LAMININ 5 REGULATES POLYCYSTIC KIDNEYS CELL PROLIFERATION AND CYST FORMATION
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Renal cysts formation is the hallmark of autosomal dominant polycystic kidney disease (ADPKD). ADPKD cyst-lining cells have an increased proliferation rate and are surrounded by an abnormal extracellular matrix (ECM). We have previously shown that Laminin 5 (Ln-5, a $\alpha_3\beta_3\gamma_2$ trimer), is aberrantly expressed in the peri-cystic ECM of ADPKD kidneys. We report that ADPKD cells in primary cultures produce and secrete Ln-5 which is incorporated to the pericystic ECM in an in vitro model of cystogenesis. In monolayers, purified Ln-5 induces Erk activation and proliferation of ADPKD cells, while upon EGF stimulation, blocking endogenously produced Ln-5 with anti-$\gamma_2$ chain antibody reduces the sustained Erk activation and inhibits proliferation. In 3D gel culture, addition of purified Ln-5 stimulates cell proliferation and cyst formation, whereas blocking endogenous Ln-5 strongly inhibits cyst formation. Ligation of $\alpha_6\beta_4$ integrin, a major Ln-5 receptor aberrantly expressed by ADPKD cells, induces $\beta_4$ integrin phosphorylation, Erk activation, cell proliferation and cysts formation. These findings indicate that Ln-5 is an important regulator of ADPKD cells proliferation and cystogenesis, and suggest that Ln-5 $\gamma_2$ chain and Ln-5 $\alpha_6\beta_4$ integrin interaction both contribute to these phenotypic changes.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited monogenic disorders, occurring in approximately 1 to 1000 live births. ADPKD is a systemic disorder characterized by the development of multiple renal cysts, and variably associated with liver, cardiovascular, gastrointestinal, and genital abnormalities. In Western countries, ADPKD accounts for 5 to 10% of end stage renal disease (1). About 85% of families with ADPKD are linked to the PKD1 locus on chromosome 16 (2). In the remaining families, the genetic defect is linked to the PKD2 locus on chromosome 4 (3). The proteins encoded by PKD1 and PKD2, polycystins 1 (Pc-1) and 2 (Pc-2), are transmembrane proteins that are able to interact, function together as a non-selective cation channel (4), and also induce several distinct transduction pathways. The “polycystin complex” may have three different subcellular localizations and associated putative functions (5) : at lateral membranes of the cells (with a role in cell-cell interaction) (6); at the basal pole of the cell (with a role in cell-extracellular matrix interaction) (7); at the apical primary cilia of the cells (with a putative role in the mechanotransduction of the urinary flux) (8,9). In ADPKD renal cysts, somatic mutations of the wild-type allele of PKD1 and PKD2 and subsequent loss of the functional polycystin complex presumably trigger a cascade of signaling and gene expression events, ultimately leading to cyst formation.

ADPKD renal cysts originate from the epithelia of the nephrons and collecting ducts. Cyst-lining cells are distinguished by increased proliferation, poor differentiation, and abnormalities in cell polarity, fluid secretion, and extracellular matrix production (1). Expanding cysts must remodel their environment as they enlarge. Morphological anomalies of the cyst-surrounding ECM have been described in human and animal models of polycystic kidney disease. Peri-cystic ECM appears thickened and multilaminated in the earliest detectable cysts (10,11). Cystic epithelial cells produce an abnormal basement membrane and have an abnormal proliferative response to ECM proteins (12-14). Thus, altered extracellular matrix has been postulated to play a role in the pathogenesis of ADPKD. However, the contribution of specific ECM proteins to the development of renal cysts is ill defined.

We have previously shown that Laminin 5 is strongly expressed by ADPKD cells and by the peri-cystic ECM of ADPKD kidneys, while no Laminin 5 expression could
be detected in adult control kidneys (15). Laminins are a family of at least 15 multi-functional αβγ heterotrimeric proteins commonly found in basement membranes of epithelia (16). The various isoforms have a cell- and tissue-specific expression and are differentially recognized by integrins. Laminins undergo a sequential series of extracellular proteolytic cleavages, which might successively turn on and off one or several of their biological and mechanical functions, though interaction with integrins and other cell-surface receptors (17). Laminin-5 (Ln-5) is a heterotrimer of α3β3 and γ2 chains. Its cell surface receptors are integrins α3β1, α6β1, αβ1, and α6β1. Mature Ln-5 plays a fundamental role in keratinocytes adherence, by promoting through its interaction with α6β1 integrin the assembly of strong and stable adhesion structures called hemidesmosomes (18). In peritumoral ECM, Ln-5 is frequently overexpressed and the proteolytic-driven modulation of its biological activity could trigger tumour progression (19) and migration properties with subsequent invasion and metastasis (20).

