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α-Actinin-4 Is Required for Normal Podocyte Adhesion*[S]

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Mutations in the α-actinin-4 gene *ACTN4* cause an autosomal dominant human kidney disease. Mice deficient in α-actinin-4 develop a recessive phenotype characterized by kidney failure, proteinuria, glomerulosclerosis, and retraction of glomerular podocyte foot processes. However, the mechanism by which α-actinin-4 deficiency leads to glomerular disease has not been defined. Here, we examined the effect of α-actinin-4 deficiency on the adhesive properties of podocytes *in vivo* and in a cell culture system. In α-actinin-4-deficient mice, we observed a decrease in the number of podocytes per glomerulus compared with wild-type mice as well as the presence of podocyte markers in the urine. Podocyte cell lines generated from α-actinin-4-deficient mice were less adherent than wild-type cells to glomerular basement membrane (GBM) components collagen IV and laminin 10 and 11. We also observed markedly reduced adhesion of α-actinin-4-deficient podocytes under increasing shear stresses. This adhesion deficit was restored by transfecting cells with α-actinin-4-GFP. We tested the strength of the integrin receptor-mediated linkages to the cytoskeleton by applying force to microbeads bound to integrin using magnetic pulling cytometry. Beads bound to α-actinin-4-deficient podocytes showed greater displacement in response to an applied force than those bound to wild-type cells. Consistent with integrin-dependent α-actinin-4-mediated adhesion, phosphorylation of β1-integrins on α-actinin-4-deficient podocytes is reduced. We rescued the phosphorylation deficit by transfecting α-actinin-4 into α-actinin-4-deficient podocytes. These results suggest that α-actinin-4 interacts with integrins and strengthens the podocyte-GBM interaction thereby stabilizing glomerular architecture and preventing disease.

It has been hypothesized for some time that glomerular visceral epithelial cells, or podocytes, are required for buttressing glomerular capillaries against the forces generated by the cardiac cycle (1, 2). Defects in podocyte structure, function, or number can lead to a pathologic lesion described as focal and segmental glomerulosclerosis or FSGS[2] (3–5). The unique podocyte-podocyte cell junction referred to as the slit-diaphragm is the distal component of the glomerular filter. Components of the slit-diaphragm appear to serve signaling as well as structural functions. Mutations in several slit-diaphragm protein-encoding genes have been shown to lead to proteinuria, nephrotic syndrome, and/or FSGS in humans or mice (including *NPHS1*, *NEPH1*, *CD2AP*, *FAT*, *TRPC6*, and *NPHS2* (6–11)). By contrast, the FSGS gene *ACTN4* encodes an actin-associated cytoskeletal protein α-actinin-4 that colocalizes with the actin bundles extending along the center of the podocyte extensions known as foot processes (12). This suggests that the mechanism of disease in α-actinin-4-deficient mice may be different from that observed in mice (or humans) with deficiency of a slit-diaphragm component.

We previously reported the development of an α-actinin-4-deficient mouse model (13). Although ACTN4-associated disease in humans is characterized by dominantly inherited gain-of-function mutations (12, 14, 15), mice lacking α-actinin-4 expression show a recessive phenotype characterized by progressive proteinuria and glomerular disease characterized by podocyte foot process retraction (13). Thus, α-actinin-4 plays an essential and non-redundant role in podocyte function. In addition to cross-linking actin filaments, α-actinin-4 binds a number of adhesion molecules including β1-integrin and vinculin (16, 17). Data from a number of experiments suggest that human ACTN4-mediated disease is the result of gain-of-function mutations in the actin binding domain of the encoded protein (14, 15, 18, 19). Interestingly, many of these mutations in ACTN4 lead to an altered subcellular localization of the protein suggesting that the mutations in ACTN4 may lead to loss-of-function of the protein (14). Both mice harboring a point mutation implicated in human ACTN4-mediated disease and

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[2] The abbreviations used are: FSGS, focal and segmental glomerulosclerosis; ACTN4, α-actinin-4; DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type; FACS, fluorescent-activated cell sorting; GFP, green fluorescent protein; GBM, glomerular basement membrane; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

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Actn4-deficient mice develop similar renal disease phenotypes, including similar levels of proteinuria and podocyte foot process effacement (13, 14). Thus, understanding the mechanism of the renal phenotype in α-actinin-4-deficient mice should provide insights into both the mechanism of ACTN4-mediated familial FSGS and the function of its gene product in podocytes.

Recent reports have also shown that one mechanism for the development of glomerular disease is podocyte depletion *in vivo*, as demonstrated by loss of podocyte markers in sclerotic glomeruli as well as the presence of shed podocytes in the urine (20–22). Loss of podocytes is a feature not just of primary glomerular disease but may be an important component in the pathogenesis of common secondary diseases such as diabetic nephropathy (23–26). Here we report that loss of α-actinin-4 leads to podocyte depletion. We used α-actinin-4-deficient mice both to characterize podocyte depletion *in vivo* as well as to generate α-actinin-4-deficient podocytes. We used these cultured podocytes as a cell system to understand the mechanism of podocyte depletion in α-actinin-4 deficiency. We demonstrate that α-actinin-4-deficient cells are less adherent than cells expressing α-actinin-4 and that this deficit can be rescued by transfecting α-actinin-4-deficient cells with α-actinin-4. We provide evidence that this altered adhesion is integrin-mediated by demonstrating that α-actinin-4 deficiency weakens the physical linkage of integrins to the cytoskeleton and alters the phosphorylation of the cytoplasmic domain of β1-integrin.

EXPERIMENTAL PROCEDURES

Animals—Actn4$^{-/-}$ mice were developed as described previously (13). Animal studies were performed in accordance with a protocol approved by the Harvard Medical Area Standing Committee on Animals.

Cell Culture and Transfection—Podocytes were maintained in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Sigma), and antibiotics/antimycotics (Invitrogen, Grand Island, NY). To differentiate cells, cells were plated for 10–14 days at 37 °C. We used the FuGENE reagent (Roche Applied Science), and 1 μg of DNA to perform transient transfections of podocytes.

Adhesion Assays—Plates (96 well) were coated with collagen I (10 μg/ml), collagen IV (10 μg/ml), laminin 10 and 11 (placental) (10 μg/ml), fibronectin (10 μg/ml), vitronectin (10 μg/ml), or laminin-1 (10 μg/ml) with 1% bovine serum albumin as the control. Podocytes were harvested with trypsin/EDTA and resuspended in serum-free medium. Cells were allowed to attach at 37 °C for 1 h. Unbound cells were removed by washing twice with phosphate-buffered saline. Attached cells were fixed in formalin, and cells in each well were counted. Two individuals recorded cell attachment for these experiments. Cell counts were obtained by averaging cell numbers from five wells. We normalized experiments to compare different repetitions of the assay on different days. Because of day-to-day variation in experimental setup, we normalized attachment assays by the average cell attachment/binding for that day.

For adhesion studies under shear stress, we plated differentiated podocytes on collagen I, cultured the cells for a minimum of 6 days, and then used a Vera Varistaltic pump for flow rates of 1–9 ml/sec to produce a shear stress of 72–649 dyne/cm^2. Observations were made using a cell chamber positioned under a Nikon Diaphot microscope equipped with a CCD camera using IPLab software for Macintosh.

