Integrin-linked kinase regulates p38 MAPK-dependent cell cycle arrest in ureteric bud development

Joanna Smeeton, Xi Zhang, Nada Bulus, Glenda Mernaugh, Anika Lange, Courtney M. Karner, Thomas J. Carroll, Reinhard Fässler, Ambra Pozzi, Norman D. Rosenblum and Roy Zent

SUMMARY
The integrin-linked kinase (ILK), pinch and parvin ternary complex connects the cytoplasmic tails of β1 integrins to the actin cytoskeleton. We recently showed that constitutive expression of ILK and alpha parvin in both the ureteric bud and the metanephric mesenchyme of the kidney is required for kidney development. In this study, we define the selective role of ILK in the ureteric bud of the mouse kidney in renal development by deleting it in the ureteric cell lineage before the onset of branching morphogenesis (E10.5). Although deleting ILK resulted in only a moderate decrease in branching, the mice died at 8 weeks of age from obstruction due to the unprecedented finding of intraluminal collecting duct cellular proliferation. ILK deletion in the ureteric bud resulted in the inability of collecting duct cells to undergo contact inhibition and to activate p38 mitogen-activated protein kinase (MAPK) in vivo and in vitro. p38 MAPK activation was not dependent on the kinase activity of ILK. Thus, we conclude that ILK plays a crucial role in activating p38 MAPK, which regulates cell cycle arrest of epithelial cells in renal tubulogenesis.

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

INTRODUCTION
Mурine kidney development begins at embryonic day (E) 10.5 when the ureteric bud (UB) grows into the metanephric mesenchyme (MM) in response to inductive cues mediated by growth factors (Reidy and Rosenblum, 2009). The UB undergoes many iterations of branching morphogenesis to create the intrarenal collecting system comprising collecting ducts (CD), calyces and the pelvis, as well as forming the ureters and the bladder trigone. The MM gives rise to the nephrons.

Integrin-linked kinase (ILK) is a cytoplasmic protein that is a component of the ILK-pinch-parvin macromolecular protein complex situated at focal adhesions. It comprises a pseudokinase domain at the C-terminus, a pleckstrin homology-like domain and ankyrin repeats at the N-terminus (Fukuda et al., 2009; Lange et al., 2009). The C-terminus of ILK binds either directly or indirectly to the cytoplasmic tail of β1- and β3-integrins. ILK plays a key role in organizing the actin cytoskeleton by recruiting actin binding and actin regulatory proteins, such as pinch, parvin, paxillin and kindlin (Legate and Fassler, 2009; Legate et al., 2006; Legate et al., 2009), and although it lacks intrinsic kinase activity (Fukuda et al., 2009; Lange et al., 2009), it can regulate the activation of cell signaling molecules such as protein kinase B (PKB/AKT) (Hannigan et al., 2007; McDonald et al., 2008; Wang et al., 2008; White et al., 2006). There is limited knowledge about the role of ILK in renal development. Deletion of ILK in mice results in early embryonic lethality due to defects in epiblast polarity and F-actin organization (Sakai et al., 2003). However, it is clear that ILK is required for renal development as we recently demonstrated that mice with point mutations in the conserved lysine residue of the potential ATP-binding site of the pseudokinase domain of ILK, which is essential for α-parvin binding, die perinatally owing to renal agenesis (Lange et al., 2009). This in vivo phenotype and our in vitro organ culture data demonstrating failure of the UB to respond to GDNF, which is a crucial growth factor required for induction of UB branching morphogenesis, suggested an intrinsic defect in UB cells. The mechanisms by which ILK deficiency abrogates or limits UB branching remain undefined.

The selective role of ILK in the developing collecting system of the kidney has only been investigated in vitro. In these studies a dominant-negative ILK (R211A) inhibited branching morphogenesis in kidney organ cultures and overexpressing a ‘kinase-dead’ ILK mutant (E359K) and treatment with an ILK inhibitor decreased the ability of inner medullary CD cells to undergo BMP7-induced branching morphogenesis by inhibiting phosphorylation of p38 MAPK and ATF2. These results suggested that ILK functions upstream of p38 MAPK during BMP7 signaling and that ILK plays a role in the BMP7-p38MAPK-ATF2 signaling pathways, which regulates epithelial cell morphogenesis (Leung-Hagelseijn et al., 2005).

