BETA1 INTEGRIN IN KIDNEY DEVELOPMENT

By

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Dedicated to my father, Sen Zhang, and my mother, Baozhen Wang, who give me everything and expect nothing in return.

ORIGINAL PUBLICATIONS

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BMP	bone morphogenic protein
ECM	extracellular matrix
EM	electron microscopy
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GBM	Glomerular basement membrane
GDNF	Glial-cell derived neurotrophic factor
GFR	growth factor receptor
HGF	Hepatocyte growth factor
ILK	integrin linked kinase
IMCD	inner medullary collecting duct
MAPK	mitogen activated protein kinase
MDCK	mardin darby canine kidney
MIDAS	metal-ion-dependent adhesion site
MM	metanephric mesenchyme
PDGF	Platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PAS	Periodic Acid Schiff's
PKA	protein kinase A

PKC	protein kinase C
UB	ureteric bud
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Integrin structure and function

Integrins are cell surface receptors that mediate the interactions between cells and extracellular matrix (ECM). They consist of non-covalently bound α □ □ and□β subunits that combine in a restricted manner to form specific α β dimers. In mammals there are 18α and 8β subunits that form more than 20 different dimers, each of which exhibits different ligand binding properties(Hynes 2002);(Legate, Wickstrom et al. 2009). β1 is the most abundantly expressed β subunit and is found in almost all cell types in the body, including the kidney. Integrins α3β1, α6β1 and α6β4 are the major laminin binding receptors, while the predominant collagen receptors are integrins α1β1and α2β1. Although they are primarily thought of as anchoring molecules, integrins play a crucial role in cell adhesion, migration, proliferation and apoptosis by transducing signals through their cytoplasmic tails following ligand binding (Ginsberg, Partridge et al. 2005); (Moser, Legate et al. 2009); (Legate and Fassler 2009).

Each integrin subunit is composed of a large extracellular domain, a single transmembrane pass and a cytoplasmic tail. The head region of the extracellular domain contains a metal-ion-dependent adhesion site (MIDAS), which is critical for ligand recognition(Shimaoka, Takagi et al. 2002); (Moser, Legate et al. 2009). Integrin β

cytoplasmic tails, with the exception of those of β4 and β8, are short. The affinity states of integrins are tightly regulated by their conformations. Integrins are usually expressed on the cell surface in a low-affinity bent conformation and quickly switch to a high affinity extended form upon binding of specific adaptors to their cytoplasmic tails. This process is called integrin activation or "inside-out" signaling (Calderwood, Zent et al. 1999; Carman and Springer 2003); (Arnaout, Goodman et al. 2007). Once integrins are activated, they are able to bind extracellular ligands and transmit a vast array of intracellular changes, which is known as the "outside –in" signaling **(Figure 1).** This bi-directional integrin signaling plays important roles in regulating cell functions (Hynes 2002; Schwartz and Ginsberg 2002); (Legate and Fassler 2009).

Integrin cytoplasmic tails provide anchors for multiple adaptor proteins with which they co-localize in focal adhesion sites(Zaidel-Bar, Itzkovitz et al. 2007). There are three categories of integrin adaptor proteins– adaptors that mainly have structural functions, scaffolding functions and catalytic functions. Talin, filamin and tensin are the main structural adaptors, which bind to F-actin and therefore connect integrins to cytoskeletal networks directly. CD98 and paxillin are examples of scaffolding adaptors, which provide binding sites for other proteins, including kinases, phosphatases and proteases. Catalytic adaptors, such as FAK, Src, ILK, Fyn, are critical for transmitting signals to the downstream upon integrin activation(Humphries, Byron et al. 2006); (Moser, Legate et al. 2009); (Zaidel-Bar, Itzkovitz et al. 2007); (Legate and Fassler 2009). Integrin downstream signaling can regulate cell functions at multiple levels. The

immediate changes following integrin activation includes increased tyrosine phosphorylation of specific adaptors such as FAK/Src complex, which is recognized as one of the central regulators of integrin signaling. Integrins also lead to increased concentrations of lipid second messengers, such as PIP2 and PIP3, which further activate AKT signaling pathways. Short term attachment to ECM leads to cell cytoskeletal rearrangement, which regulates cell migration, spreading and polarization. Long term integrin-mediated cell adhesion results in changes in signaling pathways and gene expression which mediate cell survival, growth and differentiation (Legate and Fassler 2009); (Calderwood, Fujioka et al. 2003).

Integrin-growth factor crosstalk

Integrins co-localize with multiple growth factor receptors (GFRs) in focal adhesion sites. Although both receptors can act independently, more frequently they act synergistically through a dynamic dialogue in response to the composition of extracellular matrix, its mechanical properties as well as the growth factor environment. Both integrin occupancy and aggregation are required for maximally synergistic, collaborative signaling output.

The crosstalk between integrin signaling and GFR signaling is bi-directional. Integrins are shown to work either as upstream or downstream regulators of GFR activity. For example, it was demonstrated that β1 integrin regulates EGFR expression, which is reduced upon cell detachment from ECM (Reginato, Mills et al. 2003). And EGFR

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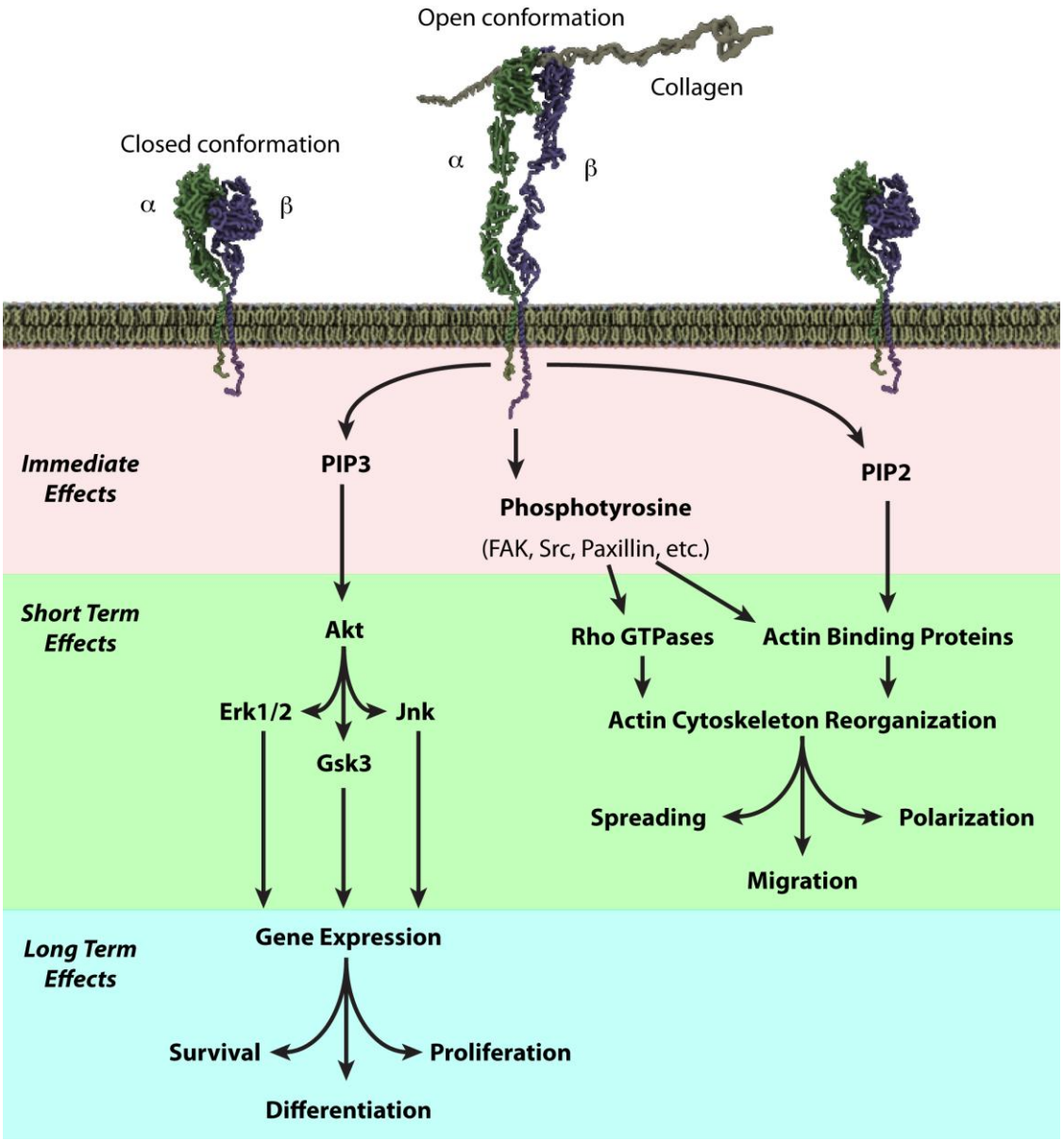

Figure 1: Integrin "outside-in" signaling Integrins usually express on cell surface in an inactive bent conformation and quickly switch to an extended conformation upon integrin activation. Integrin activation leads to the downstream signaling events that can be divided into three temporal stages, which regulate various cell functions.

Modified from (Legate, Wickstrom et al. 2009) Figure1

stimulation leads to increased β3- and β1- dependent cell adhesion (Zheng, Woodard et al. 2000). Several mechanisms have been proposed how integrins mediate GFR activity **(Figure 2).** The first mechanism is that integrins lead to aggregation of adaptor proteins such as FAK, which are recruited to the plasma membrane where they associate with GFRs. Growth factor binding activates GFRs, which function synergistically with integrins leading to enhanced downstream signaling(Alam, Goel et al. 2007). The second mechanism is that integrins alter GFR activity by changing their localization. Integrins co-localize GFRs, along with multiple signaling molecules, cytoskeletal molecules in the focal contacts. Integrins are shown to mediate IGF-1R signaling by perturbing their localization in focal contacts (Goel, Breen et al. 2005). α2β1 integrin is also demonstrated to co-localize with and activate EGFR at cell-cell contact sites(Yu, Miyamoto et al. 2000). The third hypothesized mechanism is that integrins may alter GFR rate of internalization or of degradation. A recent study has demonstrated that cell adhesion can change PDGFR rate of degradation via ubiquitination(Alam, Goel et al. 2007).

Several studies indicate that integrins are critical for the propagation of growth factor dependent cell signaling. Absence of integrins leads to diminished growth factor mediated signaling, despite normal growth factor receptor expression and activation on the cell surface (Baron and Schwartz 2000). Studies in Erk-MAPK pathway have provided us an example of the mechanisms whereby integrins regulate growth factor mediated signaling. It was shown that activation of MEK1 by FAK-Src complex, following integrin-mediated cell adhesion, is required for the signal to proceed to MAPK

activation, although growth factors are able to activate Ras independently of adhesion (Slack-Davis, Eblen et al. 2003). Also Raf1 phosphorylation is lost in non-adherent cells, which renders EGF unable to induce activation of Erk (Edin and Juliano 2005). Therefore MEK1 and Raf1 are two convergence points in integrin-mediated growth factor-dependent MAP kinase signaling.

Furthermore, studies suggest that, integrins are able to induce ligand-independent activation of GFRs, at least to the extent of enhanced tyrosine phosphorylation. For example, integrin αvβ3 activation leads to EGFR phosphorylation in the absence of EGF (Moro, Dolce et al. 2002). The biological effects of ligand-independent integrin mediated GFR activation remain unknown. It may be necessary for maximal physiological regulation of ECM and GFRs, as well as for the cells to respond to mechanical stimuli detected by integrins. Recent evidence also indicates that, integrin activation increases growth factor expression in the cellular micro-environment. For example, β1c integrin up-regulates IGF expression at both the mRNA and protein levels, leading to increased cell adhesion (Goel, Moro et al. 2006).

Many fundamental questions still need to be addressed to dissect the mechanisms of this integrin-growth factor crosstalk. For example, it remains unknown which regulators control integrin-GFR association and disassociation. Integrins may potentially serve as the primary target in pathological conditions, such as degenerative disease, inflammation and cancer, since they appear to be molecular switches controlling GFR

activity (Streuli and Akhtar 2009); (Alam, Goel et al. 2007). Furthermore, since integrin functions are tissue-specific and cell type-specific, the mechanism whereby integrins mediate growth factor-dependent signaling in different developmental processes may differ and remain unclear (Legate, Wickstrom et al. 2009).

Integrin function in development

Integrin activation is critical for dynamic processes such as embryonic development, inflammation and wound healing. The role of integrins in development has been investigated utilizing both transgenic mice models and in vitro cell culture system. Homozygous deletion of integrin subunit sometimes leads to early embryonic lethality or neonatal death in mice. For example, mice homozygous for null mutation in the gene for β1 integrin die at pre-implantation stage around E5.5. The functions of integrins in different organs, such as skin, neuron and mammary gland, have been studied utilizing tissue specific integrin deletion. All the studies have highlighted that integrins have an effect on cell survival, proliferation and matrix remodeling. These effects are integrin-type, cell type- and developmental stage-dependent. For example, ablation of integrin α3 subunit in skin only generated microblistering, whereas specific integrin β1 deletion in the epidermis resulted in severe skin blistering, massive failure of BM assembly/organization, hemidesmosome instability, and a failure of keratinocytes within the hair follicles to remodel BM and invaginate into the dermis (DiPersio, van der Neut et al. 2000); (DiPersio, Hodivala-Dilke et al. 1997). In vitro studies have revealed that skin maintains homeostasis via ECM synthesis and degradation which are regulated by

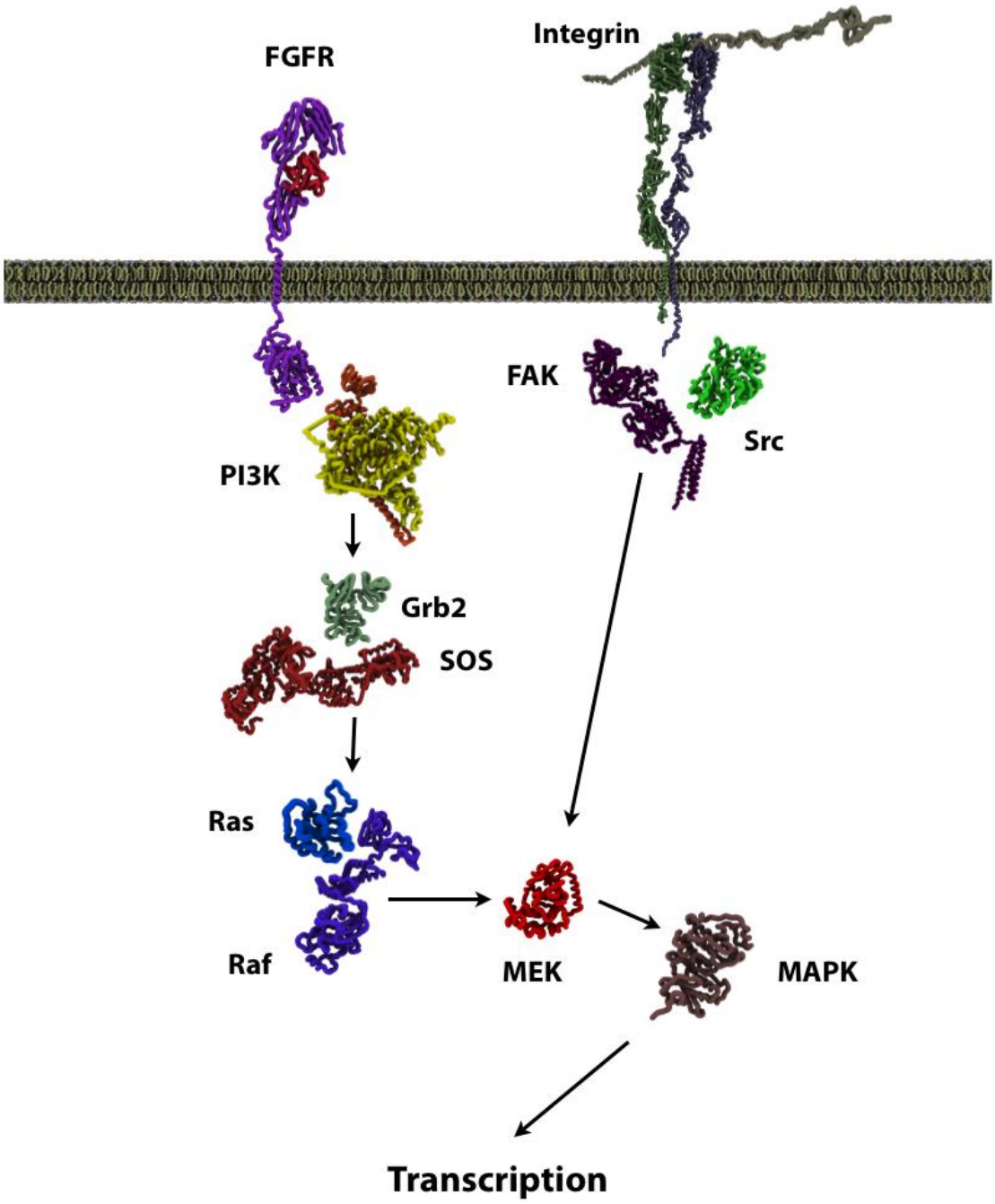

Figure 2: Regulation of GFR signaling by integrins Integrins, upon association with GFRs, modulate GFR dependent cell signaling at multiple levels. This synergism between integrins and GFRs leads to maximal signaling output.

Modified from Alam, Goel et al. 2007 Figure 1

different integrin subunits. α2β1 is the predominant collagen receptor on keratinocytes and its activation up-regulates MMP1 synthesis via p38 MAP kinase signaling pathways. In contrast, integrin α1β1 is expressed predominantly by fibroblasts and its activation leads to decreased MMP synthesis depending on ERK1/2 signaling(Pozzi and Zent 2003).

In mammary gland, deletion of α6 integrins did not result in any functional or developmental abnormalities in mice, although these integrins are expressed in development (Klinowska, Alexander et al. 2001). However when β1 is deleted early in mammary gland development the alveoli was disorganized and contained clumps of epithelial cells bulging into what would normally be luminal space, suggesting that β1 integrin is essential for normal lobuloalveolar development. When β1 integrin was deleted in already developed mammary glands, no obvious abnormalities were observed, although mice showed smaller glands and produced less milk than control mice. Furthermore in a cell culture model β1 integrin was shown to be essential for terminal differentiation of the epithelial cells. Thus in early mammary development β1 integrin is required for normal formation of the gland, while in the setting of a developed mammary gland it is required for normal function of the gland (Naylor, Li et al. 2005). Neuronal differentiation is also retarded in β1 integrin-deficient stem cell derived neurons due to limited migration and morphological differentiation (Clegg, Wingerd et al. 2003). However, the role of β1 integrins in kidney development was poorly characterized.

Kidney development

Kidney is the mammalian excretory system and contributes to the maintenance of homeostasis through urine production. Various metabolic waste products are eliminated in the urine by a complex process that involves filtration, active absorption, passive absorption and secretion. Most of the filtrates are reabsorbed in the kidneys. Kidneys also regulate the fluid and electrolyte balance of the body and are the sites of production of renin, which plays roles in the regulation of blood pressure. Each kidney can be divided into an outer cortex and an inner medulla. The renal medulla consists of pyramidal structures, the medullary pyramids， from the base of which parallel arrays of tubules, referred to as the medullary rays, penetrate the cortex. The nephron is the functional unit of the kidney. There are around one million nephrons in an adult human kidney. Each nephron includes the glomerulus, the proximal convoluted tubule, loop of Henle, and the distal convoluted tubule, which is joined to a collecting duct.

The metanephric kidney develops from the metanephric mesenchyme (MM), which is a specialized region of the caudal end of the intermediate mesoderm. MM signals to the wolffian duct, a tube running along each side of the body， which evaginates ureteric bud (UB) in response to the signal. UB invades the MM and finally gives rise to the collecting system of the kidney via a process known as branching morphogenesis. The kidney collecting system includes the collecting ducts, calyces, pelvis, and ureter. In the mouse, UB formation is initiated at day 10.5 and undergoes the first bifid branching event, forming a T-shaped bud at E11.5. After a rapid branching

stage, on E17.5 it enters a stage when the tubules elongate extensively(Costantini 2006). The signals that promote and direct UB branching morphogenesis could be derived from multiple sources, including the UB itself and any other cell population in the developing kidney, such as the MM, stroma or vascular cells(Dressler 2009).