Podocyte Number in Glomeruli—3–4-μm renal sections from Actn4$^{-/-}$ and Actn4$^{+/+}$ mice were fixed in acetone and co-stained for WT1 and DAPI. Co-localization of DAPI and WT1 signal was counted as detection of a podocyte. Two individuals made the experimental counts. The glomerulus was approximated to be a sphere, and a random sampling of 30 Actn4$^{+/+}$ and 30 Actn4$^{-/-}$ glomeruli were analyzed for spherical size. Cross-sectional areas were calculated from the number of pixels per glomerulus per field to get an average pixel number per glomerulus. Based on the articles of Sanden *et al.* (27) and Weibel and Gomez (42) describing the counting of structures of sections and approximating the glomerulus to be a sphere, we used cross-sectional area as a measure in order to compare glomeruli size from Actn4$^{+/+}$ and Actn4$^{-/-}$ mouse kidneys (27). Using ImageJ (ImageJ 1.33u Wayne Rasband, National Institutes of Health), the area of 30 Actn4$^{-/-}$ glomeruli and 30 Actn4$^{+/+}$ glomeruli were analyzed to assess the relative area of glomeruli in Actn$^{+/+}$ *versus* Actn4$^{-/-}$ mice. Area was calculated using ImageJ (pixel density): 18004.22 pixels ± 8365 for Actn4$^{+/+}$ glomeruli *versus* 17116 ± 5909 pixels for Actn4-deficient glomeruli. These relative areas were not statistically different. As area was not significantly different between the Actn4$^{+/+}$ and Actn4$^{-/-}$ mice, we estimated podocyte number per glomerulus by counting glomerular cells that were co-stained for WT1 and DAPI. Mice from both the 1 week and 6–10 week groups were subjected to statistical analysis by comparing all podocyte numbers/glomeruli for a minimum of 95 glomeruli counted per both the WT and Actn4$^{-/-}$ mice groups.

Urinary Podocytes—To estimate both the total urine protein loaded on gels and the amount of proteinuria in Actn4$^{-/-}$ mice, we measured the albumin (mg/dl) and albumin/creatinine (mg/g) ratio of urine samples of Actn4$^{-/-}$ mice and controls using a Bayer DCA 2000+ machine. Expression of WT1 was assessed by Western blot of urine samples. Wild-type podocyte cell lysates were loaded as a positive control. Mouse monoclonal anti-WT1 antibody (sc-7385, Santa Cruz Biotechnology) was used to detect WT1.

Immunoblotting—Cells were lysed in ice-cold 20 mM HEPES, pH 7.0 buffer with 0.5% Triton X-100 buffer, with 2 mM EGTA, 4 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and a protease inhibitor mixture tablet (Complete, Roche Applied Science). Anti-α3-integrin antibody was provided by Dr. Jordan Kriedberg (Children's Hospital, Boston). Anti-β1-integrin antibody was from BD Biosciences (cat. no. 610467) (San Jose, CA). Phosphospecific anti-β1-integrin antibody (serine 785) was from BIOSOURCE (cat. no. 44-870, Camarillo, CA). This antibody specifically recognizes the serine 785 phosphorylation of β1-integrin and has been tested by the manufacturer using purified phosphopeptides to verify specificity (details are at the manufacturer's website). Anti-nephrin and anti-podocin antibodies were previously described (28). Anti-synaptopodin antibody was provided by Dr. Peter Mundel (Albert Einstein Medical School). For the Kinexus signaling molecule

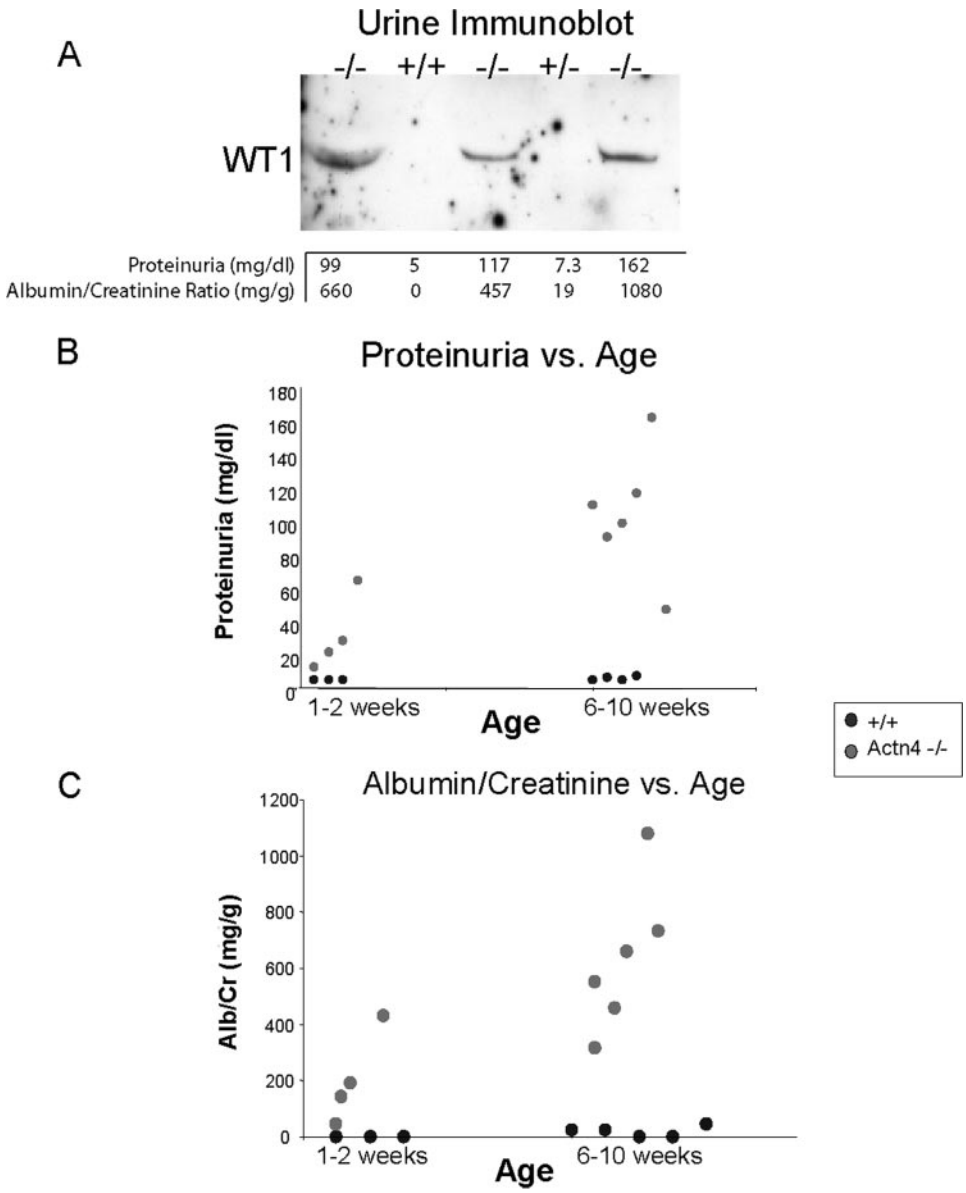

FIGURE 1. **WT1 as a marker of urinary podocyte loss in Actn4$^{-/-}$ mice.** *A*, representative Western blot of urine from Actn4$^{+/+}$ and Actn4$^{-/-}$ mice stained with WT1 antibody. Both albumin and albumin/creatinine ratios of urine samples from Actn4$^{-/-}$ mice and WT controls were measured as an estimate of total urine protein concentration. The representative protein concentration of urine is listed under the immunoblot. *B*, analysis of proteinuria (albuminuria, mg/dl) in Actn4-deficient mice at two different age groups (1–2 weeks and 6–10 weeks). *C*, analysis of albumin/creatinine ratio (mg albumin/g creatinine) in Actn4-deficient mice at two different age groups (1–2 weeks and 6–10 weeks). WT control littermates were analyzed as well for *A–C*. There was no statistically significant level of both albuminuria and albumin/creatinine ratio in WT controls ($p < 0.002$). All Actn4$^{-/-}$ mice analyzed that had WT1 in the urine also had some degree of proteinuria.

screen, podocyte cell lysates were prepared using modified radioimmune precipitation assay buffer as described above.

