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Integrin alpha6 maintains the structural integrity of the kidney collecting system

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Abstract

Laminins are a major constituent of the basement membranes of the kidney collecting system. Integrins, transmembrane receptors formed by non-covalently bound α and β subunits, serve as laminin receptors, but their role in development and homeostasis of the kidney collecting system are poorly defined. Integrin α3β1, one of the major laminin receptors, plays a minor role in kidney collecting system development, while the role of α6 containing integrins (α6β1 and α6β4), the other major laminin receptors, is unknown. Patients with mutations in α6 containing integrins not only develop epidermolysis bullosa, but also have abnormalities in the kidney collecting system. In this study, we show that selectively deleting the α6 or β4 integrin subunits at the initiation of ureteric bud development in mice does not affect morphogenesis. However, the collecting system becomes dilated and dysmorphic as the mice age. The collecting system in both null genotypes were also highly susceptible to unilateral ureteric obstruction injury with evidence of excessive tubule dilatation and epithelial cell apoptosis. Mechanistically, integrin α6-null collecting duct cells are unable to withstand high mechanical force when adhered to laminin. Thus, we conclude that α6 integrins are important for maintaining the integrity of the kidney collecting system by enhancing tight adhesion of the epithelial cells to the basement membrane. These data give a mechanistic explanation for the association between kidney collecting system abnormalities in patients and epidermolysis bullosa.

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Introduction

The multi-branched kidney collecting system develops from the ureteric bud (UB) which undergoes iterative branching morphogenesis following its interactions with the metanephric mesenchyme (MM). This process requires growth factor-mediated cell signaling and integrin-dependent cell-extracellular matrix (ECM) interactions.

Integrins are transmembrane receptors formed by non-covalently bound α and β subunits and they mediate multiple cellular processes including adhesion, migration, proliferation and tubule formation [1–3]. In mammals, 18 α and 8 β subunits combine in a restricted manner to form specific dimers with different ligand binding properties [4]. Of these heterodimers, α3β1, α6β1 and α6β4 act as primary receptors for laminins (LM) which are large heterotrimeric glycoproteins composed of one α, one β, and one γ chain [5, 6]. LMs belong to a multigenic family with five α, four β and three γ chain genes that can assemble into at least 15 different heterotrimers [7]. Preferred ligands for integrin α3β1 are LMs-111 and 332 and the α5-containing LMs, such as LM-511 and 521 [8, 9]. Integrin α6β1 was identified as a receptor for LMs-111, 511 and 521, while integrin α6β4 preferentially binds to LM-332, however it also interacts with the LMs-511 and 521 [9–11].

The contribution of α6 containing integrins to skin development is well described. Mutations of either α6 or β4 result in junctional epidermolysis bullosa with skin blistering in humans [12]. Consistent with this finding, integrin α6- and β4-null mice die at birth with epidermolysis bullosa, despite no skin morphogenesis defects [13–15]. Dysplasia of the kidney collecting system is also found in epidermolysis patients caused by mutations in the subunits of either integrin α6β4 or of LM-332[16], however due to their perinatal fatality, no causal link has been shown in mice. There is good circumstantial evidence that these integrins are important for normal development and maintenance of the kidney collecting system as integrin α6 and β4 subunits are highly expressed in the UB and antibodies directed against either subunit decreased UB branching morphogenesis in *ex vivo* organ culture models[17]. Also, selectively deleting the other major LM receptor, integrin α3β1, in renal collecting ducts at an early embryonic stage resulted in only a mild renal phenotype [18–20].

In this study, we selectively deleted the α6 or β4 integrin subunits in the developing UB and found that although morphogenesis was normal, the kidney collecting system was pathologically dilated in aged mice. Both null genotypes were also highly susceptible to tubular dilatation and tubular cell apoptosis following ureteric obstruction. Mechanistically integrin α6-null collecting duct (CD) cells cannot withstand high forces when adhered to LM-332. Thus, we conclude that α6 integrin/LM-332 interactions play a key role in maintaining the integrity of the kidney collecting system by mediating tight adhesion of the epithelial cells to the basement membrane. These data provide a mechanistic explanation for the susceptibility of epidermolysis bullosa patients to kidney injury.

Results

Integrin α6 and β4 subunits are not required for normal ureteric bud morphogenesis, but are necessary for maintaining normal kidney collecting duct integrity

We deleted either the α6 or β4 integrin subunit at the initiation of UB development (E 10.5) by crossing integrin *Itga6*$^{flox/flox}$ or integrin *Itgb4*$^{flox/flox}$ mice with the Hoxb7cre mouse. Despite successful deletion of integrin α6 (Fig. 1A), the kidneys of Hoxb7cre:*Itga6*$^{flox/flox}$ mice developed normally with no defects observed in 3 months old mice (Fig. 1B–C). Interestingly, at 6 months of age these mice had dilated collecting ducts (Fig. 1 D–E) that was worse in 10 month old mice (Fig. 1F–I). Despite these kidney defects, these mice lived a normal life span. We also achieved successful deletion of the integrin β4 in the collecting system of Hoxb7cre:*Itgb4*$^{flox/flox}$ mice (Fig. 2A) and, similar to the integrin Hoxb7cre:*Itga6*$^{flox/flox}$ mice, no kidney abnormalities were detected in 3 months old mice (Fig. 2B–C). The Hoxb7cre:*Itgb4*$^{flox/flox}$ kidneys demonstrated some dilatation of the collecting ducts at both 6 and 12 months, however it was less pronounced than that observed in Hoxb7cre:*Itga6*$^{flox/flox}$ mice (Fig. 2. D–I). These results suggest that α6 containing integrins are not required for morphogenesis of the developing kidney collecting system but are necessary for maintenance of its integrity over time.