Induced MM is aggregated around the UB tips. Aggregates proliferate, begin to exhibit evidence of epithelial cell polarity and form renal vesicles. The structure referred to as comma shaped body is generated when a single cleft forms in the vesicle. The second cleft generates the S-shaped body, which differentiates into glomerulus when it is infiltrated by endothelial cells at E14.5 in mice. Glomerular basement membrane (GBM) is formed at the boundary between podocyte and endothelial cells. The podocytes begin to extend primary and secondary foot processes, while endothelial cells become fenestrated. The fenestrated endothelia on the capillary side and interdigitated podocyte foot processes on the urinary space side separated by GBM together form the glomerular filtration barrier. A specific structure called slit diaphragm between podocyte foot processes helps to maintain a specific pore size that only allows small molecules to traverse the filtration barrier. The integrity of the glomerular filter depends in large part on the proteins of the nephrin complex including nephrin, podocin and CD2AP, which localize to the slit diaphragm (Boute, Gribouval et al. 2000; Roselli, Gribouval et al. 2002). Mutations in several proteins of the nephrin complex lead to the effacement of podocyte foot process (Dressler 2006); (Jarad and Miner 2009).

The kidney development can be regulated at both transcriptional and cellular levels. For example, *Lim1, Pax2/8, Eya1* and *Odd1* genes are known to be critical for early patterning of the kidney (Xu, Adams et al. 1999). Several transcriptional factors, such as Foxc1, FoxC2, suppress ectopic UB outgrowth (Wilm, James et al. 2004), while the *Hox* family genes are positive regulators of UB branching (Wellik, Hawkes et al. 2002). Up-regulation of *Wnt4* gene in MM aggregates is required for the formation of polarized renal vesicles, which further differentiate into nephrons (Merkel, Karner et al. 2007). At the cellular level, multiple signal transduction proteins, such as protein kinase C (PKC), protein kinase A (PKA) and Erk MAPK kinase, have been implicated in UB development. PKC and Erk MAPK signaling pathways have been shown to induce UB branching, while PKA signaling inhibits branching. Notch signaling in early aggregates and s-shaped bodies is revealed to play an essential role in proximal-distal patterning as the nephron develops (Dressler 2006). Furthermore, several growth factors and distal effector molecules, such as ECM proteins, proteases and integrins, are all critical for kidney development (Orellana and Avner 1998; Reidy and Rosenblum 2009)

Several model systems have been generated to investigate kidney development, including whole embryonic kidney organ culture system, isolated UB culture system, genetically engineered mice and cell culture models. Three renal cell lines have been used extensively for studies of branching morphogenesis – UB (ureteric bud), IMCD (inner medullary collecting duct) and MDCK (mardin darby canine kidney) cells (Pohl, Stuart et al. 2000).

Growth factor function in kidney development

Several growth factors, such as GDNF, FGF, HGF and BMP7, are essential for UB branching morphogenesis. VEGF plays fundamental roles in glomerulogenesis and maintenance of glomerular integrity. **(Figure 4)**

GDNF is first expressed in the metanephric mesenchyme adjacent to the caudal region of the WD, where the UB will emerge. GDNF signals through a receptor tyrosine kinase, Ret, together with the co-receptor Gfrα1. Knock-out of any of these genes usually leads to renal agenesis due to failure of the UB to emerge from the WD. Thus GDNF signaling promotes UB outgrowth and is also important for continued UB branching morphogenesis, based on several types of evidence in vitro and in vivo (Shakya, Watanabe et al. 2005). Among the cellular processes that might mediate epithelial branching, GDNF has been implicated in two of them, proliferation and migration, and might also have effects on cell-cell adhesion. At least three of the well-studied signaling pathways downstream of Ret contribute to UB branching - the PI3-kinase/Akt, Ras/Erk MAP kinase, and PLC-γ/calcium pathways (Tang, Cai et al. 2002; Kim and Dressler 2007).

While numerous members of the FGF family are expressed in the developing kidney(Cancilla, Davies et al. 2001), FGF7 and FGF10 have been implicated as two central players in UB branching morphogenesis (Qiao, Uzzo et al. 1999; Ohuchi, Hori et al. 2000). The kidneys of FGF10-null mice are slightly smaller than normal. Mice

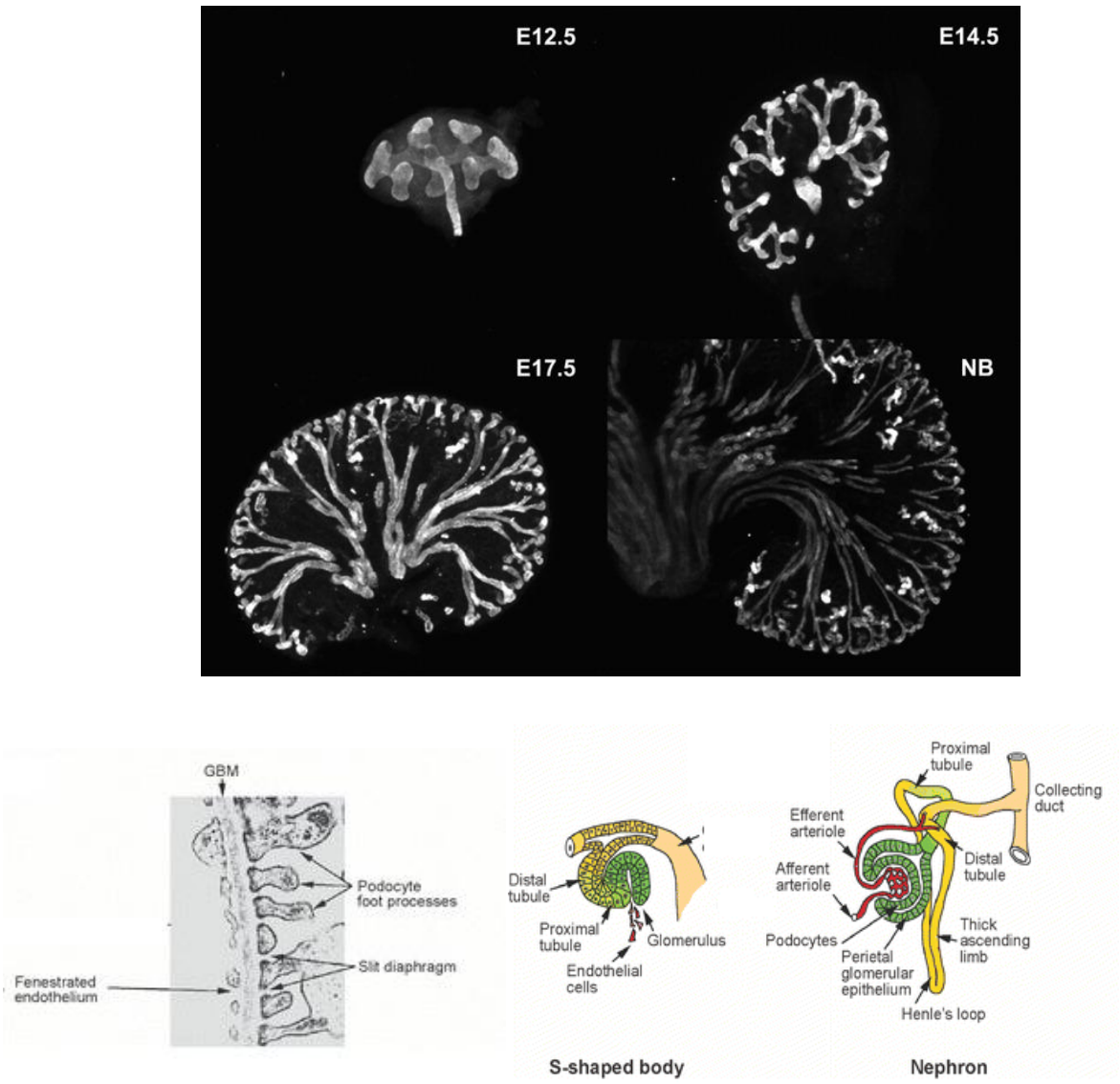

Figure 3 Development of the kidney Induction of the MM by the UB promotes aggregation of the condensed mesenchyme around UB tips. UB undergoes rounds of branching events and finally gives rise to the kidney collecting system. The aggregates become polarized as they undergo mesenchymal-to-epithelial conversion and finally differentiate into the nephrons. Glomerulogenesis starts upon invasion of endothelial cells into the proximal cleft at S-shaped body In a mature glomerulus, the filtration barrier includes the fenestrated endothelial on the capillary side and the interdigitated podocyte foot process on the urinary side separated by GBM.

Modified from Cebrián 2004 Figure2 & Dressler 2006 Figure2

lacking FGF7 have defects in basement membrane and reduced nephron numbers. These two FGFs are likely to be partially redundant in vivo, but the phenotype of FGF7/FGF10 double mutant mice has not been reported yet. FGFR2 is the receptor for both FGF7 and FGF10. Mice with deletions of FGFR1 or FGFR2 are early embryonic lethal prior to the initiation of kidney development. UB specific deletion of FGFR2 results in aberrant UB branching, thin ureteric bud stalks, and fewer ureteric bud tips with reduced proliferation and inappropriate regions of apoptosis(Zhao, Kegg et al. 2004); (Beenken and Mohammadi 2009).

Hepatocyte growth factor (HGF) is a mesenchymally derived factor and signals through its transmembrane receptor c-Met. HGF/c-Met signaling plays important roles in renal tubule branch and elongation. In vitro, when IMCD or MDCK cells are grown suspended in a collagen gel in the presence of HGF, they form an elongated, spindle-like morphology with processes extended into the matrix. However, in the absence of HGF, they form cysts consisting of a monolayer of cells which surrounds a central lumen. Following HGF stimulation, some of the cyst cells migrate away from the cyst and into the surrounding matrix where they elongate and proliferate (Karihaloo, Nickel et al. 2005).

Bone morphogenic protein 4(BMP-4) and BMP7, which are made by developing tubules, have been implicated in MM proliferation. In vitro, BMP7 inhibits apoptosis of the uninduced MM (Dudley, Lyons et al. 1995). BMP7 in coordination with fibroblast

growth factor2 (FGF2), play roles in maintaining the competence of the mesenchyme to respond to inductive signals.

During glomerular development, the podocytes express a number of vascular growth factors such as VEGF-A, and the glomerular endothelial cells express the VEGF receptors (Eremina, Sood et al. 2003). Using cell-specific gene targeting, it is shown that VEGF production by podocytes is required for glomerular endothelial cell migration, differentiation, and survival. The podocyte-specific homozygotes for VEGF usually die at birth with renal failure and grossly abnormal glomeruli that lack mature endothelial cells. Mice carrying one hypomorphic VEGF-A allele and one podocyte-specific null VEGF-A die at 3 weeks age due to renal failure, characterized by dilated capillary loops as well as severe endothelium and mesangium injuries. Over-expression of VEGF-A in podocytes also leads to end-stage renal failure. These findings demonstrate that a strict regulation of VEGF signaling is required between the podocyte and glomerular endothelium (Eremina, Cui et al. 2006). Platelet-derived growth factor (PDGF) secreted by endothelial cells may also help to maintain the mesangium integrity (Lindahl, Hellstrom et al. 1998).

ECM function in kidney development

The roles of extracellular matrix proteins in kidney development have also been investigated utilizing both genetic mice models and in vitro cell culture systems. GBM plays an important role in maintenance of glomerular filtration barrier through the

function of its major components collagen IV, laminins and proteoglycans. Changes in GBM composition occur during kidney development. A laminin α1 to α5 expression transition occurs at capillary formation stage, concurrently with the laminin β1 to β2 and collage α1, 2 to α3, 4, 5 chain switches. At the onset of metanephrogenesis, laminin α5 is mostly expressed in the Wolffian duct and in the UB basement membranes. Once the mesenchyme condensation and polarization occur, there is a significant increase in laminin α1 expression. At the S-shape stage, the developing GBM express both laminin α1 and α5 chains. But at the capillary formation stage, α1 chain is eliminated leaving α5 as the only GBM laminin α chain(Miner 2005).

Targeted mutations in ECM components significantly affect the kidney function (Muller and Brandli 1999). A striking defect in glomerulogenesis is found in laminin322 mutant embryos, characterized by an abnormal GBM and a complete absence of vascularized glomeruli(Miner and Li 2000). In mice lacking collagen IV α3 chain, there are aberrant deposition of BM components such as collagen α1, α2, perlecan and fibronectin, resulting in compromised GBM integrity over time (Miner and Sanes 1996; Miner and Li 2000). ECM proteins also play essential roles in regulating UB branching morphogenesis, either through simple adhesions or adhesion mediated signaling pathways. Matrix metalloproteases are found to contribute to UB branching, possibly by creating available spaces and modifying growth factor availability.

Most of the interactions between cells and ECM proteins are mediated through cell surface receptor integrins. Activated integrins modulate cell functions, such as cell

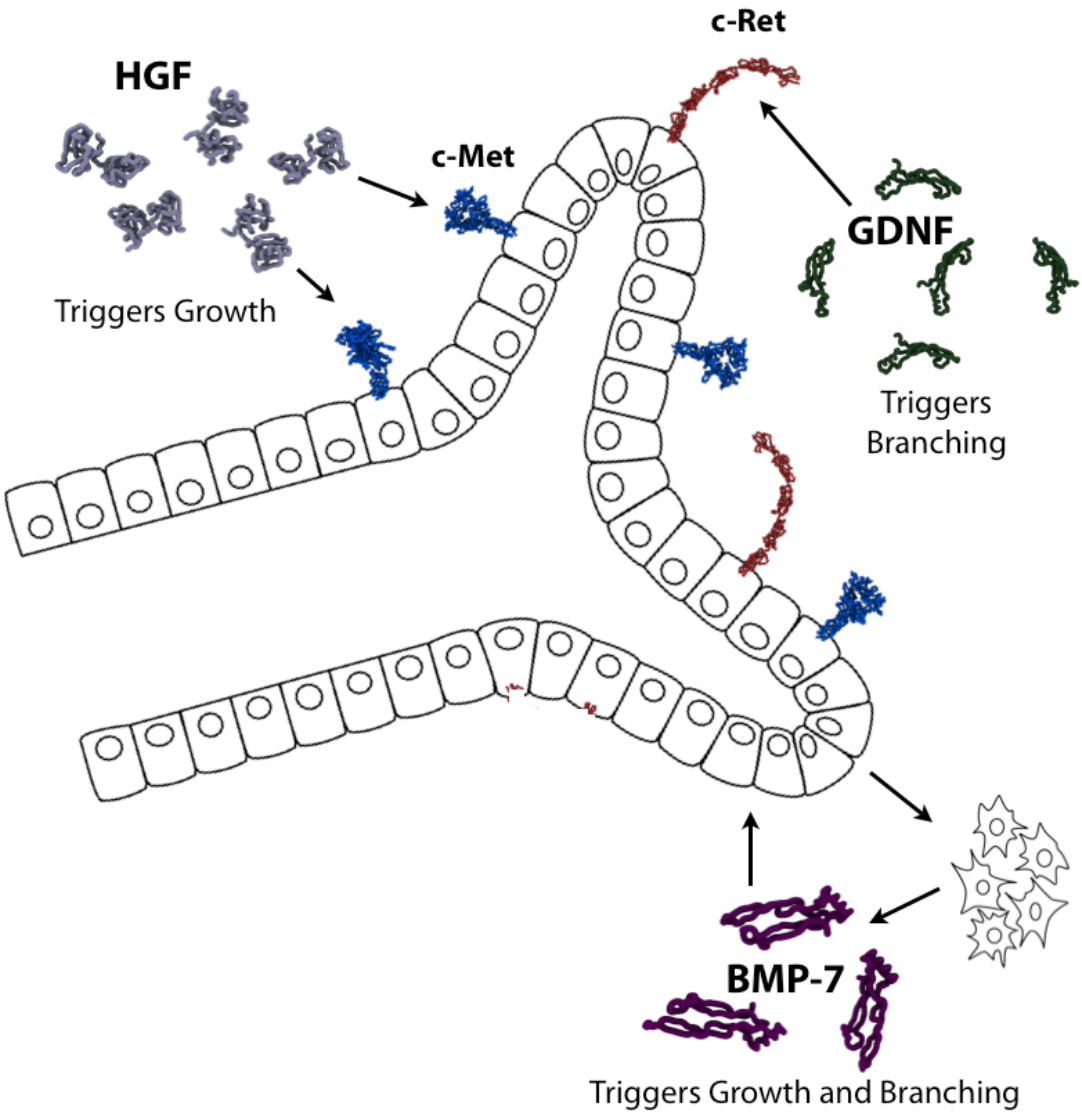

Figure 4: Growth factors in kidney development Key signals regulating the development of the ureteric bud include growth factors, distal effector molecules and genes that regulate proliferation and apoptosis. Several growth factors, such as GDNF, FGF and HGF, are all critical for this developmental process.

Modified from (Sariola and Saarma 1999) Figure3

migration, spreading, proliferation and apoptosis, which are important for kidney development. Integrins exhibit spatiotemporal expression in the mammalian kidneys. The α2β1, α3β1 and α6β1 integrins are all expressed by distal tubules and by collecting duct epithelial cells(Korhonen, Ylanne et al. 1990). The α3β1 integrin is the major ECM receptor expressed by podocytes along the GBM. Endothelial cells express α3β1, α5β1, α6β1 and αv-containing integrins(Miner 2005). α1β1 integrin is the most prominent type expressed on mesangial cells. Altered expression and localization of integrins have been observed in several renal diseases. But the mechanisms whereby integrins mediate kidney development are poorly characterized.

Integrin function in kidney development

β1 is abundantly expressed in the kidneys and it can bind to at least 12 α subunits(Hynes 2002). The specific functions of β1 integrins in kidney development are poorly characterized in genetic mouse models, as homozygous β1-deletion results in pre-implantation lethality (Fassler and Meyer 1995). As an alternative approach, mutations in α subunits were generated to identify their roles in renal development. Both integrin α3β1 (Kreidberg, Donovan et al. 1996) and α8β1 (Denda 1999) were shown to be important for UB branching morphogenesis. α3β1 is the only integrin shown to play a significant role in glomerular development *in vivo* (Kreidberg, Donovan et al. 1996). Deletion of α6 subunit leads to neonatal death, but surprisingly these mice don't show any renal phenotypes (Georges-Labouesse, Messaddeq et al. 1996).

1) αβ1 function in renal collecting system development

Both integrin α8-null and α3-null mice showed severe renal collecting system defects (Brandenberger, Schmidt et al. 2001); (Kreidberg, Donovan et al. 1996). α8-null mice have defects in growth and branching of the UB and in recruitment of mesenchymal cells into epithelial structures. Most homozygous mutants die by the first or second day after birth and have severe kidney abnormalities. Half of the mutant animals were born without ureters or kidneys. Consistent with these phenotypes, α8 expression is induced in mesenchymal cells upon contact with the ureteric bud, since inductive interactions between the ureteric epithelium and MM are essential for kidney morphogenesis in wild type animals (Brandenberger, Schmidt et al. 2001).

The α3 integrin gene is expressed during the development of many epithelial organs, including the kidney, lung and skin. The collecting system of α3-null mice is also severely affected with fewer than normal collecting ducts in the papilla and more dilated tubules, suggesting decreased UB branching morphogenesis (Rahilly and Fleming 1992; Kreidberg, Donovan et al. 1996). When the integrin α3 subunit was specifically deleted in the UB, the kidney papillaes were either absent or abnormal (Liu, Chattopadhyay et al. 2009), however the rest of the collecting system of the kidney was unaffected.