Based on our recent discovery of Ln-5 aberrant expression in the peri-cystic ECM in ADPKD, we hypothesized that this molecule could promote cystogenesis. In the present study, we characterize the Ln-5 γ2 chains isoforms produced and secreted by ADPKD cyst-lining cells, and provide evidence for a crucial role for Ln-5 in proliferation, signaling and \textit{in vitro} cystogenesis.

**EXPERIMENTAL PROCEDURES**

**Antibodies, matrix molecules and reagents** - Monoclonal antibodies were obtained as follows: anti-integrin α2 (clone P1E6), anti-integrin α3 (clone P1B5), anti-integrin α6 (clone GoH3), anti-integrin B1 (clone 6S6), anti-integrin B4 (clone 3E1) and anti-Laminin 5 γ2 chain (clone D4B5) were from Chemicon (Temecula, CA, USA); anti-PCNA (clone PC10) was from Novocastra (Newcastle, UK); anti-phosphotyrosine was from Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies were: anti-Laminin 5 L132 raised against purified native Laminin 5 was a gift from Dr Patricia Rousselle (Lyon, France); anti-B1 (clone H-101) from Santa Cruz (Santa Cruz, CA, USA); anti-phospho Erk1/2 (Thr 202/ Tyr 204) from New England BioLabs, total Erk1/2 from Sigma. Secondary antibodies and non-specific mouse IgGs were from Jackson ImmunoResearch Laboratories. FITC-goat anti-mouse antibody was from (Alexa Fluor, Molecular Probes).

Purified human ECM molecules used in this study were: laminin 5 (Ln-5; α3β3γ2, purified from SCC25 cells), a kind gift of Dr Patricia Rousselle (21); collagen I and collagen IV were purchased from Sigma. Culture plates were coated 3 hours at 37°C with 1 to 50 µg/ml laminin 5, collagen I or collagen IV in PBS; unbound sites were blocked with 1% albumin in PBS at 37°C for 2 hours. DMEM, HEPES, cell dissociation buffer were from Life technologies. Growth factor reduced Matrigel® was from Beckton Dickinson. All other reagents were from Sigma unless specified.

**Cell culture** - Primary cell cultures of cystic epithelium (“ADPKD cells”) and non-cystic epithelium (“control cells”) were established as previously described (15), subcultured and exclusively used between passages 2 and 4. Cells were grown in DMEM-based medium containing 1% foetal bovine serum, 5 µg/ml insulin, 10 µg/ml transferrin, 5 ng/ml Sodium Selenite, 6.5 ng/ml Triiodothyronin, 10 ng/ml Epidermal growth factor (EGF), 500 ng/ml Hydrocortisone, and 1% HEPES (Invitrogen, Carlsbad, CA, USA), referred as “defined medium”. For functional studies (cell proliferation, see below), cells were cultivated 24 h in serum-free DMEM, 1% HEPES (referred as “starvation”) to obtain quiescence, harvested with cell dissociation buffer (Invitrogen), washed and resuspended in basal DMEM medium (referred as “basal medium”) containing 0.1% foetal bovine serum and 1% HEPES, supplemented with EGF and various antibodies when indicated.

**In vitro cyst formation** - Gel mix was made of a mixture of ice-cold liquefied Growth factors-reduced Matrigel® and isotonic saline (80/20 %). 1.5 × 10⁴ cells were incorporated to 100 µl of gel mix, dispensed into flat-bottomed 96-well culture plate wells, and incubated at 37°C. After solidifying, gel mix was overlaid with 100 µl of defined medium. Plates were maintained at 37°C with 5% CO2 and observed daily. Cysts were visualized using a x 20 objective and counted under a light microscope at day 7. Six optic fields in triplicate wells were counted for each condition. For functional experiments, purified Ln-5 was added to the gel mix to reach the final concentration of 2 to 20 µg/ml; anti-Laminin 5 D4B5 blocking Ab (final concentration 10 µg/ml), anti-α3, α5, α6, β1 integrin antibodies (final concentration 10 µg/ml) and anti β4 integrin3E1 stimulating Ab (final concentration 2 µg/ml) were added to the defined medium every other day.

**Cell proliferation** - In monolayer culture, when ADPKD or control cells reached 70% confluence, the defined medium was replaced by basal medium to induce quiescence. After 24h, cells were harvested with enzyme-
free cell dissociation buffer (Invitrogen), washed, and seeded in a 96-well plate at the concentration of 2500 cells/100µl per individual chamber in basal medium to maintain cell viability during the experimental period. The wells had been previously left uncoated or coated with ECM molecules (as described above). After 12 h of adhesion, the basal medium was replaced by basal medium containing the indicated concentrations of EGF or antibodies., The Promega Cell titer 96 MTT assay method (Promega) was used to assess number of cells before medium replacement (T0) and the relative rate of cell proliferation after 3 days of culture (T72). This colorimetric assay system measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells, and the relative absorbance at 595 nm measured after incorporation of MTT is linearly correlated to the number of viable cells counted by a classical hemacytometer technique. Inhibition of proliferation experiments, apoptosis and anoikis were ruled out by daily microscopic analysis of the monolayers, and terminal differentiation was ruled out by the ability of cells to proliferate after addition of fresh defined medium (results not shown). In 3D gel culture, defined medium was removed at day 6, and gel mixes were submitted to Promega Cell titer 96 MTT assay to assess the rate of cell proliferation as above. A well containing the gel mix without cells was used as reference for absorbance measurement at 595 nm. Immunoblot analysis - In monolayer cultures, cells were harvested with 0.05% trypsin and 0.53 mM EDTA, lysed in buffer (150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 5% glycerol, and protease inhibitor tablet [Roche Laboratory]) for 10 min at 4°C. Supernatants from cells cultured for 48 h were collected and concentrated 40 times using Microcon membranes (Amicon®). Cyst fluids retrieved from ADPKD patients and concentrated 40 times using Microcon membranes.