The only cell type in the kidney where ILK has been specifically deleted is in podocytes of the glomerulus and these mice develop massive proteinuria and end-stage renal failure resulting in death at approximately six to eight weeks of age (Dai et al., 2006; El-Aouini et al., 2006; Kanasaki et al., 2008). The phenotype is very similar, although less severe, to when β1-integrin is deleted in podocytes (Kanasaki et al., 2008; Pozzi et al., 2008), suggesting that β1-integrin-dependent signaling is at least in part dependent on the ILK.
To define whether ILK regulates similar pathways to β1-integrin in UB development in vivo, we selectively deleted it in the ureteric bud cell lineage starting before the onset of UB branching morphogenesis. Unlike the similar phenotypes observed when ILK or β1-integrin was deleted in podocytes, there was a significant difference between mice where these proteins were deleted in the UB. In contrast to the severe branching morphogenesis abnormality in mice lacking β1 expression in the UB, mice lacking ILK expression have a moderate branching phenotype. Despite this, these mice die at 8 weeks of age from severe obstruction caused by cell accumulation within the tubule lumens. Although multiple signaling pathways were dysregulated in the CDs of β1-null mice, only p38MAPK signaling is disrupted in the collecting system of ILK-null mice. In vitro studies show that ILK-null CD cells are unable to undergo contact inhibition owing to their inability to activate p38 MAPK, which occurs independent of the role for ILK as a kinase. These data suggest that the obstruction in the CDs of the ILK-null mice in vivo is due to the key role of ILK as a regulator of epithelial cell contact inhibition, which is mediated by activating p38 MAPK. Thus, deleting β1 and ILK in the UB results in markedly different phenotypes because ILK only transduces a distinct subset of signaling pathways activated by β1-integrin.

**MATERIALS AND METHODS**

**Generation of Hoxb7Cre;ILKlox/lox mice**

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. ILKlox/lox mice (Terpstra et al., 2003) were crossed with the Hoxb7Cre (generous gift of Dr A. McMahon, Harvard University) (Kobayashi et al., 2005) or Hoxb7creEGFP mice (generous gift from Dr C. Bates, University of Pittsburgh) (Zhao et al., 2004). Mice were a F4-F6 generation toward the C56/Black6 background. Aged-matched littermates homozygous for the floxed ILK gene, but lacking Cre (ILKlox/lox mice), were used as controls.

**Morphologic analysis**

For morphological and immunohistochemical analysis, kidneys were removed at different stages of development and (1) fixed in 4% formaldehyde and embedded in paraffin; (2) embedded in OCT compound (Thermo Fisher Scientific) and snap frozen in liquid nitrogen; or (3) fixed in 2.5% glutaraldehyde, post-fixed in OsO4, dehydrated in ethanol and embedded in resin. Paraffin tissue sections were stained with either Hematoxylin and Eosin 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). UB branching was imaged in whole kidneys by fluorescence microscopy for the expression of GFP.

For immunofluorescence of ILK and β1-integrin expression, 8 μm thick cryosections of E11.5, E12.5 and E14.5 embryos were fixed for 1 hour in 3% PFA at 4°C and frozen in OCT (Thermo Shandon). Sections were permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with 3% BSA for 1 hour and incubated overnight with the primary antibody, followed by incubation with the secondary antibody. The fluorescent images were collected by laser scanning confocal microscopy (DMIRE2; Leica) using Leica Confocal software, version 2.5, Build 1227. The primary antibodies were an rabbit anti-ILK antibody (Cell Signaling, 3862), rabbit anti-β1 antibody (Millipore, AB1952) and a rat anti-nitrogen antibody (Millipore, MAB1883).

Immunohistochemistry for GSK3β was performed on 5 μm kidney sections of E12.5 embryos that were cut from paraffin blocks using a rabbit monoclonal antibody (Cell Signaling, 27C10).

**Ureteric bud isolation and real-time reverse-transcriptase PCR**

Kidneys from E11.5 Hoxb7Cre;ILKlox/lox and ILKlox/lox mice were dissected as previously described (Cain et al., 2009). RNA was isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion) and cDNA was generated using First Strand cDNA Synthesis (Invitrogen) from total RNA. Real-time PCR reaction mix contained a CDNA sample and primers against ILK. Real-time PCR amplification was performed using the Applied Biosystems 7900 HT Fast RT-PCR system. Relative levels of mRNA expression were determined using the standard curve method. Individual expression values were normalized by comparison to β-2 microglobulin.

**In situ TUNEL and BrdU incorporation assays**

Embryonic kidney tissue was formalin-fixed and paraffin-embedded prior to sectioning (4 μm). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) was performed as described in the manufacturer’s instructions (Promega). Cell proliferation was assayed by incorporation of 5-bromo-2-deoxyuridine (BrdU, Roche Molecular Biochemicals), as previously described (Cano-Gauci et al., 1999). Briefly, a single intraperitoneal injection of BrdU (100 mg/g body weight) was given to pregnant females 2 hours prior to sacrifice. BrdU-positive cells were identified using anti-BrdU peroxidase-conjugated antibody (Boehringer Mannheim). Immunoreactivity was visualized using aminoethyl carbazole horseradish peroxidase chromogen and substrate solution (Zymed Laboratories).

**In situ mRNA hybridization**

Whole embryos were fixed in 4% PFA in PBS for 16 hours at 4°C. In situ hybridization was performed as described (Ding et al., 1998) on paraffin-embedded sections (4 μm) using DIG-labeled cDNA probes encoding Wnt4 and Wnt9b.

**Generation of ILK-null cell line**

CD cells were isolated from ILKlox/lox mice following the methodology described by Husted et al. (Husted et al., 1988) and ILK was deleted by infecting the cells with an adenovirus in vitro. Deletion of ILK was verified by immunoblotting. Generation of the CD cells expressing the ILK mutants was described previously elsewhere (Lange et al., 2009).