2) αβ1 function in glomerular development

Integrin α3-null mice (Kreidberg, Donovan et al. 1996) show severe abnormalities in glomeruli formation and structure as well. In mature glomerulus, α3β1 integrin is the predominant integrin expressed by glomerular podocytes in a polarized pattern along the

GBM. It is primarily a receptor for laminins322 and 511/521 but it can also interact with collagen IV and is expressed in all glomerular and tubule cells(Kreidberg 2000). α3-null mice show abnormal glomeruli formation, with alterations in glomerular capillary loops, disorganized glomerular BMs and inability of the podocyte foot processes to mature properly. When integrin α3 subunit was specifically deleted in the podocyte (Sachs, Kreft et al. 2006), α3-null pod mice develop massive proteinuria in the first week of life and nephrotic syndrome by 5–6 weeks of age. The kidneys of the 6 week old mice contained sclerosed glomeruli, a disorganized GBM and prominent protein casts in dilated proximal tubules. Electron microscopy revealed complete effacement of podocyte foot processes in newborn mice and widespread lamination and protrusions of the GBM in 6 week-old mice. Integrin α1β1 is also highly express in the glomerulus and plays a minor role in glomerular development, as integrin α1- mice show subtle glomerular phenotypes characterized by mild mesangial hypercellularity and matrix deposition (Hartner, Cordasic et al. 2002; Haas, Amann et al. 2003; Chen, Moeckel et al. 2004). Glomeruli of integrin α2-null mice do not exhibit any overt glomerular phenotypes.

In contrast, when integrin α6 was deleted in mice, no renal phenotypes were seen (Georges-Labouesse, Messaddeq et al. 1996). α6 is highly expressed in both the tubules and glomerulus and can heterodimerize with either β1 or β4(Hemler, Crouse et al. 1989). α6β1 is predominantly a receptor for laminins111 and 511/521, while α6β4 appears to interact specifically with laminins 511/521 and 322, where it participates in the formation of hemidesmosomes(Colognato and Yurchenco 2000). These ECM components are

highly expressed in the BMs of both the tubules and glomerulus. α3/α6 double-deficient mice die at late embryonic stage; have a similar renal phenotype to that seen in the α3-null mice, with the exception that the double mutants did not develop ureters (De Arcangelis, Mark et al. 1999). This may possibly be explained by the fact that the α3/α6 null mice die at late embryonic stage when the renal phenotype is not easily seen.

In contrast to these in vivo data there is considerable evidence that α6 integrins are important for renal development in vitro and that inhibiting α3 and α6 function has an additive effect with respect to branching morphogenesis (Zent, Bush et al. 2001). Utilizing organ culture models, integrin α6β1 was shown to be required for normal nephrogenesis by interacting with its ligand laminin111(Falk, Salmivirta et al. 1996). And laminin111 has previously been shown to be of major importance for the development of kidney tubules. In contrast, the collagen receptors, integrins α1β1 and α2β1, do not play a major role in this process in vitro (Zent, Bush et al. 2001), and the role of the RGD binding receptors α5β1 and αv integrins is unknown.

From the above data it is clear that integrins that interact with BMs (i.e. α1β1, α3β1, α6β1) do play a role in both renal collecting system development and glomerular development; however there is conflict between the in vivo and in vitro data. In addition, with the exception of the α3- or α8-null mice, integrin α-null mice did not exhibit as severe a phenotype as expected. This is most likely because multiple α integrins can interact with the same ligands thus compensating for the lack of a particular α subunit. This phenomenon was observed in skin development and mammary gland development.

Specific integrin β1 deletion leads to much more severe skin blistering than α3-null mice, which suggest that multiple α1 integrins contribute to keratinocyte proliferation and differentiation (DiPersio, van der Neut et al. 2000); (DiPersio, Hodivala-Dilke et al. 1997). Also in mammary gland, deletion of α3β1 or α6 integrins did not result in any functional or developmental abnormalities in mice, while deletion of β1 integrin in early mammary gland development results in severe lobuloalveolar phenotypes (Klinowska, Alexander et al. 2001);(Naylor, Li et al. 2005). These results suggest that αsβ1 integrins other than α3β1 and α6β1 play a role in their development. The roles played by β1 are organ specific and cell type-specific. β1 integrin function in kidney development was unknown and, as demonstrated by results from the studies indicated above, it was difficult to predict. Although integrin α3β1 is the major ECM receptor required for renal development, it is possible that deleting β1 integrin would result in a more profound phenotype, as multiple BM-interacting αβ1 integrins would be deleted simultaneously and the conflict between in vitro and in vivo data does suggest a likely functional redundancy among α subunits. Based on these observations, we hypothesize that β1 integrin is critical for kidney development.

Aims of dissertation

The overall aims of this dissertation are:

1) To determine the role of β1 integrin in collecting system development

The kidney collecting system develops from branching morphogenesis of the ureteric bud. This process requires signaling by growth factors such as GDNF and FGF, as well as cell-ECM interactions mediated by integrins.

To investigate the function of β1 integrin in UB development we selectively deleted integrin β1 at initiation (E10.5) and late (E18.5) stages of development. We analyzed morphological and physiological properties of these two β1-null mice kidneys in vivo. Furthermore, we explored the underlying mechanisms via in vitro cell culture system.

2) To determine the role of β1 integrin in glomerular development

β1 containing integrins are highly expressed in the glomerulus of the kidney; however their roles in glomerular morphogenesis and maintenance of glomerular filtration barrier integrity are poorly understood.

To study these questions we selectively deleted β1 integrin in the podocyte at capillary formation stage (E14.5). We analyzed morphologies of β1-null mice kidneys at different developmental stages utilizing both light microscopy and electron microscopy. As podocyte is an important component of glomerular filtration barrier, we focused on investigation of glomerular structural integrity.

CHAPTER II

CHARACTERIZATION OF β1 INTEGRIN FUNCTION IN KIDNEY DEVELOPMENT

Introduction

Formation, growth and branching morphogenesis of the collecting system of the kidney require interactions between the UB and the MM. The UB ultimately forms the multi-branched collecting system within the kidney, as well as the ureter and the bladder trigone. Development of the collecting system initially involves many iterations of branching morphogenesis followed by a period where kidney growth predominates. This complex developmental process is dependent on numerous factors, including growth factor-dependent cell signaling induced by GDNF and FGFs as well as interactions between cells and ECM components (Dressler 2006).

β1 is one of the most abundantly expressed β subunit in the kidney (Kreidberg and Symons 2000). Integrins have the potential to play important roles in organ morphogenesis by modulating cell growth, motility and shape(Schwartz and Ginsberg 2002). The role of β1 integrins in kidney collecting system development is unclear. Among the different integrin α-null mice generated, only integrin α3- and α8-null mice show a severe collecting system phenotype (Muller, Wang et al. 1997); (Linton, Martin et al. 2007); (Liu, Chattopadhyay et al. 2009). Interestingly, integrin α6-null mice do not display a collecting system phenotype (Georges-Labouesse, Messaddeq et al. 1996),

although the α3/α6 double-deficient mice fail to develop ureters (De Arcangelis, Mark et al. 1999). In contrast to the *in vivo* data, in organ and cell culture models, α6 integrins (i.e. α6β1 and α6β4) have been found to be important for UB branching morphogenesis and blocking the α6 subunit alone or in combination with the α3 subunit affects UB branching morphogenesis(Zent, Bush et al. 2001).

To determine whether β1 integrins other than α3β1 play a role in UB development *in vivo*; we selectively deleted β1 integrin in the UB at two time points. A severe branching phenotype was observed when β1 integrin was deleted at E10.5, at the time that UB branching morphogenesis is initiated. In contrast, when β1 integrin was deleted in collecting ducts at E18.5, development was normal; however severe collecting system injury was observed in adult animals following ureteric obstruction. We found that canonical signaling pathways activated by FGFs require β1 integrin expression in CD cells *in vitro* and most importantly, activation of the same pathways was decreased in β1-null UBs *in vivo*, despite the normal activation state of the FGF receptor. Thus in addition to their well characterized roles in adhesion and migration, β1 integrins play a critical role in transducing growth factor-dependent signals required for UB branching morphogenesis and maintaining collecting tubule integrity following injury.

Material and methods

Generation of Hox7b-Cre;$\beta1^{flox/flox}$ mice, AQP2-Cre$\beta1^{flox/flox}$ mice

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. Integrin $\beta1^{flox/flox}$ mice (generous gift of Dr. E. Fuchs, Howard Hughes Medical Institute, The Rockefeller University) or integrin $\beta1^{flox/flox}$ lacZ mice, in which a promoterless lacZ reporter gene was introduced after the downstream loxP site (Brakebusch, Grose et al. 2000)were crossed with the Hox7b-Cre mice (Hox-Cre) (generous gift of Dr.A.McMahon) or aqp2-Cre mice mice (Ouvrard-Pascaud, Puttini et al. 2004). Mice varied between 4^{th} and 6^{th} generation C57BL6. Age-matched littermates homozygous for the floxed integrin β1 gene, but lacking Cre ($\beta1^{flox/flox}$ mice), were used as negative controls.

Morphologic analysis

For morphological and immunohistochemical analysis, kidneys at different stages of development were removed immediately at sacrifice and fixed in 4% formaldehyde and embedded in paraffin, or embedded in OCT compound and stored at -80^{o}C until use, or fixed in 2.5% glutaraldehyde, post-fixed in OsO_4, dehydrated in ethanol and embedded in resin. Paraffin tissue sections were stained with either hematoxylin and eosin (H&E) or Periodic Acid Schiff's (PAS) for morphological evaluation by light microscopy. For electron microscopy, ultrastructural assessments of thin kidney sections were performed using a Morgagni transmission electron microscope (FEI, Eindhoven, Netherlands).

Organ Culture

Embryonic kidneys were isolated from E12.5 mice and placed on the top of Transwell filters and cultured (37°C and 5% CO_2/100% humidity) in DMEM/F12 media

supplemented with 10% FBS. After 3 days, the kidneys were fixed in 4% paraformaldehyde and stained with fluorescein-conjugated E-cadherin antibodies (BD Transduction Laboratories, Lexington, KY). The number of branching structures was counted.

Generation of β1-null cell line

CD cells were isolated from $\beta1^{flox/flox}$ mice following the methodology described by Husted et al (Husted, Hayashi et al. 1988) and β1 was deleted by infecting the cells with an adenocre virus. To verify adequate deletion the cells were subjected to flow cytometry as described below.

Flow Cytometry

$\beta1^{flox/flox}$ IMCD cells and β1-null cells were incubated with anti-β1,β4,α1,α2,α6,αv integrin antibodies (BD Transduction Laboratories, Lexington, KY), followed by FITC-conjugated secondary antibodies. Expression levels of different integrins in these two cell lines were detected by flow cytometry.

Cell adhesion

Cell adhesion assays were performed in 96 well plates. Briefly plates were coated with different concentrations of ECM components and blocked with BSA. 10^5 cells were placed in each well in serum-free DMEM for 60 minutes and non-adherent cells were removed and the remaining cells were fixed, stained with crystal violet, solubilized and the O.D. of the cell lysates was read at 570 nm. Four independent experiments were performed in triplicate.

Cell migration

Cell migration was assayed on transwells with 8-µm pores that were coated with different ECM components. 10^5 cells were added to the upper part and that migrated through the filter were counted 4 hours later. Four independent experiments were performed in triplicate.

Cell Proliferation

5 x 10^3cells were plated per well in 96-well plates on different ECM components and maintained in DMEM (10% FBS) overnight followed by DMEM (2% FBS) for another 24 hrs. Cells were then incubated for 24hrs with 1 µCi/well [^{3}H] thymidine (PerkinElmer Life Sciences). The cells were solubilized and radioactivity was measured using a scintillation counter. For sequential counting assays, 6 X 10^4 cells were plated per plated on collagen or vitronectin and maintained in a serum-free environment. Cells were counted 48 hrs after FGF (10ug/ml) administration.

Cell Polarity

Cells were grown on transwells consisting of polyvinylpyrolidone-free polycarbonate filters with 0.4µm pores. When the cells were confluent, they were fixed in 3.7% formaldehyde and incubated with anti-ZO-1 (1:200) (BD Transduction Laboratories, Lexington, KY) antibodies followed by the appropriate FITC-conjugated secondary antibody. Chamber slides were mounted and viewed using a confocal microscope.

Cell Spreading

Cells were plated onto slides coated with Collagen-I (10ug/ml) or Vitronectin for 30mins after which FGF (10ug/ml) or GDNF (10ug/ml) was added. Forty five minutes later cells were fixed, permeabilized and exposed to Rhodamine-Phallodin (1:5000). For p-FGFR1 and t-FGFR1 staining, cells were incubated with anti-pFGFR1 (directed against Y654) (Abcam, Cambridge, MA) and tFGFR1 antibody (Cell Signaling, Beverly, MA) overnight, then incubated with a FITC conjugated secondary antibody for 2hrs and visualized under a microscope.

Results

Deleting β1 integrin in the UB at E 10.5 results in a major branching morphogenesis phenotype

To define the role of β1 integrin in the developing UB, we crossed HoxB7Cre mice, which express Cre in the Wolfian duct and UB from E10.5, with integrin $\beta1^{flox/flox}$ mice, in which a promoterless lacZ reporter gene was introduced after the downstream loxP site (Brakebusch, Grose et al. 2000). As demonstrated in **Figure 5A**, strong β-galactosidase staining was evident in the collecting system of the kidney. We confirmed β1 integrin deletion by performing immunoblotting on isolated papillae of newborn HoxB7Cre;$\beta1^{flox/flox}$ mice with an antibody directed at the integrin β1 subunit (**Figure 5A**). Although these mice were born in the normal Mendelian ratio, all the HoxB7Cre;$\beta1^{flox/flox}$ mice died between four and six weeks of age. The kidneys of 6 week old were smaller and slightly cystic compared to those isolated from $\beta1^{flox/flox}$ mice (**Figure 5B and C**). On microscopic examination, we found that HoxB7Cre;$\beta1^{flox/flox}$

kidneys had far fewer nephrons than $\beta1^{flox/flox}$ kidneys. Moreover, there were many more dilated tubules in both the cortex and medulla, and there was marked interstitial fibrosis (**Figure 1 D-I**). The most severe tubular dilatation was seen in the CDs located in both the medulla and the cortex (**Figure 5 D-I**).

To further define the defects present in these mice, we performed studies on embryonic kidneys from E11.5 until birth. At every stage analyzed, we observed clearly a branching morphogenesis defect in HoxB7Cre;$\beta1^{flox/flox}$ mice. At day E11.5 the UB was less branched and smaller in HoxB7Cre;$\beta1^{flox/flox}$ mice (**Figure 6A and B**). Kidneys of HoxB7Cre;$\beta1^{flox/flox}$ mice at E13.5 (**Figure 6C and D**) as well as E15.5 (**Figure 6E and F**) were significantly smaller with a decreased number of UB branches and nephrons. We further verified the branching phenotype observed in HoxB7Cre;$\beta1^{flox/flox}$ mice by performing *in vitro* cultures of E12.5 embryonic kidney. After 48 hours in culture, HoxB7Cre;$\beta1^{flox/flox}$ E12.5 kidneys formed approximately 5 branches per structure relative to the 50 detected in the $\beta1^{flox/flox}$ kidneys (**Figure G-I**).

Kidneys of newborn HoxB7Cre;$\beta1^{flox/flox}$ were also smaller and had less collecting ducts than $\beta1^{flox/flox}$ mice (**Figures 6J and K**). Interestingly the tubules, although somewhat disorganized, did not appear to have abnormalities with respect to polarity (Figures 6L and M). We confirmed this observation by staining the newborn kidneys with antibodies directed against E-cadherin (**Figure N and O**) and ZO1. Electron microscopy on non-perfused newborn kidneys also established that cells forming the tubules were polarized and that the tubular basement membranes were normal, despite the finding that

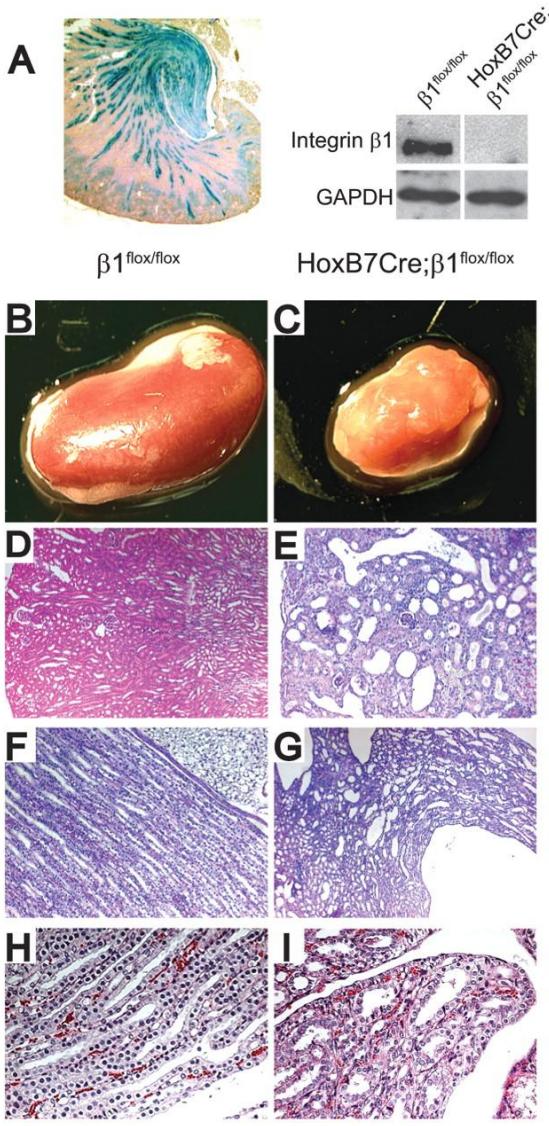

Figure 5. HoxB7Cre;β1$^{flox/flox}$ mice develop severe end stage renal failure. (A) LacZ staining of HoxB7Cre;β1$^{flox/flox}$ mice at P10 demonstrating excision of β1 integrin (left panel). Deletion of β1 integrin in the HoxB7Cre;β1$^{flox/flox}$ mice was confirmed by immunoblotting. (right panel). **(B-C)** Gross appearance of kidneys of 6 week old HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice . **(D-E)** Microscopy of PAS stained kidney slides (100x). **(F-G)** The collecting ducts in the medulla and papilla of the HoxB7Cre;β1$^{flox/flox}$ mice are dilated and disorganized (100x). **(H-I**) Dilatation of the collecting ducts is present in the papilla (200x).

the tubular lumens were dilated (**Figure 6P and Q**). A significant decrease in nephrons was also present in newborn HoxB7Cre;$\beta1^{flox/flox}$ mice as there were 1.6 vs 4.2 glomeruli/mm^2 of cortex in the HoxB7Cre;$\beta1^{flox/flox}$ and $\beta1^{flox/flox}$ mice respectively (**Figure 6R**).

Taken together our data demonstrate that deleting β1 integrin in the UB of the kidney at the time of initiation of branching morphogenesis results in a profound branching phenotype with fewer nephrons and a severely dysmorphic dysplastic collecting system.

Deleting β1 integrin in the CDs at E18.5 results in normal kidney development but an abnormal response to renal injury

To determine the role of β1 integrin in CD development, we utilized the aquaporin 2 Cre (Aqp2Cre) mouse to delete β1 in CDs at E18.5 (Stricklett, Nelson et al. 1999; Zhang, Zhang et al. 2005). We confirmed gene deletion in the Aqp2Cre; $\beta1^{flox/flox}$ mice by immunoblots on isolated papillae of 6 week old mice (**Figure 7A**). These mice were born in the normal Mendelian ratio and lived a normal lifespan. Despite intensive investigation, no gross or microscopical abnormalities were found at any age in these mice (**Figure 7B-G**). Due to this surprising lack of phenotype, we investigated the timing of the β1 integrin subunit deletion and found that it was still expressed in the CDs at P21, suggesting that β1 integrin was still present when development was complete.