The integrin α6 and β4 subunits protect the kidney collecting system from injury

Based on the finding that the collecting system of the Hoxb7cre:*Itga6*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice developed dysplastic papillae over time, we tested the hypothesis that α6-containing integrins play a critical role in maintaining kidney structure following injury by subjecting them to unilateral ureteric obstruction (UUO). Three days after UUO the Hoxb7cre:*Itga6*$^{flox/flox}$ kidneys displayed increased tubular dilation, flattened collecting duct epithelium and proteinacious casts relative to kidneys from *Itga6*$^{flox/flox}$ mice (Fig. 3 A–B). On day 5 after UUO, we also observed an increase in cellularity at the corticomedullary junction of the kidneys of the Hoxb7cre:*Itga6*$^{flox/flox}$ mice (Fig. 3 C–D). By day 10 the increased cellularity in the Hoxb7cre:*Itga6*$^{flox/flox}$ kidneys was easily seen (Fig. 3 E–F). Despite this phenotype, kidneys of injured Hoxb7cre:*Itga6*$^{flox/flox}$ mice did not develop worse fibrosis than *Itga6*$^{flox/flox}$ mice as assessed by trichrome staining (Fig 3G–H) and immunoblotting for collagen I in isolated kidneys (Fig. 3I–J). The degree of tubular injury was scored (see Concise Methods) at days 3, 5 and 10 after UUO, and the Hoxb7cre:*Itga6*$^{flox/flox}$ mice always had a significantly worse injury score than the *Itga6*$^{flox/flox}$ mice. The respective scores for the Hoxb7cre:*Itga6*$^{flox/flox}$ and *Itga6*$^{flox/flox}$ mice were 3.25 ± 0.5 (SE) versus 2.00 ± 0.15 (SE) at 3 days; 3.75 ± 0.00 (SE) versus 2.25 + 0.25 (SE) at 5 days and 4.00 ± 0.00 (SE) versus 3.00 + 0.25 (SE) at 10 days. Furthermore, the kidneys of Hoxb7cre:*Itga6*$^{flox/flox}$ mice developed a significant increase in tubular dilatation compared to *Itga6*$^{flox/flox}$ mice (Fig. 3 K). here was also a significant increase in TUNEL staining in the Hoxb7cre:*Itga6*$^{flox/flox}$ collecting duct epithelium 2 days after UUO (Fig. 3 L–N) and this difference persisted at 3 and 5 days (data not shown). Taken together, α6-containing integrins protect the kidney collecting system from injury in the setting of UUO model by decreasing collecting duct dilatation and apoptosis, but not the fibrotic response.

We next investigated the relative contribution of integrin α6β4 in mediating this protection in the setting of UUO injury model by performing the same experiments in the *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice. Similar to the Hoxb7cre:*Itga6*$^{flox/flox}$ mice, the Hoxb7cre:*Itgb4*$^{flox/flox}$ mice developed more severe injury than the *Itgb4*$^{flox/flox}$ mice at 3, 5 and 10 days after injury (Fig 4A–F), which was characterized by increased tubular dilation, flattened collecting duct epithelium, proteinacious casts and excessive cellularity but no increase in fibrosis. When the injury was scored it was significantly worse in the Hoxb7cre:*Itgb4*$^{flox/flox}$ when compared with the *Itgb4*$^{flox/flox}$ mice at 3, 5 and 10 days respectively. (3.5 ± 0.5 (SE) versus 2.25 ± 0.15 (SE) at 3 days; 4 ± 0.00 (SE) versus 2.5 + 0.25 (SE) at 5 days and 4.00 ± 0.00 (SE) versus 3.00 + 0.25 (SE) at 10 days). There were no significant differences in fibrosis between the two genotypes as assessed by trichrome staining and immunoblotting for collagen I (Fig. 4G–J). The tubular dilatation was significantly increased in the Hoxb7cre:*Itgb4*$^{flox/flox}$ relative to the *Itgb4*$^{flox/flox}$ mice in all three days (Fig. 4K). There was also a significantly increased amount of apoptosis in the collecting ducts of the injured Hoxb7cre:*Itgb4*$^{flox/flox}$ when compared to *Itgb4*$^{flox/flox}$ mice at 2, 3 and 5 days (Fig. 4 L–N). Thus the differences in severity of injury in the α6 and β4 null mice relative to their wild type controls were very similar, suggesting the protection from injury after UUO was primarily mediated by integrin α6β4.

Integrin α6-null collecting duct cells adhere and signal normally on LM-511 but not on LM-332

To study the mechanisms whereby integrin α6-LM interactions regulate the homeostasis of the kidney collecting system during aging and after UUO, we isolated collecting duct cells from *Itga6*$^{flox/flox}$ mice (Itga6$^{f/f}$ cells) and deleted *Itgα6 in vitro* using Cre adenovirus (Itga6$^{-/-}$ cells). The α6 integrin subunit was efficiently deleted resulting in no surface expression on CD cells (Fig. 5A). This deletion also decreased the surface expression of β4 but did not significantly alter β1 surface expression or total expression of the α3 (Fig. 5A–B) or αv integrin subunits (data not shown). We next assessed the functional consequences of deleting integrin α6 on CD cell functions on LM-511, the principal LM in the collecting duct. Surprisingly, CD cells lacking the integrin α6 subunit did not show significant changes in cell adhesion, migration and proliferation (Fig. 5 C–E). Furthermore, when Itgα6$^{-/-}$ CD cells were plated on purified LM-511, there were no differences in adhesion-dependent Akt, p38-MAPK or ERK activation (Fig. 5F). Thus, in cells expressing the LM-binding integrin α3β1, loss of the integrin α6 does not play a role in mediating interactions between CD cells and LM-511.