**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. 1 × 10⁵ cells were placed in each well 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 × 10⁵ 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 each treatment. Four independent experiments were performed in triplicate.

**Cell proliferation**

5 × 10⁵ cells were plated per well in 96-well plates on collagen I 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 [³H] thymidine (PerkinElmer Life Sciences). Twenty-four hours later, the cells were solubilized and radioactivity was measured using a scintillation counter.

**Cell spreading**

Cells were plated onto slides coated with collagen I (10 μg/ml) for 1 hour, after which they were fixed, permeabilized and exposed to Rhodamine Phallolidin (1:5000) and visualized under a microscope.

**Cell polarity**

Cells were grown on transwells comprising polyvinylpyrrolidone-free polycarbonate filters with 0.4 μm pores. After reaching confluency, cells were fixed in 4% formaldehyde and incubated with anti-ZO1 (1:200; BD Transduction Laboratories) antibodies followed by the appropriate FITC-conjugated secondary antibody. Chamber slides were mounted and viewed using a confocal microscope.
Tubulogenesis assays

CD cells were placed in 3D gels comprising rat tail collagen I and Matrigel (Becton Dickinson) and Dulbecco’s Minimal Essential Media (DMEM) containing 20 mM HEPES (pH 7.2) as previously described (Pozzi et al., 2006). Briefly, $1.5 \times 10^5$ cells were suspended in 100 μl of gel and plated onto 96 wells. After 1 hour at 37°C, an equal volume of DMEM supplemented with 10% FBS was added to the gels. The cells were allowed to grow for 7 days, at which time the gels were stained with Rhodamine Phalloidin and photographed on a confocal microscope.

Cell viability assays

One thousand CD cells were seeded in 96-well plates and grown in medium with 15% FBS. At the timepoints indicated, 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] stock solution was added to each well in an amount equal to 10% of the culture volume in 8 replicates and incubated for 4 hours at 37°C. After the incubation period, the resulting formazan crystals were dissolved in MTT solvent (0.1 M HCl in anhydrous isopropanol) in an amount equal to the original cell culture volume. Absorbance was measured at a wavelength of 570 nm and background absorbance at 690 nm was subtracted.

Immunoblotting

The replating assays were performed as previously described (Abair et al., 2008). In brief, serum-starved CD cells were plated in serum-free medium on collagen I (20 μg/ml) for 0, 30 and 60 minutes after they were lysed and subjected to immunoblotting.

The effects of growth factors on CD cells were examined as previously described (Zhang et al., 2009). In brief, cells were trypsinized into 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 timepoints 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 a 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were blocked in 5% milk with 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), cyclin D1 (1:1000; 2922), p27kip1 (1:1000; 2552) and phospho-CDK2 (1:1000; 2561S) were purchased from Cell Signaling. FAK (sc-558) and pFAK (sc-16662-R) antibodies were purchased from Santa Cruz Biotechnology.

Statistics

The 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

Deletion of ILK in the UB results in obstruction of the collecting system

To define the role of ILK in the developing ureteric bud (UB), we crossed HoxB7Cre mice (Zhang et al., 2009), which express Cre in the Wolffian duct and UB from E10.5, with ILKlox mice (Terpstra et al., 2003). The mice were born in the predicted Mendelian ratio; however, all the HoxB7Cre;ILKlox mice died by eight weeks of age. ILK deletion was confirmed in mutant mice by performing immunoblotting on isolated papillae of HoxB7Cre;ILKlox mice with an antibody directed at ILK (Fig. 1A). The predominant phenotype was that of progressive obstruction within the papilla of the kidney resulting in destruction of the cortex and medulla (Fig. 1B-E). Approximately 73% (22/30) of the kidneys displayed evidence of gross obstruction, whereas 27% (8/30) were severely hypoplastic. In HoxB7Cre;ILKlox mice that were sacrificed prior to developing end-stage renal failure, there was preservation of the cortex with severe tubular abnormalities within the medulla (Fig. 1E), whereas no abnormalities were seen in the HoxB7Cre;ILKlox mice (40×). The arrows denote the regions of the kidney that are shown at higher power in panels F-I. (F-I) The collecting ducts (CDs) of the HoxB7Cre;ILKlox mouse is disorganized and hypercellular (G,I) when compared with ILKlox controls (F,H). F,G, 100×; H,I, 400×.

Fig. 1. HoxB7Cre;ILKlox mice develop end-stage renal failure due to obstruction in the CDs. (A) Deletion of ILK in the HoxB7Cre;ILKlox mouse was confirmed by immunoblotting the kidney medulla of newborn mice with an anti-mouse ILK antibody (right panel). (B,C) Gross appearance of kidneys of 6-week-old HoxB7Cre;ILKlox and ILKlox mice. (D,E) Microscopy of Hematoxylin and Eosin (H&E) stained kidney slides showing destruction of the medulla and corticomedullary junction in HoxB7Cre;ILKlox kidneys but not in the ILKlox mouse (40×). The arrows denote the regions of the kidney that are shown at higher power in panels F-I. (F-I) The collecting ducts (CDs) of the HoxB7Cre;ILKlox mouse is disorganized and hypercellular (G,I) when compared with ILKlox controls (F,H). F,G, 100×; H,I, 400×.
Deletion of ILK in the UB results in a moderate branching morphogenesis defect and impaired metanephric mesenchyme induction

