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β1 integrin is necessary for ureteric bud branching morphogenesis and maintenance of collecting duct structural integrity

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The kidney collecting system develops from branching morphogenesis of the ureteric bud (UB). This process requires signaling by growth factors such as glial cell line derived neurotrophic factor (GDNF) and fibroblast growth factors (FGFs) as well as cell extracellular matrix interactions mediated by integrins. The importance of integrin signaling in UB development was investigated by deleting integrin β1 at initiation (E10.5) and late (E18.5) stages of development. Deletion at E10.5 resulted in a severe branching morphogenesis phenotype. Deletion at E18.5 did not alter renal development but predisposed the collecting system to severe injury following ureteric obstruction. β1 integrin was required for renal tubular epithelial cells to mediate GDNF- and FGF-dependent signaling despite normal receptor localization and activation in vitro. Aberrations in the same signaling molecules were present in the β1-null UBs in vivo. Thus β1 integrins can regulate organ branching morphogenesis during development by mediating growth-factor-dependent signaling in addition to their well-defined role as adhesion receptors.

KEY WORDS: Kidney, Growth factor receptors, Renal development, Branching morphogenesis, Mouse

INTRODUCTION

Formation, growth and branching morphogenesis of the collecting system of the kidney require interactions between the ureteric bud (UB) and the metanephric mesenchyme (MM). The UB ultimately forms the multibranched 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 in which kidney growth predominates. This complex developmental process is dependent on numerous factors, including growth-factor-dependent cell signaling induced by glial cell line derived neurotrophic factor (GDNF) and fibroblast growth factors (FGFs) as well as interactions between cells and extracellular matrix (ECM) components (Dressler, 2006).

Integrins are cell-surface receptors that mediate the interactions between cells and ECM. They consist of non-covalently bound α and β subunits that combine in a restricted manner to form specific αβ dimers. In mammals there are 18α and 8β subunits that form more than 20 different dimers, each of which exhibits different ligand binding and signaling properties (Hynes, 2002). β1 is the most abundantly expressed β subunit and is found in almost all cell types in the body, including the kidney (Hynes, 2002; Kreidberg and Symons, 2000). Integrins α3β1, α6β1 and α6β4 are the major laminin-binding receptors, whereas 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 (Moser et al., 2009; Pozzi and Zent, 2003). Thus, integrins have the potential to play important roles in organ morphogenesis by modulating cell growth, motility and shape (Hynes, 2002; 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 (Kreidberg et al., 1996; Muller et al., 1997). Integrin α8-null mice have defects in UB growth and branching as well as recruitment of mesenchymal cells into epithelial structures, because integrin α8β1 expression is induced in mesenchymal cells upon contact with its specific ligand, nephronectin, which is expressed by the UB (Linton et al., 2007). Mice lacking the integrin α3 subunit have a reduced number of collecting ducts (CDs) in the papilla, suggesting decreased branching morphogenesis of the UB (Kreidberg et al., 1996). When the integrin α3 subunit was specifically deleted in the UB, the kidney papillae were either absent or abnormal (Liu et al., 2009); however, the rest of the collecting system of the kidney was unaffected. Interestingly, integrin α6-null mice do not display a collecting system phenotype (Georges-Labouesse et al., 1996), although α3/α6 double-deficient mice fail to develop ureters (De Arcangelis 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. Thus, the relative importance of integrins α6β1 and α6β4 in UB branching is currently uncertain (Zent 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

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when β1 integrin was deleted at E10.5, at the time that UB branching morphogenesis is initiated. By 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 crucial role in transducing growth-factor-dependent signals required for UB branching morphogenesis and maintaining collecting tubule integrity following injury.

MATERIALS AND METHODS

Generation of HoxB7Cre;β1$^{flox/flox}$ mice and Aqp2Cre; β1$^{flox/flox}$ mice

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. Integrin β1$^{flox/flox}$ mice (generous gift of Dr E. Fuchs, Howard Hughes Medical Institute, The Rockefeller University) (Raghavan et al., 2000) and integrin β1$^{flox/flox}$ mice, in which a promoterless *lacZ* reporter gene was introduced after the downstream loxP site (Brakebusch et al., 2000) were crossed with the HoxB7Cre mice (generous gift of Dr A. McMahon) (Kobayashi et al., 2005) or Aqp2Cre mice (Ahn et al., 2004; Stricklett et al., 1999b). Mice were a F4-F6 generation toward the C56/Black6 background. Aged-matched littermates homozygous for the floxed integrin β1 gene, but lacking Cre (β1$^{flox/flox}$ mice), were used as controls.

Morphological analysis

For morphological and immunohistochemical analysis, kidneys were removed at different stages of development and were: (1) fixed in 4% formaldehyde and embedded in paraffin; (2) embedded in OCT compound without fixation and stored at –80°C until use; or (3) 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 or periodic acid-Schiff (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, The 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, USA), as described (Zent et al., 2001). The number of branching structures was counted and quantified as number of branches/kidney. Ten kidneys per phenotype were analyzed.

Generation of integrin β1-null cell line

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

Flow cytometry

β1$^{flox/flox}$ CD cells and β1$^{-/-}$ cells were incubated with anti-mouse β1, β4, α1, α2, α6 and αv integrin antibodies (BD Transduction Laboratories), 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, as described (Chen et al., 2004). Briefly plates were coated with different concentrations of ECM components and blocked with BSA. In each well, 1×10^5 cells were placed in serum-free DMEM for 60 minutes; non-adherent cells were removed and the remaining cells were fixed, stained with Crystal Violet, solubilized and the optical density of the cell lysates was read at 540 nm. Four independent experiments were performed in triplicate.

Cell migration

Cell migration was assayed as previously described (Chen et al., 2004). Briefly, 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. The cells that migrated through the filter after 4 hours were counted. Three random fields were analyzed per treatment. Four independent experiments were performed in triplicate.