Although LM-511 is the principal LM in the collecting duct, we showed that LM-332 is also expressed in the developing collecting system, where it is required for normal CD development [18]. As both integrins α6β1 and α6β4 are receptors for LM-332, we defined the functions of Itga6$^{-/-}$ CD cells on LM-332. Unlike on LM-511, these cells had significant adhesion and migration defects on LM-332 (Fig. 6A, B) with no overall changes in their ability to proliferate on this substrate (Fig. 6C). When we determined whether there were adhesion-dependent signaling defects of the *Itga6*$^{-/-}$ CD cells on LM-332, none were found (Fig. 6D). Finally, based on the severe dilatation of the tubules seen in the UUO model in the Hoxb7cre:*Itga6*$^{flox/flox}$ mice, we assessed the adhesion strength of Itga6$^{-/-}$ CD cells on

LM-332 by performing the spinning disc assay. The adhesive strength of these cells was significantly less compared to Itga6$^{f/f}$ CD cells on LM-332, but not on fibronectin, a ligand for α5β1 and αv integrins (Fig. 6E). These results suggest that integrin Itga6$^{-/-}$ CD cells have an adhesion and migration defect on LM-332 and they cannot withstand high forces when adhered to LM-332.

Discussion

Normal UB development requires integrin-dependent cell-ECM interactions, however the role of specific integrins in this process is poorly defined. Deleting the integrin β1 subunit (which deletes 12 integrins) at the initiation of the developing UB resulted in a severe UB branching phenotype that ultimately caused lethal kidney failure [21]. Surprisingly, deleting the α3 subunit in the UB only results in a mild UB development disorder [18–20] and integrin α1 and α2 null mice have normal kidney collecting systems [3, 4]. The addition of blocking α6 and β4 antibodies to *in vitro* kidney and UB cultures inhibited branching morphogenesis suggesting these LM-binding receptors are important for collecting system development [17, 22]. In this study we directly investigated the role of the α6 integrins in UB development by selectively deleting the α6 or β4 subunit at the initiation of UB development. We show that normal UB morphogenesis does not require expression of either of these integrin subunits, however as the mice age the collecting system becomes dilated and dysmorphic, suggesting a role for the α6 integrins in maintenance of the normal architecture of the kidney collecting system. Consistent with this finding the collecting systems of both the α6- and β4-null mice were highly susceptible to UUO injury, exhibiting excessive tubule dilatation and apoptosis of the kidney tubules. Mechanistically integrin α6-null CD cells have an adhesion and migration defect on LM-332 and they cannot withstand high forces when adhered to this LM. Thus, α6 integrins are important for maintaining the integrity of the kidney collecting system by providing sufficient adhesive strength to epithelial cells on BMs. In the absence of α6 integrins, there is less tight adhesion of CD cells to the BM leading to tubular dilatation over time and increased susceptibility to injury. These data suggest a mechanistic explanation for the higher incidence of renal abnormalities in patients with epidermolysis bullosa [16, 23–25].

Our result that deleting the α6 or β4 integrin subunit from the developing UB did not alter branching morphogenesis was consistent with the observation that newborn constitutive integrin α6-null mice had no renal phenotype [13]. These data contrast with the minor UB branching abnormalities seen in the integrin α3 and LM γ2, or α5 null mice[18–20], which suggests that integrin α3β1 is the principal LM receptor required for normal UB development *in vivo*. As the renal phenotype of the integrin α3-null mouse is so mild and the α6, α1 and α2 null mice are normal there does not appear to be a major αβ1 integrin that regulates UB morphogenesis. This contrasts with the glomerulus, where deleting the α3 or β1 integrin subunit causes very similar phenotypes [26–29].

The kidney collecting system in both the Hoxb7cre:*Itga6*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice become dilated and dysmorphic over time. This phenotype is worse in the α6-null mice, suggesting that both integrin α6β1 and α6β4 play a role in this process. Both mice were equally susceptible to injury after UUO where they exhibited excessive tubular

dilatation and epithelial cell apoptosis. These data are consistent with the primary role of α6β4 as an integrin that mediates tight adhesion to a basement membrane. Integrin α6β4 is a major component of both the type I and II hemidesmosome structures [30, 31]. Type I are found in stratified and pseudostratified epithelia such as the skin, while type II are found in simple epithelia such as the uroepithelial layers [32–34]. There are reports of junctional epidermolysis bullosa patients with either α6 or β4 mutations that have a dilated renal pelvis and hydronephrosis[16, 23–25], features similar to those seen in the aged mice. Interestingly, the urinary tract signs in patients do not always correlate with the skin manifestations. Thus our mouse data provides mechanistic insights as to why patients with epidermolysis bullosa caused by α6β4 integrin mutations have kidney collecting system defects. Furthermore, it suggests these patients are potentially susceptible to exacerbated injury in conditions with excessive intraluminal tubular pressure such as obstruction or after the passage of renal stones.

Deleting the integrin α6 subunit from CD cells resulted in a mild adhesion and migration defect as well as decreased adhesion strength on LM-332. Surprisingly, α6 integrins do not play a role in CD cell interactions with LM-511, nor do they regulate any form of ligand-dependent cell signaling. These results are consistent with findings that LM-332 supports stable adhesion of many cell types by interacting with both integrin α6β4 and α3β1, while the latter is the principal integrin that promotes CD cell adhesion to LM-511[9–11]. Thus in CD cells that express α3β1 at high levels, α6 integrins play a minor role in supporting adhesion to LM-332 and do not alter adhesion to LM-511. These data suggest that the principal role of integrin α6β4, like in the skin, is probably to mediate tight adhesion and adhesive strength of CD cells to the tubular basement membrane.