We previously showed that the HoxB7Cre;β1flox mice have a severe branching morphogenesis phenotype that is present as early as E11.5 (Zhang et al., 2009). We therefore determined the expression pattern of ILK and β1-integrin during early kidney development by performing immunostaining on E11.5 kidneys. Both ILK and β1-integrin are expressed in the developing UB and the metanephric mesenchyme (MM) at E11.5 (Fig. 2A,B) and this expression is increased at day E12.5 and E14.5, respectively (Fig. 2C,D). Thus, ILK is expressed in the UB at E11.5 and its expression levels increase with time.

To define the temporal relationship of branching abnormalities in the HoxB7Cre;ILKflox mice, we studied embryonic kidneys starting from E11.5. In contrast to the HoxB7Cre;β1flox mice, at this developmental stage, both HoxB7Cre;ILKflox and ILKflox mice had developed T-shaped UBs (Fig. 2E,F). It was only at E12.5 that a significant branching phenotype was observed in HoxB7Cre;ILKflox mice, with an average of 6 branch points compared with the 13 branch points present in the ILKflox mice (Fig. 2G-I). A similar branching defect was seen when HoxB7Cre;ILKflox and ILKflox E12.5 kidneys were cultured in vitro (data not shown). In addition to the branching phenotype, at E13.5, there was less comma- and S-shaped body formation in the HoxB7Cre;ILKflox kidneys (Fig. 2J,K) and these defects were even more conspicuous at E14.5 (Fig. 2L,M).

To further define the UB branching defect as well as the mechanism for defective induction of the MM in the HoxB7Cre;ILKflox kidneys, we performed proliferation and apoptosis assays on the embryonic kidneys of HoxB7Cre;ILKflox and ILKflox mice. There was no difference in proliferation or apoptosis in E12.5 kidneys in the different genotypes (data not shown); however, there was a significant decrease in proliferation at E13.5 HoxB7Cre;ILKflox embryonic kidneys, which was predominantly found in the MM (Fig. 2N). There was also a significant increase in apoptosis at the E14.5 HoxB7Cre;ILKflox kidneys, which was also mainly found in the MM (Fig. 2O). Thus, deleting ILK early in UB development results in a moderate branching morphogenesis phenotype.
characterized by both decreased proliferation and increased apoptosis mainly in the MM, which commence at days E13.5 and 1E4.5, respectively.

Owing to the unexpected finding that the abnormalities in kidney development only commenced at E12.5, we examined whether ILK was being efficiently deleted in the UB at the initiation of UB development. To do this, E11.5 UBs were dissected out of HoxB7Cre;ILKflox kidneys and quantitative real-time PCR was performed. Almost no ILK mRNA transcripts were present in the HoxB7Cre;ILKflox UBs (Fig. 3A), suggesting that the Cre was effectively deleting the ILK gene at this timepoint. Owing to the low turnover rate of ILK (Lorenz et al., 2007), we performed immunofluorescence to define the expression levels of ILK in E12.5 kidneys from HoxB7Cre;ILKflox mice. In contrast to the almost total absence of ILK mRNA in the UB at E11.5, there was still expression of ILK in the UB in the HoxB7Cre;ILKflox mice, although it was decreased compared with ILKflox mice (Fig. 3B,C). Thus, the moderate branching phenotype of the UB at E12.5 in the HoxB7Cre;ILKflox mice correlates with the moderately decreased expression of ILK in the UB at this timepoint.

ILK regulates multiple signal pathways including the WNT signaling pathways (Oloumi et al., 2010; Oloumi et al., 2006), which are required for renal development (Pullkainen et al., 2008). As both WNT4 (Chi et al., 2004; Itaranta et al., 2006; Naillat et al., 2010; Shan et al., 2009) and WNT9b (Carroll et al., 2005; Karner et al., 2009) play vital roles in renal development, we assessed whether deleting ILK in the UB affects their expression by performing in situ hybridization on E12.5 kidneys from HoxB7Cre;ILKflox and ILKflox mice. No differences in WNT4 or WNT9b expression were seen in the HoxB7Cre;ILKflox (Fig. 3D,F) and ILKflox (Fig. 3E,G) mice. A key component in mediating WNT-dependent signaling is GSK3β (Pullkainen et al., 2008), which has been shown to be regulated by ILK (Hannigan et al., 2007). We therefore determined whether there were any alterations in GSK3β expression in the E12.5 kidneys of HoxB7Cre;ILKflox mice. When immunohistochemistry using antibodies directed against GSK3β was performed, equal expression of this protein was observed in E12.5 kidneys of HoxB7Cre;ILKflox (Fig. 3H) and ILKflox (Fig. 3I) mice. Thus, the defects in kidney development caused by deleting ILK in the UB at the initiation of its development are not due to alterations in WNT4, WNT9b or GSK3β expression in the kidney.