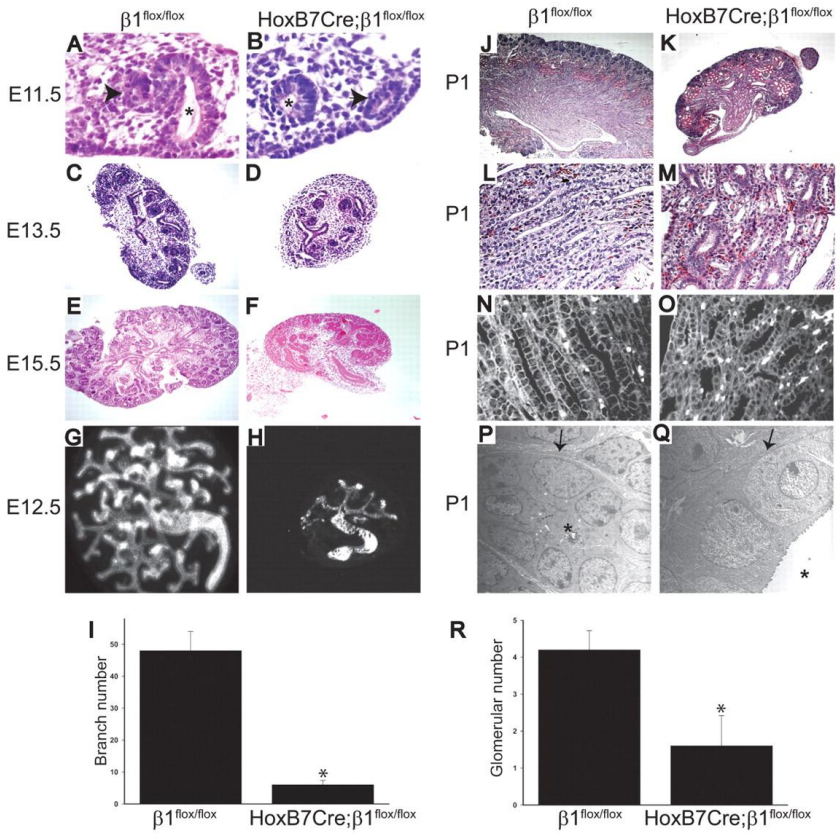

Figure 6. HoxB7Cre;β1$^{flox/flox}$ mice have a severe branching morphogenesis phenotype. (A-B) Decreased branching of the UB is present in E11.5 HoxB7Cre;β1$^{flox/flox}$ embryos (600x). **(C-F)** Decreased UB branching and nephron number is present in E13.5 (C and D) and E15.5 (E and F) HoxB7Cre;β1$^{flox/flox}$ kidneys when compared to β1$^{flox/flox}$ kidneys (40x). **(G-I)** Cultures of E12.5 kidneys of HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice were performed on transwells. The kidneys were stained with antibodies directed against E-cadherin (G and H). The number of branches was counted in 10 kidneys from both genotypes and expressed as mean +/- SD. $p<0.01$ (I). **(J-K)** PAS staining of P1 HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice kidneys (25x). **(L-M)** Collecting ducts within the papilla of P1 HoxB7Cre;β1$^{flox/flox}$ mice were dilated and irregular (200x). **(N-O)** E-cadherin staining of P1 kidneys (**P-Q)** Electron microscopy of collecting ducts. **(R)** The number of glomeruli in the cortices from similar sections of 10 P1 HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice were counted and expressed as glomeruli/mm^2. The mean and +/- SD are shown. $p<0.01$.

These mice provided us a model to determine whether β1 integrin played a protective role to obstructive injury of the collecting system. When 6 week old $\beta1^{flox/flox}$ and Aqp2Cre; $\beta1^{flox/flox}$ mice were subjected to unilateral ureteric obstruction, we observed markedly increased tubular dilatation and flattening of tubular epithelial cells in the Aqp2Cre; $\beta1^{flox/flox}$ mice at both 5 (**Figure 7H-K**) and 10 days following injury. When tubular injury was scored at day 5, it was significantly worse in the Aqp2Cre; $\beta1^{flox/flox}$ than the $\beta1^{flox/flox}$ mice (4.8+/-0.6 versus 1.8+/-0.3 $p<0.01$). We also found in these mice significantly more trichrome blue positive matrix (**Figure 7L and M**), consistent with interstitial fibrosis. Moreover, we found that injured Aqp2Cre; $\beta1^{flox/flox}$ mice had more tubular apoptosis by performing TUNEL staining (**Figure 7N-P**), which was further verified by increased activation of caspase-3 (**Figure 7Q**). Thus, deleting β1 integrins at late stages of UB development renders mice susceptible to severe renal injury following ureteric obstruction.

Deleting β1 integrin in renal IMCD cells results in adhesion, migration and proliferation defects

We next isolated IMCD cells from 3 week old $\beta1^{flox/flox}$ mice and deleted β1 *in vitro* utilizing adeno-cre virus to define its role in IMCD cell function. IMCD cells are an ideal *in vitro* model to study integrin-dependent functions in both adult and embryonic tubular epithelial cells derived from the UB (Chen, Roberts et al. 2004). We verified successful deletion of β1 in IMCD ($\beta1^{-/-}$) cells by flow cytometry utilizing an antibody directed at the extracellular domain of the mouse β1 integrin **(Figure 8A)**. Similarly, all

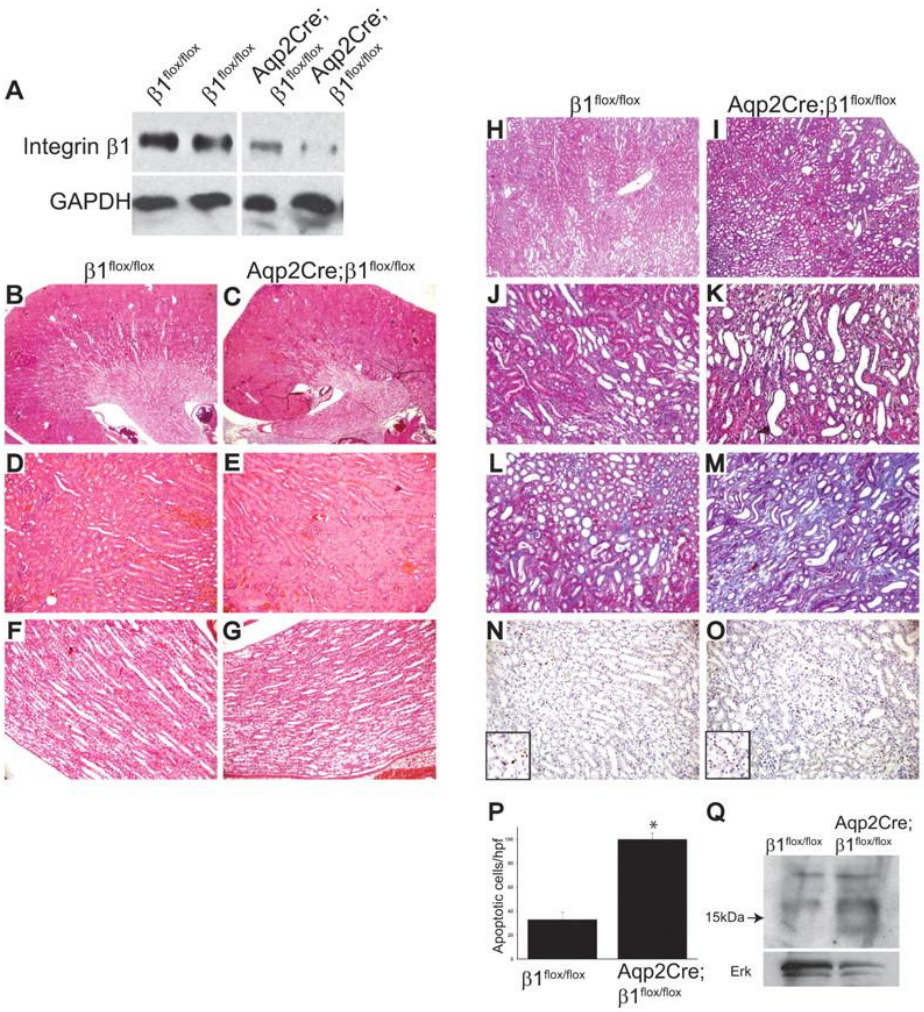

Figure 7. Aqp2Cre;β1$^{flox/flox}$ mice develop severe injury following unilateral ureteric obstruction. **(A)** Papillae of 6 week old Aqp2Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice were isolated and immunoblotted for β1 integrin. **(B-G)** Microscopy of PAS stained kidney slides (40x) **(B-C)** or high (100) **(D-G)** power of either the cortex **(D-E)** or medulla **(F-G)**. **(H-K)** Kidneys of 6 week old Aqp2Cre;β1$^{flox/flox}$ mice and β1$^{flox/flox}$ mice (H-I; 100x and J-K; 200x). **(L-M)** More intense and abundant trichrome blue staining was evident in 5 day injured Aqp2Cre;β1$^{flox/flox}$ than β1$^{flox/flox}$ mice. **(N-P)** Increased TUNEL staining was evident in 5 day injured Aqp2Cre;β1$^{flox/flox}$ compared to β1$^{flox/flox}$ mice. Insets emphasize the degree of apoptosis in the kidneys. The degree of apoptosis was quantified and expressed as the mean of apoptotic cells/microscopic field +/- SD (10 fields of 10 kidneys from either genotype were analyzed). $p<0.01$. **(Q)** Immunoblots with an antibody directed against caspase-3 were performed on medullas of 5 days injured Aqp2Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$.

the α subunits that heterodimerize exclusively with β1 were not expressed on the $\beta1^{-/-}$ cells. No differences in β4 or αv expression (**Figure 8A**) were observed between the two cell types. Next we investigated cell adhesion to various matrices and found that $\beta1^{-/-}$ IMCD cells adhered poorly to collagen I and laminin 1 (**Figure 8B**). In contrast, adhesion to fibronectin (ligand for both integrin α5β1 and αv containing integrins) was only partially impaired, while binding to vitronectin (ligand for αvβ3 and αvβ5 integrins) was unaffected. Similar results were observed for cell migration (**Figure 8C**). $\beta1^{-/-}$ IMCD cells also proliferated significantly less than $\beta1^{flox/flox}$ cells when plated on β1 integrin-dependent substrates (**Figure 8D**), an effect that was not observed on fibronectin, vitronectin or plastic substrata. To determine the role of β1 integrin in the regulation of IMCD cell polarity, we grew cells on transwells till confluent and stained them with ZO-1 (**Figure 8E**) and E-cadherin antibodies. Confocal microscopy revealed no difference in ZO-1 or E-cadherin localization on Z sectioning between the two cell lines. Thus, deleting integrin β1 from IMCD cells results in decreased cell adhesion, migration and proliferation on β1-integrin dependent substrates; however it does not significantly alter the ability of the cells to polarize.

β1 integrin expression is essential for growth factor signaling required for UB branching morphogenesis

UB branching morphogenesis *in vivo* is regulated by critical growth factors, including GDNF and FGF family members. As the major UB developmental defect in the HoxB7Cre;$\beta1^{flox/flox}$ mice occurred during the rapid branching phase known to be

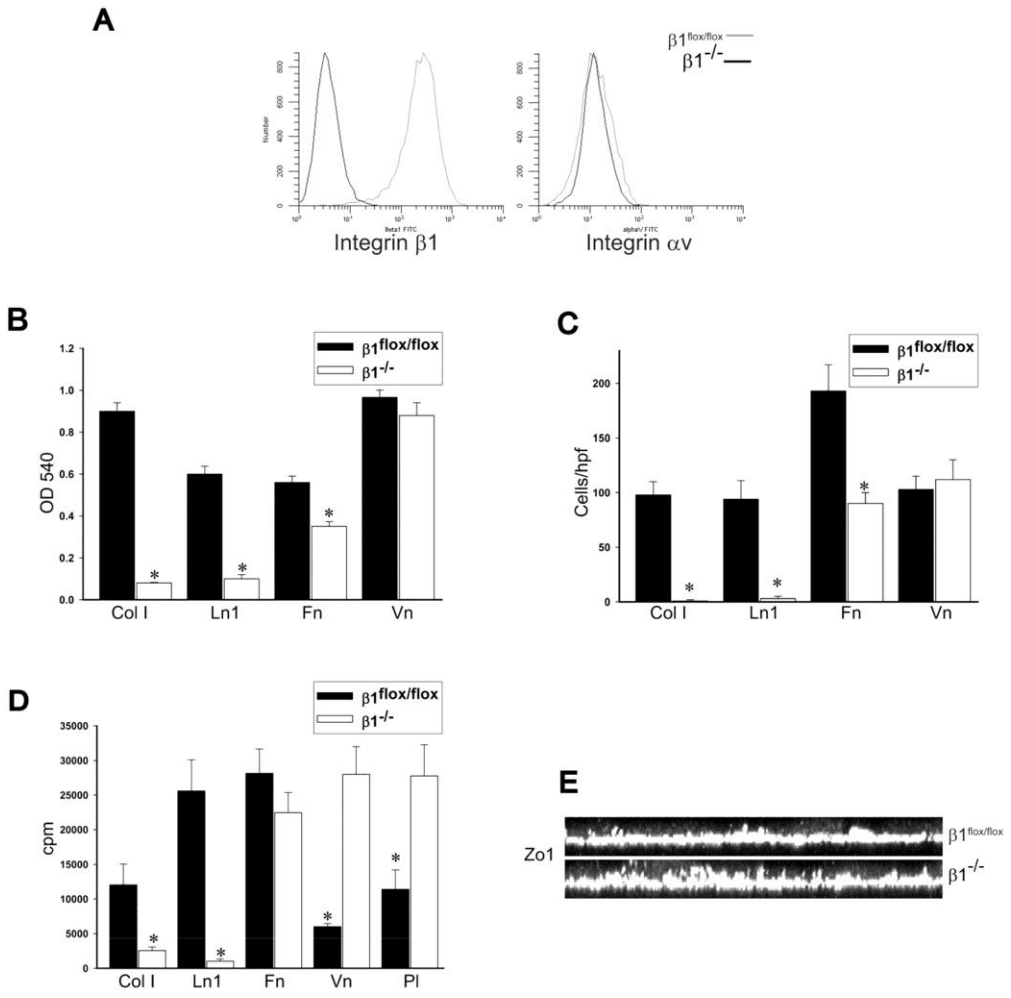

Figure 8. Deleting the β1 integrin from CD cells results in abnormal adhesion, migration and proliferation on β1 integrin-dependent substrates. (A) Flow cytometry was performed on $\beta1^{flox/flox}$ and $\beta1^{-/-}$ cells utilizing antibodies directed against the extracellular domain of the β1 and αv integrin subunits. **(B)** CD cell populations were allowed to adhere to collagen I (Col I) , laminin-1 (Ln I), fibronectin (Fn) or vitronectin (Vn) (all at 10 ug/ml) and cell adhesion was evaluated 1 hour after plating. Values are the mean ± SD of three experiments performed in triplicates. $p<0.05$ **(C)** CD cells were plated on transwells coated with the matrices indicated (all at 10 µg/ml) and migration was evaluated after 4 hours. $p<0.05$ **(D)** The CD cell populations were plated on plastic (PL) or the ECM matrices denoted (all at 10 µg/ml). After 24 hours cells were treated with ^{3}H-Thymidine and incubated for a further 24 hours. ^{3}H-Thymidine incorporation was then determined as described in the Methods. $p<0.05$ **(E)** CD cell populations were grown on transwells and stained with antibodies directed against ZO-1.

dependent on these growth factors, we investigated the role of β1 integrin in GDNF- as well FGF2- and 10-mediated signaling *in vitro*. Since GDNF and FGF2 can induce cell spreading (Klint, Kanda et al. 1999; Murakami, Iwashita et al. 1999), we determined the requirement of β1 integrin for growth factor-mediated IMCD cell spreading on collagen I or laminin I. We found that, unlike $\beta1^{flox/flox}$, $\beta1^{-/-}$ IMCD cells adhered poorly when plated on collagen I and the few that did adhere were unable to spread **(Figure 9A**). Addition of GDNF, FGF2, FGF10 (Figure 5A) or HGF (data not shown) induced much less spreading of $\beta1^{-/-}$ cells compared to $\beta1^{flox/flox}$ IMCD cells. In contrast both $\beta1^{-/-}$ and $\beta1^{flox/flox}$ CD cells spread equally when plated on vitronectin, and this effect was increased to a comparable degree in both genotypes following the addition of GDNF (**Figure 9A**).

To determine the requirement of β1 integrin for GDNF, FGF2, FGF10 and HGF signaling on collagen I, we placed $\beta1^{flox/flox}$ and $\beta1^{-/-}$ IMCD cells on collagen I for 2 hours, and stimulated them with the growth factor afterwards. We found that, compared to $\beta1^{flox/flox}$ IMCD cells, FAK, ERK, p38MAPK and Akt phosphorylation was markedly decreased in the $\beta1^{-/-}$ IMCD cells in response to GDNF, FGF2, FGF10 (**Figures 9B-D**) as well as HGF. As expected, no difference in GDNF-dependent signaling was observed between the $\beta1^{flox/flox}$ and $\beta1^{-/-}$ IMCD cells plated on vitronectin (**Figure 9E**). These results suggest that β1 integrin expression is required for CD cells to spread and mediate signaling induced by GDNF, FGF2, FGF10 and HGF when plated on β1 integrin-dependent substrates.

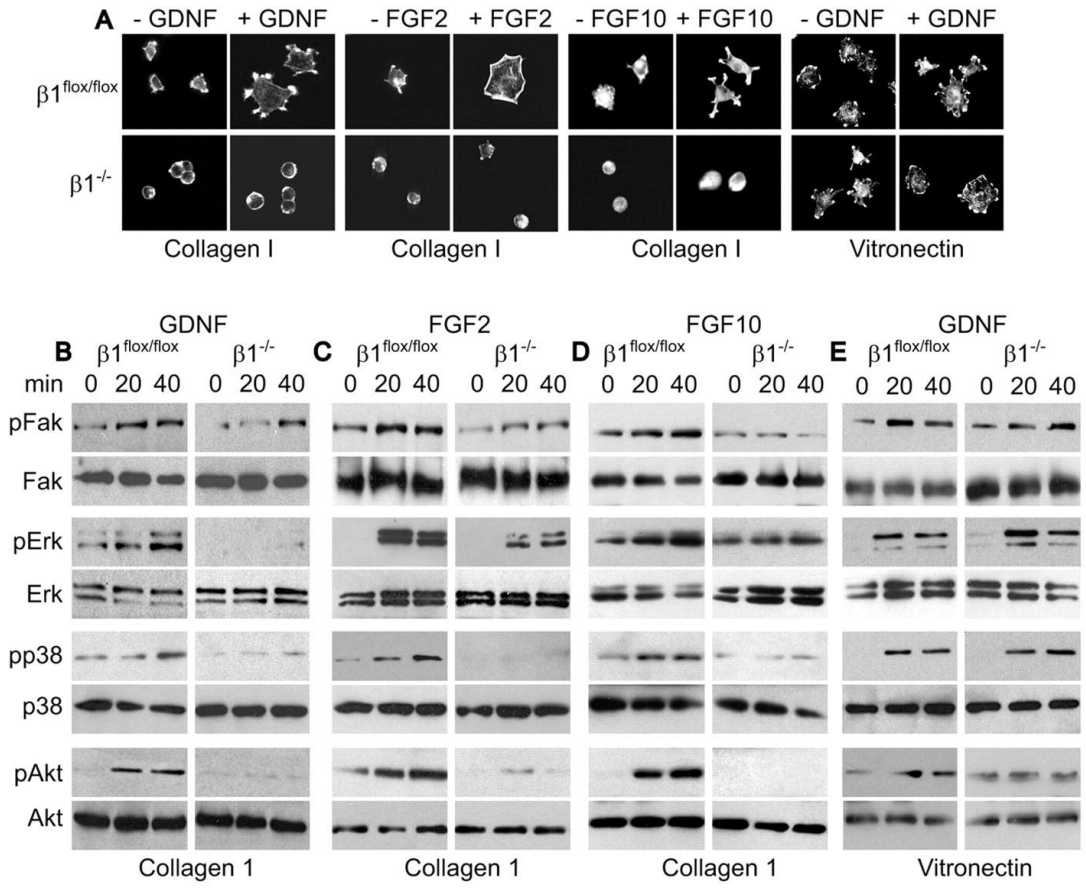

Figure 9. β1$^{-/-}$ CD cells are unable to spread or signal in response to growth factors. (A) β1$^{flox/flox}$ and β1$^{-/-}$ CD cells were allowed to adhere to collagen I or vitronectin (both at 10 μg/ml) for 45 minutes Cells were then incubated with or without FGF2, FGF10 or GDNF (all at 10 ng/ml) and after 1 hour they were stained with rhodamine phalloidin. **(B-E)** β1$^{flox/flox}$ and β1$^{-/-}$ CD cells were allowed to adhere to collagen I (B-D) or vitronectin (E) for 2 hours after which they were treated with the growth factor designated for various times. The cells were then lysed and 20 μg of total cell lysates were analyzed by western blot for levels of activated and total FAK, ERK, p38 MAPK, and Akt. A representative experiment is shown.