Our results showing that integrin α6β4 is required for normal maintenance of the kidney collecting system adds to its described functions. Other than the severe blistering of the skin despite normal morphogenesis, all the phenotypes are subtle and occur postnatally in an organ specific manner. The only other branching organ where integrin α6β4 was shown to be important is the mammary gland. Deleting β4 resulted in small glands that had increased apoptosis in the surrounding mesenchyme due to decreased PTHrP expression and signaling[35], however these results contrast with another study where mammary glands were shown to develop normally in integrin α6-null mice[36]. Similar to our results, deleting the β4 integrin subunit in Schwann cells in peripheral nerves resulted in abnormal myelin folding and slower nerve recovery after injury because integrin α6β4 is required to anchor Schwann cells to the basal lamina[37, 38]. Other defects in the nervous system associated with the loss of α6, but not β4, have also been described. Deletion of the α6 subunit in the brain using the nestin-Cre mouse, did not result in major abnormalities in the laminar organization of the cerebral cortex and only mild defects in the cerebellar foliation. Compensation by other LM-binding proteins was advanced as the reason for this surprisingly subtle phenotype [39]. Similarly, when the α6 subunit was specifically deleted from Schwann cells using the mP0TOT-Cre mice, there were no abnormalities in axonal sorting and only minor ensheathment and myelin anomalies. The LM receptor, integrin α7β1, partially compensated for α6β1 in Schwann cells [40]. Integrin α6 was also shown to play a role in the development of the olfactory bulb where it is required for neural migration[41]. Thus, with the exception of the skin where it is absolutely required for the

tight adhesion of the epidermis to the basement membrane, it appears that other organs can compensate for the lack of integrin α6.

In conclusion our data suggests that α6 integrins provide adhesion strength for CD cell attachment to BMs and that deleting α6 containing makes the kidney collecting system susceptible to age related degeneration and injury due to increased intraluminal pressure of the tubules. These data provide a molecular explanation for the increased incidence of urogenital abnormalities in patients with epidermolysis bullosa due to integrin α6β4 mutations and suggests physicians should protect the kidney collecting system of these patients from trauma and injury.

Concise Methods

Reagents and antibodies

Human LMs 332 and 511 were produced as previously described[42, 43]. Collagen I was purchased from BD Biosciences (San Jose, CA, USA); fibronectin and vitronectin were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The following antibodies were used in Western immunoblot analyses: integrin α3 (AB1920, Millipore, Temecula, CA, USA and AF2787, R&D Systems); integrin β1 (AB1952, Millipore); integrin β1 (MAB1997, Millipore); integrin β4 (AF4054, R&D Systems); integrin α6 (3750), phospho-AktThr308 (9275), phospho-AktSer473 (9271), total Akt (9272), phospho-p38Thr180/Tyr182 (9211) and total p38 (9212), phospho-ERK1/2Thr202/Tyr204 (9101), total ERK1/2 (9102), (all from Cell Signaling Technologies) and collagen I (MD Biosciences). Antibody to β-actin (A4700, Sigma-Aldrich) and alpha-tubulin (3873, Cell Signaling Technologies) were used to evaluate protein loading. Anti-mouse β1 (550530), α1 (555001), α2 (553819), α5 (553350), α6 (555734) and αv (550024) integrin antibodies were purchased from BD Biosciences. R-phycoerythrin-conjugated secondary antibodies were bought from Invitrogen (Carlsbad, CA, USA).

Generation of Hoxb7cre:Itgα6$^{flox/flox}$ and HoxB7cre:Itgβ4$^{flox/flox}$ mice

Integrin α6$^{flox/flox}$ (Itgα6$^{flox/flox}$) mice [13, 39], 1996) and integrin β4$^{flox/flox}$ (Itgβ4$^{flox/flox}$) mice[15] were crossed with the HoxB7cre mice (generous gift of Dr. A. McMahon)[44]. Age-matched littermates homozygous for the integrin Itgα6$^{flox/flox}$ or Itgβ4$^{flox/flox}$ gene but lacking cre were used as controls. The expression of integrin α6 and β4 in the developing mouse collecting ducts was determined using Western immunoblot analysis.

Unilateral ureter obstruction

All procedures using animals were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Unilateral ureteric obstruction was performed on mice aged 8 weeks. Mice were anesthetized with ketamine and administered pre-operative buprenorphine and isotonic saline. The right ureter was ligated with 4.0 silk tie sutures. Mice were euthanized 2, 3, 5, 7, 10 and 14 days' post-surgery and obstructed kidneys were collected for histology and western blot analysis. Tubular dilatation was quantified using the ImageJ NIH software (Rasband, W.S., ImageJ, U. S. National

Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). At least 10 different animals were analyzed per each group.

Protein extraction and immunoblotting

Protein from the renal papilla of individual 3-day old pups or the whole kidney after UUO was isolated and lysed using a Polytron homogenizer in T-PER reagent (Thermo Scientific, Waltham, MA, USA) with protease inhibitors (P8340 Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors cocktails 1 and 2 (P5726 and P0044, Sigma-Aldrich). The homogenate was centrifuged at 17,000×g for 20 min at 4°C. The supernatant was collected and stored at 80°C. Cell lysates were prepared using M-PER reagent (Thermo Scientific, Waltham, MA, USA). Lysates were centrifuged at 17,000×g for 15 min at 4°C. Protein concentration in the supernatants was measured using BCA reagent (Thermo Scientific). Protein extracts were subjected to Western immunoblot analysis and developed using the Western Lightning Chemiluminescence Plus detection system (Perkin Elmer-Cetus, Wellesley, MA) according to the manufacturer's protocol. Densitometry was performed using the ImageJ program. Normalization of each protein of interest was performed relative to α-tubulin or β-actin value.