Cell proliferation

For each well, 5×10^3 cells were plated in 96-well plates on different ECM components and maintained in DMEM (10% FBS). After 12 hours, the cells were incubated in DMEM (2% FBS) for 24 hours and then pulsed with 1 μCi/well [^{3}H] thymidine (PerkinElmer Life Sciences). Twenty-four hours later, the cells were solubilized and radioactivity was measured using a scintillation counter. For manual counting assays, 6×10^4 cells were plated per plate on collagen or vitronectin and maintained in a serum-free environment. Cells were counted 48 hours after FGF (10 ng/ml) administration.

Cell polarity

Cells were grown on transwells consisting of polyvinylpyrolidone-free polycarbonate filters with 0.4 μm pores. After reaching confluency, cells were fixed in 4% formaldehyde and incubated with anti-ZO-1 (1:200; BD Transduction Laboratories, Lexington, KY, USA) 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 (10 μg/ml) or Vitronectin (10 μg/ml) for 30 minutes, after which FGF (10 ng/ml) or GDNF (10 ng/ml) was added. Forty-five minutes later cells were fixed, permeabilized and exposed to rhodamine phallodin (1:5000). For analysis of phosphorylated and total FGFR1 cells were incubated with anti-pFGFR1 (Y654; Abcam, Cambridge, MA, USA) and anti-FGFR1 antibody (Cell Signaling, Beverly, MA, USA) for 12 hours at 4°C, followed by incubation with a FITC-conjugated secondary antibody for 2 hours, and visualized under a microscope.

Immunoblotting

The CD cells were trypsinized, washed, suspended in serum-free DMEM and then plated on Collagen I or Vitronectin (10 μg/ml) for 45 minutes. Growth factors (FGF or GDNF, 10 ng/ml) were added to the medium and the cells were lysed at different time points following growth-factor stimulation. For analysis on kidney tissues, the medullas were removed and lysed with RIPA buffer. Lysates were clarified by centrifugation and 30 μg total protein was electrophoresed onto an 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were blocked in 5% milk/TBS Tween and then incubated with the different primary antibodies followed by the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were identified using enhanced chemiluminescence according to the manufacturer's instructions. AKT (1:1000, 9272), pAkt (1:1000, 9271S), ERK (1:1000, 9102), pERK (1:1000, 9101S), p38 MAPK (1:1000, 9212), pp38 MAPK (1:1000, 9211S) antibodies were purchased from Cell Signaling. FAK (sc-558) and pFAK (sc-16662-R) antibodies were purchased from Santa Cruz Biotechnology.

Immunostaining

For immunofluorescence, 5 μm frozen kidney sections were fixed in 4% paraformaldehyde for 10 minutes at room temperature, blocked with 1% BSA in PBS and then stained with the following primary antibodies: anti-E-cadherin (1:200); anti-FGF2 (1:100, Upstate 05-118), anti-FGFR1 (1:100, Santa Cruz, sc121) and anti-FGFR2 (1:100, Santa Cruz, sc122). Detection of bound primary antibodies was accomplished with Alexa Fluor 647 goat anti-rabbit IgG or Alexa Fluor 488 anti-mouse IgG (Molecular Probes). Slides were then analyzed under an epifluorescence microscope.

Unilateral ureteric obstruction

The right ureters of 6-week-old Aqp2Cre; $\beta1^{flox/flox}$ mice and $\beta1^{flox/flox}$ mice were ligated and mice were sacrificed 5, 7 and 10 days after the surgery. Kidneys were removed and fixed in 4% formaldehyde and embedded in paraffin. The degree of tubular injury was then scored by the following criteria. Histological changes consistent with tubular necrosis were quantified by calculating the percentage of tubules with cell necrosis, loss of brush border, cast formation or tubular dilatation as follows. Degree of injury was scored as 0 (none), 1 (1-10%), 2 (11-25%), 3 (26-45%), 4 (46-75%) and 5 (76-100%). At least 10 fields were analyzed for each slide. Degree of interstitial fibrosis and inflammatory infiltrate were judged using the same scale.

Statistics

Student's *t*-test was used for comparisons between two groups, and analysis of variance using Sigma Stat software was used for statistical differences between multiple groups. $P<0.05$ was considered statistically significant.

RESULTS

Deleting β1 integrin in the UB at E10.5 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 Wolffian 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 et al., 2000). As demonstrated in Fig. 1A, 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 (Fig. 1A). Although these mice were born in the normal Mendelian ratio, all the HoxB7Cre;$\beta1^{flox/flox}$ mice died between 4 and 6 weeks of age. The kidneys of 6-week-old mice were smaller and slightly cystic compared with those isolated from $\beta1^{flox/flox}$ mice (Fig. 1B,C). On microscopic examination, 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 (Fig. 1D-I). The most severe tubular dilatation was seen in the CDs located in both the medulla and the cortex (Fig. 1D-I).

To further define the defects present in these mice, studies were performed on embryonic kidneys from embryonic day 11.5 (E11.5) until birth. At every stage analyzed a branching morphogenesis defect was clearly present in HoxB7Cre;$\beta1^{flox/flox}$ mice. At day E11.5 the UB was less branched and smaller in HoxB7Cre;$\beta1^{flox/flox}$ mice (Fig. 2A,B). Kidneys of HoxB7Cre;$\beta1^{flox/flox}$ mice at E13.5 (Fig. 2C,D) as well as E15.5 (Fig. 2E,F) were significantly smaller, with a decreased number of UB branches and nephrons. The branching phenotype observed in HoxB7Cre;$\beta1^{flox/flox}$ mice was verified by performing in vitro cultures of E12.5 embryonic kidney. After 48 hours in culture, HoxB7Cre;$\beta1^{flox/flox}$ E12.5 kidneys formed approximately five branches per structure relative to the 50 detected in the $\beta1^{flox/flox}$ kidneys (Fig. 2G-I).