**HoxB7Cre;ILKflox mice develop obstruction of the CDs due to intraluminal cell growth**

To further define the obstruction phenotype in the mice, we sacrificed mice every day during embryonic development and at birth (Fig. 4A-I). We found a variable phenotype in newborn HoxB7Cre;ILKflox mice from a moderate branching phenotype in approximately 48% of mice (10/21; Fig. 4E) to severe obstruction with a destroyed medulla and markedly dilated CDs in the medullary rays in 52% (11/21; Fig. 4D,F). Evidence of excessive cellularity and morphological abnormalities of the CDs was evident even when the renal phenotypes were relatively mild (Fig. 4G,H). This phenotype became evident at approximately E17 (Fig. 4I).

To determine whether the morphological abnormalities were due to alterations in epithelial cell polarity, we performed staining for epithelial cell markers on newborn mice. No differences in ZO1, β-catenin (Fig. 4J,K), Par3 or E-cadherin (Fig. 4L,M) localization was present in the HoxB7Cre;ILKflox and ILKflox mice. There were also no differences in membrane localization of E-cadherin between the two genotypes, as determined by immunoblotting of membrane and nuclear fractions of isolated medullas from the two genotypes (data not shown), further suggesting no abnormalities in cellular polarity in the HoxB7Cre;ILKflox mice. To obtain high-definition visualization of the CDs, we performed electron microscopy on the CDs. As suggested by the polarity marker staining, there were no obvious morphological differences in the cells in the HoxB7Cre;ILKflox and ILKflox mice; however, it was clear that the tubules were dilated and that the epithelial cells grew on top of each other within the lumen of the CDs (Fig. 4N,O). When cell proliferation in the medulla was determined in newborn mice by Ki67 staining, there was more than double the number of proliferating cells in the HoxB7Cre;ILKflox (B) when compared with ILKflox (C) mice; however, there was still expression of the protein within the UBs. Arrows indicate the ureteric bud (UB). (D-I) In situ hybridization (D-G) or immunostaining (H,I) revealed no differences in Wnt4 (D,E), Wnt9b (F,G) or Gsk3β (H,I) expression in E12.5 kidneys isolated from HoxB7Cre;ILKflox (D,F,H) and ILKflox (E,G,I) mice.

**ILK-deficient renal CD cells have adhesion, migration and proliferation defects and are unable to undergo tubulogenesis in vitro**

We undertook two approaches to define the role of ILK in renal tubule cell tubulogenesis. ILK was depleted in a UB cell line utilizing siRNA, and CD cells were isolated from 6-week-old mice.
and infected with adeno Cre recombinase to delete ILK in vitro (Fig. 5A). As the results of siRNA-depleted UB and ILK−/− CD cells are virtually identical, we only show findings obtained for the ILK−/− CD cells.

We initially determined whether there were differences in the ability of the ILKflox/flox and ILK−/− CD to adhere, migrate or proliferate on β1-integrin-dependent matrices. Deleting ILK resulted in a decrease in all three of these cell functions on collagen I (Fig. 5B-D). Similar results were observed for laminin 111 (data not shown). The cells also demonstrated a marked spreading defect on collagen I (Fig. 5E,F) and laminin 111 (data not shown). No differences in integrin expression were detected (data not shown). To determine the role for ILK in the regulation of CD cell polarity, cells were grown on transwells until confluent and stained with ZO1 (Fig. 5G,H) and E-cadherin (data not shown) antibodies. Confocal microscopy revealed no difference between the two cell lines in ZO1 or E-cadherin localization on Z-sectioning. We also determined the effects of deleting ILK in vitro CD cell tubulogenesis in either 3D collagen I (data not shown) or collagen and Matrigel gels (Fig. 5I,J). In contrast to the ILKflox CD cells, which formed well-defined branching tubules with lumens, the ILK−/− cells were only able to form small cyst-like structures. Thus, deleting ILK from CD cells results in a decrease in cell adhesion, spreading, migration and proliferation, and inhibits in vitro tubulogenesis without altering the ability of the cells to polarize.

ILK is required for specific integrin- and growth-factor-dependent signaling required for UB branching morphogenesis

ILK plays a central role in regulating cell signal transduction induced by integrin ligation and growth factors (Legate and Fassler, 2009; Legate et al., 2006; Legate et al., 2009). Owing to the moderate adhesion, migration and spreading defects observed in the ILK−/− CD cells, we investigated which signaling pathways were altered when these cells were plated onto collagen I (Fig. 6A) or laminin 111 (data not shown). Consistent with the decreased ILK−/− CD cell adhesion, there was a decrease in FAK phosphorylation 20 and 40 minutes after cell plating (Fig. 6A); however, there was no alteration in AKT or ERK phosphorylation. Surprisingly, the most dramatic change in signaling was the inability of ILK−/− CD cells to phosphorylate p38 MAPK when plated on collagen I.