Deleting β1 integrin in the UB results in diminished cell proliferation and signaling

As $\beta1^{-/-}$ IMCD cells showed a significant decrease in activation of signaling pathways stimulated by GDNF and FGFs, we assessed the activation status of the same pathways in the developing collecting system *in vivo*. We performed western blot analysis of isolated medullas of newborn mice and observed drastically decreased activation of FAK, ERK, p38 MAPK and Akt in HoxB7Cre;$\beta1^{flox/flox}$ compared to $\beta1^{flox/flox}$ mice **(Figure 10A**). As expected the decreased signaling activation correlated with decreased cellular proliferation in the UB, which was particularly evident in E15.5 where the proliferation in $\beta1^{flox/flox}$ mice was 10 fold greater than that of HoxB7Cre;$\beta1^{flox/flox}$ mice (**Figure 10B**). Similarly, we observed decreased proliferation in the collecting ducts of newborn HoxB7Cre;$\beta1^{flox/flox}$ relative to the $\beta1^{flox/flox}$ mice (**Figure 10B**). As FGFs play an important role in mediating proliferation and branching of the UB at E15 (Bates 2007), we determined FGF2, FGFR1, and FGFR2 expression in renal tubules at this time point. We didn't observe any differences in FGF2 and the FGFR expression between HoxB7Cre;$\beta1^{flox/flox}$ and $\beta1^{flox/flox}$ kidneys (**Figure 10C**), suggesting that decreased proliferation and signaling were not due to decreased expression of the growth factors or their receptors.

To further investigate whether β1 expression might alter FGF receptor localization and/or activation, we plated $\beta1^{-/-}$ and $\beta1^{flox/flox}$ IMCD cells on collagen I and subsequently stimulated with FGF2. We observed equal amounts of FGFR1 surface expression and phosphorylation by IF in both $\beta1^{-/-}$ and $\beta1^{flox/flox}$ IMCD cells, despite the

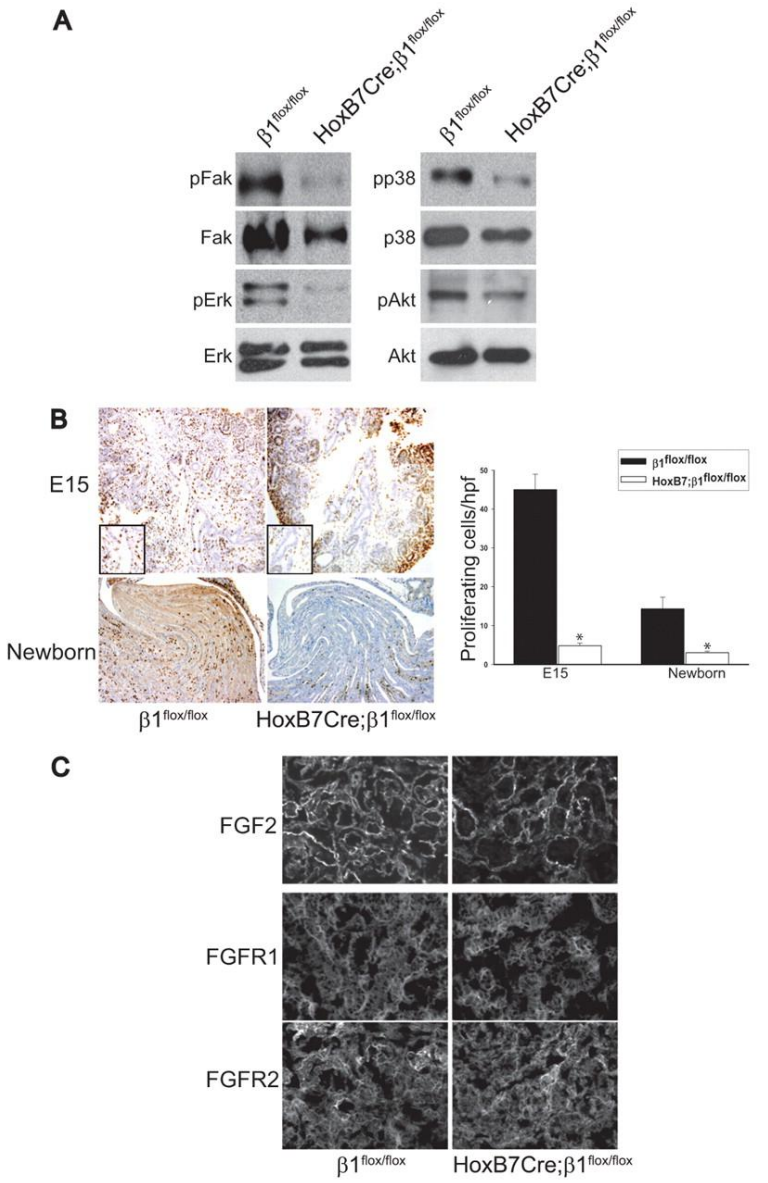

Figure 10. HoxB7Cre;β1$^{flox/flox}$ mice have severe signaling and proliferative defects. (A) Medullas of P1 β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ kidneys were lysed and 20 μg of total protein was analyzed by western blot for levels of activated and total FAK, ERK, p38 MAPK, and Akt. **(B)** Ki67 staining was performed on kidneys of β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ E15.5 and newborn mice. The number of Ki67 positive cells in the UB (E15.5) or collecting ducts (newborn) of the mice was quantified and expressed as mean ± SD of five high power fields of three different mice. (*) indicates statistically significant differences ($p<0.05$) between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice. **(C)** Tubules derived from both the metanephric mesenchyme and ureteric bud of E15.5 β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ kidneys were immunostained for FGF2, FGFR1 and FGFR2.

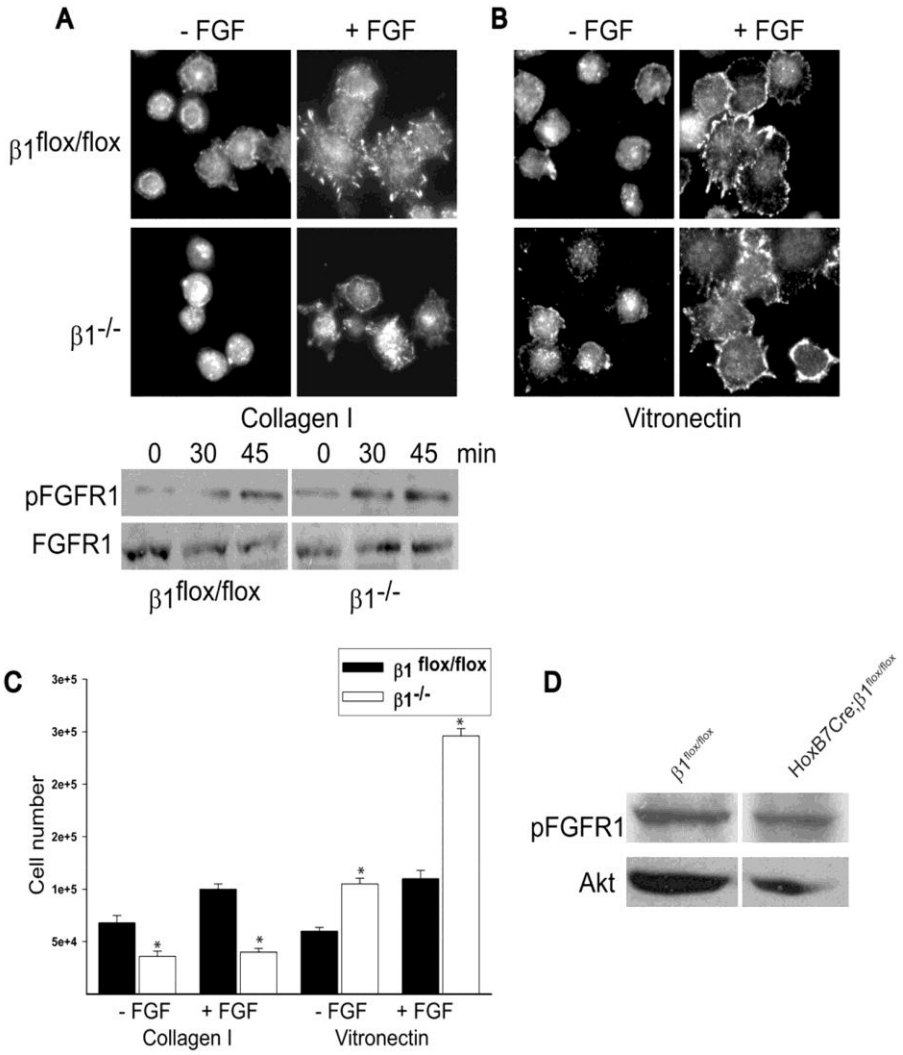

Figure 11. FGFR1 is localized to the cell membrane and is equally phosphorylated in $\beta1^{flox/flox}$ and $\beta1^{-/-}$ cells. (A-B) $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD cells were allowed to adhere for 30 minutes to collagen I (A) or vitronectin (B) (both 10 μg/ml), after which they were incubated with or without FGF2 (FGF) (10 ng/ml) for 1 hour and then stained with an anti-pFGFR1 antibody. The lower panel of A shows the levels of pFGFR1 and total FGFR1 in $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD that were allowed to adhere to collagen I for 30 minutes and then treated with FGF2 for the times indicated. **(C)** 3×10^5 $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD cells were grown in 6 well plates coated with collagen I or vitronectin (10 μg/ml) with or without FGF2 (10 ng/ml). Forty-eight hours later the cells were trypsinized and counted. Values are the mean ± SD of three different experiments. $p<0.05$ **(D)** Medullas of P1 $\beta1^{flox/flox}$ and HoxB7Cre;$\beta1^{flox/flox}$ mice were lysed and 20 μg of total cell lysates were immunoblotted for levels of pFGFR1. Equal protein loading was verified by incubating the blots with anti-Akt antibodies.

inability of the $\beta1^{-/-}$ cells to spread on this matrix (**Figure 11A**). Our immunoblot analysis also confirmed comparable FGFR1 phosphorylation and expression in $\beta1^{-/-}$ and $\beta1^{flox/flox}$ CD cells plated on collagen (**Figure 11A**). As expected, localization and phosphorylation of FGFR1 was the same in the $\beta1^{-/-}$ and $\beta1^{flox/flox}$ IMCD cells plated on vitronectin (**Figure 11B**). Next we determined the proliferative response of $\beta1^{-/-}$ and $\beta1^{flox/flox}$ IMCD cells to FGF2. $\beta1^{-/-}$ IMCD cells plated on collagen I did not proliferate, while both cell populations responded to this growth factor when grown on vitronectin (**Figure 11C**). Thus, although the FGFR1 is expressed on the cell surface of $\beta1^{-/-}$ IMCD cells and can be activated by FGF2, ligation of β1 integrin is required for this growth factor receptor to signal normally and induce cell proliferation.

To determine whether similar findings were seen *in vivo*, we performed immunoblotting on renal papillae isolated from newborn $\beta1^{flox/flox}$ and HoxB7Cre;$\beta1^{flox/flox}$ kidneys. Similar to the *in vitro* results, we found equal phosphorylation levels of FGFR1 in $\beta1^{flox/flox}$ and HoxB7Cre;$\beta1^{flox/flox}$ mice (**Figure 11D**), verifying that UBs lacking β1 integrins have severe abnormalities in branching morphogenesis, without alterations in the levels of growth factor receptor phosphorylation/activation.

Conclusions

In this study we demonstrate that deleting β1 integrin in the UB at E10.5 results in severe branching morphogenesis abnormalities and decreased nephron formation, with

death of the mice by 4 to 6 weeks of age. These results confirm the recent observation that deleting β1 integrin in the developing UB results in small kidneys with hypoplastic renal medullary collecting ducts (Wu, Kitamura et al. 2009). The abnormality in branching morphogenesis was significantly worse in mice where β1 integrin rather than α3 was specifically deleted in the UB (Liu, Chattopadhyay et al. 2009), suggesting that other αβ1 integrins play a role in this process. This was exemplified by the concomitant decrease in nephron number in the hoxb7Cre;□β1$^{flox/flox}$ kidneys, which was not seen when α3 was either deleted in the whole mouse or specifically in the UB. The only other branching organ where α3, α6 and β1 integrin subunits have been deleted is in the mammary gland (Klinowska, Alexander et al. 2001); (Naylor, Li et al. 2005);

(Taddei, Deugnier et al. 2008). Mammary gland development was normal when the α3 or α6 integrin subunits were deleted and β1 deletion resulted in a loss of epithelial integrity and displacement of cells from the basement membrane, but no branching phenotype was evident. Similar to the kidney phenotype there were no abnormalities in epithelial cell polarity or basement membrane deposition.

When β1 integrin was deleted in the collecting ducts at E18.5 using the Aqp2Cre mouse, no developmental phenotype was observed. This likely occurs because sufficient β1 integrin is expressed in the collecting ducts until P21 when normal development is complete and suggests that early but not late loss of β1 integrin expression regulates terminal differentiation and collecting duct function (Wu, Kitamura et al. 2009). The lack

of phenotype in the Aqp2Cre;β1$^{flox/flox}$ mice could be explained by the fact that either β1 was inefficiently deleted or its turnover is slow. We would suggest that the latter explanation is more likely, since deleting this matrix receptor rendered the adult kidney more susceptible to injury following ureteric obstruction. Thus β1 integrins are required to maintain structural integrity when the collecting system is subjected to the increased hydrostatic pressure induced by tying the ureter.

Utilizing cultured IMCD cells, we found that deleting β1 integrin significantly impaired canonical signaling pathways activated by FGFs and GDNF, both of which are known to be important in UB branching (Pachnis, Mankoo et al. 1993; Zhao, Kegg et al. 2004). Similar alterations in signaling pathways were seen in the HoxB7Cre;β1$^{flox/flox}$ mice despite normal expression levels of FGF2 and the FGF receptors 1 and 2. As the phenotype of the HoxB7Cre;β1$^{flox/flox}$ kidney is primarily that of decreased UB branching morphogenesis, which phenocopies many of the features seen in the GDNF, Ret (the GDNF receptor) and FGFR2 null mice, we attribute the phenotype, at least in part to abnormalities in growth factor-dependent signaling. To the best of our knowledge, this is the first illustration that β1 integrin mediates growth factor-dependent branching morphogenesis during a developmental process.

Growth factor receptors and integrin-stimulated pathways undergo cross talk at many levels. We demonstrate the requirement of β1 integrins for FGF2 and FGF10 signaling in polarized epithelial cells, despite normal FGFR1 activation and expression

on the cell membrane. FGFR1 phosphorylation is also normal in HoxB7Cre; $\beta1^{flox/flox}$ and $\beta1^{flox/flox}$ mice despite a severe branching phenotype suggesting that β1 integrins are not required for normal phosphorylation of the receptor *in vivo*. Thus in the context of UB development we propose that β1 is required for the propagation of the signals downstream of receptors like the FGF receptors rather than for receptor phosphorylation *per se*.

In conclusion, our data demonstrates that β1 integrins play a role in mediating growth factor-dependent branching morphogenesis in the developing UB. This novel role for β1 integrins in developmental processes is in addition to their well defined function in maintaining structural integrity of tubules by promoting adhesion to basement membranes.

CHAPTER III

CHARACTERIZATION OF β1 INTEGRIN FUNCTION IN GLOMERULAR DEVELOPMENT

Introduction

The glomerulus is the principal filtering unit of the kidney and its filtration barrier consists of endothelial cells and podocytes separated by a GBM comprised primarily of collagen IV and laminins (Miner 2005). Both the cellular components and the GBM are required to maintain the integrity of the filtration barrier, and perturbations of any of these components results in developmental or functional aberrations of the glomerulus. The importance of ECM components in glomerular development and integrity has been demonstrated in genetically engineered mice (Noakes, Miner et al. 1995; Cosgrove, Meehan et al. 1996; Miner and Sanes 1996; Miner, Patton et al. 1997; Miner and Li 2000); however the role of integrins is far less clear.

α3β1 is the only integrin shown to play a significant role in glomerular development *in vivo* (Kreidberg, Donovan et al. 1996). Mice lacking the integrin α3 subunit die in the neonatal period and show severe glomerular abnormalities. Similar glomerular phenotypes have also observed in mice with podocyte specific deletion of α3 integrin. In contrast to these mice, the total integrin α6-null mice do not have an overt

renal phenotype (Georges-Labouesse, Messaddeq et al. 1996), although integrin α6β1 is expressed with its major ligands laminins-111, -511, and -521 (Aumailley, Bruckner-Tuderman et al. 2005) during various stages of glomerular development (Miner 1999; Kreidberg and Symons 2000). Integrins α1β1 and α2β1 are highly expressed in the glomerulus where they play a minor role in glomerular development (Hartner, Cordasic et al. 2002; Chen, Moeckel et al. 2004).

Although integrin α3β1 is the major ECM receptor required for glomerular development, we hypothesized that deleting β1 integrin in podocytes would result in a more profound phenotype than that found in mice lacking the α3 integrin subunit in the podocytes as multiple β1 (αsβ1) integrins that interact with the GBM would not be expressed.

To determine the role of all the αsβ1 integrin heterodimers in glomerular development, we deleted β1 integrin selectively in glomerular podocytes by crossing integrin $\beta1^{flox/flox}$ mice (Raghavan, Bauer et al. 2000) with the same podocin-Cre (pod-Cre) mice (Moeller, Sanden et al. 2003) used to delete the integrin α3 subunit (Sachs, Kreft et al. 2006). We provide evidence that pod-Cre;$\beta1^{flox/flox}$ mice develop end stage renal failure by 3 to 5 weeks of age due to glomerular abnormalities characterized by podocyte loss followed by degeneration of the glomerulus. Thus, mice in which integrin β1 was deleted from the podocytes have for the most part a similar but worse phenotype than mice where only the α3 integrin subunit was deleted, suggesting that α3β1 is the

principal but not the only podocyte integrin required to maintain the glomerular filtration barrier.

Materials and methods

Generation of pod-Cre; β1flox/flox mice

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee and are housed in a pathogen free environment. Integrin $\beta1^{flox/flox}$ mice (generous gift of Dr. E. Fuchs, Howard Hughes Medical Institute, The Rockefeller University)(Raghavan, Bauer et al. 2000) or integrin $\beta1^{flox/flox}$ lacZ mice, in which a promoterless lacZ reporter gene was introduced after the downstream loxP site (Brakebusch, Grose et al. 2000) were crossed with the podocin-Cre mice (pod-Cre) generated as previously described (Moeller, Sanden et al. 2003). Mice varied between 4^{th} and 6^{th} generation C57BL6. Aged-matched littermates homozygous for the floxed integrin β1 gene, but lacking Cre ($\beta1^{flox/flox}$ mice), were used as negative controls for pod-Cre; $\beta1^{flox/flox}$ mice.

Clinical parameters and morphologic analysis

Proteinuria was determined by analyzing 2 µl of urine per mouse on 10% SDS-PAGE gels that were subsequently stained by Comassie Brilliant Blue.

The mesangial cell number was evaluated in a blind fashion by a renal pathologist counting the number of mesangial cells in 100 random glomerular sections per mouse. Glomeruli were counted in 5 individual mice with a total of 500 glomeruli examined in each group. The mesangial cell number was expressed as a mean +/- standard deviation.

GBM thickness was assessed by point to point measurements using a 2Kx2K camera (Advanced Microscopy Techniques Corp., Danvers, MA) with the associated digital imaging software. Ten different segments of GBM per mouse from 3 mice were measured and values were expressed as mean +/- standard deviation.

The number of podocytes was determined by counting their number in 10 randomly chosen EM sections of glomeruli with 3 different mice per genotype analyzed. The number of podocytes was expressed as mean +/- standard deviation.