Analyses of kidney tissue morphology, apoptosis and fibrosis

Kidneys were cut in half, fixed on 10% formalin and embedded in paraffin. Severity of tubular injury was assessed on hematoxylin and eosin (H&E) sections. The percentage of tubules with cell necrosis, loss of brush border, cast formation, and tubular dilation as follows: 0, none; 1, 10%; 2, 11 to 25%; 3, 26 to 45%; 4, 46 to 75%; and 5, >76%. At least 10 fields (×200) were reviewed for each slide in a blinded manner. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed as described by the manufacturer's instructions (Promega Corporation, Madison WI). TUNEL-positive (apoptotic) cells were counted from 10 randomly selected high-power fields using five kidney samples per genotype. All slides were analyzed in a blinded fashion. Trichrome staining was performed according to the kit's instructions (Sigma-Aldrich).

Generation of Itgα6$^{f/f}$ and Itgα6 $^{/}$ CD cells

CD cells were isolated from 5- to 6-wk-old Itgα6$^{flox/flox}$ mice as previously described[45] and immortalized with pSV40 plasmid. Loci for the α 6 integrin subunit in CD cells were deleted with adenovirus expressing cre recombinase. CD cells were grown in DMEM/F12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

Flow cytometry

Flow cytometry analysis was performed as previously described[21]. CD cells were incubated with anti-mouse β1, β4, α1, α2, α6 and αv integrin antibodies followed by FITC-conjugated secondary antibodies.

Cell adhesion

Cell adhesion assays were performed in 96-well plates as previously described [46]. Cells (1×10^5) were seeded in serum-free medium onto plates containing different concentrations

of ECM for 60 min. Non-adherent cells were removed and the remaining cells were fixed, stained with crystal violet, and solubilized, and the optical densities of the cell lysates were read at 570 nm (OD570). Adhesion was calculated as percent of positive control (adhesion to serum).

Cell migration

Cell migration was assayed as previously described [46]. Transwells with 8-μm pores were coated with different ECM components, and 1×10^5 cells were added to the upper well in serum-free medium. Cells that migrated through the filter after 8 h were counted.

Cell proliferation

Cell proliferation was assessed by measuring incorporation of 5-bromodeoxyuridine (BrdU) in an enzyme-linked immunosorbent assay–based 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III (Roche Applied Science, Indianapolis, IN) as previously described [47]. BrdU incorporation was quantified by a change of absorbance (optical density) at 405 nm.

Cell replating assay

Cell replating assays were performed on CD cells that were trypsinized, washed, suspended in serum-free DMEM, plated on LM-332 or LM-511 (1 μg/ml) and harvested at 0, 30 and 60 min later. Cells were washed in PBS and lysed using M-PER reagent with protease and phosphatases inhibitor cocktails (Sigma). Protein extracts (20–40 μg) were subjected to Western immunoblot analysis.

Spinning disk adhesion assay

Mean cell adhesion strength was measured using a spinning disk system as previously described[48, 49]. Briefly, coverslips with adherent cells were mounted on the spinning platform and spun. After spinning, remaining cells were fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton X-100, stained with ethidium homodimer-1, and counted. Cell counts were normalized to the number of cell counts at the center of the disk, where the applied force is zero. The shear stress for 50% detachment (τ50) was used as the mean cell adhesion strength.

Statistical analyses

The mean and SEM of each treatment group were calculated for all experiments. At least 4 independent experiments (some in triplicates each) were performed. Student's t test was used to compare two groups. All statistical tests were two-sided and statistical analysis was done with the use of SigmaStat software (Systat Software Inc., San Jose, CA). Statistical significance was defined as p less than or equal to 0.05.

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Highlights

This manuscript highlights the fact that although integrins α6β1 and α6β4 are highly expressed in the ureteric bud if the kidney, they are not required for its normal development. However, these integrins are vital in protecting the kidney collecting system against injury and deterioration with the ageing process. It also highlights the possibility that patients with mutations in the α6 or β4 integrins that develop bullous skin diseases are more susceptible to injury of the kidney collecting system.

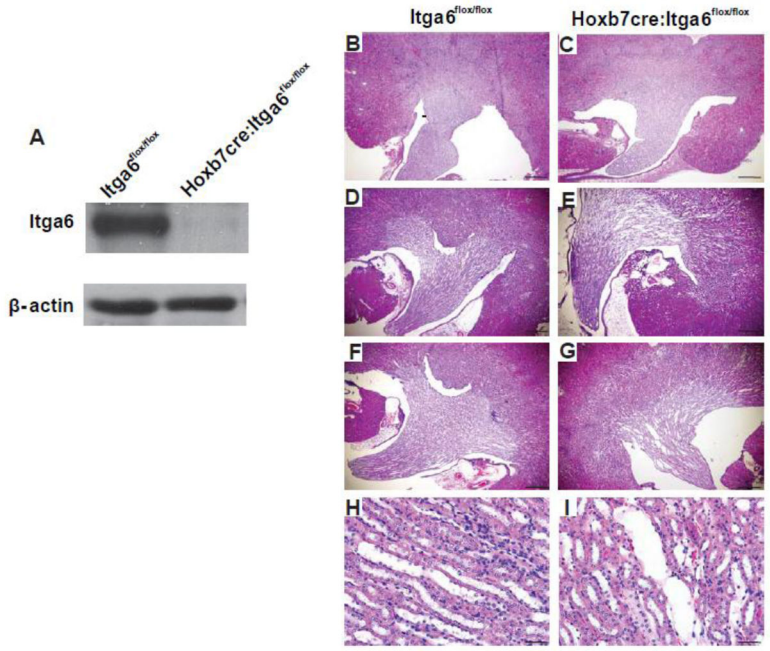

Figure 1. The integrin α6 subunit is not required for normal UB morphogenesis but is necessary to maintain normal kidney collecting duct morphology