Kidneys of newborn HoxB7Cre;$\beta1^{flox/flox}$ were also smaller and had fewer collecting ducts than $\beta1^{flox/flox}$ mice (Fig. 2J,K). Interestingly the tubules, although somewhat disorganized, did not appear to have abnormalities with respect to polarity (Fig. 2L,M). This observation was confirmed when the newborn kidneys were stained with antibodies directed against E-cadherin (Cdh1 – Mouse Genome Informatics) (Fig. 2N,O) and ZO1 (Tjp1 – Mouse Genome Informatics) (data not shown). 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 the tubular lumens were dilated (Fig. 2P,Q). A significant decrease in nephrons was also present in newborn HoxB7Cre;$\beta1^{flox/flox}$ mice, as there were 4.2 versus 1.6 glomeruli/mm^2 of cortex in the HoxB7Cre;$\beta1^{flox/flox}$ and $\beta1^{flox/flox}$ mice, respectively (Fig. 2R). The difference in absolute glomerular number between HoxB7Cre;$\beta1^{flox/flox}$ and $\beta1^{flox/flox}$ mice is even bigger when one considers that kidneys of the HoxB7Cre;$\beta1^{flox/flox}$ are much smaller than those of $\beta1^{flox/flox}$ mice.

Taken together, these 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.

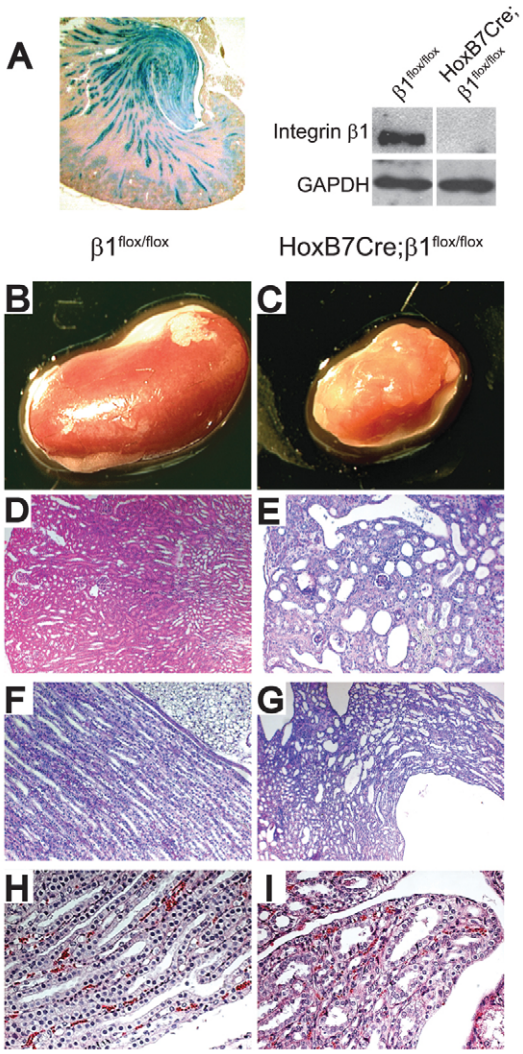

Fig. 1. HoxB7Cre;$\beta1^{flox/flox}$ mice develop severe end-stage renal failure. (**A**) *lacZ* staining of HoxB7Cre;$\beta1^{flox/flox}$ mice at P10, demonstrating excision of β1 integrin (left panel). Deletion of β1 integrin in the HoxB7Cre;$\beta1^{flox/flox}$ mice was confirmed by immunoblotting the kidney medulla of newborn mice with an anti-mouse β1 integrin subunit (right panel). (**B**,**C**) Gross appearance of kidneys of 6-week-old $\beta1^{flox/flox}$ and HoxB7Cre;$\beta1^{flox/flox}$ mice. (**D**,**E**) Microscopy of PAS-stained kidney slides, showing dilatation of cortical collecting ducts in HoxB7Cre;$\beta1^{flox/flox}$ but not $\beta1^{flox/flox}$ mice (100×). (**F**,**G**) The collecting ducts in the medulla and papilla of the HoxB7Cre;$\beta1^{flox/flox}$ mice are dilated and disorganized (100×). (**H**,**I**) Dilatation of the collecting ducts is present in the papilla (200×).