FACS—To specifically assess cell surface expression of β1-integrin, we employed FACS with the antibody FITC-CD29 from BD Biosciences. We also used antibodies PE-α3-integrin (Santa Cruz Biotechnology), PE-αV-integrin (BD Biosciences), and PE-β3-integrin (BD Biosciences). Geometric means of replicate samples were analyzed for statistical significance. A minimum of three replicates were analyzed for both WT and Actn4$^{-/-}$ podocyte cell lines.

Apoptosis Assays—In vitro apoptosis assays measured histone-complexed DNA fragments: Cell Death Detection Plus ELISA (Roche Applied Science) at absorbance 405 nm following the manufacturer's protocol. Caspase 3 activation was measured by assessing cleavage of caspase 3 (caspase 3 antibody, Stressgen, Victoria, Canada) (29). *In vivo* studies used the TUNEL assay to end-label DNA strand breaks: *In situ* Cell Death Detection kit, TMR Red (Roche Applied Science). Nuclear staining of kidney sections was also used to assess *in vivo* apoptosis by staining sections with DAPI. Apoptotic nuclei are detected as small, condensed, symmetrical spheres next to a nucleus; this technique has been used to analyze kidney glomerular apoptosis (30–32). Kidney sections derived from the anti-mouse mesangial cell serum glomerulonephropathy model (33) were provided by Dr. Jeremy Duffield (Brigham and Women's Hospital, Boston, MA).

Magnetic Bead-pulling—4.5 μm Dynal (Invitrogen) magnetic beads were coated with RGD peptide (Integra LS, San Diego, CA). Podocytes were serum-starved in RPMI-alone medium for 24 h prior to experiments. RGD-coated beads were then applied to cells for 10–20 min and an electromagnet (350 pN force) was applied to beads for a 2-s pulse. Bead displacement was measured using IP Lab as described by Matthews *et al.* (34).

Analysis of Results—All results depicted in graphs are averages ± S.E. or S.D. Comparisons were analyzed by the two-tailed Student's *t* test. $p < 0.05$ was considered significant.

RESULTS

WT1, a Podocyte Marker, Detected in the Urine of Actn4-deficient Mice—Urinary podocytes have been reported in various human and rodent glomerulopathies (20, 35). We looked for urinary podocyte markers as an indicator of podocyte detachment *in vivo* in Actn4$^{-/-}$ mice. WT1 is a transcription factor implicated in glomerular disease that specifically localizes to glomerular podocytes (36–40). We detected WT1 protein in the urine of Actn4$^{-/-}$ mice but not in urine from wild-type littermates. Fig. 1*A* shows a representative Western blot performed using mouse urinary protein. To detect WT1 expression by Western blot, we used a mouse monoclonal antibody previously shown to be specific for podocytes of mature kidneys (21, 27). We have detected urinary podocytes from Actn4$^{-/-}$ mice as early as 8 days of age. We

have only observed urinary WT1 in Actn4$^{-/-}$ mice (80%) but never in Actn4-expressing mice. In the early age mice group (1 week), we detected urinary WT1 in 75% of Actn4$^{-/-}$ mice and in the late age mice cohort (6–10-week-old), we detected urinary WT1 in 83% of mice. We also detected the presence of podocin, another podocyte-specific marker, in a subset of urine samples collected from Actn4$^{-/-}$ mice (data not shown). We previously reported that Actn4$^{-/-}$ mice have progressive proteinuria (13). We assayed urine for protein and compared the age of onset of proteinuria with the age of onset of podocyturia. Fig. 1*B* summarizes the proteinuria (albuminuria) in Actn4$^{-/-}$ mice as compared with controls and Fig. 1*C* summarizes the albumin/creatinine ratio. Both albuminuria and albumin/creatinine ratio are used as clinical parameters in determining the extent of urine protein concentration in patient's urine. Again, only Actn4$^{-/-}$ mice had proteinuria as compared with WT control littermates at both age groups ($p < 0.002$) (Fig. 1, *B* and *C*). Moreover, all Actn4$^{-/-}$ mice exhibiting podocyturia showed some degree of proteinuria and *vice versa*. Thus, podocyturia and proteinuria appear to develop concurrently. None of the wild-type control littermates exhibited detectable proteinuria (Fig. 1, *B* and *C*).

Podocyte Number per Glomerulus Is Decreased in Actn4-deficient Mice—To correlate urine podocytes with podocyte detachment *in vivo*, we compared relative podocyte number per glomerulus of Actn4$^{-/-}$ mice *versus* Actn4$^{+/+}$ mice. There have been numerous reports detailing methods to estimate podocyte number and podocyte loss (5, 21, 27, 41). It is difficult to determine an exact number of podocytes per glomerulus given the complex capillary loop structure on which the podocytes reside. Most techniques rely on using many cross-sections from a given kidney. To obtain a relative measurement of podocytes per glomerulus for comparisons, we used WT1 staining as a marker of podocytes in 3–4-micron thick kidney sections. WT1 has been used before for counting podocytes by approximating the renal glomerulus to be a sphere and assessing volume using equations derived by Weibel and Gomez (22, 27, 42). Kidney sections were also co-stained for DAPI. Two different investigators used colocalization of WT1 and DAPI to identify podocytes (Fig. 2*A*). We found a 9.9% ($p = 0.02$) decrease in podocytes per glomerulus in day 8 Actn4$^{-/-}$ mice compared with day 8 Actn4$^{+/+}$ mice (Fig. 2*B*). At 6–10 weeks of age, similar assays revealed an increase in the difference in podocyte number per glomerulus (18.8%, $p = 0.008$) (Fig. 2*B*). The 10–20% podocyte loss in podocyte number/glomeruli of young Actn4$^{-/-}$ mice, while not large in absolute magnitude of the change, is similar to what has been observed in other related models. Wharram *et al.* (22) recently described the direct effect of podocyte loss in rat kidneys. They were able to control podocyte loss because rats were engineered to express the diphtheria toxin receptor only on podocytes, rendering these cells susceptible to the toxin. 0–20% podocyte loss caused clear glomerular changes including proteinuria and mesangial cell expansion (22). 21–40% podocyte loss in this model leads to focal segmental glomerulosclerosis and >40% leads to global glomerulosclerosis (22). While we believe the trend toward podocyte loss in Actn4$^{-/-}$ mice would increase with age, we limited our analysis of glomeruli studied to prior to onset of major glomerulosclerosis because significant scarring of glomeruli and altered podocyte morphology makes interpretation of the presence or absence of podocytes unreliable. Other groups have also found small but reproducible podocyte losses in other animal models of glomerular disease. For example, Petermann *et al.* (35) also used WT1 to count podocytes in a rat model of experimental membranous nephropathy and by 1 month of age, observed a 20% loss of glomerular podocytes *in vivo*. Susztak *et al.* (43) observed podocyte depletion in type I and type II diabetic mouse models in the range of 27–37%.

α-Actinin-4 Mediates Adhesion of Podocytes to Components of Glomerular Basement Membrane—To understand the mechanism of glomerular disease, we generated Actn4-deficient podocytes from Actn4-deficient mice according to the protocol of Mundel *et al.* (38). We generated two Actn4$^{-/-}$ lines and two Actn4$^{+/+}$ lines from littermates. The podocytes were conditionally immortalized using a temperature-sensitive, interferon-γ (IFN-γ) inducible SV40 transgene. With this system, SV40 expression is turned off when cells are grown at the restrictive temperature of 37 °C and IFN-γ is removed from the medium (Fig. 3*A*). In addition, synaptopodin expression is increased as cells are switched to the restrictive temperature and adopt a differentiated phenotype (Fig. 3*B*). The Actn4$^{-/-}$ cells express podocyte specific markers, including nephrin, podocin, and synaptopodin. Similar to our previous reports of immunostaining in Actn4$^{-/-}$ kidneys (13), we did not observe a difference in expression of podocyte markers between wild-type and Actn4$^{-/-}$ podocyte cell lines (Fig. 3*C*).