We next defined whether this signaling abnormality was specific to integrin ligation or whether it was also present when cells were activated by growth factors known to be important for UB
branching morphogenesis. GDNF is crucial for the initial branching phase of the UB, whereas the FGFs are required for later phases of branching. The requirement of ILK for GDNF and FGF10 signaling was assessed on the β1-integrin-dependent matrix collagen I, as well as plastic and the β1-integrin-independent matrix, vitronectin. ILKflox/flox and ILK−/− CD cells were placed on these matrices for 2 hours, after which they were stimulated with GDNF, FGF10 or FGF2. No differences in signaling were observed between ILKflox/flox and ILK−/− CD cells when they were plated on collagen I (Fig. 6B), vitronectin (data not shown) or plastic (data not shown) and exposed to GDNF. When placed on either vitronectin (Fig. 6B) or plastic (data not shown) and exposed to FGF10, both ILKflox/flox and ILK−/− CD cells were able to phosphorylate FAK, AKT and ERK equally. However, the ILK−/− CD cells did not phosphorylate p38 MAPK, whereas ILKflox/flox cells showed robust p38 MAPK phosphorylation. Similar results were observed with FGF2 (data not shown). When placed on collagen I, the ILK−/− CD cells could not phosphorylate p38 MAPK and there was also decreased phosphorylation of AKT and ERK when compared with ILKflox/flox CD cells. These results suggest that ILK is not required for normal GDNF-dependent signaling of CD cells; however, it differentially regulates certain FGF-dependent signaling pathways on different matrices, with the biggest effect seen on the p38 MAPK pathway.

As p38 MAPK activation was markedly impaired in CD cells following integrin ligation and exposure to FGFs, we determined whether this pathway was altered in the developing collecting system in vivo. When isolated collecting systems from newborn HoxB7Cre;ILKflox and ILKflox mice were immunoblotted for FAK, ERK, p38 MAPK and AKT, only p38 MAPK phosphorylation was markedly decreased in the HoxB7Cre;ILKflox mice (Fig. 6C).

Taken together, these results suggest that deleting ILK in cells derived from the collecting system of the kidney either in vivo or in vitro has profound effects on p38 MAPK activation.

Inability of ILK−/− CD cells to activate p38 MAPK results in the loss of CD cell contact inhibition

The intraluminal cellular filling of the CDs and the inability to activate p38 MAPK were the most striking phenotypes observed in the HoxB7Cre;ILKflox mice. Because ILK−/− cells proliferate slower than ILKflox CD cells in vitro and there is a marked proliferative defect in the HoxB7Cre;ILKflox kidneys during the rapid phase of UB development, we hypothesized that ILK regulation of p38 MAPK was required to induce cell cycle arrest in the developing UB. A study demonstrating that p38 MAPK activation is required for cells to undergo contact inhibition in vitro (Faust et al., 2005) further supported this hypothesis. When non confluent, ILK−/− CD cells were placed in 10% FBS and they grew slower than ILKflox/flox (data not shown); however, they did not stop proliferating at confluence and grew on top of each other (Fig. 7A), although they were not transformed as they failed to grow in soft agar (data not shown). To formally test the ability of ILK−/− CD cells to exit the cell cycle, we performed MTT assays on ILKflox/flox and ILK−/− CD cells grown in 10%
FBS. As shown in Fig. 7B, 6 days after reaching confluence, almost a twofold-higher density saturation was achieved by the ILK–/– compared with the ILKflox/flox CD cells. An immunoblot of total pFAK is shown to verify equal protein loading. The CD cells were allowed to adhere to collagen I or vitronectin for 1 hour, after which they were cultured 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 pFAK, pAKT, pERK and pP38 MAPK. An immunoblot of total pFAK is shown to verify equal protein loading. A representative experiment is shown. (C) Medullas of newborn ILKflox/flox and HoxB7Cre;ILKflox/flox kidneys were lysed and 20 μg of total protein was analyzed by western blot for levels of phosphorylated and total FAK, ERK, p38 MAPK and AKT.

Fig. 6. ILK–/– CD cells and HoxB7Cre;ILKflox mouse renal medulla is unable to activate P38 MAPK. (A) ILKflox/flox and ILK–/– CD cells were either left in suspension or plated onto collagen I, after which they were lysed at 20 and 40 minutes, respectively, and immunoblotted for pFAK, pAKT, pERK and pP38 MAPK. An immunoblot of total FAK is shown to verify equal protein loading. (B) ILKflox/flox and ILK–/– CD cells were allowed to adhere to collagen I or vitronectin for 1 hour, 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 pFAK, pAKT, pERK and pP38 MAPK. An immunoblot of total pFAK is shown to verify equal protein loading. A representative experiment is shown. (C) Medullas of newborn ILKflox/flox and HoxB7Cre;ILKflox/flox kidneys were lysed and 20 μg of total protein was analyzed by western blot for levels of phosphorylated and total FAK, ERK, p38 MAPK and AKT.