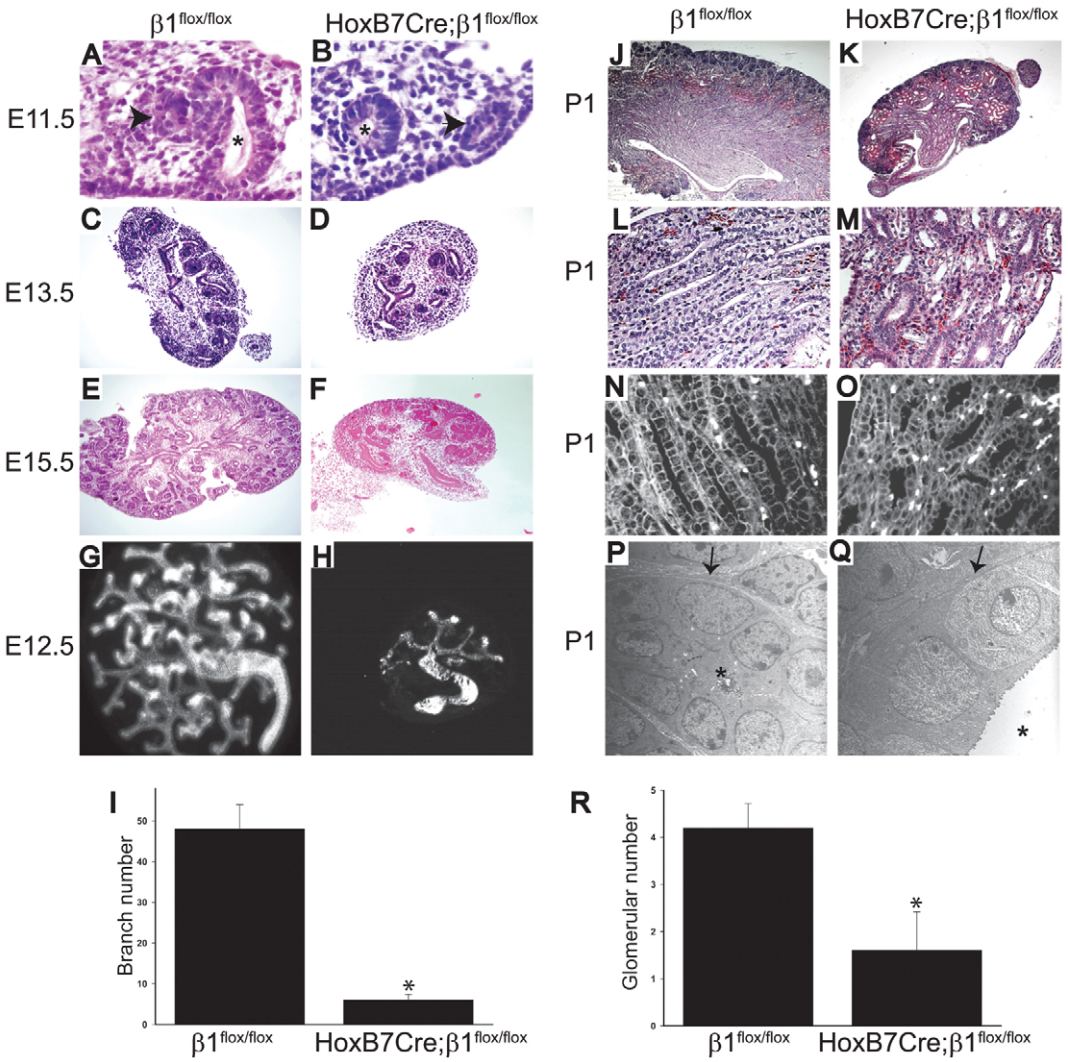

Fig. 2. HoxB7Cre;β1$^{flox/flox}$ mice have a severe branching morphogenesis phenotype. (**A**,**B**) Decreased branching of the UB is present in E11.5 HoxB7Cre;β1$^{flox/flox}$ embryos (600×). The Wolffian duct is shown with an arrowhead and the UB by an asterisk. (**C-F**) Decreased UB branching and nephron number is present in E13.5 (C,D) and E15.5 (E,F) HoxB7Cre;β1$^{flox/flox}$ kidneys when compared with β1$^{flox/flox}$ kidneys (40×). (**G-I**) Cultures of E12.5 kidneys of HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice were performed on transwells, as described in the Materials and methods. The kidneys were stained with antibodies directed against E-cadherin (G,H). The number of branches was counted in 10 kidneys from both genotypes and expressed as mean ± s.d. Differences between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice (*) were significant with P<0.01 (I). (**J**,**K**) PAS staining revealed that P1 HoxB7Cre;β1$^{flox/flox}$ mice have small kidneys, fewer nephrons and dilated tubules in both the cortex and medulla when compared with β1$^{flox/flox}$ mice (25×). (**L**,**M**) Collecting ducts within the papilla of P1 HoxB7Cre;β1$^{flox/flox}$ mice were dilated and irregular (200×). (**N**,**O**) E-cadherin staining of P1 kidneys showed no differences in localization and cell polarity between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice. (**P**,**Q**) Electron microscopy of collecting ducts demonstrated dilatation of the tubules in P1 HoxB7Cre;β1$^{flox/flox}$ mice, but there were no abnormalities in epithelial cell polarization or basement membrane structure (8500×). The arrows mark the tubular basement membrane and the asterisks demonstrate the tubular lumen. (**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 ±s.d. are shown. Differences between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice (*) were significant, with P<0.01.

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 (R. Nelson and D.E.K., unpublished) (Ahn et al., 2004; Stricklett et al., 1999a; Zhang et al., 2005). Gene deletion in the Aqp2Cre; β1$^{flox/flox}$ mice was confirmed by immunoblots on isolated papillae of 6-week-old mice (Fig. 3A). These mice were born in the normal Mendelian ratio and lived a normal life span. Despite intensive investigation, no gross or microscopical abnormalities were found at any age in these mice (Fig. 3B-G). Owing 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 postnatal day 21 (P21) (data not shown), suggesting that β1 integrin was still present when development was complete.

These mice provided a model to determine whether β1 integrin played a protective role to obstructive injury of the collecting system. When 6-week-old β1$^{flox/flox}$ and Aqp2Cre; β1$^{flox/flox}$ mice