We measured the attachment of differentiated podocytes to various extracellular matrix proteins similar to the procedures previously described for rat glomerular epithelial cells (44). Compared with the two Actn4$^{+/+}$ podocyte lines, both of the Actn4$^{-/-}$ podocyte lines were less adherent to two glomerular basement membrane components: collagen IV (2.7-fold decrease, $p < 0.01$) and laminin 10/11 (3.1-fold decrease, $p < 0.03$) (Fig. 4*A*) (45). We also observed a trend toward decreased adhesion of Actn4 knock-out podocytes to collagen I (2.7-fold, $p < 3.7 \times 10^{-5}$), laminin I (1.6-fold, $p = 0.03$), fibronectin (1.9-fold, $p = 0.03$), and vitronectin (1.9-fold, $p = 0.01$) (supplementary Fig. S1).

As a complementary test of cell adhesion that does not involve cell trypsinization, we applied shear stress to cells allowed to differentiate on a collagen I-coated plate. We subjected cells to high shear stress via fluid flow, and using time-lapse microscopy, observed the ability of cells to adhere to substrate under this flow. Again Actn4-deficient cells were markedly less adherent when subjected to shear stress as compared with Actn4-deficient cells rescued by stable expression of α-actinin-4-GFP (Fig. 4, *B* and *C*). We cannot rule out the possibility that matrix secretion by Actn4 deficient cells *versus* rescued Actn4-deficient cells may in fact be changed as podocytes differentiate and that loss of Actn4 may affect podocyte attachment as well as podocyte matrix formation.

We ruled out increased apoptosis as a significant cause of decreased cell adhesion in Actn4$^{-/-}$ podocytes as there was no significant change in DNA fragmentation ELISA assays between wild-type and knock-out podocytes (WT = 0.33 *versus* Actn4$^{-/-}$ 0.35, $p = 0.8$) (supplemental Fig. S2*A*). As a test of *in vivo* apoptosis, we also tested Actn4$^{-/-}$ mouse glomerular

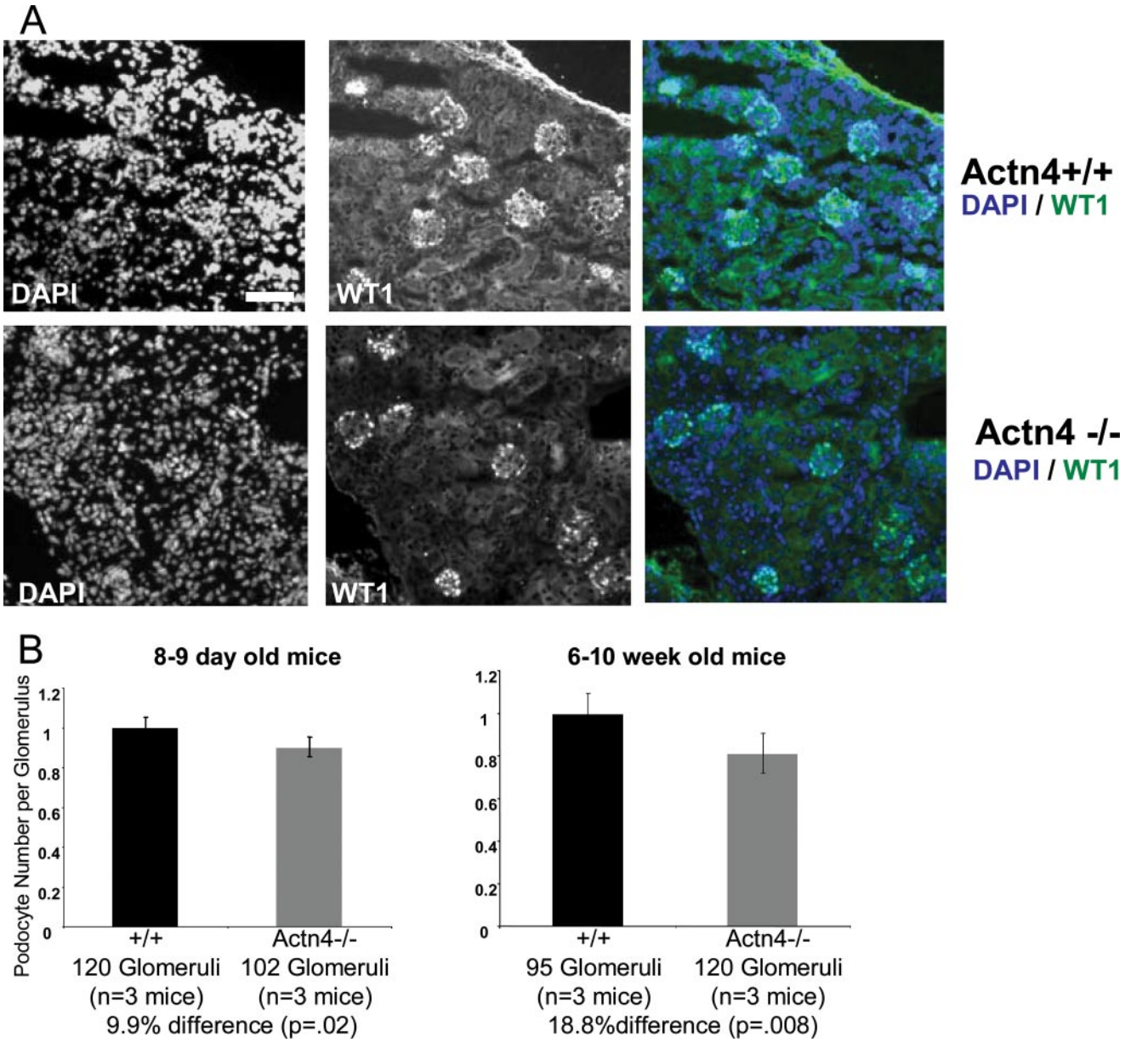

FIGURE 2. **Podocyte number per glomerulus is decreased in Actn4$^{-/-}$ mice.** *A*, representative immunohistochemistry of glomeruli in 3–4-micron sections stained for WT1 and DAPI in both control and Actn4−/− mice. *Scale bar*, 50 microns (×200 magnification). *B*, we estimated podocyte number per glomerulus by counting glomerular cells that were co-stained for WT1 and DAPI. The figure shows summary of podocyte numbers per glomerulus in both control and Actn4$^{-/-}$ mice at two different ages. At 8–9 days of age, Actn4-deficient mice had 9.9% ($p = 0.02$) less podocytes per glomerulus compared with control littermates. At 6–10 weeks of age, Actn4-deficient mice had 18.8% ($p = 0.008$) less podocytes per glomerulus compared with control littermates. The graph has been normalized with average control podocyte number denoted as 1 (*i.e.* normal podocyte per glomerulus). Both mice at the 1 week and 6–10 week cohort were subjected to statistical analysis by comparing all podocyte numbers/glomeruli with a minimum of 95 glomeruli per group of mice analyzed.

lysates and again found no statistically significant change (WT = 0.14 *versus* Actn4$^{-/-}$ 0.19, $p = 0.7$) (supplemental Fig. S2*A*). As a complementary test of apoptosis in the Actn4$^{-/-}$ podocytes, we assessed caspase 3 activation. Caspase 3 is cleaved when activated by a number of upstream effectors including caspase 8, 10, and TGF-β (29, 32, 46). We observed no significant caspase 3 cleavage in either the two WT or the two Actn4$^{-/-}$ podocyte cell lines. As a positive control, we serum-starved control podocytes and were able to detect cleaved/activated caspase 3 (20 and 18 kDa on immunoblot) in these cells as well as from dead podocytes collected from the medium.