β₃ integrin phosphorylation - To obtain selective ligation of α₆β₄ in the absence of any co-stimulus, cells were serum starved, detached with cell dissociation buffer, resuspended at 5.10⁶ cells per 200 µl aliquots and incubated at 37°C in suspension with the 3E1 monoclonal antibody (50µg/ml) coupled with protein sepharose A/G beads for the indicated times. After cell lysis at 4°C in lysis buffer, beads were pelleted, washed 3 times with lysis buffer, and eluted with 1 x sample buffer at 95°C for 5 min. The eluted proteins were subjected to SDS-PAGE and immunoblot analysis (as described above) with anti phospho tyrosine mAb. Membranes were stripped and reprobed with anti-β₃ polyclonal Ab.

Immunofluorescence - Gel mixes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature followed by 100% methanol at −20°C for 20’, and saturated for 30 minutes with 2% BSA in PBS, with intermediate washes in PBS. Gel mixes were immunostained with either anti Laminin5 D4B5 mAb (1 hour at 4°C, 1:100), or anti Laminin 5 L132 pAb (1 hour at room temperature, 1:100) and anti-β1 6S6 mAb (1h, room temperature, 1:100), or anti-PCNA PC10 mAb (2h, room temperature, 1:50), a FITC-goat anti-mouse antibody, then probed 60’ at room temperature with the appropriate secondary antibodies coupled to Alexafluor 488 or 594 (Molecular Probes). Nuclei were counterstained with DAPI (Sigma). For Laminin5 and β1 integrin stainings, gel mixes were observed with an inverted fluorescence microscope. For PCNA staining, semi-liquefied gels were transferred and spread on coverslips, mounted with Aquapolymount antifading solution (Agar, UK) onto glass slides and observed under a Leica fluorescence microscope. For each culture condition, PCNA positive nuclei were counted in at least 150 multicellular structures and results were expressed as a percentage of nuclei identified by DAPI counterstaining.

Statistical and density scanning analyses - Experiments were performed at least three times with primary cultures originating from different ADPKD kidneys. Density scanning was performed with NIH image 6.02 software. Data are presented as means ± SEM or SD and analyzed by one-way ANOVA with the unpaired t-test. Values considered as significant were P < 0.05 (*), P < 0.01 (**) or P < 0.001 (***)
RESULTS

Ln5 expression and secretion by ADPKD cystic epithelia

In a previous study, we reported that Ln-5 is strongly expressed by the peri-cystic ECM of ADPKD kidneys, while no Ln-5 expression could be detected in non-cystic adult control kidneys. Ln-5 aberrant expression was similarly observed in primary cultures of epithelial cells derived from ADPKD cysts, when compared to control renal tubular epithelial cells (15).

To study Ln-5 production during in vitro cyst formation, ADPKD cells were incorporated in 3D Matrigel-based gels, and incubated at 37°C for 7 to 10 days. Multicellular cystic structures became apparent at day 3-4 and were allowed to grow until day 6 (fig 1A). Immunofluorescent staining with anti-Ln5 1L132 pAb and integrin β1 showed exclusive basal staining around cysts, suggesting that Ln-5 is incorporated in the ECM (fig 1A, panels a,b). A similar pattern was observed with the anti-Ln5 γ2 chain D4B5 mAb, suggesting that Ln-5 chains are assembled together in the pericystic ECM (fig 1A, panel f).

To further assess Ln-5 production by ADPKD cells, we performed immunoblotting with the anti Ln5 γ2 chain D4B5 mAb in various 2D and 3D cell culture conditions. Known proteolytic fragments of the γ2 chain are depicted in figure 1B. Ln-5 γ2 chain was detected in cell lysates of quiescent and EGF-stimulated ADPKD cells (fig 1C, lanes 1 and 2), both in its mature form (155 kDa) and in its processed isoform γ2'(domains I-III, 100 kDa). Ln-5 γ2 chains were also found in the supernatant of ADPKD cells cultured for 48h, suggesting a cellular secretion (fig 1C, lanes 3 and 4). Of note, mature γ2 was not detectable, and in addition to the γ2' isoforms, we identified a 60 kDa fragment, which was dramatically overexpressed after EGF stimulation (fig 1C, lane 4). These result suggests an overall increased production and secretion of Ln5 after EGF stimulation. In 3D culture conditions, whereas our gel mix contained very little Ln-5 (fig 1C, lane 5), ADPKD cells undergoing cystogenic growth produced significant amounts of Ln-5 with major bands at 100, 60 and 30 kD and a faint band at 150 kDa corresponding to unprocessed γ2 chain (fig 1C, lane 6).

