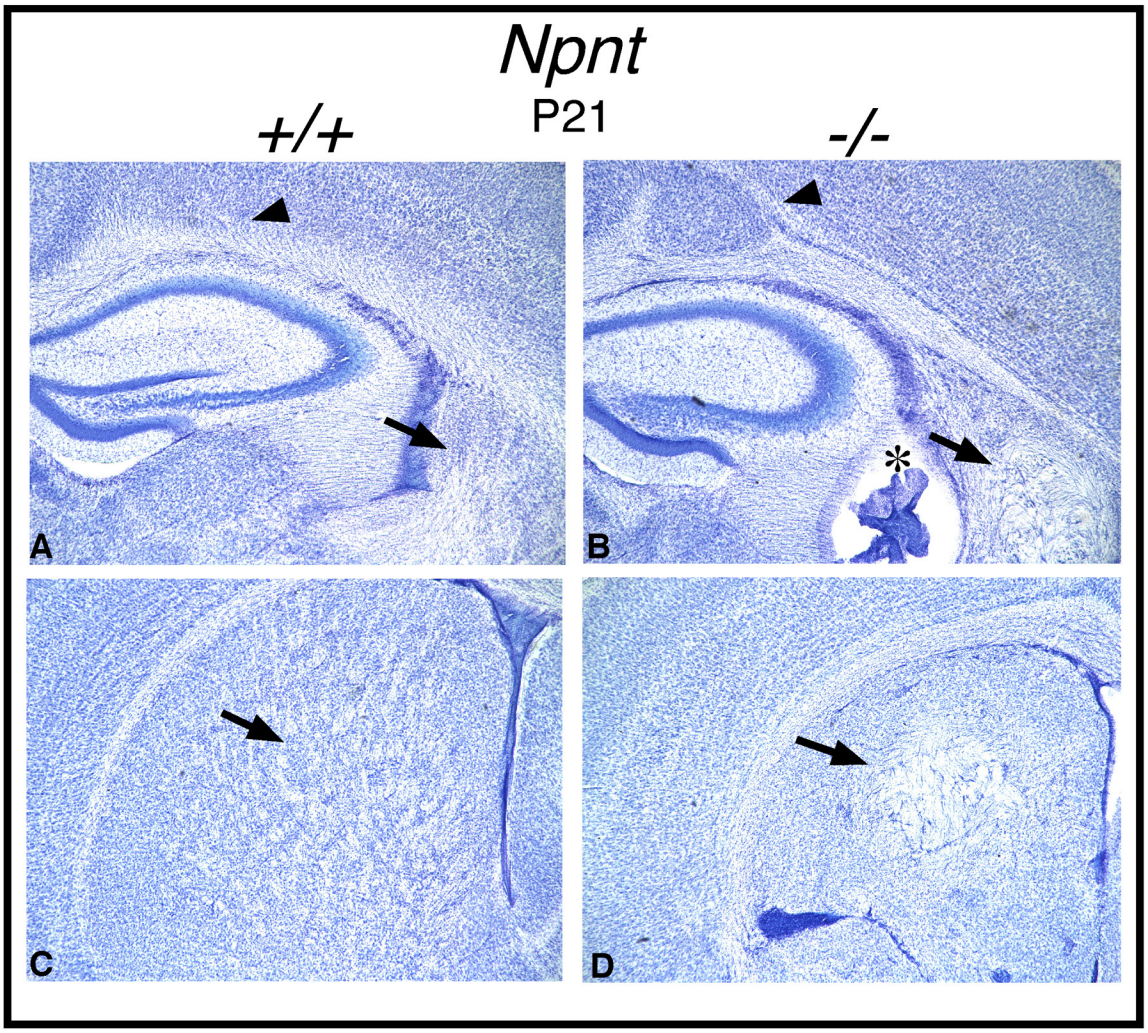

Fig. A.2

Appendix B

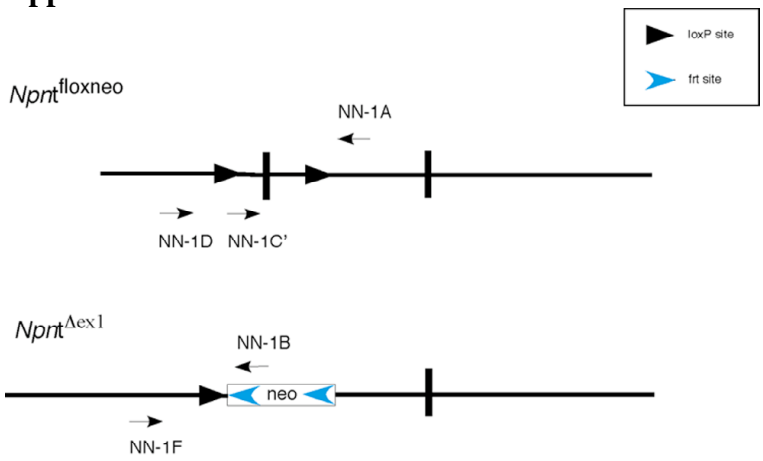

Fig. B.1. Sites for primers used to genotype *Npnt* alleles.

***Npnt*^{WT}** NN-1A: 5'-AGT CCA TCC TGA TCA CTG GCT-3' Band size: 279 bp

NN-1C' 5'-GCA ACC TTC AGC GTC CC-3'

***Npnt*^{floxed}** NN-1A: 5'-AGT CCA TCC TGA TCA CTG GCT-3' Band size: 312 bp

NN-1C' 5'-GCA ACC TTC AGC GTC CC-3'

***Npnt*^{floxed}** NN-1A: 5'-AGT CCA TCC TGA TCA CTG GCT-3' Band size: 920 bp

NN-1D: 5'-ACG CGT ACT TCC ACT TCC ACC-3'

***Npnt*^{floxed};Cre** Band size: 111 bp

***Npnt*^{Δex1}** NN-1B: 5'-TAT GGC TTC TGA GGC GGA AAG AAC-3'

NN-1F: 5'-AAG TGG AGC TTC AGG ACA CAG-3' Band size: 509 bp

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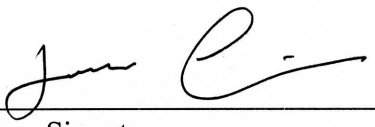

Author Signature

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