In addition, *in vivo* TUNEL assays also showed no difference between wild-type and Actn4-deficient mice (supplementary Fig. S3). As a positive control for the TUNEL assay, we treated WT control kidney sections with DNAseI and were able to detect positive TUNEL stain in the kidney. Yet another method to detect significant apoptosis *in vivo* is to observe the presence of nuclear condensation, a characteristic morphologic change in apoptotic nuclei. Apoptotic nuclei are observed to be small, condensed, symmetrical spheres next to a nucleus. This technique has been widely used to analyze kidney glomerular apoptosis (30–32, 47–51). As a positive control, we also stained the nuclei of a known

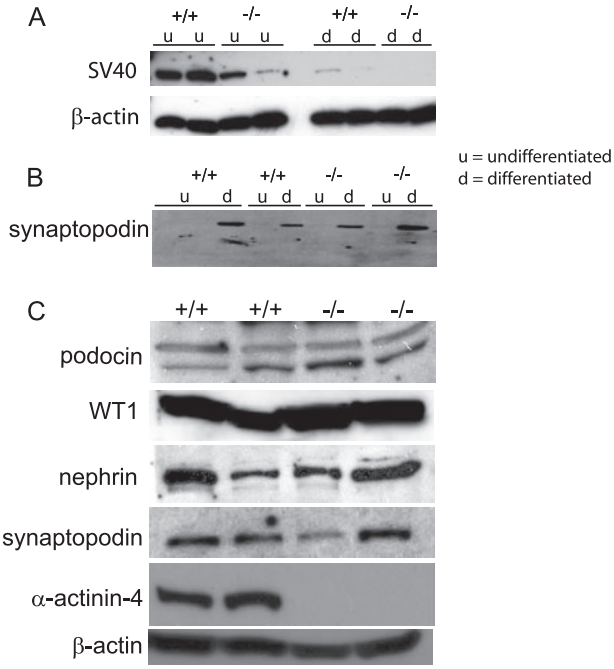

FIGURE 3. **Conditionally immortalized Actn4$^{-/-}$ podocytes express characteristic podocytes markers.** *A*, differentiation of podocytes: SV40 expression is down-regulated when temperature is switched from 33 to 37 °C and IFN-γ is removed from medium in podocyte cell lines (*u*, undifferentiated cells grown at 33 °C, *d*, differentiated cells). *B*, synaptopodin expression is increased as podocytes differentiate in cell culture at the restrictive temperature of 37 °C. *C*, immunoblot of podocyte markers. Expression of podocyte markers (podocin, nephrin, synaptopodin) is unchanged in differentiated Actn4−/− podocytes (2 separate clonal lines) as compared with wild-type control podocytes (2 separate clonal lines). Actn4-deficient podocytes have no detectable expression of α-actinin-4 protein.

model of kidney glomerular apoptosis (anti-mouse mesangial cell serum glomerulonephropathy (33)) as a reference for morphological identification of apoptotic nuclei in the kidney sections. Two investigators examined the Actn4$^{-/-}$ kidney sections and found no apoptotic nuclei (supplementary Fig. S4).

In addition, both the control and Actn4$^{-/-}$ cell lines were found to proliferate at similar rates while progressing from undifferentiated to the differentiated state (data not shown). Thus, we conclude that the decreased adhesive nature of the Actn4-deficient podocytes is specific and not because of changes in cell death or proliferation.

Integrin Expression in Actn4-deficient Podocytes—The integrin family of cell adhesion receptors is critical in mediating attachment of cells to basement membranes. α3β1-Integrin has been shown to be expressed on glomerular podocytes and to be important in attaching podocytes to the glomerular basement membrane components (44, 52–57). In particular, anti-Fx1A, an α3β1-integrin blocking antibody was found to block adhesion of rat glomerular epithelial cells to collagen, laminin, and fibronectin with the greatest observed effect on blocking adhesion to collagen IV (44). α3-Integrin knock-out mice have both abnormal kidney and lung development and survive only until the neonatal period (58). Within the glomerulus, α3-integrin knock-out mice have disorganized glomerular basement membranes and podocytes with immature foot processes (58). We therefore tested the hypothesis that altered expression of this receptor in Actn4$^{-/-}$ podocytes may mediate the decreased adhesion observed in cell culture and *in vivo*. Immunoblotting showed no difference in expression of either the α3- and β1-integrin subunits (supplementary Fig. S5).

We next tested the hypothesis that loss of Actn4 may lead to improper cell surface expression of α3β1-integrin. FACS analysis again revealed no difference in β1-integrin between wild-type and Actn4−/− podocytes (Fig. 5*A*). We observed a small trend toward increased expression of α3-integrin (Fig. 5*B*) and αV-integrin (Fig. 5*C*) in Actn4$^{-/-}$ podocytes. These results were not statistically significant (α3-integrin, 22% increase, $p = 0.16$, αV, 30% increase, $p = 0.16$) (Fig. 5*E*). We also screened β3-integrin expression as this integrin has been reported in the glomerulus and associates with αV-integrin (59, 60). We observed no difference in cell surface expression of β3-integrin (Fig. 5*D*). While αV-integrin showed robust expression in wild-type and Actn4-deficient podocytes, β3-integrin was not significantly expressed in either cell type (Fig. 5*D*).

Actn4-deficient Podocytes Have Weaker Integrin-Cytoskeleton Linkages—α-Actinin-4 is expressed in the foot processes of human podocytes near the basement membrane, as detected by immunoelectron microscopy (61). α-Actinins also interact directly with the cytoplasmic domain of β-integrins (16, 19) within focal adhesion anchoring complexes that govern the strength of cell adhesions (62). We tested the strength of integrin-cytoskeleton linkages in control and Actn4$^{-/-}$ podocytes by applying controlled mechanical stresses (2 s, 350 pN) to magnetic microbeads (4.5-μm diameter) coated with a synthetic RGD peptide that binds to integrin receptors on the cell surface of podocytes using magnetic pulling cytometry (Fig. 6, *A* and *B* and supplementary Video 1A) (34).

Past studies have demonstrated that stress-induced bead displacements are directly related to the degree of structural and mechanical coupling between integrins and the cytoskeleton through bead-associated focal adhesion proteins (34). Immunofluorescence microscopy confirmed that vinculin, a focal adhesion protein that binds α-actinin (17, 63), was recruited to the site of RGD bead binding on the podocyte membrane (Fig. 6*B*), confirming that binding of these beads induces focal adhesion formation as observed in many other cell types (47–49). Bright field images recorded before and during force application to beads bound to control and Actn4-deficient cells revealed that the integrin-cytoskeleton linkages in knock-out cells were significantly weaker, and hence displaced more than those in control cells (Fig. 6, *C* and *D* and supplementary Video 1, *B* and *C*). We performed these displacement measurements in 69 individual wild-type cells and in 84 different Actn4$^{-/-}$ cells. The difference we observed was striking in that RGD beads displaced 4-fold more in Actn4-deficient podocytes compared with control podocytes ($p = 3 \times 10^{-6}$). These results suggest that α-actinin-4 and integrins normally interact to strengthen focal adhesions and cell-matrix adhesion in podocytes, and that loss of α-actinin-4 results in reduced structural stability.