Immunostaining

Rat anti-mouse β1 integrin (MAB1997) was purchased from Chemicon. Rat anti-mouse laminin α1 mAb 8B3 was a gift from Dr. D. Abrahamson (St John and Abrahamson 2001)and rabbit anti-human podocin was a gift from Dr. Corinne Antignac (Roselli, Gribouval et al. 2002). Rabbit anti-laminin β5 (Miner, Patton et al. 1997), rabbit anti-sera specific for the mouse collagen type IV α4 chain (Miner and Sanes 1994)and rabbit anti-nephrin(Holzman, St John et al. 1999) have been described. Rabbit anti-chick integrin α3 was a gift from Mike Dipersio, Albany Medical College (DiPersio, Shah et al. 1995), CD2AP antibody was a gift from Andrey Shaw (St. Louis, Washington University); and ILK antibody (3862) was purchased from Cell Signaling. Anti-mouse CD31, rabbit polyclonal antibodies to WT1 and anti-VEGF antibodies were purchased from Santa Cruz Biotechnology. Monoclonal antibodies to entactin (clone ELM1 Rat monoclonal) were purchased from Chemicon. Alexa 488- and Cy3- conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR) and Chemicon (Temecula, CA), respectively.

Seven µm kidney frozen sections were incubated with the antibodies described above diluted in PBS with 1% BSA followed by incubation with the appropriate secondary antibody. The sections were subsequently mounted in 90% glycerol containing 0.1X PBS and 1 mg/ml *p*-phenylenediamine and viewed under epifluorescence with a Nikon Eclipse 800 compound microscope. Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI).

Podocyte apoptosis was determined on kidney paraffin sections utilizing Apoptag Apoptosis Detection Kit, as described by the manufacturer (Chemicon). Positive apoptotic cells were counted as podocytes when residing on the outer aspect of PAS-positive basement membrane. Apoptotic cells were determined in 25 random glomeruli per kidney with 4 different mice per genotype analyzed. The number of podocytes was expressed as mean +/- standard deviation.

In situ hybridization

Kidneys were dissected from mice on postnatal day 0, 10 and 21, washed briefly in RNase-free PBS and fixed overnight in DEPC-treated 4% paraformaldehyde. The tissues were then placed in 30% sucrose for 12–24 hours, embedded in Tissue-Tek OCT 4583 compound (Sakura Finetek USA Inc.) and snap frozen. Ten-micron tissue samples were cut on a Leica Jung cryostat (model CM3050; Leica Microsystems Inc.) and transferred to Superfrost microscope slides (Fisher Scientific Co.). The slides were stored at –20°C until needed. Digoxigenin-labeled VEGF-A (kind gift of A. Nagy, Samuel

Lunenfeld Research Institute, Toronto, Canada) probes were prepared according to the Roche Molecular Biochemicals protocol (Roche Molecular Biochemicals).

Slides were dried at room temperature for 2 hours, treated with 15ng/ml proteinase K in depc-PBS at 37^0C for 5 minutes, washed in depc PBS, fixed in 4% PFA at room temperature for 7 minutes and washed first in depc PBS and then in 2x SSC (PH 7). Slides were prehybridized in mailers for 1 hour at 60^0C using hybridization buffer (2.5 ml - 20x SSC, 5 ml formamide, 250 µl – 20% CHAPS, 100 µl – 10% Triton X-100, 50 µl – 10 mg/ml yeast RNA, 25µl – 20 mg/ml Heparin, 100 µl – 0.5 EDTA, 0.2 g blocking powder, 1.2 ml depc H2O) after which they were hybridized with the probe (1 ng/ml) diluted in hybridization buffer at 60^0C overnight. The next day the slides were washed sequentially in 0.2XSSC and formamide/0.2x SSC. The slides were blocked with blocking solution from Roche as per instructions and then incubated with anti-DIG antibody for 2 hours. Slides were washed and substrate was added as per instructions from Roche. Slides were washed again and then immersed in Fast Red, dehydrated and mounted.

Results

Loss of β1 integrin in the podocyte results in massive proteinuria and end stage renal failure

To determine the function of αsβ1 integrins expressed by podocytes in the glomerulus, we crossed mice carrying the floxed β1 integrin gene ($\beta1^{flox/flox}$)

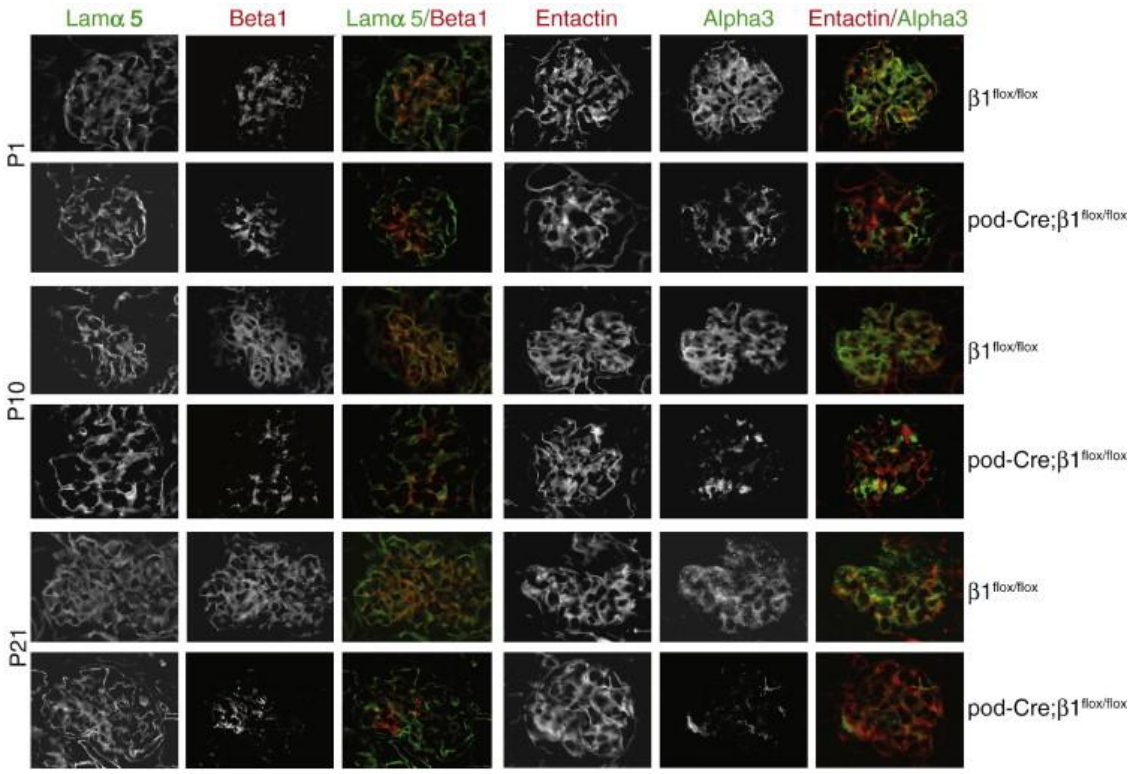

Fig. 12. β1 integrin subunit is deleted in pod-Cre;β1$^{flox/flox}$ mice. Frozen sections of kidneys from P1, P10 and P21 β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice were co-stained with anti-mouse integrin β1 (red) and anti laminin-α5 chain (green) or anti-mouse integrin α3 (green) and anti-entactin (red), respectively.

(Raghavan, Bauer et al. 2000) with mice expressing Cre recombinase under the control of the podocin promoter (pod-Cre)(Moeller, Sanden et al. 2003), in which recombination occurs during the capillary loop stage in glomerular development. To verify that the β1 integrin subunit was deleted in the podocytes, we performed immunostaining for the β1 and α3 integrin subunits in P1, P10 and P21 mice. Expression of both of these subunits was significantly reduced in a segmental pattern in P1 pod-Cre;$\beta1^{flox/flox}$ mice (**Figure 12**) and were further decreased in the glomeruli of P10 and P21 pod-Cre;$\beta1^{flox/flox}$ mice.

Pod-Cre;$\beta1^{flox/flox}$ mice were born in the expected Mendelian ratio, however the pod-Cre;$\beta1^{flox/flox}$ mice became less physically active than their littermate $\beta1^{flox/flox}$ controls and developed severe edema 3 weeks after birth. Ninety percent (18/20) of the pod-Cre;$\beta1^{flox/flox}$ mice were euthanized between 4 and 5 weeks of age due to end stage renal failure and nephrotic syndrome and only 10% (2/20) survived to 6 weeks of age (**Figure 13A**). The six week old pod-Cre;$\beta1^{flox/flox}$ mice had massive proteinuria, with the predominant band running at the molecular weight of albumin (~66Kd) (**Figure 13B**). Autopsy of the mutant mice revealed smaller and paler kidneys than those isolated from age matched control animals, which are characteristic features of end stage disease (**Figure 13C**). Numerous Bowmans capsules in pod-Cre;$\beta1^{flox/flox}$ mice were either empty or had partially disintegrated glomeruli as shown in **Figures 13G**. Interestingly the mesangium was only mildly hypercellular with little matrix expansion, and segmental areas of mesangiolysis were noted (**Figure 13I**). In addition to the glomerular pathology, we observed marked tubular dilatation and flattening of epithelial cells with extensive proteinaceous tubular casts (**Figure 13E**). Thus, all the mutant mice developed end stage

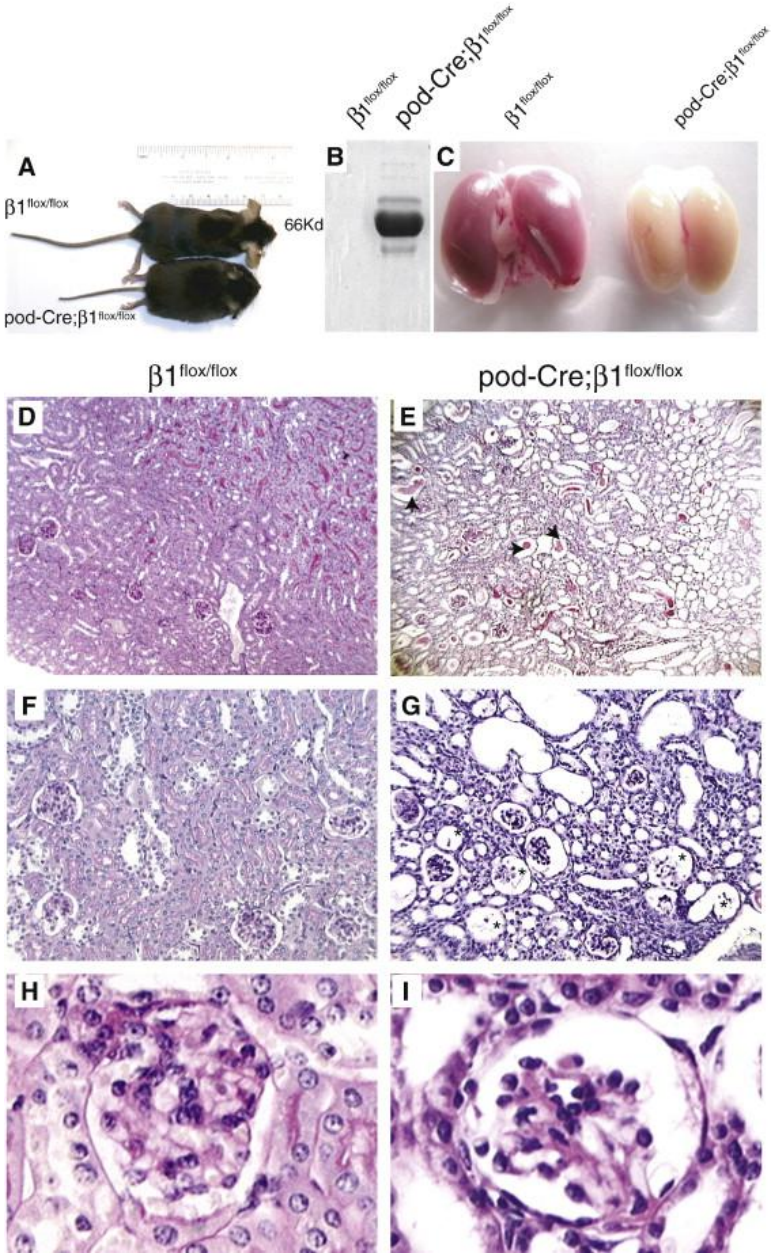

Fig. 13. Six-week-old pod-Cre;β1$^{flox/flox}$ mice develop severe proteinuria and end stage renal disease. (A–C) Six-week-old pod-Cre;β1$^{flox/flox}$ mice are smaller with evidence of severe edema (A), albuminuria (2 μl urine/lane) (B) and end stage kidneys (C) compared to aged matched β1$^{flox/flox}$ mice. (D–I) PAS staining of kidneys derived from the mice described above showing glomerular and tubular interstitial abnormalities in the mutant group. The arrows in panel E show dilated tubules filled with hyaline material and the asterisks in panel G show the remnants of glomeruli in the pod-Cre;β1$^{flox/flox}$ mice. (D, E = 100×; F, G = 200×; H, I = 630×).

kidney disease characterized by pathological changes in the glomeruli and tubulo-interstitium.

Glomerular capillary morphogenesis is normal in embryonic and newborn pod-Cre;$\beta1^{flox/flox}$ mice but becomes abnormal 10 days after birth

Due to the severity of the renal phenotype in the mutant mice we investigated when the abnormalities first became apparent. The podocin promoter is activated during the capillary formation stage (Moeller, Sanden et al. 2003), so we initially determined the histology of kidneys derived from E15.5 embryos from 10 wild type and mutant mice. Glomeruli were indistinguishable from the wild type controls in all the pod-Cre;$\beta1^{flox/flox}$ mice (**Figures 14A and 14B**). Also in mutant P1 (n=10) mice, no overt abnormalities were apparent (**Figures 14C and 14D**), however even at this early age the mice exhibited proteinuria (**Figure 14M**) but no hematuria.

In contrast, kidneys from mutant P10 mice demonstrated tubular dilatation and multiple cytoplasmic vacuoles within the tubular epithelial cells (**Figures 14E-H**), which are consistent with heavy proteinuria. In addition, some glomeruli from mutant mice showed segmentally expanded capillary lumens (**Figures 14H**). By 3 weeks of age the tubules showed increased dilatation and there were a number of expanded capillary loops and mesangial hypercellularity (**Figures 14J and 14L**). When mesangial cell number was assessed, there were significantly more in the pod-Cre;$\beta1^{flox/flox}$ mice compared to the $\beta1^{flox/flox}$ mice (17.1+/-3.6, vs 11.3+/-2.3. $p< 0.05$).

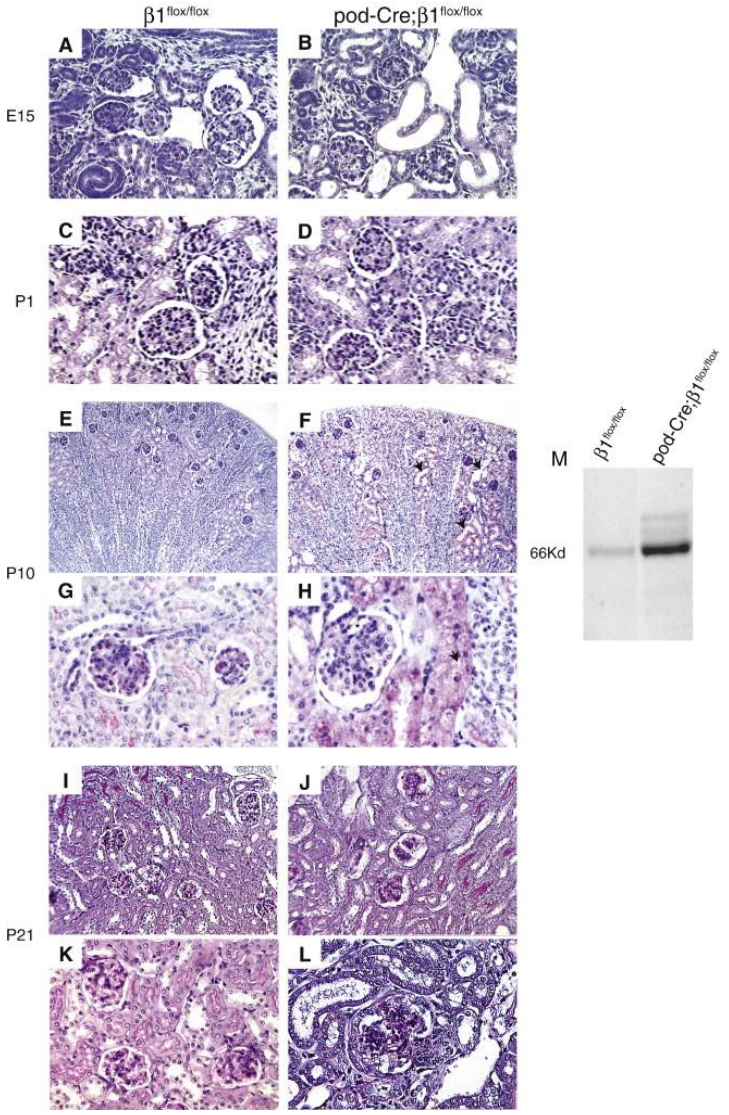

Fig. 14. Kidneys from pod-Cre;β1$^{flox/flox}$ mice exhibit severe abnormalities in the glomerulus and tubulointerstitium. (A, B) PAS staining of kidneys derived from E15.5 β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice (200×). (C, D) PAS staining of kidneys derived from newborn β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice (200×). (E, F) PAS staining of kidneys derived from P10 β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice (100×). (G, H) In P10 mutant mice there was evidence of "ballooned" glomerular capillary loops (asterisk) and protein containing vacuoles (arrow) in the tubules of the pod-Cre;β1$^{flox/flox}$ mice (400×). (I–L) PAS staining of kidneys derived from 3-week-old β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice revealed dilated tubules, evidence of "ballooned" capillary loops and mesangial hypercellularity (I, J = 200×; K, L = 400×). (M) Comassie staining of urine (2 μl/lane) from newborn β1$^{flox/flox}$ and pod-Cre;β1$^{flox/flox}$ mice showing albuminuria in the latter group.

Deletion of β1 integrin in the podocyte results in foot process effacement

To further evaluate the integrity of the glomerular filtration barrier, we examined glomeruli of mice at various ages by electron microscopy. At E15 we observed evidence of foot process effacement in the pod-Cre;$\beta1^{flox/flox}$ mice but surprisingly the GBM was intact in both genotypes (**Figure 15**). Similar ultrastructural findings were present in the kidneys isolated from P1 mice (**Figure 15**). Also no significant difference in thicknessof the GBM was seen in $\beta1^{flox/flox}$ and pod-Cre;$\beta1^{flox/flox}$ P1 mice (130+/-25.9 nm vs. 143+/-39.5: p=0.16). In P10 pod-Cre;$\beta1^{flox/flox}$ mice, we observed extensive foot process effacement and early segmental splitting of the GBM. In the P21 mutant mice, these features became more evident (**Figure 15**).

To determine whether the abnormalities in the filtration barrier in newborn mice was due to altered expression or localization of proteins involved in slit diaphragm formation or structural proteins known to be associated with nephrotic syndrome, we performed immunofluorescence for nephrin, podocin and CD2AP (**Figure 16A**). We found similar expression and localization of these proteins in control and mutant mice. As integrins are thought to be critical for normal BM development, we further examined expression of collagen IV and laminins in glomeruli. Unexpectedly, no differences in collagen IV α1, α3, α4, α5 and α6 chain expression were observed (**Figure 16B**). Expression of both laminin α1 and α5 chains as well as α2 and β2 chains was also similar in both genotypes (**Figure 16C**).

