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ScienceDirect
Developmental Biology 313 (2008) 584–593
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

# Integrin β1-mediated matrix assembly and signaling are critical for the normal development and function of the kidney glomerulus

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Received for publication 19 July 2007; revised 8 October 2007; accepted 29 October 2007
Available online 12 November 2007

**Abstract**

The human kidneys filter 180 l of blood every day via about 2.5 million glomeruli. The three layers of the glomerular filtration apparatus consist of fenestrated endothelium, specialized extracellular matrix known as the glomerular basement membrane (GBM) and the podocyte foot processes with their modified adherens junctions known as the slit diaphragm (SD). In this study we explored the contribution of podocyte β1 integrin signaling for normal glomerular function. Mice with podocyte specific deletion of integrin β1 (podocin-Cre β1-fl/fl mice) are born normal but cannot complete postnatal renal development. They exhibit detectable proteinuria on day 1 and die within a week. The kidneys of podocin-Cre β1-fl/fl mice exhibit normal glomerular endothelium but show severe GBM defects with multilaminations and splitting including podocyte foot process effacement. The integrin linked kinase (ILK) is a downstream mediator of integrin β1 activity in epithelial cells. To further explore whether integrin β1-mediated signaling facilitates proper glomerular filtration, we generated mice deficient of ILK in the podocytes (podocin-Cre ILK-fl/fl mice). These mice develop normally but exhibit postnatal proteinuria at birth and die within 15 weeks of age due to renal failure. Collectively, our studies demonstrate that podocyte β1 integrin and ILK signaling is critical for postnatal development and function of the glomerular filtration apparatus.


*Keywords:* Beta1 integrin; ILK; Glomerular basement membrane; Podocyte; Slit diaphragm; Filtration barrier; Podocin Cre; Permselectivity; Proteinuria; FAK

## Introduction

The glomerular endothelium, the GBM and podocyte foot processes/slit diaphragm are three distinct components of the filtration apparatus of the kidney. Structural and functional insufficiency of podocytes is implicated as a key determinant in the pathogenesis of several nephritic glomerular diseases including focal segmental glomerulosclerosis (FSGS). Podocytes are highly differentiated and polarized cells characterized by actin-rich foot processes that likely interact with the glomerular basement membrane (GBM) and play an important role in the filtration properties of the glomerulus. Previous studies have suggested that α3β1 integrin mediated interaction of podocytes with the GBM is necessary for proper function of the glomerulus (Kreidberg et al., 1996).

Integrin β1 and the associated integrin linked kinase (ILK) play a crucial role in cell survival, tissue homeostasis and carcinogenesis (Hannigan et al., 2005; Legate et al., 2006). Integrin β1 forms at least 12 different kinds of integrins via their binding to different α chains of the integrin family (Brakebusch et al., 2002; Hynes, 2002). Glomerular podocytes express integrin α3β1 and some data suggest that this integrin facilitates binding to laminin in the GBM (Baraldi et al., 1994). Decreased expression of α3β1 integrin is demonstrated in human diabetic

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doi:10.1016/j.ydbio.2007.10.047

kidney disease, FSGS and several animal models of experimental glomerulonephritis (Chen et al., 2006; Regoli and Bendayan, 1997). Integrin β1 binds to the ILK via its cytoplasmic domain (Hannigan et al., 1996). Integrin-activated ILK induces anti-apoptotic signals (Hannigan et al., 2005; Legate et al., 2006). Such observations suggest that ILK mediated signaling via integrin β1 is likely important for podocyte function.

In this report we demonstrate that specific deletion of integrin β1 in the podocytes of mice (podocin-Cre β1-fl/fl mice) leads to postnatal death with massive proteinuria and podocyte defects. The GBM in the newborn podocin-Cre β1-fl/fl mice exhibits many structural defects including multi-laminations and splitting. Next, we demonstrate that specific deletion of ILK in podocytes (podocin-Cre ILK-fl/fl mice) leads to proteinuria starting at birth, the development of glomerulosclerosis by 8 weeks of age and death due to renal failure by 15 weeks of age. Collectively, these results further demonstrate a critical role for podocyte–GBM interactions mediated by β1 integrin and ILK in the normal assembly of the GBM and also proper function of the glomerular filtration apparatus.

## Materials and methods

### *Animals*

Podocin-Cre mice were kindly provided by Dr. Jordan A. Kreidberg, at the Children's Hospital, Boston. β1 integrin flox mice were purchased from the Jackson Laboratory (Bar Harbor, ME). ILK-flox mice were kindly provided by Dr. Shoukat Dedhar and Dr. Robert Gerszten. For podocyte specific deletion of the target gene, podocin-Cre positive/heterozygous (flox/wt) mice were mated with homozygous (flox/flox) mice. Mice were maintained at the Beth Israel Deaconess Medical Center animal facility under standard conditions. All animal studies were reviewed and approved by the animal care and use committee of the Beth Israel Deaconess Medical Center.