Integrin Phosphorylation Is Altered in Actn4-deficient Podocytes—To understand the mechanism of podocyte detachment, we performed a screen for alterations in the activation of signaling molecules in Actn4$^{-/-}$ podocytes and control cells

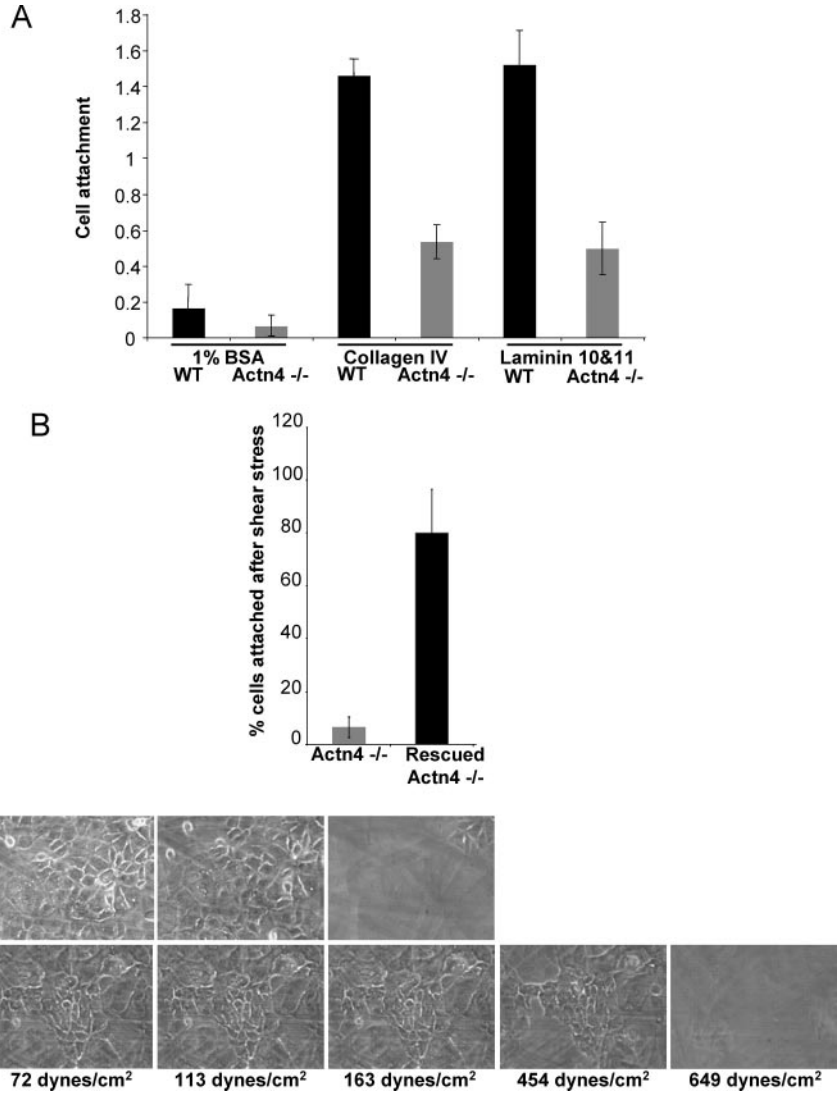

FIGURE 4. **Decreased attachment of Actn4$^{-/-}$ podocytes to components of the glomerular basement membrane.** Podocytes were allowed to differentiate at subconfluent levels for a minimum of 10 days at the restrictive temperature, 37 °C. Afterward, cells were replated on various matrix proteins for 1 h: matrix proteins tested included: collagen I, collagen IV, laminin 10/11 (placental), fibronectin, vitronectin, or laminin-1 with 1% bovine serum albumin as the negative control. Because of day-to-day variation in experimental setup, we normalized experiments to compare repetitions of the assay for three independent experiments. *A*, results of Collagen IV, laminin 10 and 11, and 1% bovine serum albumin are shown. Actn4-deficient podocytes had statistically significant decreased adherence to collagen IV (2.7-fold, $p = 0.01$) and laminin 10 and 11 (3-fold, $p = 0.03$). *B*, cells were allowed to differentiate for a minimum of 6 days at the restrictive temperature. We applied increasing shear stress to cells until point of detachment. Graph shows average results of three independent experiments. Again, graph shows that Actn4-deficient podocytes were markedly less adherent than rescued Actn4-deficient podocytes stably transfected with Actn4-GFP (*rescued cells*). The percentage of cells remaining adherent after flow was 6.5% for Actn4-deficient podocytes compared with 80% for rescued cells ($p = 0.01$). *C*, example of shear stresses applied to both Actn4-deficient cells and rescued cells. Actn4-deficient cells detached at a lower shear stress than rescued cells.

podocytes rescued the expression of the α-actinin-4 protein as well as the phosphorylation of β1-integrin (Fig. 7, *B* and *C*). Our signaling molecule screen showed no significant differences in the activation of other signaling pathways implicated in adhesion, including the integrin signaling pathway FAK (specific epitopes analyzed in Kinexus screen included Ser^{722}, Ser^{843}, Tyr^{576}, and Tyr^{861}).

DISCUSSION

Taken together, our results suggest that the interaction between α-actinin-4 and integrin adhesion receptors is critical for maintaining strong podocyte adhesions to the glomerular basement membrane. We have performed a number of experiments to examine both the proximal biochemical and the downstream phenotypic features of the role of α-actinin-4 in regulating this physical interaction. *In vivo*, we were able to detect WT1, a podocyte protein, in the urine of Actn4$^{-/-}$ mice. This correlated with a decrease in podocyte number per glomerulus in the kidneys of these mice. We furthermore showed that podocytes generated from Actn4$^{-/-}$ had decreased adhesion to glomerular basement membrane components. Additionally, we were able to rescue the adhesion deficit by transfecting Actn4-deficient podocytes with α-actinin-4-GFP and observing that the rescued cells were more resistant to shear stress than Actn4-deficient cells. Both the *in vivo* and cell line studies suggest that α-actinin-4 is important in podocyte attachment to matrix. We observed that while Actn4 deficient podocytes show no change in the total cell surface expression of β1-integrin, the major integrin implicated in mediating podocyte-matrix adhesion, the integrin-cytoskeleton linkage in Actn4$^{-/-}$ cells was less resistant to stress as measured using magnetic pulling cytometry. Lastly, we observed a marked difference in the phosphorylation state of β1-integrin in Actn4$^{-/-}$ cells, suggesting that α-actinin-4 is required for proper integrin signaling. This alteration was reversible upon transient transfection of the Actn4$^{-/-}$ cells with wild-type Actn4.

Although human ACTN4 mutations cause disease in the heterozygous state via dominant inheritance, it is clear from the

(Kinexus). Phosphorylation of serine 785 within the intracellular domain of β1-integrin has been reported to promote attachment to laminin in two different cell lines: GD25 (fibroblast cell line) and F9 (teratocarcinoma cell line) (64, 65). In differentiated α-actinin-4-deficient podocytes, we observed loss of phosphorylation of β1-integrin at serine 785. In repeated experiments, we consistently found that phosphorylation of this site was decreased in Actn4$^{-/-}$ podocytes (Fig. 7*A*). Similar results were obtained for undifferentiated Actn4$^{-/-}$ podocytes (data not shown). Transient transfection of Actn4 into Actn4$^{-/-}$