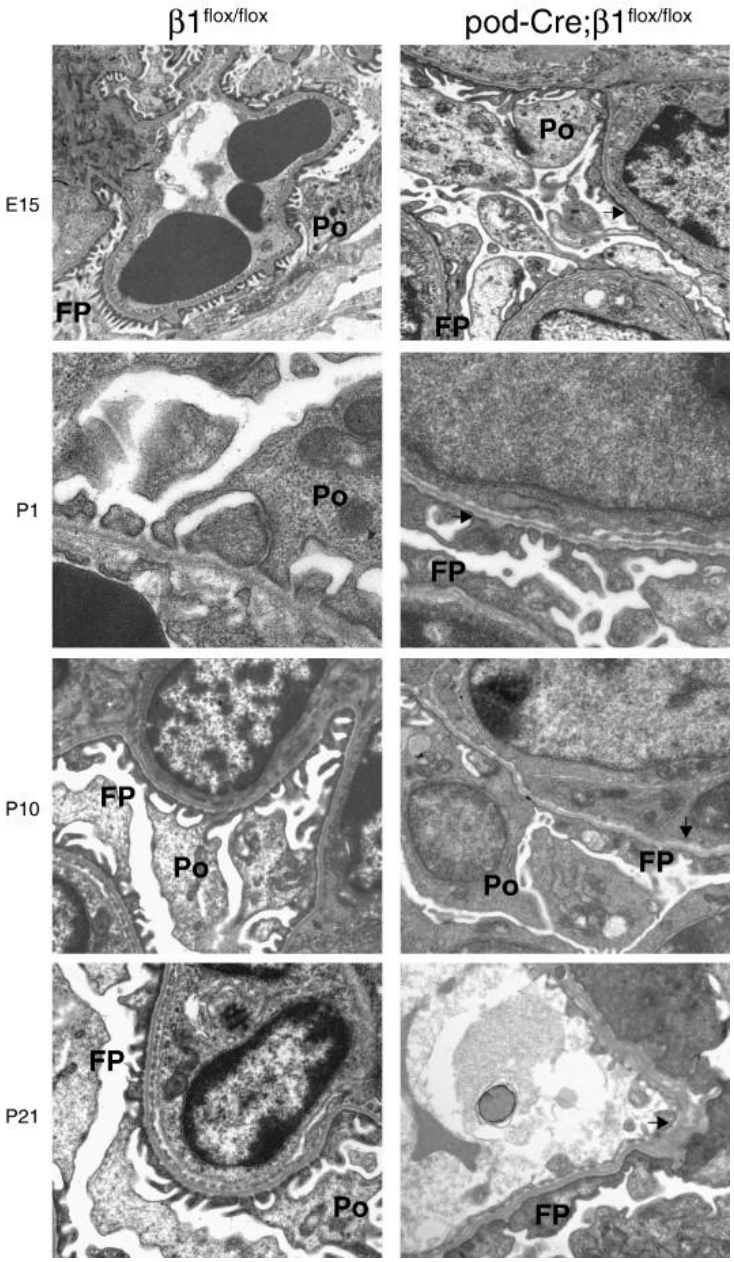

Fig. 15. Glomeruli from pod-Cre;β1$^{flox/flox}$ mice demonstrate podocyte foot process effacement. EM analysis was performed on kidneys of mice at various ages. In the E15 mutant mice there is evidence of foot process effacement of the podocytes but the GBM (arrow) was normal. Similar findings are present in P1 kidneys. In P10 mice, in addition to the foot process effacement, there was evidence of very mild segmental splitting of the GBM (arrow) which progressed by day P21. Abbreviations: FP = Foot Process; Po = Podocyte.

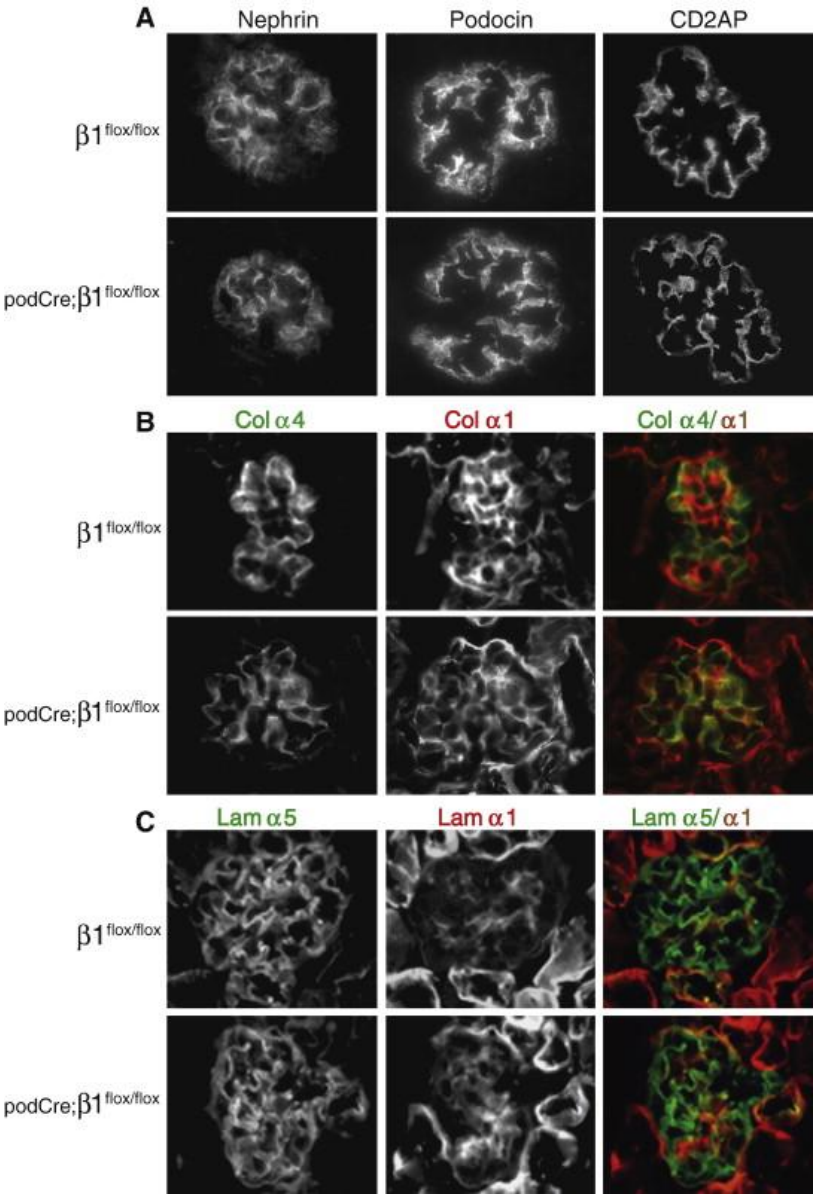

Fig 16. Glomeruli from newborn pod-Cre;β1$^{flox/flox}$ mice demonstrate normal expression of podocyte-specific structural proteins glomerular basement membrane components. Frozen sections of kidneys derived from newborn mice were stained with antibodies to (A) Nephrin, podocin or CD2AP, (B) the α4 (green) or α1 (red) chains of collagen IV and (C) the α5 (green) or α1 (red) laminin chains.

Taken together these data suggest that during embryogenesis lack of integrin β1 results in podocyte abnormalities characterized by dysmorphic foot processes, with no gross abnormalities in GBM composition or ultrastructure.

Podocyte apoptosis occurs in pod-Cre;$\beta1^{flox/flox}$ mice within 10 days of birth

Based on the phenotypes of the P21 and 6 week old pod-Cre;$\beta1^{flox/flox}$ mice, where there were dilated glomerular capillaries and subsequent glomerular disintegration, we determined whether the podocytes in the mutant mice were undergoing apoptosis. As seen in **Figure 17A**, a significant number of apoptotic podocytes were present in the P10 and P21 mutant mice. Furthermore, we determined expression of the specific podocyte markers WT1 (**Figure 17B**), podocin, CD2AP and synaptopodin by immunofluoresence and found that they were significantly decreased in P10 and P21 mutant glomeruli suggesting the possibility of podocyte loss in the pod-Cre;$\beta1^{flox/flox}$ mice.

As deletion of the β1 integrin binding protein, integrin linked kinase (ILK), in podocytes results in focal segmental glomerulosclerosis and alteration in the distribution of integrin α3β1 starting at 4 weeks of age (Dai, Stolz et al. 2006; El-Aouni, Herbach et al. 2006), we investigated whether deleting the β1 integrin subunit from podocytes altered glomerular ILK expression. As shown in **Figure 17B**, we didn't observe any differences in glomerular ILK expression in P1 mutant and control mice. However, by P10 the mutant mice showed a marked decrease in glomerular ILK expression. By P21 virtually

no ILK was detected in glomeruli. The pattern and time course of ILK expression in the mutant mice was very similar to that seen for β1 integrin (**Figure 12**), suggesting that expression of the β1 integrin subunit might play a specific role in regulating ILK expression in the podocyte.

To specifically confirm podocyte loss in the P21 pod-Cre;$\beta1^{flox/flox}$ mice, we determined the number of podocytes per glomerular section utilizing electron microscopy. An example of a low power EM picture from P21 pod-Cre;$\beta1^{flox/flox}$ and Cre;$\beta1^{flox/flox}$ mice (**Figure 17C**) illustrates the decreased number of podocytes in a section of a glomerulus of the mutant mice. When formally quantified, P21 pod-Cre;$\beta1^{flox/flox}$ had about one third of the number of podocytes compared to their $\beta1^{flox/flox}$ litter mates (**Figure 17C**). All together, these data suggest that there are significantly less podocytes in the P21 pod-Cre;$\beta1^{flox/flox}$ mice.

<u>Deletion of β1 integrin in the podocyte results in abnormalities of both capillary loops and the mesangium</u>

One of the most surprising findings of this study was that despite the obvious abnormalities of the podocytes, the principal lesions seen in P21 mutant mice was the degeneration of the capillary loops and mesangium with little glomerulosclerosis. To determine the possible mechanism underlying these capillary loop abnormalities, we initially investigated the integrity of the endothelium by staining the kidneys with antibody to CD31, a specific endothelial cell marker. As shown in **Figure 18A**, in P1

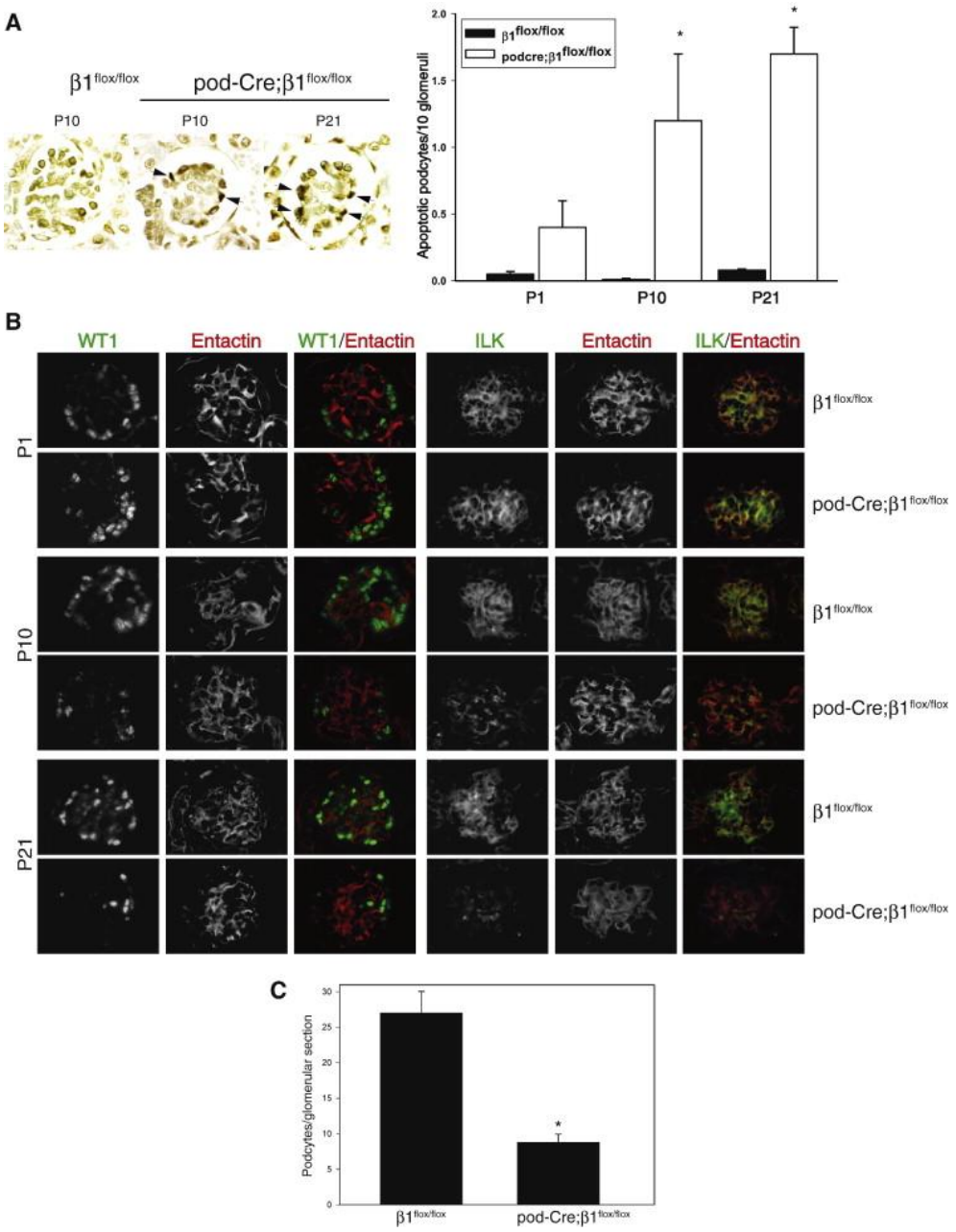

Fig. 17. Podocytes of pod-Cre;β1$^{flox/flox}$ mice undergo apoptosis. (A) Examples of apoptosis, as determined by TUNEL assay, detected in the glomeruli of β1$^{flox/flox}$ or pod-Cre;β1$^{flox/flox}$ mice at the time points indicated. The number of apoptotic podocytes expressed per 10 glomeruli is demonstrated graphically. The (*) indicates significant differences ($p < 0.01$) between the two genotypes. (B) Frozen sections of glomeruli were co-stained for WT-1 (green) and entactin (red) or ILK (green) and entactin (red) as described in Materials and methods. (C) The number of podocytes in EM sections were evaluated as described in Materials and methods and expressed as mean ± S.D. (*) indicates significant differences ($p < 0.05$) between P21 β1$^{flox/flox}$ and P21 pod-Cre;β1$^{flox/flox}$ mice.

$\beta1^{flox/flox}$ and pod-Cre;$\beta1^{flox/flox}$ mice, the intensity and distribution of CD31 positive cells was similar. By P10 there was significantly less CD31 staining in the glomeruli of mutant mice and by P21 the CD31 staining was virtually undetectable in the mutant mice, suggesting that endothelial cells had undergone cell death during this time period. To verify these findings, we performed electron microscopy on the glomerular capillary loops. At day 10, we observed significant vacuolation, a sign of cell damage, in the endothelial cells of mutant mice (**Figure 18B**), and by 3 weeks of age only a nuclear remnants of capillary endothelial cells were evident in many capillary loops (**Figure 18B**).

As mesangial injury was evident in the mutant mice starting at 3 weeks of age (**Figure 14**), we studied the mesangium in detail by EM. We found multiple cytoplasmic vacuoles, indicative of cellular damage, in the mesangial cells of mutant mice starting at 10 days of age (**Figure 18C**). By 3 weeks the chromatin material of mesangial cells was clumped, suggestive of severe cellular injury and there were lucent areas within the mesangial matrix indicative of defective matrix assembly. These results suggest that the mesangium, like the endothelium, was injured in the mutant mice.

Vascular endothelial growth factor-A (VEGF), which is primarily produced by the podocytes, is a growth factor required for both normal capillary loop development as well as mesangial cell survival (Eremina, Sood et al. 2003; Eremina, Cui et al. 2006). In this context, mice lacking podocyte-produced VEGF develop grossly abnormal glomerular capillary loops. Furthermore, in mice hypomorphic for podocyte-produced

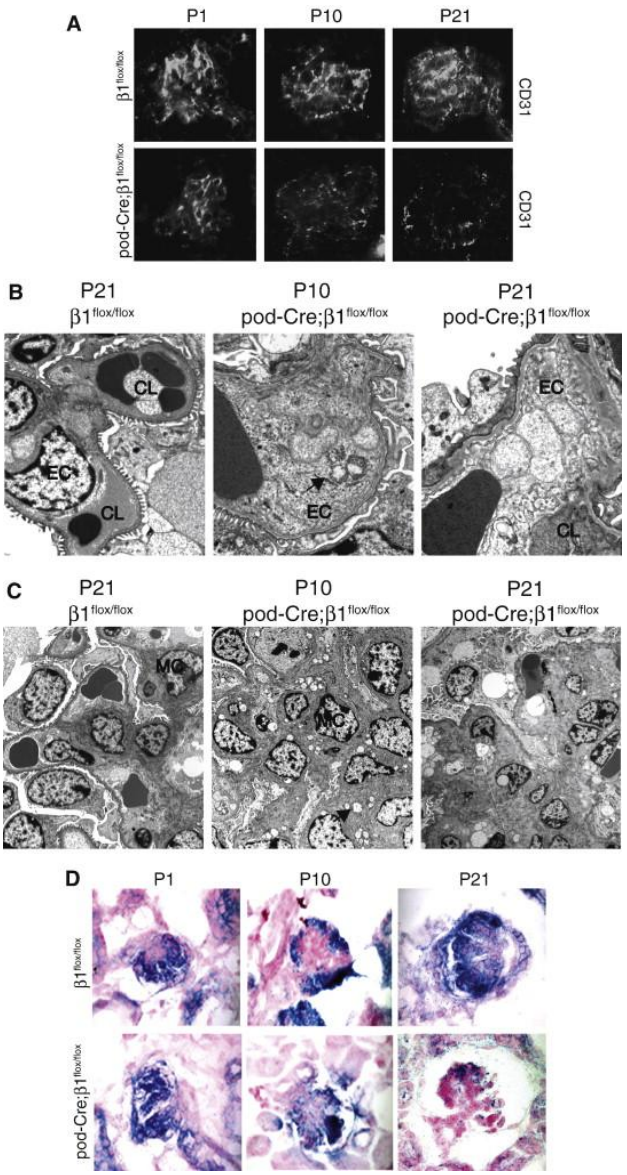

Fig. 18. Glomerular capillary and mesangium injury in pod-Cre;β1$^{flox/flox}$ mice. (A) Frozen sections of kidneys derived from newborn, P10 and P21 mice were stained with CD31 antibodies to visualize the glomerular vasculature. (B) EM of kidneys from P21 β1$^{flox/flox}$ (3000×) as well as P10 (11,000×) and P21 (11,000×) pod-Cre;β1$^{flox/flox}$ mice revealed normal morphology of endothelial cells in the β1$^{flox/flox}$ mice. Vacuoles (arrow) in the endothelial cells (arrow) were evident in the P10 and P21 mutant mice. Abbreviations: EC = endothelial cells; CL = capillary loops; (C) EM of kidneys emphasizing the mesangium. Note the presence of vesicles (arrow) in the mesangial cells of mutant mice by P10 (8900×) and the increased mesangial matrix in the P21 pod-Cre;β1$^{flox/flox}$ mice (4th panel) (2200×). Abbreviations: MC = mesangial cell. (D) In situ hybridizations on kidneys of newborn, P10 and P21-day-old mice showing VEGF mRNA expression.

VEGF, there is ballooning of the glomerular capillaries and mesangiolysis(Eremina, Sood et al. 2003). This finding suggests that VEGF is required for the normal development and maintenance of glomerular integrity, especially with respect to the capillary loops and the mesangium. As the pod-Cre;$\beta1^{flox/flox}$ mice reduce podocyte number with time, we analyzed the levels of VEGF in the glomeruli of $\beta1^{flox/flox}$ and pod-Cre;$\beta1^{flox/flox}$ mice. The mRNA of this growth factor was similar in both genotypes at birth (**Figure 18D**). In 10 days old mutant mice, we observed decreased mRNA expression (**Figure 18D**). By 3 weeks significantly less VEGF message was detected in the glomeruli of the pod-Cre;$\beta1^{flox/flox}$ mice (**Figure 18D**). Thus, the timing of glomerular degeneration correlates with the lack of VEGF expression by the podocytes.