Interestingly, the Ln-5 γ2 chain isoforms profiles differed when ADPKD cells were grown in 2D and in cystogenic 3D conditions, and the 30 kD band appearance was restricted to 3D culture conditions, suggesting that a specific proteolytic cleavage of the γ2 chain occurs in this setting. In cyst fluid retrieved from ADPKD patients, 60 and 30 kd Ln-5 γ2 chain bands were detected (fig. 1D), indicating that Ln-5 secretion and proteolysis also occurred during in vivo cystogenesis.

ADDIN-Ln-5 induces ADPKD cell proliferation - One key feature associated with cyst formation in ADPKD is enhanced cell proliferation. ADPKD cells displayed a higher proliferation rate compared to control cells when grown in defined medium (figure 2A) or in basal medium supplemented with 10 or 20 ng/ml EGF (figure 2B).

Conversely, in basal medium, ADPKD cells did not display any significant proliferation advantage over control cells (Figure 2A). To selectively assess the effects of Ln-5 on proliferation in the absence of any co-stimulus, quiescent cells were plated on plastic or dishes coated with purified ECM components (purified Ln-5, collagen I, collagen IV) and cultured in basal medium for 3 days. Ln-5, but not collagen I or IV, stimulated cell proliferation in a dose-dependant manner, with a more pronounced effect on ADPKD cells compared to control cells (figure 2C). Furthermore, a dose dependent effect was observed with increasing concentration of Ln-5 coated dishes from 1 to 50 µg/ml (figure 2C).

Upon EGF stimulation, ADPKD cells both produce Ln-5 and proliferate. To investigate if endogenously produced Ln-5 could participate in ADPKD cell proliferation, quiescent ADPKD cells were stimulated by EGF and incubated with the anti-Ln-5 D4B5 blocking antibody for 3 days. As shown in figure 2D, D4B5 did not reduce cell number at T0 (12h after plating), ruling out a major effect on cell adhesion. By contrast, D4B5 blunted the pro-proliferative effects of EGF stimulation. No cellular apoptosis or necrosis was detected in D4B5 treated ADPKD cells. Additionally, D4B5 decreased EGF-stimulated proliferation dose-dependently, with a maximal effect (~75%) obtained at 10 µg/ml (figure 2E).

Taken together, these data suggest that aberrant expression of Ln-5 confers a proliferative advantage to ADPKD cells, with a specific role for the γ2 chain of Ln-5.

Ln-5-induced Erk (extracellular signal-regulated kinase) activation in ADPKD cells - Upon ligation to their ECM ligands and subsequent activation, integrins induce signaling events and mitogen-activated protein (MAP) kinase Erk1 and Erk2 activity (22). Activation of the MAP kinase pathway in turn leads to transcriptional control of genes important for cell proliferation (23). We examined if Ln-5 could activate Erk in quiescent ADPKD cells. After 30' of adhesion, Erk1,2 phosphorylation was barely detectable on a plastic support, significant on collagens I and IV, and more robust on Ln-5 coated dishes (figure 3A). In addition, a dose-dependent effect of
Ln-5 induced Erk activation was observed (5 and 10 μg/ml). Time-course experiments revealed that the level of Erk activation peaked at 30’ of adhesion and declined thereafter (not shown).

We next examined the ability of the anti-Ln-5 D4B5 antibody to disrupt EGF-mediated Erk activation. Time course experiments revealed that quiescent ADPKD cells, grown on plastic and stimulated by EGF, displayed a more sustained ERK1/2 phosphorylation (up to 16 h) when compared to control cells (figures 3B and 3C). As shown on figure 3, Ln-5 blocking by D4B5 antibody reduced EGF-induced prolonged Erk activation, while no effect was observed on early ERK1/2 phosphorylation (Figures 3D and 3E). Taken together, these results suggest that endogenously produced Ln-5 may contribute to a sustained ERK activation and therefore increase proliferation of ADPKD cyst-lining cells.

Ln-5 regulates in vitro cyst formation - To adress the role of Ln-5 in cyst formation and cyst enlargement, we used the in vitro system described above, in which ADPKD cells incorporated in 3D Matrigel®-based gels progressively form multicellular cystic structures. Incorporation of various amounts of purified Ln-5 to the gel mix enhanced cystic formation, as assessed by the number of cysts per optic field at day 7 (figure 4A). Ln-5 incorporation to the gel mix was also associated with an increased number of viable cells, as assessed by MTT test (figure 4B).