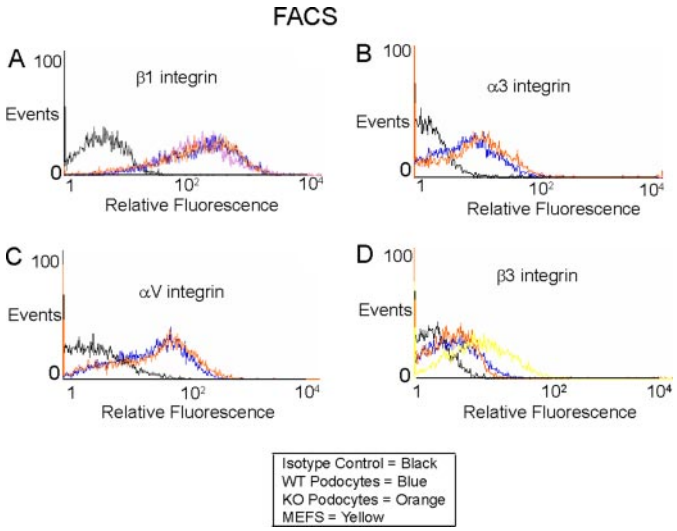

FIGURE 5. **Integrin expression in Actn4$^{-/-}$ podocytes.** We used FACS analysis to detect cell surface expression of integrins. *Black*, isotype control; *blue*, WT podocytes; *orange*, KO Podocytes; *yellow*, mouse embryo fibroblasts (MEFs). *A*, FACS was used to assess proper membrane expression of β1-integrin receptor. We found that cell surface expression of β1-integrin is essentially unchanged in Actn4$^{-/-}$ podocytes (geometric mean (mean fluorescence index) of replicates for WT *versus* KO: 170 *versus* 183, $p = 0.57$). *B*, FACS showed that Actn4-deficient podocytes had 22% more cell surface specific expression of α3-integrin but that this result was not statistically significant ($p = 0.16$). *C*, FACS also showed a similar increase in αV-integrin (30% increase) but again this was not statistically significant ($p = 0.16$). *D*, there was no difference in cell surface expression of β3-integrin expression. As a positive control for β3-integrin, β3-integrin expression in wild-type MEFs (mouse embryo fibroblasts) is also depicted.

present as well as our earlier studies that α-actinin-4 plays a critical and non-redundant role in glomerular function. The marked alteration in integrin-mediated adhesion also suggests different cellular functions for the two highly homologous proteins α-actinin-1 and α-actinin-4, both of which are expressed in mouse podocytes (13).

Does the absence of α-actinin-4 cause glomerulosclerosis as a direct effect of decreased podocyte adhesion to GBM and consequent podocyte loss? Recent genetic studies in rodents have convincingly demonstrated that podocyte loss itself can lead to glomerulosclerosis (22, 66). Thus while α-actinin-4 deficiency may have other effects, loss of podocyte adhesion is sufficient to explain the renal disease in Actn4$^{-/-}$ mice. Although apoptosis has been suggested to be an important mechanism for podocyte detachment in some animal models and human disease states (41, 67–70), we did not find evidence for increased apoptosis in α-actinin-4-deficient cells. Moreover, we did not detect any glomerular apoptosis in Actn4-deficient mice. Furthermore, we did not observe alterations in the slit-diaphragm complex proteins either in cells or *in situ*. Rather, the data presented suggest that podocyte loss can be a direct effect of altered adhesive properties of cells.

How does absence of α-actinin-4 cause altered podocyte adhesion to the glomerular basement membrane? Although the best defined function of α-actinin-4 is to crosslink and bundle actin filaments, the members of the α-actinin family interact with a large number of other molecules (71). Among these is a well-described interaction with the intracellular domain of β1-integrin. In the absence of α-actinin-4 expression, serine 785 phosphorylation of β1-integrin is greatly reduced. This

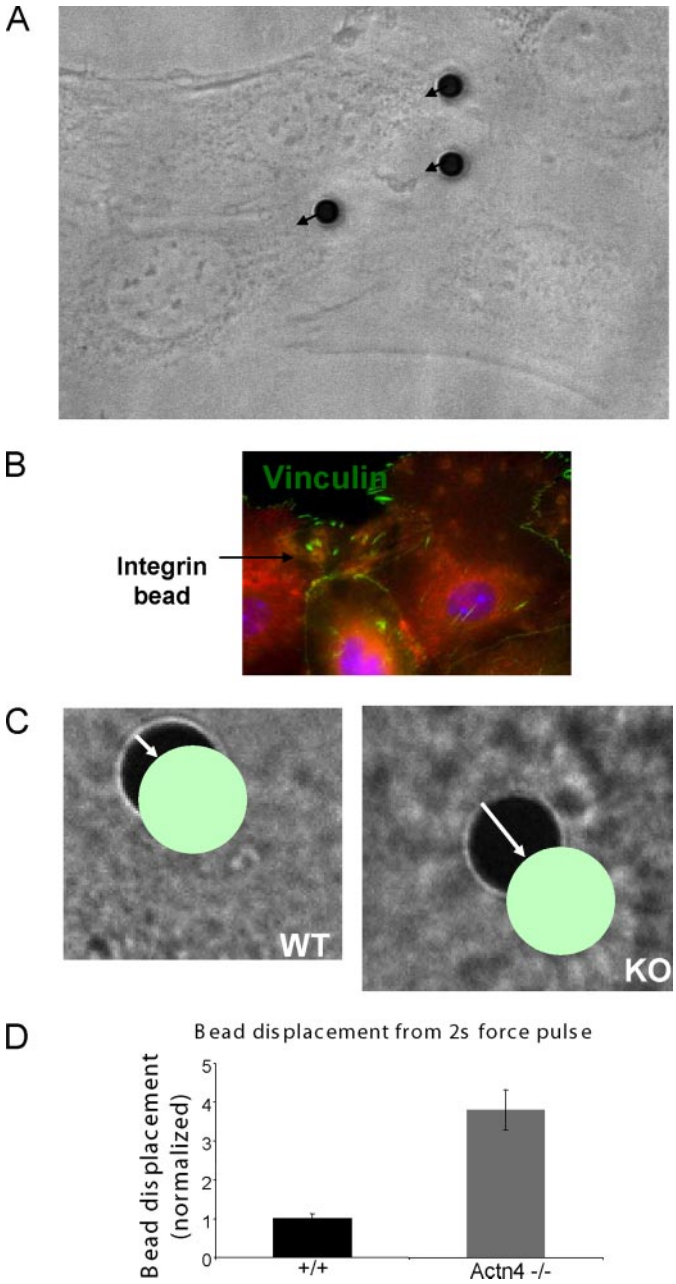

FIGURE 6. **Actn4$^{-/-}$ podocytes have weaker integrin-cytoskeleton linkages.** To test the strength of integrin-cytoskeleton linkages in Actn4-deficient podocytes, RGD-coated beads were applied to cells for 10–20 min and an electromagnet (350 pN force) was applied to beads for a 2-s pulse. 69 independent measurements were performed in control cells; 84 measurements were made in Actn4$^{-/-}$ cells. *A*, representative figure showing three beads binding to the apical surface of podocytes grown in a confluent monolayer. *Arrows* depict the movement of the beads in response to force. (Supplementary Video 1A shows movement of beads to the left.) *B*, example of an RGD-coated bead recruiting vinculin, a focal adhesion protein known to bind α-actinins, to the apical surface of a podocyte. *C*, representative images of one RGD-coated bead bound to wild-type and Actn4-deficient podocytes. Actn4$^{-/-}$ podocytes had more bead displacement (*greater arrow length*) suggesting weaker integrin-cytoskeleton linkages on Actn4$^{-/-}$ podocytes. *Arrows* indicate bead displacement (see also supplementary Video 1B and 1C). *D*, graph depicts average bead binding of Actn4−/− *versus* controls. Actn4-deficient podocytes had 4-fold more bead displacement as compared with wild-type podocytes ($p = 3 \times 10^{-6}$).