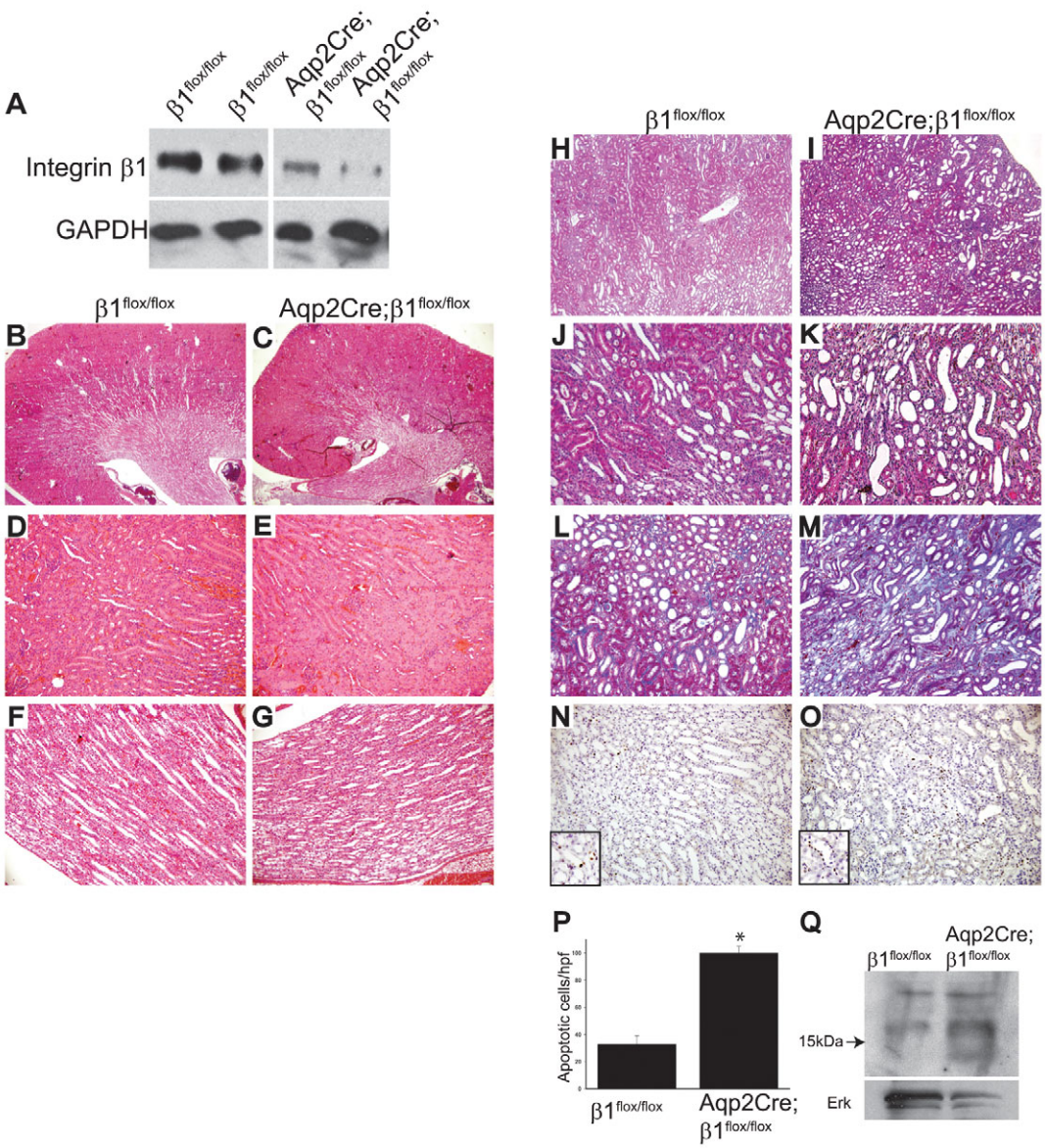

Fig. 3. Aqp2Cre; β1$^{flox/flox}$ mice develop severe injury following unilateral ureteric obstruction. (**A**) Papillae of 6-week-old Aqp2Cre; β1$^{flox/flox}$ and β1$^{flox/flox}$ mice were isolated and immunoblotted for β1 integrin. (**B-G**) Microscopy of PAS-stained kidney slides showed no differences in the morphology of Aqp2Cre; β1$^{flox/flox}$ and β1$^{flox/flox}$ at low (40×) (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 show more severe tubular dilatation and injury 5 days after unilateral ureteric obstruction when compared with the β1$^{flox/flox}$ mice (H,I 100× and J,K 200×). (**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 ± s.d. (10 fields of 10 kidneys from either genotype were analyzed). Differences between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ mice (*) were significant, with P<0.01. (**Q**) Immunoblots with an antibody directed against caspase-3 were performed on medullas of 5 day injured Aqp2Cre; β1$^{flox/flox}$ and β1$^{flox/flox}$. This is an immunoblot of a pool of five kidneys from each genotype.

were subjected to unilateral ureteric obstruction, there was markedly increased tubular dilatation and flattening of tubular epithelial cells in the Aqp2Cre; β1$^{flox/flox}$ mice at both 5 (Fig. 3H-K) and 10 (data not shown) days following injury. When tubular injury was scored at day 5, it was significantly worse in the Aqp2Cre; β1$^{flox/flox}$ than the β1$^{flox/flox}$ mice (4.8±0.6 versus 1.8±0.3 P<0.01). These mice also had significantly more Trichrome-Blue-positive matrix (Fig. 3L,M), consistent with interstitial fibrosis. Moreover, injured Aqp2Cre; β1$^{flox/flox}$ mice had more tubular apoptosis, as shown by TUNEL staining (Fig. 3N-P) and verified by increased activation of caspase-3 (Fig. 3Q). 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 CD cells results in adhesion, migration and proliferation defects

We next isolated CD cells from 3-week-old β1$^{flox/flox}$ mice and deleted β1 in vitro, utilizing adeno-cre virus to define its role in CD cell function. CD cells are an ideal in vitro model to study integrin-dependent functions in both adult and embryonic tubular epithelial