Inversely, addition of anti-Ln-5 D4B5 mAb (10 μg/ml every other day) to the gel mix supernatant drastically reduced the number of cysts formed at day 7 by 73 ± 9 % (figure 4C). In 3D gel cultures, initially isolated ADPKD cells treated with D4B5 proliferated and ultimately formed predomininatly non-cystic multicellular structures (figure 4D). In this setting, the number of viable cells determined by MTT test was reduced by 19 ± 28 % (non significant) when compared to cells treated with isotypic IgG (figure 4E), with no morphologic signs of apoptosis or necrosis. To further delineate the role of Ln5 in proliferation of 3D cultured cells, we studied PCNA staining of nuclei in multicellular (either cystic or non-cystic) structures. As shown in figure 4F, D4B5 significantly reduced the percentage of PCNA positive nuclei in gels mixes stimulated by EGF.

Overall, these results suggest that Ln-5 not only maintains its proliferative effects in 3D, but also is a mandatory ECM component to maintain cystic architecture of ADPKD cells.

Among Ln-5 integrin receptors, αβ4 is an important mediator of ADPKD cell proliferation and cystogenesis - We have previously shown that integrin chains α2, α3, α6, and β1 are strongly expressed by both ADPKD and control kidneys; conversely, β4 integrin is not detectable in adults tubular epithelial cells, while strongly expressed by ADPKD cyst lining cells, along with an αβ chain. β4 immunoblotts also suggested that α6β4 integrin aberrant expression was maintained in vitro, in both quiescent and growing primary cultures of ADPKD cells (15). We thus elected to focus on the role of Ln-5-αβ4 integrin interaction, which is likely to be a distinctive feature of ADPKD cyst-lining cells compared to normal tubular epithelial cells.

To mimic Ln5-αβ4 interaction, we used the stimulating anti-β4 antibody 3E1 (24). Incubation of 3E1-coupled beads with ADPKD cells in suspension, which ligates and clusters β4 at the cell surface, induced β4 integrin tyrosine-phosphorylation (figure 5A).

To confirm that α6β4 integrin receptor was involved in Ln-5-induced Erk activation, quiescent ADPKD cells were plated on dishes pre-coated with anti- β4 3E1 mAb. As shown on figure 5B, β4 integrin ligation by 3E1 strongly induced Erk1,2 phosphorylation. Taken together, these results suggest that among Ln-5 receptors, integrin α6β4 is able to activate ERK signaling pathway.

Incubation of 3E1 soluble antibody in basal medium induced proliferation of quiescent ADPKD cells, with a dose-response between .125 and 2 μg/ml (figure 5C). We then assessed the role of α6β4 integrin and other Ln5 integrin receptors in cyst formation. In 3D culture conditions, incorporation of stimulating anti-β4 mAb 3E1to the gel supernatant increased the number of cysts formed (+34 ± 31 %) whereas cyst formation was reduced by anti-α6 (-23±20%) and anti-β1 (-32±6%) and not statistically modified by anti-α2 and anti-α3 blocking antibodies (figure 5D). Overall, these results indicate that overexpressed Ln-5 and α6β4 form a functional axis that contributes to proliferation and cystogenesis of ADPKD cells, and that among other Ln5 receptors, α6β1 integrin also participates in cyst formation.

DISCUSSION

Following our finding that Ln-5 is aberrantly expressed in the pericystic ECM of ADPKD kidneys (15), the immunofluorescence and immunoblot data described in the present study (Figure 1) indicate that ADPKD cyst-lining cells are able to produce and secrete Ln-5 specific γ2 chains. In our in vitro cyst formation assays, ADPKD cells continue to produce Ln-5 and incorporate it in the ECM component to maintain cystic architecture of ADPKD cells.
cell proliferation has been documented in several ADPKD cells. The promoting role of purified Ln-5 on stimulates their growth, with a more marked effect on tubular cells on dishes coated with purified Ln-5. We report that plating ADPKD and control renal epithelial migration and invasion, a role on cell proliferation. Here may play, on top of its well studied effects on cell tumor cell populations suggest that this ECM molecule stimulating autocrine cell signaling.