(A) Expression of integrin α6 in papillary lysates from a 3-day old *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice was analyzed by Western blotting analysis. α6 is indicated by the arrowhead. (B–I) Morphology of H&E stained kidney sections from *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice at 2 months (magnification 40×, scale bar =500um) (B, C), 6 months (magnification 40×, scale bar =500um), (D, E) and 10 months of age (F, G) (magnification 40×, scale bar =500um) and (H, I) (magnification 200×, scale bar =100um).

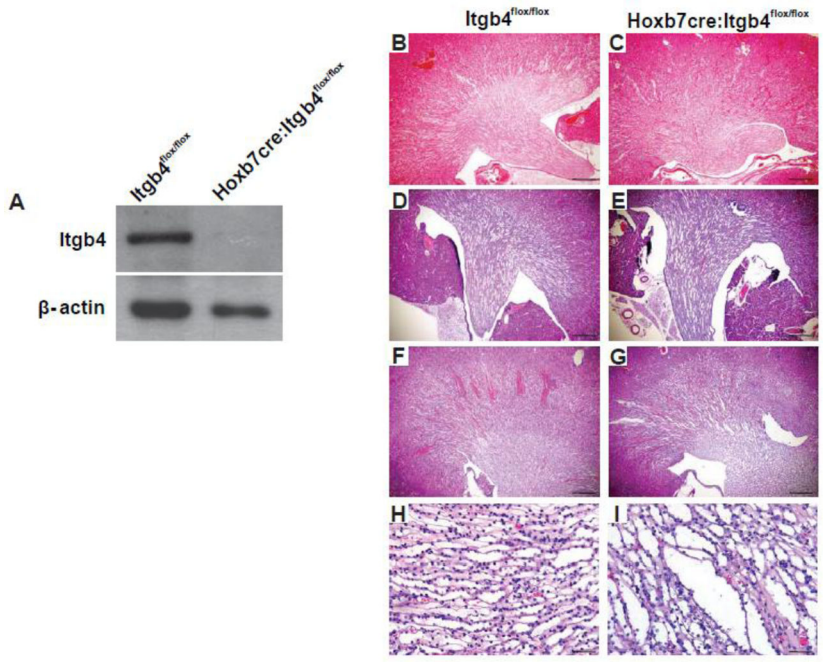

Figure 2. The integrin β4 subunit is not required for normal UB morphogenesis but is necessary to maintain normal kidney collecting duct morphology

A) Expression of integrin β4 in papillary lysates from a 3-day old *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice analyzed by Western blotting analysis. (B–I) Morphology of H&E stained kidney sections from *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice at 2 months (magnification 40×, scale bar =500um) (B, C), 6 months (magnification 40×, scale bar =500um), (D, E) and 12 months of age (F, G) (magnification 40×, scale bar =500um) and (H, I) (magnification 200×, scale bar =100um).