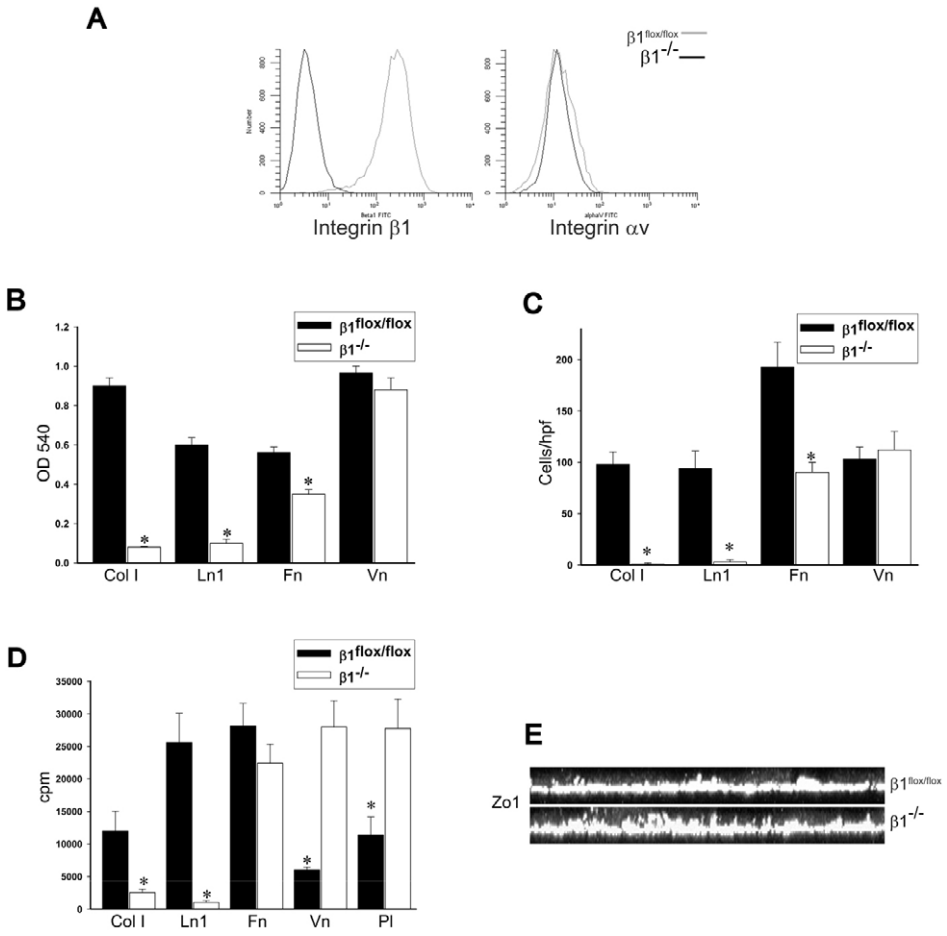

Fig. 4. Deleting the β1 integrin from CD cells results in abnormal adhesion, migration and proliferation on β1 integrin-dependent substrates. (**A**) Flow cytometry was performed on $\beta1^{flox/flox}$ and $\beta1^{-/-}$ cells utilizing antibodies directed against the extracellular domain of the β1 and αv integrin subunits. (**B**) CD cell populations were allowed to adhere to collagen I (Col I), laminin-1 (Ln1), fibronectin (Fn) or vitronectin (Vn) (all at 10 μg/ml) and cell adhesion was evaluated 1 hour after plating. Values are the mean ± s.d. of three experiments performed in triplicate. Asterisks denote statistically significant differences ($P<0.05$) between $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD cells. (**C**) CD cells were plated on transwells coated with the matrices indicated (all at 10 μg/ml) and migration was evaluated after 4 hours. Values are the mean ± s.d. of three experiments performed in triplicate. Asterisks denote statistically significant differences ($P<0.05$) between $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD cells. (**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 Materials and methods. Values are the mean ± s.d. of three experiments performed in triplicate. Asterisks indicate statistically significant differences ($P<0.05$) between $\beta1^{flox/flox}$ and $\beta1^{-/-}$ CD cells. (**E**) CD cell populations were grown on transwells and stained with antibodies directed against ZO-1.

cells derived from the UB (Chen et al., 2004). Successful deletion of β1 in CD ($\beta1^{-/-}$) cells was verified by flow cytometry utilizing an antibody directed at the extracellular domain of the mouse β1 integrin (Fig. 4A). Similarly, all the α subunits that heterodimerize exclusively with β1 were not expressed on the $\beta1^{-/-}$ cells (not shown). No difference in β4 (data not shown) or αv expression (Fig. 4A) were observed between the two cell types. When cell adhesion to various matrices was investigated, $\beta1^{-/-}$ CD cells adhered poorly to collagen I and laminin 1 (Fig. 4B). By contrast, adhesion to fibronectin (ligand for both integrin α5β1 and αv-containing integrins) was only partially impaired, whereas binding to vitronectin (ligand for αvβ3 and αvβ5 integrins) was unaffected. Similar results were observed for cell migration (Fig. 4C). $\beta1^{-/-}$ CD cells also proliferated significantly less than $\beta1^{flox/flox}$ cells when plated on β1 integrin-dependent substrates (Fig. 4D), an effect that was not observed on fibronectin, vitronectin or plastic substrata. To determine the role of β1 integrin in the regulation of CD cell polarity, cells were grown on transwells till confluent and stained with ZO-1 (Fig. 4E) and E-cadherin (data not shown) 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 CD 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 crucial 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 dependent on these growth factors, we investigated the role of β1 integrin in GDNF- as well FGF2- and 10-mediated signaling in vitro. As GDNF and FGF2 can induce cell spreading (Klint et al., 1999; Murakami et al., 1999),

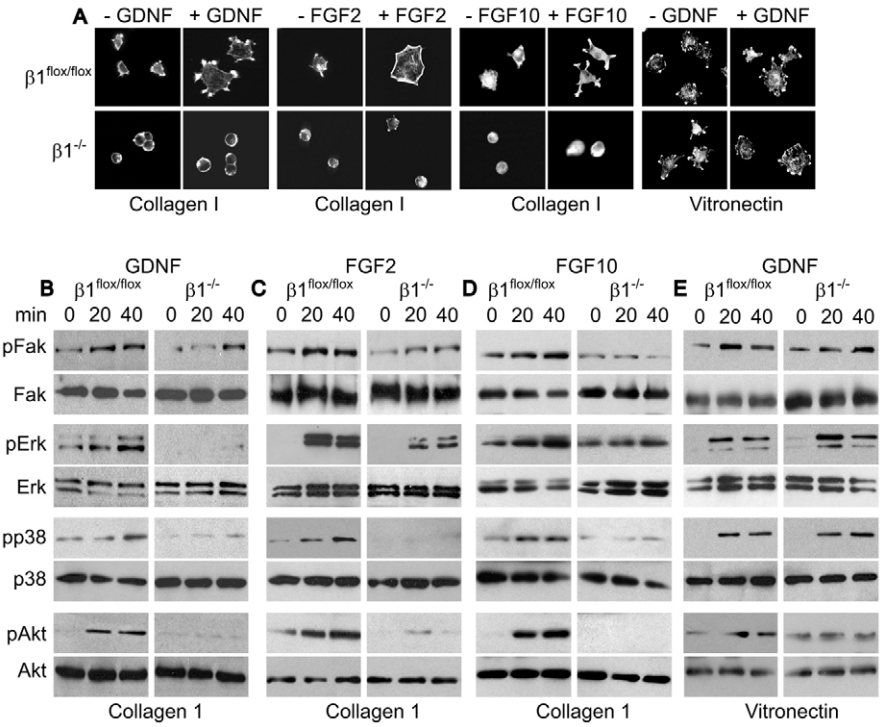

Fig. 5. β1$^{-/-}$ CD cells are unable to spread or signal in response to growth factors. (**A**) β1$^{flox/flox}$ and β1$^{-/-}$ CD cells were allowed to adhere to collagen I or vitronectin (both at 10 μg/ml) for 45 minutes. Cells were then incubated with or without FGF2, FGF10 or GDNF (all at 10 ng/ml) 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.

we determined the requirement of β1 integrin for growth-factor-mediated CD cell spreading on collagen I or laminin I (data not shown). Unlike β1$^{flox/flox}$, β1$^{-/-}$ CD cells adhered poorly when plated on collagen I, and the few that did adhere were unable to spread (Fig. 5A). Addition of GDNF, FGF2, FGF10 (Fig. 5A) or HGF (data not shown) induced much less spreading of β1$^{-/-}$ cells compared with β1$^{flox/flox}$ CD cells. By contrast, both β1$^{-/-}$ and β1$^{flox/flox}$ 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 (Fig. 5A).