Fig. 7. Confluent ILK–/– CD cells are unable to undergo contact inhibition or activate P38 MAPK. (A) ILK–/– CD cells grow on top of each other under normal culture conditions in 10% FBS. (B) ILK–/– CD cells proliferate when cultured under confluent conditions as assessed by the MTT assay. Values are the mean ± s.d. of three experiments performed in triplicates. (C) Under confluent conditions, ILK–/– CD cells are unable to activate P38 MAPK. They also have increased expression of cyclin D1 and decreased expression of p27, as well as increased pCDK2. An immunoblot of total p38 MAPK is shown to verify equal protein loading. (D) ILKflox/flox CD cells lose the ability to undergo contact inhibition, as assessed by the MTT assay when treated with the p38 MAPK inhibitor SB203580. (E) ILK–/– CD cells stably expressing S343A, K220A and K220M mutants proliferate like ILKflox/flox CD cells when cultured under confluent conditions, as assessed by the MTT assay. (F) S343A-, K220A- and K220M-expressing ILK–/– CD cells activate p38 MAPK to similar levels as ILKflox/flox CD cells.

We next defined whether the putative ILK kinase activity is required for ILK to activate p38 MAPK by transfecting the ILK–/– CD cells with wild-type ILK or point mutants including a serine (S343A) kinase-dead mutant of the potential autophosphorylation site (Persad et al., 2001), as well as a lysine 220 mutant to alanine (K220A) or methionine (K220M) in the ATP-binding site of ILK reported to lead to a kinase-dead ILK associated with diminished binding to β-parvin. We recently demonstrated that none of these mutants altered the kinase activity of ILK with respect to phosphorylating MBP (Lange et al., 2009). When MTT assays were performed on the mutant cells at 6 days of confluency, they all underwent contact inhibition (Fig. 7E) and phosphorylated p38 MAPK-like ILK–/– CD cells constituted with full-length ILK (Fig. 7F). We also found that ILK does not coimmunoprecipitate with p38 MAPK (data not shown). Together, these results suggest that ILK and p38 MAPK do not form a complex and, in line with previous findings, we show that ILK-dependent p38 MAPK activation is not due to ILK kinase activity.
DISCUSSION

We recently demonstrated that ILK is required for kidney development. In these studies, mice expressing constitutive K220A or K220M point-mutations in the α-parvin binding site of ILK developed severe renal agenesis and/or dysgenesis due to intrinsic abnormalities of the UB and MM, as well as defective inductive tissue interactions between them (Lange et al., 2009). In the current study, we explored the specific role of ILK expression in the UB in renal collecting system formation. Unlike studies where ILK and β1-integrins were deleted in podocytes, the HoxB7Cre;ILKflox mice did not phenocopy the HoxB7Cre;β1flox mice (Dai et al., 2006; El-Aouni et al., 2006; Kanasaki et al., 2008; Zhang et al., 2009). HoxB7Cre;β1flox kidneys demonstrate a lethal branching phenotype starting at UB induction (Zhang et al., 2009); however, the branching defect in the HoxB7Cre;ILKflox mice is only moderate and starts at E12.5. Both genotypes die at a similar age but the predominant abnormality in the HoxB7Cre;ILKflox mice is obstruction due to cells that have grown into the tubular lumen of the CDs. We further show that, in contrast to β1–/– CD cells, which are unable to activate any signals induced by HGF, FGF and GDNF (Zhang et al., 2009), ILK–/– CD cells transduce signals from select growth factors and the signaling defects are primarily restricted to the P38 MAPK pathway. Thus, ILK appears to mediate the transduction of a limited number of signals activated by β1-integrin that underly the major phenotypical differences in UB development.

The moderate branching defect that was delayed until E12.5 in the HoxB7Cre;ILKflox mice was unexpected. In light of our recent data that renal agenesis occurs in the ILK (K220A) and (K220M) mice (Lange et al., 2009), it is highly unlikely that ILK expression in the UB is not required for the initial induction and branching of the UB (Costantini, 2006; Costantini and Shaya, 2006). The explanation for the moderate early defects is most likely the slow turnover of ILK following ILK gene deletion, which was also observed in other organs such as skin (Lorenz et al., 2007). We could clearly rule out an inefficient deletion by the HoxB7Cre mouse because there was almost no ILK message in UBs isolated from E11.5 kidneys.

There are big differences in the renal phenotypes between the ILK (K220A) and (K220M) mice and the HoxB7Cre;ILKflox mice. Renal agenesis is the principal defect in the K220A or M mutant ILK mice, which is due to intrinsic abnormalities of both the UB and MM and defective inductive tissue interactions between them (Lange et al., 2009). By contrast, renal agenesis did not occur in the HoxB7Cre;ILKflox mice because ILK is present in the UB at the initiation of UB development. The principal findings of excessive intraluminal cell growth in the collecting ducts in the HoxB7Cre;ILKflox mice is not seen in the ILK (K220A) and (K220M) mice (Lange et al., 2009). This suggests that this phenotype is not due to the interaction of ILK with α-parvin, as this is disrupted by the K220A and K220M mutations (Lange et al., 2009). We do not currently have a clear molecular explanation for the phenotype of the HoxB7Cre;ILKflox mice; however, ILK binds more than ten proteins (McDonald et al., 2008) and many of these interactions have physiological significance (Grashoff et al., 2004; Legate et al., 2006; Wickstrom et al., 2010). We are in the process of defining the molecular mechanism for this phenotype by making specific point ILK mutations expressed exclusively in the UB in vivo as well as by generating mice where ILK-binding partners are deleted in the UB and comparing their phenotypes with the HoxB7Cre;ILKflox mice.