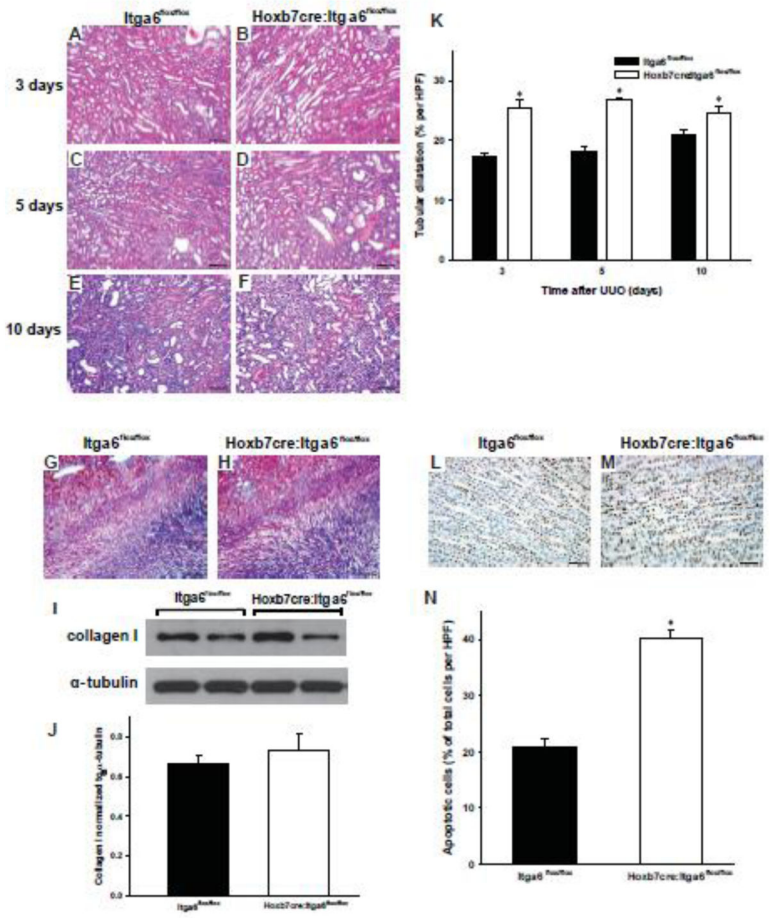

Figure 3. Hoxb7cre:*Itga6*$^{flox/flox}$ mice have increased injury after UUO

(A–F) Morphology of H&E stained kidney sections from *Itga6*$^{flox/flox}$ (A, C, E) and Hoxb7cre:*Itga6*$^{flox/flox}$ (B, D, F) 2 month old mice subjected to UUO for 3 (A, B), 5 (C, D) or 10 (E, F) days (magnification 200×, scale bar =100um). (G, H) Fibrillar collagen was detected 7 days after UUO by Trichrome staining. (I, J) Levels of collagen I in kidney lysates of *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice at 7 days after UUO was assessed by Western analysis and quantified by densitometry, normalized to α-tubulin and reported as mean measurements ±SEM. (K) Quantitative analysis of tubular dilatation in renal sections of *Itga6*$^{flox/flox}$ (black bars) and Hoxb7cre:*Itga6*$^{flox/flox}$ (white bars) mice subjected to UUO for 3, 5 or 10 days. Values are means with SEM from 6 mice; *p<0.01between *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice. (L, M) Kidney papilla sections from UUO-treated *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice for 2 days were stained for TUNEL (apoptosis) (scale bar= 100um). (N) Quantitative analysis of apoptosis in renal papilla sections of UUO-treated *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice. Apoptosis was quantified and expressed as percent of apoptotic cells per microscopic field (9 fields of 9 kidneys from either genotype were analyzed. *p<0.01 between *Itga6*$^{flox/flox}$ and Hoxb7cre:*Itga6*$^{flox/flox}$ mice.

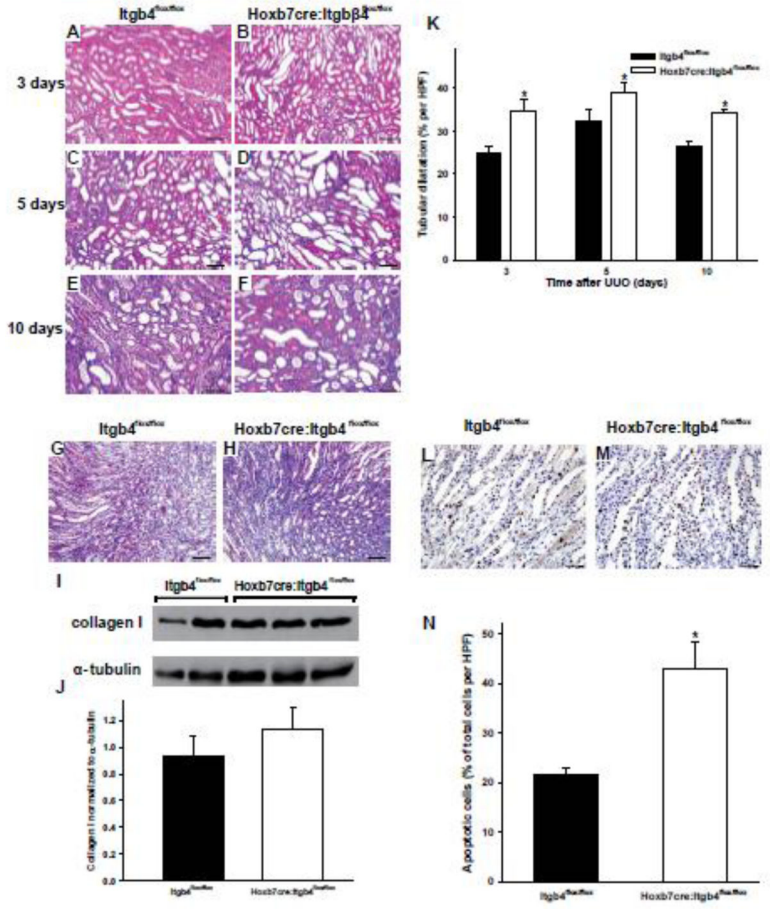

Figure 4. Hoxb7cre:*Itgb4*$^{flox/flox}$ mice have increased injury after UUO

(A–F) Morphology of H&E stained kidney sections from *Itgb4*$^{flox/flox}$ (A, C, E) and Hoxb7cre:*Itgb4*$^{flox/flox}$ (B, D, F) 2 month old mice subjected to UUO for 3 (A, B), 5 (C, D) or 10 (E, F) days (magnification 200×, scale bar =100um). (G, H) Fibrillar collagen was detected 7 days after UUO by Trichrome staining. (I, J) Levels of collagen I in kidney lysates of *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice at 7 days after UUO was assessed by Western analysis and quantified by densitometry, normalized to α-tubulin and reported as mean measurements ±SEM. (K) Quantitative analysis of tubular dilatation in renal sections of *Itgb4*$^{flox/flox}$ (black bars) and Hoxb7: Itgβ4$^{flox/flox}$ (white bars) mice subjected to UUO for 3, 5 or 10 days. Values are means with SEM from 6 mice;a significant *p<0.01 between *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice. (L, M) Kidney sections from UUO-treated *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice for 2 days were stained for TUNEL (apoptosis)(scale bar =100um). (N) Quantitative analysis of apoptosis in renal sections of UUO-treated *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice. Apoptosis was quantified and expressed as percent of apoptotic cells per microscopic field (9 fields of 9 kidneys from either genotype were analyzed *p<0.01 between *Itgb4*$^{flox/flox}$ and Hoxb7cre:*Itgb4*$^{flox/flox}$ mice.