To determine the requirement of β1 integrin for GDNF, FGF2, FGF10 and HGF signaling on collagen I, β1$^{flox/flox}$ and β1$^{-/-}$ CD cells where placed on collagen I for 2 hours, after which they were stimulated with the growth factor. Compared with β1$^{flox/flox}$ CD cells, FAK, ERK, p38MAPK and Akt phosphorylation was markedly decreased in the β1$^{-/-}$ CD cells in response to GDNF, FGF2, FGF10 (Fig. 5B-D) and HGF (data not shown). As expected, no difference in GDNF-dependent signaling was observed between the β1$^{flox/flox}$ and β1$^{-/-}$ CD cells plated on vitronectin (Fig. 5E). 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.

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

As β1$^{-/-}$ CD 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. Western blot analysis of isolated medullas of newborn mice showed drastically decreased activation of FAK, ERK, p38 MAPK and Akt in HoxB7Cre;β1$^{flox/flox}$ compared with β1$^{flox/flox}$ mice (Fig. 6A). As expected, the decreased signaling activation correlated with decreased cellular proliferation in the UB, which was particularly evident at E15.5, when the proliferation in β1$^{flox/flox}$ mice was 10-fold greater than that of HoxB7Cre;β1$^{flox/flox}$ mice (Fig. 6B). Similarly, there was decreased proliferation in the collecting ducts of newborn HoxB7Cre;β1$^{flox/flox}$ relative to the β1$^{flox/flox}$ mice (Fig. 6B). As FGFs play an important role in mediating proliferation and branching of the UB at E15 (Bates, 2007; Bush et al., 2006), FGF2, FGFR1 and FGFR2 expression were determined in renal tubules at this time point. No differences in FGF2 and FGFR expression were observed between HoxB7Cre;β1$^{flox/flox}$ and β1$^{flox/flox}$ kidneys (Fig. 6C), suggesting that decreased proliferation and signaling were not due to decreased expression of the growth factors or their receptors.

To investigate whether β1 expression might alter FGF receptor localization and/or activation, β1$^{-/-}$ and β1$^{flox/flox}$ CD cells were plated on collagen I and subsequently stimulated with FGF2. Equal amounts of FGFR1 surface expression and phosphorylation were observed by immunofluorescence in both β1$^{-/-}$ and β1$^{flox/flox}$ CD cells, despite the inability of the β1$^{-/-}$ cells to spread on this matrix (Fig. 7A). Immunoblot analysis confirmed comparable FGFR1 phosphorylation and expression in β1$^{-/-}$ and β1$^{flox/flox}$ CD cells plated on collagen (Fig. 7A). As expected, localization and phosphorylation of FGFR1 was the same in the β1$^{-/-}$ and β1$^{flox/flox}$ CD cells plated on vitronectin (Fig. 7B). When the proliferative response of β1$^{-/-}$ and β1$^{flox/flox}$ CD cells to FGF2 was determined, β1$^{-/-}$ CD cells plated on collagen I did not proliferate, whereas both cell populations responded to this growth factor when grown on vitronectin (Fig. 7C). Thus, although the FGFR1 is expressed on the cell surface of β1$^{-/-}$ CD 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, immunoblotting was performed on renal papillae isolated from newborn β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ kidneys. Similar to the in vitro results, FGFR1 was phosphorylated equally in β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ mice (Fig. 7D), verifying that UBs lacking β1 integrins have severe abnormalities in branching morphogenesis, without alterations in the levels of growth factor receptor phosphorylation/activation.

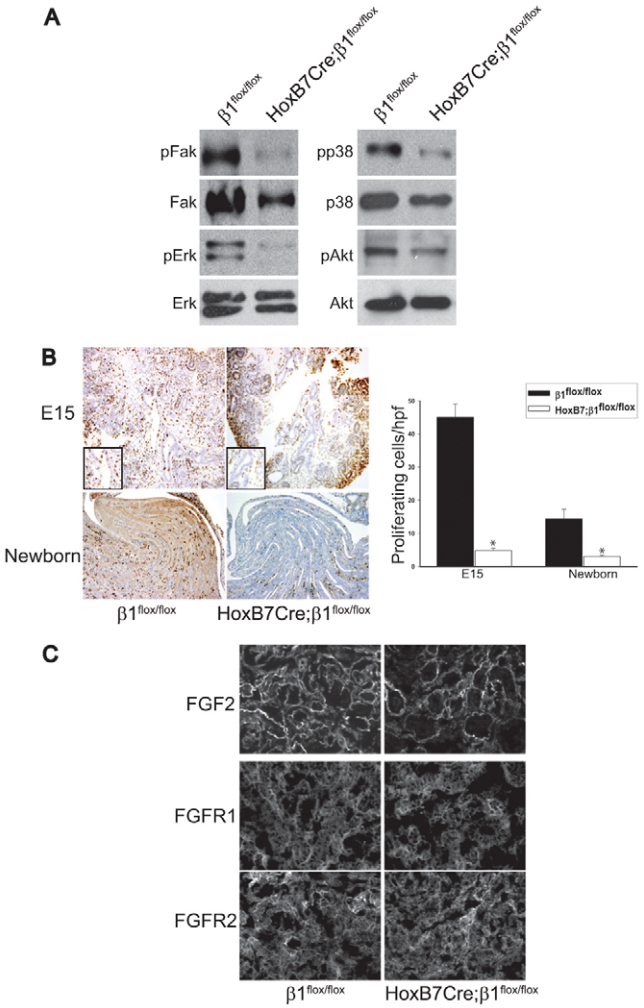

Fig. 6. HoxB7Cre;β1$^{flox/flox}$ mice have severe signaling and proliferative defects. (**A**) Medullas of P1 β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ kidneys were lysed and 20 μg of total protein was analyzed by western blot for levels of activated and total FAK, ERK, p38 MAPK and Akt. (**B**) 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 ± s.d. of five high power fields of three different mice. Asterisks indicate 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.