misocalized and overexpressed EGF receptors, thus of ADPKD cyst lining cells could interact with speculate that DIII fragments secreted at the apical pole of the cystic fluid has already been reported (28). One could observe that D4B5 mAb does not block keratinocyte α3β1 integrin ligation to laminin 5 and subsequent adhesion, but strongly inhibits haptotactic migration of these cells suggest that the γ2 chain of Ln5 or DIII containing fragments of it, may exert biological effects independently of integrin α3β1 ligation (32). Further studies should adress the potential effect of recombinant DIII on ADPKD cell proliferation and cyst formation. Ln-5 may also regulate cell proliferation upon ligation of its α3 chain to its receptor integrins α6β4 and α6β4. In response to Ln5, both integrins were shown to stimulate epithelial cell proliferation (30,33). ADPKD cells express α6β4 and α6β4 integrins whereas control tubular epithelial cells α6β4 but not α6β4 integrin (15). Such a difference in integrin receptors distribution could at least partially explain why ADPKD cells proliferate more than control cells when plated on Ln5 (figure 2C). Indeed, we observed that β4 integrin ligation to the stimulating anti-β4 3E1 mAb was sufficient to induce ADPKD cells proliferation (figure 5C). These data suggest that Ln-5-α6β4 integrin interaction is likely to be a distinctive feature of ADPKD cells that is able to promote proliferation. Our MAPK analyses suggest that this enzyme is a key component of the pathway that transduces signals from Ln-5 in ADPKD cells. Upon adhesion and ligation to purified Ln-5, quiescent ADPKD cells display Erk1/2 activation. β4 integrin ligation by 3E1 stimulating mAb also induced β4 integrin tyrosine phosphorylation and Erk activation (figure 5A,B) suggesting the existence of a functional Ln5-α6β4-MAPK signaling pathway in ADPKD cells, in accordance with results published in
several epithelial cell lines (30,33). Although the role of α,β integrin was not addressed in this study, others have found that α, integrin function-inhibiting antibodies reduce early ERK activation in epithelial cell lines plated on Ln-5 suggesting the existence of a Ln5-α,β,-MAPK signaling pathway (30).

In EGF-stimulated ADPKD cells, Ln-5 blocking by D4B5 antibody did not impact the intensity of early Erk activation, but significantly reduced late ERK1/2 phosphorylation (Figures 3D,E). This suggests that the endogenously produced γ2 chain of Ln-5 contributes to the sustained ERK activation of EGF stimulated ADPKD cells. The duration of ERK signaling is critical to generating specific biological responses to MAP kinase pathway signaling. Prolonged ERK activation promotes its sustained nuclear localization, stabilization of intermediate early gene products, and cell cycle progression (34-36). It is well established that cell interaction with growth factors and ECM components ultimately lead to Ras-ERK activation, and several studies suggest that both stimuli may operate in a coordinated and sequential fashion, with growth factors being responsible for transient ERK activation and subsequent integrin signaling being responsible for delayed and sustained ERK activation (37,38).

In our study however, the mechanism by which D4B5 antibody impacts ERK activity remains uncertain. D4B5 may prevent cell interaction with cryptic fragments of the γ2 chain produced and released upon EGF stimulation, although we cannot exclude that this mAb perturbs α,β integrin signaling by steric hindrance.

To address the functional role of Ln-5 during cystogenesis, we modulated the ECM environment of ADPKD cells growing in 3D gels. Adding purified exogenous Ln-5 to the gel mix increased both the number of viable cells and cysts formed (fig 4A,B). Inversely, incorporation of blocking anti-Ln-5 mAb D4B5 drastically reduced cystogenesis (-73%) and was able to prevent EGF-stimulated cell proliferation assessed by PCNA staining (fig 4C,F). Although endogenously produced Ln-5 was reported to protect epithelial tumor cells survival from apoptosis in 3D gel culture conditions (39), we found that D4B5-induced inhibition of cyst formation could occur with no morphological evidence of cell apoptosis and no major reduction in the number of viable cells (fig 4E). These data mainly suggest that cell proliferation contribute to Ln-5 driven cystogenesis. Other mechanisms, such as maintenance of cell apical polarity by recuitment and assembly of laminins at their basal pole may also participate in cyst formation but were not assessed in this study. As discussed above, several non-exclusive mechanism may account for the promoting role of Ln-5 γ2 chain on cystogenesis. EGF receptor was reported to be overexpressed and mislocalized to the apical pole of the cells in humans and several animal models of polycystic kidney diseases (42). Furthermore, inactivating EGF pathway dramatically improved the cystic phenotype (43). Thus, cryptic Ln-5 γ2 fragments produced and secreted by ADPKD cells, such as DIII, could activate EGF receptor and promote cyst formation.