### *Antibodies*

Hamster anti-mouse integrin β1, β3 and rat anti-mouse integrin α3 antibody, anti-CD31-FITC conjugated antibody and anti-fibronectin monoclonal antibody were purchased from Becton Dickinson (Franklin Lakes, NJ). The polyclonal anti-podocin antibody was a gift from Dr. Peter Mundel, Mount Sinai School of Medicine, New York. The polyclonal anti-nephrin and anti-COL4A3, -COL4A4 and -COL4A5 antibodies were previously described (Sugimoto et al., 2006). Rat anti-laminin β1, β2 and anti-entactin antibodies were purchased from CHEMICON international (Temecula, CA). Anti-WT1 and anti-YFP polyclonal antibodies are purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-FAK$^{Tyr397}$ for immunofluorescence and anti-actin antibodies were purchased from Sigma Aldrich (St. Louis, MI). Anti-phospho-FAK$^{Tyr397}$ for both western blot and immunofluorescence was purchased from Invitrogen (Carlsbad, CA). The rabbit polyclonal antibody against total FAK was purchased from Upstate Biotechnology (Lake Placid, NY).

### *Immunofluorescence*

Immunofluorescence was performed as previously described (Hamano et al., 2003). Briefly, frozen sections were fixed in 100% acetone at −20 °C for 10 min. After blocking, sections were incubated with primary antibodies for 1 h at room temperature and subsequently labeled with secondary antibodies (Jackson Immunoresearch, West Grove, PA).

### *SDS–page and western blot analysis*

Samples (1 μl of urine samples or purified protein from outer cortex of kidney with lysis buffer (Tris 50 mM PH7.5, NaCl 0.15M, SDS 0.1%, Triton X-100 1%, Deoxycholate 1% with protease inhibitor)) were denatured with SDS sample buffer in boiling water. Denatured samples were separated on 8 or 10% SDS–polyacrylamide gels and blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon, Bedford, MA) by semi-dry method. The transferred protein was visualized with Coomassie Brilliant Blue (CBB). After blocking with TBS-T (Tris-buffered saline, 0.05% Tween 20) containing 5% non-fat milk, the membranes were incubated with anti-WT1 (1:500 diluted), anti-podocin (1:2000), anti-phospho FAK$^{Tyr397}$ (1:500), anti-total-FAK (1:1000) or anti-actin (1:1000) antibodies room temperature 1 h (anti-WT1 and anti-podocin) or 4 °C overnight (others). The membranes were washed three times and incubated with 1:10000 diluted horseradish peroxide (HRP)-conjugated anti-rabbit secondary antibody (Promega, Seattle, WA) at room temperature for 1 h. The immunoreactive bands were detected with an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Rockford, IL).

### *Electron microscopy*

Kidney tissues were fixed in 0.1 M cacodylate acid with 2% glutaraldehyde. Electron microscopy (transmission electron microscopy: TEM and scanning electron microscopy: SEM) was performed as previously described (Sugimoto et al., 2006).

### *Detection of LacZ expression*

Kidney samples (1 mm$^2$) from 6 week old R26Rstop LacZ flox mice (Mao et al., 1999) with or without podocin-Cre were fixed at 4 °C for 4 h in 4% paraformaldehyde. Samples were washed 3 times with PBS pH 7.3 and then stained overnight at 37 °C with LacZ staining buffer (1 mg/ml X-gal, 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate in PBS). After washing with PBS pH 7.3, samples were embedded into paraffin. Sections (10 μm) were then deparaffinized and counterstained with eosin.

### *Detection of yellow fluorescence protein (YFP) expression*

For the YFP staining, kidneys were pre-fixed overnight with 4% PFA, and then samples were allowed to equilibrate in 30% sucrose in PBS. The fixed samples were mounted in OCT mounting media. The frozen sections were then blocked directly with 2% BSA PBS and then incubated with 1:200 diluted anti-YFP antibody followed by rhodamine-conjugated secondary antibody. The detection was performed by immunofluorescence microscopy.

## Results

### *Podocin specific gene deletion*

To demonstrate podocin specific gene deletion by Cre-recombinase, R26Rstop LacZ flox mice were mated with podocin-Cre mice. In the progeny, Cre-recombinase activates the LacZ reporter gene by the excision of a stop cassette between two loxP sites. LacZ labeling reveals that LacZ Rosa-flox/podocin-Cre mice specifically express LacZ in the glomerular podocytes (Fig. 1A) when compared to control mice (Fig. 1B). To further validate this expression pattern, we also performed similar experiments with R26Rstop EYFP floxed mice (Srinivas et al., 2001). As in R26Rstop LacZ floxed mice, EYFP reporter gene is induced by the excision of a stop cassette in the presence of Cre-recombinase in the R26Rstop EYFP flox mice. Immunofluorescence analysis clearly reveals that podocytes in EYFP Rosa-flox/podocin-Cre mice express YFP protein (Figs. 1C and D). These results indicate specific activity of podocin promoter driven Cre-recombinase in glomerular podocytes.