Conclusions

In our study we showed that selectively deleting αsβ1 integrins in the podocyte resulted in 1) a defective glomerular filtration barrier present at birth; 2) podocytes loss over time; 3) capillary loop and mesangium degeneration with little evidence of glomerulosclerosis and 4) the development of end stage kidneys characterized by both tubulointerstitial and glomerular pathology by 3 to 6 weeks of age. Taken together these results demonstrate that although the injury in the β1 integrin null mouse is more severe and has some differences in pattern, the overall phenotype is similar to that found in mice where the α3 integrin subunit is selectively deleted in podocytes. This suggests that integrin α3β1 is the principal integrin required to maintain the structural integrity of the glomerulus and other αsβ1 integrins play a relatively minor role.

The end stage kidneys of the pod-Cre;$\beta1^{flox/flox}$ mice were characterized by severe tubulointerstitial disease in addition to the glomerular pathology. This was likely a consequence of the heavy glomerular proteinuria that results in interstitial mononuclear cell accumulation and activation of interstitial fibroblasts to deposit collagens in the tubulointerstitial compartment of the kidney (Remuzzi 1995); (Eddy 1994)

The mutant mice in this study showed normal glomerular morphogenesis, GBM formation as well as normal expression of slit diaphragm and key cytoskeletal proteins. In contrast, in the newborn integrin α3-null mouse, the GBM was disorganized and capillary loop numbers were reduced in addition to the podocyte abnormalities (Kreidberg, Donovan et al. 1996). The differences in glomerular morphogenesis between these two mice may mostly be because: i) sufficient αsβ1 integrins are expressed by the podocytes prior to Cre-mediated excision, as podocin is expressed at the S-shape body stage of development(Moeller, Sanden et al. 2003). ii) αsβ1 integrins might still be expressed by the podocytes following cre expression due to inefficient deletion of β1 integrin. Also pod-Cre; $\alpha3^{flox/flox}$ mice showed abnormally thickened GBM and severe lamellations, compared with normal GBM formation in our pod-Cre; $\beta1^{flox/flox}$ mice. A possible explanation for this discrepancy is that integrin α3β1 might negatively regulate integrin α2β1-dependent glomerular collagen production. This hypothesis is based on the observation that integrin α2β1 is a positive regulator of collagen synthesis (Ivaska, Reunanen et al. 1999); integrin α2β1 function can be negatively regulated by the expression of other integrin family members.

Overall, the renal phenotypes we observed in pod-Cre;$\beta1^{flox/flox}$ mice are more severe than that of α3-null mice. We observed earlier onset and increased severity of injury in the pod-Cre;$\beta1^{flox/flox}$ compared to α3-null mice. This could be due to the loss of interactions of podocytes with the GBM via the collagen binding receptors, integrins α1β1and α2β1 and the laminin receptor integrin α6β1 which are expressed by podocytes during development (Korhonen, Ylanne et al. 1990; Rahilly and Fleming 1992). This finding suggests that other αβ1 integrins also play minor roles in maintenance of glomerular structural integrity. Another one of the most interesting features of the podocyte β1-null mouse was the rapidity with which the glomeruli degenerated and the lack of glomerular fibrosis relative to the glomerular injury. We tried to link this feature with the phenotypes we observed in pod-Cre;$\beta1^{flox/flox}$, such as foot process effacement, slit diaphragm abnormalities or podocyte loss overtime. However, mice deficient for ILK (Dai, Stolz et al. 2006; El-Aouni, Herbach et al. 2006)or the integrin α3 subunit in the podocytes as well as lacking proteins required for normal slit diaphragm formation (i.e. podocin and CD2-associated protein) (Shih, Li et al. 1999) also had foot process effacement, proteinuria, but developed severe glomerulosclerosis prior to developing end stage kidney. Similarly, In addition to these genetic models, when 40% of podocytes were destroyed in rats by diphtheria toxin, the primary lesion observed was glomerulosclerosis (Wharram, Goyal et al. 2005), which is consistent with the theory that if sufficient podocytes detach leaving a naked GBM, a circumscribed region of focal segmental sclerosis will initially form and eventually result in global glomerulosclerosis(Kriz 2002). Thus foot process and slit diaphragm abnormalities or

loss of up to 40% of podocytes in glomeruli does not explain why pod-Cre; $\beta 1^{flox/flox}$ did not develop glomerulosclerosis.

In an attempt to explain the lack of glomerulosclerosis and the severe mesangiolysis in our mouse model, we noted that the glomerular phenotype of the pod-Cre;$\beta 1^{flox/flox}$ mice at 3 weeks of age had many features similar to those seen in mice expressing one hypomorphic VEGF-A allele. Both these mice have dilated capillary loops and severe abnormalities within the mesangium. The phenotype in the VEGF hypomorphic mouse is proposed to be due to a requirement of podocyte-dependent VEGF production for both endothelial cell proliferation and survival, and disruption of the endothelial compartment leads to the mesangial defects (Eremina, Cui et al. 2006). Since the pod-Cre; $\beta\ 1^{flox/flox}$ mice demonstrate significant podocyte loss and the principal source of VEGF in the glomerulus is the podocytes, we postulate that when sufficient podocytes are lost in this mouse, the endothelial cells undergo apoptosis due to their dependence on this angiogenic factor, or other factors produced by the podocyte, for survival. The lack of VEGF would not explain the mesangium phenotype as mesangial cells do not express receptors for VEGF. However mesangial cells do require PDGF-β secretion by endothelial cells for their survival (Bjarnegard, Enge et al. 2004). Thus with progressive podocyte loss in the pod-Cre; $\beta 1^{flox/flox}$, we propose that the glomerulus loses VEGF and probably other podocyte-specific growth factors production, which results in endothelial cell death. The loss of growth factor production from both these cells types

subsequently results in the inability of the glomerulus to maintain integrity of the mesangium.

In conclusion we provide evidence that podocyte expression of αsβ1 integrins is required for the normal formation and integrity of the glomerular filtration barrier. Although the GBM appears to form relatively normally in newborn pod-Cre;$\beta1^{flox/flox}$ mice, podocyte foot process effacement and proteinuria is seen. With the increase in glomerular hydrostatic pressure, podocytes are lost from the glomerulus, which promotes rapid destruction of the capillary loops and mesangium with little glomerulosclerosis. The rapidly degenerating glomeruli promote tubulointerstitial disease which is likely due to the increased proteinuria. This phenotype is for the most part similar but more severe than that seen in mice lacking the α3 integrin subunit in podocytes, where proteinuria and glomerulosclerosis are the primary features, suggesting that in addition to integrin α3β1, other αsβ1 integrins play a role in maintaining the glomerular filtration barrier.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Overview

The development of an organism requires precise spatiotemporal coordination of cell behaviors， which are regulated by a complex network of regulatory factors, including growth factors and ECM proteins. Morphogenesis of the kidney serves as an excellent model system to analyze the cellular and molecular mechanisms underlying organogenesis. β1 integrin is one of the most widely expressed integrins in kidneys. The major goal of this thesis was to examine the role of β1 integrin in kidney development. We illustrated the novel role of β1 integrins in transducing growth factor-dependent signals required for UB branching morphogenesis, in addition to their well characterized roles in adhesion and migration. We also demonstrated that β1 integrin is required for normal development and maintenance of glomerular filtration barrier. These findings have significantly improved the current knowledge of the function of β1 integrins. They also facilitated further in depth investigation focusing on several important issues that we have found but not fully addressed in this study

Discussion and future directions

First, in our study to investigate the role of β1 integrin in collecting system development, we observed significantly worse renal phenotypes in our

Hoxb7Cre;□β1$^{flox/flox}$ than in mice where α3 was specifically deleted in the UB (Liu, Chattopadhyay et al. 2009), suggesting that other αβ1 integrins play a role in this process. Collagen receptor α2β1 and laminin receptor α6β1 integrins are candidates as minor players since they are both highly expressed in the collecting tubules. The different α integrin subunits may work synergistically or negatively regulate each other, maintaining homeostasis of a developmental process. For example, α3 and α6 integrins work synergistically to regulate apical ectodermal ridge formation and organogenesis (De Arcangelis, Mark et al. 1999), while α1β1 negatively regulates integrin α2β1-dependent functions in renal epithelial cells (Abair, Sundaramoorthy et al. 2008). Homozygous deletion of either α2 or α6 subunit results in subtle if any renal phenotypes, suggesting a possible functional redundancy. The relative importance of these integrin α subunits during collecting system development could be investigated by generating UB-specific double knockout mice utilizing Hox7bCre. Furthermore, the integrin α8 subunit may play a role during this process as well. α8 subunit associates specifically with β1 integrin and is expressed at both the mesenchyme aggregates and UB tips from E10.5 to E14.5. Deletion of β1 integrin in our Hoxb7Cre;□β1$^{flox/flox}$ mice leads to diminished α8 integrin expression in the UB tips, which may contribute to the severe branching defect and also the decreased nephron numbers since α8 is important for epithelization of MM (Muller, Wang et al. 1997).

We did not observe any overt renal phenotypes in our Aqp2Cre;□β1$^{flox/flox}$ mice when β1 integrin is deleted at the tubule growth and elongation stage. As we discussed

previously, this lack of phenotype could be explained by inefficient deletion or the slow turnover of β1 integrins which are abundantly expressed in the collecting system before E18.5. We could utilize a β1 knock-out, lacZ knock-in mice model and immunostaining analysis to further test this possibility. It is also possible that the function of β1 integrin is dispensable for tubule elongation and growth stage when other integrins serve as major players or compensate for loss of β1 integrin expression due to a functional redundancy. αv containing integrins and α6β4 integrin are potential candidates since they are also expressed in the tubules. α6β4 is a major laminin receptor and a functional redundancy possibly exists between β1 and β4 integrins. The role of these integrin subunits could be investigated by generating mice carrying null mutations for αv or β4 integrin subunits specifically in the collecting system utilizing AQP2Cre. UB-specific double β1/β4 knockout mice could also be generated to investigate if these two β subunits compensate for each other.

We found that, deletion of β1 integrin at the tubule growth and elongation stage still renders the adult mice kidneys more susceptible to injury when we performed ureteric obstruction on Aqp2Cre;□β1$^{flox/flox}$ mice. The similar effect has been observed in mice carrying null mutations for integrin α subunits. For example, lack of α1β1 leads to severe glomerulosclerosis after injury although no overt renal phenotypes are seen in α1-null mice kidneys (Chen, Moeckel et al. 2004). These results indicate that β1 integrin plays a role in maintaining tubule integrity. Several cell behaviors, including cell adhesion, migration and proliferation, are known to be important for the process of tissue

repair following injury. β1 integrin has a well defined role in promoting cell adhesion. Thus deletion of β1 integrin in collecting tubules leads to decreased cell adhesion to basement membrane, resulting in impaired tubule reconstitution. Furthermore, β1 integrin may also play a role in regulation of cell survival and proliferation under stress, via a tyrosine switch in its cytoplasmic tail. Previous structure function analysis shows that the tyrosine residue within the well conserved NPXY motif in β1 cytoplasmic tail functions as a switch, the phosphorylation of which occurs under stressed conditions such as inflammation and wound healing, activating downstream signaling by regulating adaptor binding(Czuchra, Meyer et al. 2006). Therefore, deletion of β1 integrin in our Aqp2Cre;□$\beta1^{flox/flox}$ mice leads to decreased cell adhesion to basement membrane and possibly decreased cell survival following obstruction, resulting in more severe injuries. Mice carrying collecting system-specific non-phosphorylatable point mutation in the tyrosine residues could be generated utilizing AQP2Cre to test if the tyrosine switch in β1 cytoplasmic tail is important for maintaining kidney collecting tubule integrity,.

In our vitro study, we explored the mechanism underlying the branching defect in Hoxb7Cre;□$\beta1^{flox/flox}$ mice. As expected, we found that deleting β1 integrin leads to significantly decreased cell proliferation on β1-dependent matrices. In contrast, one interesting finding is that $\beta1^{-/-}$ IMCD cells proliferate faster when plated on vitronectin, a ligand for αv-containing integrins such as αvβ3 and αvβ5. αv-containing integrins and β1 integrins share a lot of downstream signaling pathways in common. This increased proliferation of β1-null cells could be explained by: 1) the activation level of αv integrins

is higher in $\beta1^{-/-}$ IMCD cells than that in $\beta1^{flox/flox}$ cells, although FACS analysis reveals equal αv integrin expression levels between these two cell lines; 2) αvβ3 and αvβ5 work synergistically leading to maximal proliferative signaling output; 3) β1-containing integrins may have trans-dominant inhibitory effects on αv integrin mediated cell proliferation; 4) glucose transporters express at a higher level on $\beta1^{-/-}$ IMCD cell surface based on immunostaining. αv integrin activation levels could be detected via FACS analysis utilizing an antibody that specifically recognizes αv activation epitopes on its extracellular domain. Inhibitory antibodies could be added into the cell culture medium to determine if blocking β3 or β5 subunit has an additive effect in decreasing cell proliferation. And re-transfection of β1 integrin into our $\beta1^{-/-}$ IMCD cells as well as generation of a β1 over-expressing cell line could be performed to investigate whether increased β1 integrin expression has an inhibitory effect on αv integrin-dependent cell proliferation. Further investigation could also be done to determine if increased glucose transporter expression level contributes to increased cell proliferation of $\beta1^{-/-}$ IMCD cells. β1 integrins co-localize with CD98, an amino acid transporter, on basolateral cell surface (Feral, Nishiya et al. 2005). Deletion of β1 integrin possibly leads to decreased cell surface CD98 expression level along with reduced amino acid intake. Alternatively, $\beta1^{-/-}$ IMCD cells express higher levels of glucose transporters, leading to increased cell proliferation. CD98 expression levels on $\beta1^{flox/flox}$ and $\beta1^{-/-}$ IMCD cell surfaces could be assessed along with measurement of their amino acid uptake and glucose uptake abilities. In our study, we also observed increased ZO-1 expression levels on $\beta1^{-/-}$ IMCD cells according to confocal microscopic examination on Z sectioning. We propose that it is in

large part due to increased cell confluency on transwells at the time of staining, because of the higher proliferation rates of β1-null cells.

Furthermore, we found that several signaling pathways, such as FAK, EKR, AKT and p38, show drastically decreased activation in $\beta1^{-/-}$ IMCD cells following growth factor stimulation. Different pathways may correlate with different growth factor-mediated cell behaviors. ERK and p38 signaling pathways are mostly correlated with cell proliferation, while decreased FAK expression usually leads to decreased cell migration and adhesion. Therefore decreased signaling activation leads to the decreased proliferative and spreading responses in our β1-null cells. Since integrin function is very cell type-specific, we could further utilize signaling inhibitors to dissect the correlation between a single signaling pathway and cell function in our culture system. Cell adhesion, migration and proliferation abilities could be accessed following growth factor stimulation in the presence of a certain signaling inhibitor. Decreased signaling activation and decreased cell proliferation were also evident on HoxB7Cre;$\beta1^{flox/flox}$ mice kidneys at E15.5, despite normal expression levels of growth factor and GFRs. This decreased proliferation can be explained at several levels: 1) input from both integrin and GFRs is required to stimulate progression through the G1 phase of the cell cycle via induction of G1 cyclins through sustained ERK activation; 2) loss of integrin signaling leads to decreased translation of cyclin D1 mRNA ; 3) integrin signals are important for the down regulation of p21 family CDK inhibitors(Schwartz and Assoian 2001).

Our study has initiated future in-depth structure function analysis to dissect the mechanism whereby β1 integrin cytoplasmic tail mediates growth factor-dependent UB branching morphogenesis. Three regions are found to be preferred binding sites for most adaptors and are conserved among β-integrin tails - membrane proximal HDRK motif, membrane proximal NPXY motif and membrane distal NxxY motif (Legate and Fassler 2009). Both in vivo and in vitro studies suggest that membrane proximal HDRK sequence is not important for β1 integrin activation and function (Czuchra, Meyer et al. 2006), while the two NPXY motifs as well as a serine-threonine rich region are critical for β1 integrin(Chen, Zou et al. 2006); (Takala, Nurminen et al. 2008); (Nurmi, Autero et al. 2007). We could conduct both in vitro and in vivo studies to explore the mechanism whereby β1 integrin cytoplasmic tail regulates UB branching. In vitro, IMCD cells could be generated carrying point mutations in the two NPXY motifs and the serine-threonine rich region in β1 cytoplasmic tail. In vivo, knock-in mice with β1 cytoplasmic tail point mutants expressing specifically in the ureteric bud could be generated utilizing Hoxb7Cre. More future study may involve dissection of the complex integrin-adaptor protein network, utilizing proteomics and system biology strategies, which will give us a much clearer view of the cellular mechanism of renal development.

In the second part of our study, we investigated the function of β1 integrin in glomerular development and proposed that glomerulus loses VEGF following progressive podocyte loss in the pod-Cre; $\beta1^{flox/flox}$ mice, leading to endothelial cell death and mesangium injuries. However, we proposed this hypothesis mostly based on

morphological analysis and the role of VEGF during this process could be explored more extensively. In vivo, immunostaining could be performed to assess VEGFR expression and activation levels on endothelial cell surface, PDGF expression levels in endothelial cells as well as PDGFR levels on mesangial cell surface, in both $\beta1^{flox/flox}$ and pod-Cre; $\beta1^{flox/flox}$ mice glomeruli at different developmental stages(Eremina, Baelde et al. 2007). VEGF overexpression/β1-null double mutant mice could be generated to investigate if increased VEGF expression in podocytes is able to, at least in part, compensate for loss of podocyte numbers, and thereby extenuate the endothelium and mesangium injuries. We could further test this hypothesis in vitro by co-culture of β1-null podocytes and endothelial cells. The endothelial cell apoptosis and proliferation rates could be assessed over time. VEGFR as well as endothelial PDGF expression levels could be investigated by immunostaining. Possible alterations in signaling pathways, such as p38 MAPK, ERK1/2, PKC and PI3-kinase, could also be investigated after co-culture(Bohnsack and Hirschi 2004); (Chen, Luque et al. 2010); (Foster 2009).

Loss of expression of other growth factors following podocyte detachment may also contribute to endothelium and mesangium injuries in our pod-Cre; $\beta1^{flox/flox}$ mice. FGF is a strong candidate. Several in vitro studies have demonstrated that FGF stimulates endothelial cell proliferation via stimulation of MAPK pathway which results in activation of cyclin D1 and therefore cell cycle progression. FGF signaling has also been shown to regulate actin filament reorganization of endothelial cells during wound healing and thus helps to maintain endothelium integrity (Davidson, Dono et al. 2001). αvβ3

integrin on endothelial cell surface helps to sustain signaling activation by FGF stimulation. Blocking either αv or β3 subunit abrogates downstream proliferative signaling (Generscb, Ferletta et al. 2003). Furthermore, αvβ3 integrin is shown to directly associate with PDGFR β and help to maintain the mesangium integrity. Thus it is possible that deletion of β1 integrin in podocytes leads to podocyte detachment over time followed by decreased FGF expression, resulting in impaired αvβ3-dependent endothelial cell signaling and proliferation. Decreased proliferation and survival of endothelial cells may further lead to decreased cell surface αvβ3 expression, impaired αvβ3-PDGFR signaling and mesangium injury(Somanath, Malinin et al. 2009). FGF expression levels in the podocytes and αvβ3 expression levels on endothelial cell surfaces could be assessed by immunostaining on kidneys of our pod-Cre; $\beta1^{flox/flox}$ mice. In our study, we also demonstrated decreased ILK expression levels in pod-Cre; $\beta1^{flox/flox}$ mice glomeruli. ILK is known to form a ternary signaling complex with Pinch and Parvin, which participates in regulating various cell functions. Thus the role of β1 integrin in maintenance of glomerular integrity may involve active signaling networks mediated by ILK (Shi, Qu et al. 2008).

Furthermore, the correlation between podocyte injury and the progression of diabetic kidney disease is evident in a lot of recent studies (Sulikowska and Manitius 2007). Formation of proteinuria not only predicts the pace of renal decline; it also indicates cardiovascular disease (CVD) progression (Ratto, Leoncini et al. 2006). It would be interesting to investigate if loss of β1 integrins in podocytes increases risks of

diabetic kidney disease and cardiovascular disease. Mice muscle samples could be collected for investigation and physiological studies could be performed on $\beta1^{flox/flox}$ and pod-Cre; $\beta1^{flox/flox}$ mice.

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