Ln-5 stimulation of integrin receptors signaling may also promote cystogenesis. Although it has been reported that loss of α,β integrin, the Ln-5 γ2 receptor, resulted in reduced cyst formation by MDCK cells grown in collagen gels (44), blocking α, integrin did not reduce cystogenesis in our model (fig 5D), suggesting that the γ2 chain of Ln-5 or its fragments stimulate cystic growth independently of α, integrin ligation. Conversely, experiments conducted with anti-integrin α, α, β, blocking antibodies and anti-β, stimulating antibody suggest that α,β, α,β, and to a lesser extent α,β, integrins, all integrin receptors of Ln-5, participate in ADPKD cells cystogenic growth (fig 5D). We specifically studied the role of α,β, signaling in cystogenesis, as this integrin is overexpressed in ADPKD (15). We show that stimulating anti-β, mAb 3E1 is sufficient to increase the number of cysts formed by ADPKD cells. Autocrine Ln-5 α,β, signaling was shown to be critical for epithelial tumors survival both in 3D gel cultures and in vivo (20,39,41). Of note, in primary culture, control renal epithelial cells which do not express α,β, soon underwent apoptosis in 3D gels and never develop cysts in our hands (not shown). Thus, Ln-5 α,β, interaction and subsequent signaling may be one of the mechanisms involved in ADPKD cell cystogenic growth. Collectively, our results indicate that Ln-5 is overexpressed in the pericystic ECM of ADPKD kidneys, produced, secreted and processed by ADPKD cells. Ln-5 role in regulating ADPKD cell growth, prolonged Erk activation and in vitro cystogenesis suggest that this ECM molecule may contribute to the development of renal cysts. Thus, interfering with cyst-lining cells-Ln-5 interaction may define a new therapeutic target in ADPKD.
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig 1. Ln-5 production by ADPKD cells. A, ADPKD cells formed multicellular cystic structures in 3D Matrigel-based gels visualized under phase contrast microscopy (a). Nuclei are stained with DAPI (b). Immunofluorescent staining with L132 anti Ln-5 pAb (c), anti-β1 integrin (d) shows colocalization surrounding cyst forming cells (e). On a different cyst, immunofluorescent staining with D4B5 anti Ln-5 γ2 chain mAb show a similar pattern (f). B, Schematic representation of known Ln-5 γ2 chain isoforms. C, 40µg of proteins were extracted from cell lysates (WCL, lanes 1 and 2), 10 x concentrated supernatants (SN, lanes 3 and 4) and 3D gel mixes (3D, lanes 5 and 6) of ADPKD cells in culture for 48h. Proteic extracts were resolved by SDS-page, western blotted and probed with anti-Ln-5 γ2 chain antibody D4B5. D, Proteins were extracted from the cyst fluid of an ADPKD kidney (100 µg after 10 x concentration), resolved by SDS-page, western blotted and probed with anti-Ln-5 γ2 chain antibody D4B5.

Fig 2. Ln-5-induced proliferation of ADPKD cells. A, MTT incorporation of control cells (empty bars) and ADPKD cells (grey bars) cultivated for 3 days in basal medium or defined medium.
Each point represents the mean of triplicate wells (± SD). Representative of 3 independent experiments. B, MTT incorporation of control cells (empty bars) and ADPKD cells (grey bars) cultivated for 3 days in basal medium supplemented with EGF at the indicated concentrations. Results are expressed as mean ± SEM (n=3) of relative cell proliferation, with cell proliferation in basal medium set as 1. C, MTT incorporation of control cells (empty bars) and ADPKD cells (grey bars) cultivated for 3 days in basal medium after plating on plastic or the indicated ECM purified components: P = plastic, C.I = collagen I, C.IV = collagen IV. Results are expressed as mean ± SEM (n=3) of relative cell proliferation, with cell proliferation on plastic set as 1. D, MTT incorporation of quiescent ADPKD cells plated in basal medium for 12h (T0) and after 3 days (T72) of culture in basal media (0%) ± EGF (20 ng/ml) ± isotypic control IgG (10 µg/ml) or anti Ln5 D4B5 antibody. Each point represents the mean of triplicate wells (± SEM). Representative of 3 independent experiments. E, MTT incorporation of quiescent ADPKD cells cultivated for 3 days in basal medium containing EGF (20 ng/ml) ± anti-Ln-5 D4B5 antibody at the indicated concentrations. Results are expressed as mean ± SEM (n=3) of relative cell proliferation, with control IgG set as 100%.

Fig 3. Ln-5-induced Erk activation in ADPKD. A, Serum-starved cells were detached and kept in suspension or cultured for 60’ on 6-well plates coated with 10 µg/ml purified Ln-5, collagen I or collagen IV. Total cellular proteins were subjected to immunoblot analysis using mAb specific for the phosphorylated isoform of ERK 1,2. To evaluate loading efficiency, membranes were stripped and reprobed with actin Ab. B, After EGF stimulation (10 ng/ml), total cellular proteins from quiescent ADPKD and control cells were subjected to immunoblot analysis using mAb specific for the phosphorylated isoform of ERK 1,2 (pp44/42). Membranes were stripped and reprobed with Ab recognizing total ERK1/2 (p44/42). C, Bar graph from 6 experiments as in B: after densitometric analysis, the pp42/p42 ratio was plotted to assess the degree of ERK1/2 activation at the indicated times; results are expressed as mean ± SEM (n=6) of relative ERK2 activation, with ERK activation at 5’ of EGF treatment set as 100% (* p < 0.05). D, Quiescent ADPKD cells were stimulated by EGF (10 ng/ml) for the indicated times, with addition of either anti-D4B5 mAb or isotypic IgG control Ab (10 µg/ml). Total cellular proteins from were subjected to immunoblot analysis as in B. E, Bar graph from 3 experiments as in D: after densitometric analysis, the pp42/p42 ratio was plotted to assess the degree of ERK2 activation at the indicated times; results are expressed as mean ± SEM (n=3) of relative ERK2 activation, with ERK2 activation of cells treated with control Ab at 5’ of EGF treatment set as 100%.