The branching and growth abnormalities observed in the kidneys after E12.5 is consistent with the defects in proliferation, adhesion and migration, which are crucial processes required for UB branching morphogenesis and tubule formation (Chen et al., 2004). The defects in the HoxB7Cre;ILKflox mice and ILK–/– CD cells are less marked than those found in the HoxB7Cre;β1flox mice and β1–/– CD cells respectively, which is likely to be explained by our observations that ILK only regulates restricted signaling pathways, whereas all growth factor signaling was abrogated in β1–/– CD cells and HoxB7Cre;β1flox mice (Zhang et al., 2009). p38 MAPK pathway is the major signaling pathway regulated by ILK in UB development and it only has a moderate effect on regulating UB branching in vitro organ cultures (Pozzi et al., 2006).

Another interesting observation in the HoxB7Cre;ILKflox kidneys was the relatively severe defect in metanephric mesenchyme induction compared with the UB branching abnormality. The reasons for this are unclear; however, we demonstrated that it is not due to alterations in WNT4 or WNT9b expression in the embryonic kidney. These vital paracrine pathways required for renal development might have been ILK-dependent as ILK can regulate WNT signaling (O loumi et al., 2010; Oloumi et al., 2006). It is probable that ILK affects expression of any one of the many crucial UB-derived growth factors that induce the MM and we are in the process of defining them; however, these studies are beyond the scope of this manuscript.

The continued intraluminal cellular proliferation in the CDs, which leads to intrarenal obstruction, is unprecedented. Its correlation with decreased p38 MAPK activation in vivo suggests that a defect in this signaling pathway is at least in part the mechanism for the observed phenotype. Supporting this was our in vitro evidence that integrin ligation, together with FGF treatment, was unable to activate p38 MAPK in ILK–/– CD cells and that ILK–/– CD cells were unable to undergo contact inhibition or activate p38 MAPK. Furthermore, inhibiting p38 MAPK decreased the ability of ILKflox CD cells to undergo contact inhibition. p38 MAPK activity is increased in confluent human fibroblast cultures compared with proliferating cultures and p38 MAPK–/– fibroblasts show a high saturation density, which is reversed by reconstituted expression of p38 MAPK (Faust et al., 2005). p38 MAPK also regulates both the G2/M as well as G1/S cell cycle checkpoints (Lavoie et al., 1996; Thornton and Rincon, 2009). Consistent with our ILK–/– CD cells, which have increased cyclin D1 expression, p38 MAPK regulates G1/S cell cycle checkpoints by reducing the levels of cyclin D1 at the level of transcription and by increasing cyclin D1 ubiquitination and proteosomal degradation. p38 MAPK phosphorylates and activates p53, which leads to the inactivation of CDK2 and induction of apoptosis (Huang et al., 1999; Thornton and Rincon, 2009). Together, these data strongly argue that although ILK–/– CD cells proliferate slower than ILKflox CD cells during early UB development, they fail to undergo cell cycle arrest when tubulogenesis is complete because they cannot activate p38 MAPK.

Almost nothing is known about the relationship between ILK and p38 MAPK. In N1E-115 cells, p38 MAPK activation was reported to be involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth (Ishii et al., 2001). In other studies utilizing wild-type embryonic kidney organ cultures and IMCD cells transfected with mutants that were proposed to alter the kinase activity of ILK, ILK was shown to be required for BMP7-induced branching morphogenesis and a dominant-negative ILK decreased BMP7-dependent phosphorylation of p38 MAPK and phospho-ATF2. In our vitro
studied that the kinase activity of ILK is not required for p38 MAPK activation. In addition, activation of p38 MAPK does not appear to require ILK-α-parvin interactions as do the K220A and K220M ILK mutants, which cannot interact with α-parvin but are able to activate p38 MAPK like wild-type ILK. There is also no physical interaction between p38 MAPK and ILK as they could not be coimmunoprecipitated. Together, these data suggest that either ILK or one of its binding proteins might be required to target p38 MAPK to focal adhesions, where it is activated by one of the many kinases present in this signaling hub.

In conclusion, we demonstrated that ILK and β1-integrins have distinct roles in mediating UB development. Furthermore, deleting ILK in renal tubular epithelial cells results in their inability to activate p38 MAPK and exit the cell cycle, giving rise to a unique obstructive renal phenotype. Thus, we have demonstrated a functionally significant phenotype associated with a novel role for ILK as a cell cycle regulator.

Acknowledgements

We would like to thank Shoukat Dedhar for the kinases present in this signaling hub. We thank Sean Seuffer for technical help with confocal microscopy. This work was supported by NIH grants DK065123 (A.P. and R.Z.); DK075594 (R.Z.); DK65123 (R.Z.); DK083187 (R.Z.); AHA established investigator award (R.Z.); a Merit award from the Department of Veterans Affairs (A.P. and R.Z.) and the George O’Brien Center Grant (A.P. and R.Z.) and grants from the Canadian Institutes of Health Research and Canada Research Chairs Program (to N.D.R.). Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

References