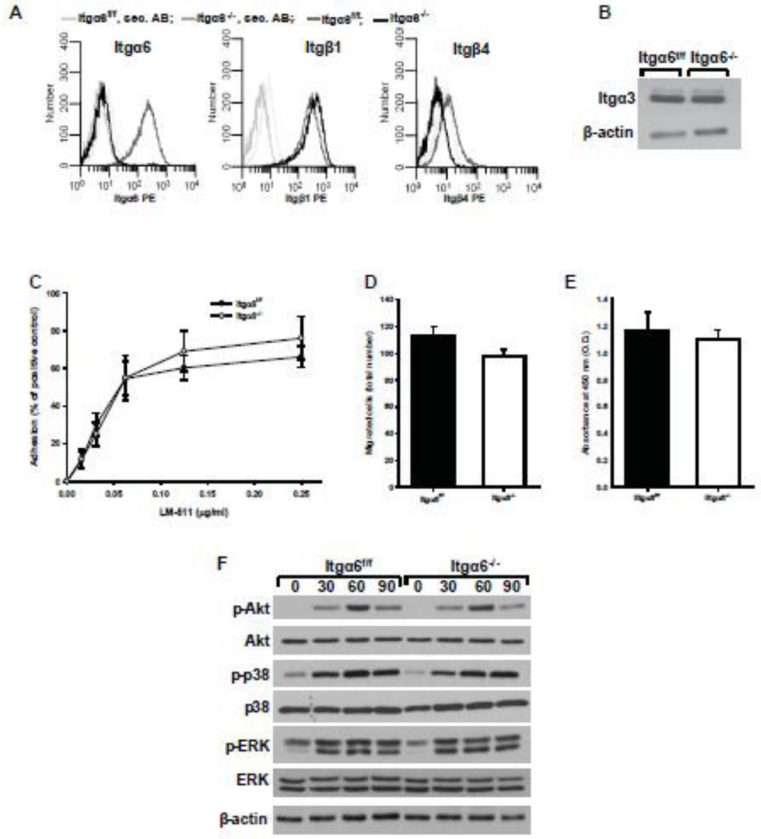

Figure 5. Itgα6$^{-/-}$ CD cells adhere, migrate, proliferate and signal normally on LM-511

(A) Surface expression of integrin α6, β1 and β4 subunits were determined on Itgα6$^{f/f}$ and Itgα6$^{-/-}$ CD cells by flow cytometry using R-phycoerythrin (PE) conjugated secondary antibodies. (B) Lysates from Itgα6$^{f/f}$ and Itgα6$^{-/-}$ CD cells (20 μg total protein/lane) were immunoblotted for integrin α3 subunits or β-actin (loading control). Adhesion (C), migration (D) and proliferation (E) on LM-511 were evaluated as described in the Methods. For migration and proliferation, 1 μg/ml LM-511 was used. Shown are mean measurements ±SEM of 4–6 independent experiments. (F) Itgα6$^{f/f}$ and Itgα6$^{-/-}$ CD cells were plated in serum-free medium on LM-511 (1 μg/ml). Cells were lysed at 30, 60 and 90 min after plating and lysates (20 μg total protein/lane) were analyzed by Western blot for levels of phosphorylated and total Akt, p38, and ERK1/2 or β-actin (loading control).

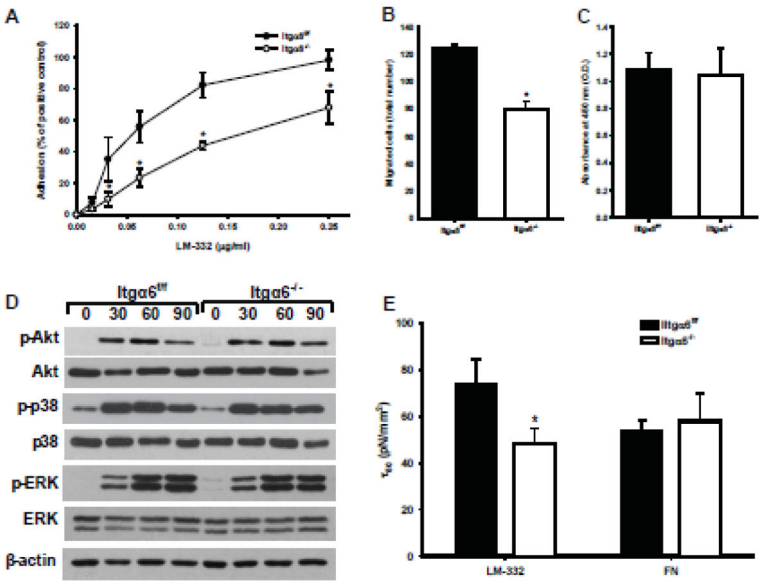

Figure 6. Itgα6$^{-/-}$ CD cells show adhesion, migration and adhesion strength defects on LM-332

Adhesion (A), migration (B), proliferation (C), and replating assay (D) on LM-322 (1 μg/ml) were evaluated as described in the Methods and in Fig. 5. Shown are mean measurements ±SEM of 4–6 independent experiments; *p<0.01between Itgα6$^{-/-}$ and Itgα6$^{f/f}$ CD cells. (E) The shear stress for 50% detachment (τ50), which represents the mean cell adhesion strength, was determined for Itgα6$^{-/-}$ and Itgα6$^{f/f}$ CD cells on LM-332 and fibronectin (which is not a ligand of a6 containing integrin) coated cell culture plates. Shown are the averaged τ50 values of 3 independent experiments; *p<0.01between Itgα6$^{-/-}$ and Itgα6$^{f/f}$ CD cells.