Fig 4. ADPKD cells were incorporated in 3D Matrigel®-based gels enriched with the indicated concentrations of purified Ln-5. After 7 days, the number of cystic structures per optic field was numerated. Representative of 4 independent experiments. B, ADPKD cells were cultivated as in A. After 7 days, the number of viable cells was assessed by MTT test. Representative of 4 independent experiments. C, ADPKD cells were cultivated as in A. In control wells, ADPKD cells were incubated with isotypic IgG mAb (10µg/ml), and the number of cysts per field was set as 100%. Incorporation of anti-Ln-5 D4B5 mAb to the gel supernatant (10µg/ml every other day) reduced cysts formation by 73 ± 9 % (n=4 experiments). D, Microphotograph (magnification x 20) showing representative multicellular structures from fig. 4C. E, ADPKD cells were cultivated as in A, with incorporation of either isotypic IgG mAb (10µg/ml) or anti-Ln-5 D4B5 mAb to the gel supernatant (10µg/ml every other day). At day 7, the number of viable cells treated with isotypic IgG (set as 100%) or D4B5 was assessed by MTT test. F, ADPKD cells were cultivated as in A in basal media ± EGF (20 ng/ml) ± isotypic control IgG (10 µg/ml) or anti Ln5 D4B5 antibody (10 µg/ml); after 5 days, cells were stained with DAPI, anti-PCNA PC10 Ab and observed under fluorescent microscopy. For each culture condition, more than 150 multicellular structures were examined and the PCNA/DAPI positive nuclei ratio was determined. Top, graph is representative of 3 independent experiments. Bottom, microphotograph showing PCNA and
DAPI stainings of one 3D-multicellular structure for each condition. All results for figure 4 are expressed as mean ±SD.

**Fig 5.** A, ADPKD cells were incubated in suspension with anti-β4 mAb 3E1 coupled with prot. G-sepharose beads for the indicated times. β4 integrin immunoprecipitates were subjected to western blot analysis using anti- phospho-tyrosine mAb. B, 12-well plates were precoated with anti-β4 mAb 3E1 or control isotypic mAb (10 μg/ml). ADPKD cells were allowed to attach for 60’, and ERK activation was detected by immunoblotting of protein extracts with a phospho Erk Ab. C, ADPKD cells grown on a plastic support were serum starved 24h and incubated in the presence of various concentrations of 3E1 mAb in basal medium. Proliferation was assessed by MTT test performed after 72h. D, ADPKD cells were cultivated in 3D gels as in Figure 4 in basal media + isotypic control IgG (10 μg/ml) or the indicated anti-integrin antibodies (all 10 μg/ml except anti-β4, 2 μg/ml). After 7 days, the number of cystic structures per optic field was numerated. Results for 5C,D are expressed as mean ± SD (number of experiments indicated in brackets) of relative cyst number per optic field, with control IgG set as 100%.
FIGURE 1

A

a

b

c
d

e
f

B

III  IV  V

γ₂  γ₂'  γ₂x

150kD  100kD  80kD

III-V (60kD)

IV-V

III (30kD)

C

WCL  SN  3D gel

1  2  3  4  5  6

EGF -  +  -  +

Cells -  +

D

60

30
FIGURE 3

A

Control cells

p44/42

pp 44/42

actin

ADPKD cells

pp44/42

p44/42

B

EGF

0 5' 15' 60' 120' 5h 8h 16h

Relative Erk activation (%)

C

Relative Erk activation (%)

EGF 0 5' 1h 8h 16h

D

ADPKD cells

pp44/42

p44/42

EGF

0 5' 4h 8h 16h

D4B5 control IgG

E

Relative Erk activation (%)

EGF 0 5' 1h 8h 16h
FIGURE 4

A

Number of cysts per field

Ln-5 (µg/ml)

0  2  10  20

**  **  **

B

OD at 595 nm

Ln-5 (µg/ml)

0  2  10  20

**  *

C

Cysts per field (%)

Control  D4B5

***

D

Control  D4B5

E

Relative absorbance at 595 nm

Control  D4B5

F

PCNA positive nuclei (%)

0%  EGF + IgG  EGF + D4B5

***

PCNA  DAPI
FIGURE 5

A
IP: β₄
IB: pTyr
IB: β₄
3E1 15' 60' 120' 240'

B
pp44/42
p44/42
IgG 3E1

C
Absorbance at 595 nm (%)

D
Cysts per field (%)

Control IgG α2 α3 α6 β1 β4

* (5) ** (7) ** (5) ** (7)

* (6) ** (6) *** (9)