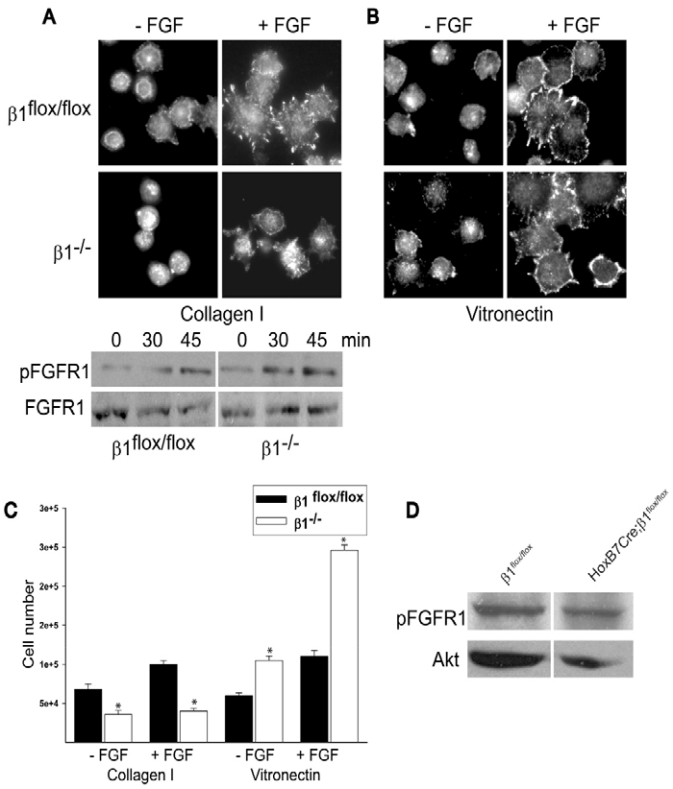

Fig. 7. FGFR1 is localized to the cell membrane and is equally phosphorylated in β1$^{flox/flox}$ and β1$^{-/-}$ cells. (**A**,**B**) β1$^{flox/flox}$ and β1$^{-/-}$ CD cells were allowed to adhere for 30 minutes to collagen I (A) or vitronectin (B) (both 10 μg/ml), after which they were incubated with or without FGF2 (FGF) (10 ng/ml) for 1 hour and then stained with an anti-pFGFR1 antibody. The lower panel in A is an immunoblot showing the levels of pFGFR1 and total FGFR1 in β1$^{flox/flox}$ and β1$^{-/-}$ CD that were allowed to adhere to collagen I for 30 minutes and were then treated with FGF2 for the times indicated. (**C**) In six-well plates coated with collagen I or vitronectin (10 μg/ml) with or without FGF2 (10 ng/ml), 3×10^5 β1$^{flox/flox}$ and β1$^{-/-}$ CD cells were grown. Forty-eight hours later the cells were trypsinized and counted. Values are the mean ± s.d. of three different experiments. Asterisks indicate statistically significant differences (P<0.05) between β1$^{flox/flox}$ and β1$^{-/-}$ CD cells. (**D**) Medullas of P1 β1$^{flox/flox}$ and HoxB7Cre;β1$^{flox/flox}$ 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.

DISCUSSION

The complex process of UB development requires both regulated growth factor signaling and cell-ECM interactions. There is in vitro and in vivo evidence that the laminin receptor, integrin α3β1, is required for collecting system development (Kreidberg et al., 1996; Liu et al., 2009), whereas requirement of the α6 integrin subunit has only been demonstrated in vitro (Zent et al., 2001). The collagen receptors, integrins α1β1 and α2β1, do not play a major role in this process (Zent et al., 2001) (R. Zent and A. Pozzi, unpublished data), and the role of the RGD-binding receptors α5β1 and αv integrins is unknown. 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 et al., 2009). The abnormality in branching morphogenesis was significantly worse in mice in which β1 integrin rather than α3 was specifically deleted in the UB (Liu 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 (Kreidberg et al., 1996; Liu et al., 2009). The only other

branching organ in which α3, α6 and β1 integrin subunits have been deleted is in the mammary gland (Klinowska et al., 2001; Li et al., 2005; Naylor et al., 2005; Taddei 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 probably 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 et al., 2009). The lack of phenotype in the Aqp2Cre; $\beta1^{flox/flox}$ 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, as 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. A similar phenomenon was observed when β1 integrin was deleted in podocytes, where glomerular morphogenesis occurred relatively normally until birth, but the glomeruli underwent rapid destruction when intraglomerular pressure was increased with the advent of glomerular filtration (Kanasaki et al., 2008; Pozzi et al., 2008).

Utilizing cultured CD 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 (Durbec et al., 1996; Pachnis et al., 1993; Zhao et al., 2004). Similar alterations in signaling pathways were seen in the HoxB7Cre;$\beta1^{flox/flox}$ mice, despite normal expression levels of FGF2 and the FGF receptors 1 and 2. As the phenotype of the HoxB7Cre;$\beta1^{flox/flox}$ 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 (Durbec et al., 1996; Pachnis et al., 1993; Zhao et al., 2004), 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 are very similar and undergo crosstalk at many levels. Growth factors can directly bind to integrins (Mori et al., 2008), integrins can directly interact with receptor tyrosine kinases (Cascone et al., 2005; Wang et al., 2008) and integrin and receptor tyrosine kinase signaling pathways intersect at various levels downstream of the respective receptors (Parise et al., 2000). 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 demonstrate 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.

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