β1-integrin phosphorylation is restored by transient transfection of Actn4 into Actn4$^{-/-}$ cells. We suspect that in podocytes, α-actinin-4 may act as a scaffolding protein, allowing

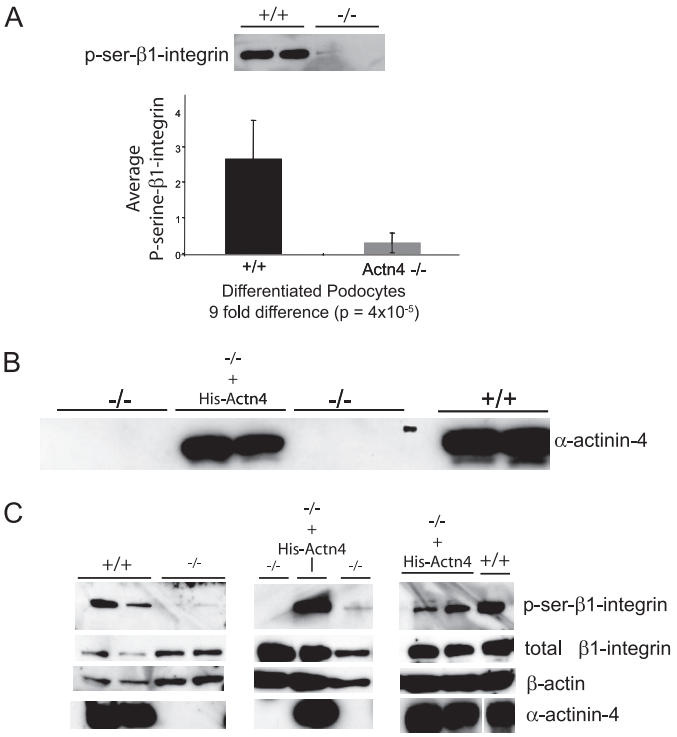

FIGURE 7. **Serine 785 phosphorylation of β1-integrin is altered in differentiated Actn4$^{-/-}$ podocytes.** *A*, graph depicting average serine 785 phosphorylation levels of β1-integrin in differentiated Actn4$^{-/-}$ podocytes as compared with controls. The graph summarizes results from four independent experiments. Representative immunoblot of serine 785 phosphorylation of β1-integrin in differentiated Actn4$^{-/-}$ podocytes and wild-type controls is provided (see also *D*) as an illustrative reference for graph. Phosphospecific antibody to serine 785 of β1-integrin was from BIOSOURCE and tested by the manufacturer for specificity using purified phosphopeptides and cells expressing mutant S785A as negative control. *B*, transient transfection of His-tagged-Actn4 into Actn4$^{-/-}$ podocytes restores Actn4 expression though not to the level of the Actn4$^{+/+}$ control podocytes. *C*, transfection of Actn4 into Actn4$^{-/-}$ podocytes as shown in *C* rescues the serine 785 phosphorylation of β1-integrin (though not to the level of the Actn4$^{+/+}$ control). This figure compares levels of serine 785 phosphorylation of β1-integrin between WT and Actn4−/− podocytes using total β1-integrin as a control and β-actin as a loading control. For reference, α-actinin-4 blots are also depicted.

proper regulation of β1-integrin activation. β1-integrin has previously shown to be phosphorylated at intracellular tyrosine, serine, and threonine sites although the significance of the various phosphorylation sites appear to have different implications for adhesion and cell motility in different cell lines (72–78). Serine 785 has been shown to promote attachment to laminin in two different cell types (fibroblast and cancer cell lines) and to inhibit cell migration in these two cell lines (65). Interestingly, Reiser *et al.* (90) have also observed that podocyte migration may be involved in puromycin mediated detachment suggesting that podocyte attachment and cell migration are two distinct processes, the first being beneficial while the second being detrimental to the glomerulus. The decreased phosphorylation of serine 785 in Actn4-deficient podocytes is a mechanism that supports decreased adhesion of Actn4-deficient podocytes when compared with wild-type control podocytes.

ILK, a protein initially described as binding and phosphorylating the β1-integrin cytoplasmic domain may also play a role in Actn4-mediated attachment of podocytes (79, 80). In ILK-β1 immune complex kinase assays, ILK phosphorylated the serine and threonine resides of β1-integrin and these complexes colocalized with FAK complexes suggesting a link with serine/threonine phosphorylation of β1-integrin and adhesion at focal contact sites (79). One focal adhesion molecule, affixin, which has been shown to be phosphorylated by ILK *in vitro*, has also been shown to bind and recruit α-actinin to focal complexes when stimulated by cell-substrate adhesion; the recruitment of α-actinin then leads to reorganization and polymerization of F-actin at focal adhesions (81, 82). Yamaji *et al.* (82) further show that the affixin-α-actinin interaction is in part dependent on functional ILK kinase activity. Our results showing decreased serine phosphorylation of β1-integrin also implicate functional ILK signaling in proper Actn4-mediated podocyte attachment.

Structural studies also suggest potential mechanisms by which α-actinins bind and activate β1-integrins. α-Actinin-4 forms a head-to-tail rod shaped homodimer, with actin binding domains at both ends. The integrin binding domain lies within the central rod domain (16). In a recent report, Kelly and Taylor (83) reconstituted focal adhesions in lipid bilayers and demonstrated that one α-actinin dimer can bind to two β1-integrin cytoplasmic domains, suggesting one possible mechanism by which α-actinin may facilitate integrin clustering and signaling. In an embryonic carcinoma cell line, α-actinin-4 has been shown to promote mechanical stiffness and stabilization of focal adhesions (84). Rajfur *et al.* (85) used CALI to show that inactivation of α-actinin-4-GFP in the focal adhesions of NIH 3T3 cells causes stress fiber retraction but not loss of focal adhesion integrity. Recently, Zhang and Gunst (86) proposed that α-actinin recruitment to adhesion complexes in adhesion sites is critical for acetylcholine mediated smooth muscle contraction; they also show that while inhibition of α-actinin/β1-integrin interactions does not prevent recruitment of vinculin, paxillin, or talin, it does prevent "active tension development" of smooth muscle cells (86). This is consistent with the renal phenotype we observe in Actn4$^{-/-}$ mice: normal podocytes at birth, but later deterioration and podocyte loss, which, we suggest, results from weaker α-actinin-4 mediated integrin-cytoskeleton linkage.

In summary, our data suggest that α-actinin-4 is required for proper β1-integrin signaling and for β1-integrin-mediated adhesion. In addition, our results support the hypothesis that glomerulosclerosis and proteinuria in α-actinin-4-deficient mice is a result of decreased podocyte adhesion, loss of glomerular podocytes, and its consequences. Further studies will be required to elucidate the precise biochemical mechanism by which α-actinin-4 regulates β1-integrin activation. Such studies should be of significant value, as they may suggest ways in which the podocyte-GBM interaction can be modulated for therapeutic benefit.

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REFERENCES

1. Kriz, W., Gretz, N., and Lemley, K. V. (1998) *Kidney Int.* **54,** 687–697
2. Kriz, W. (1997) *Adv. Nephrol. Necker Hosp.* **27,** 1–13
3. Ichikawa, I., and Fogo, A. (1996) *Pediatr. Nephrol.* **10,** 374–391
4. Somlo, S., and Mundel, P. (2000) *Nat. Genet.* **24,** 333–335
5. Wiggins, J. E., Goyal, M., Sanden, S. K., Wharram, B. L., Shedden, K. A.,

Misek, D. E., Kuick, R. D., and Wiggins, R. C. (2005) *J. Am. Soc. Nephrol.* **16,** 2953–2966
6. Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C. E., Peltonen, L., Holmberg, C., Olsen, A., and Tryggvason, K. (1998) *Mol. Cell* **1,** 575–582
7. Donoviel, D. B., Freed, D. D., Vogel, H., Potter, D. G., Hawkins, E., Barrish, J. P., Mathur, B. N., Turner, C. A., Geske, R., Montgomery, C. A., Starbuck, M., Brandt, M., Gupta, A., Ramirez-Solis, R., Zambrowicz, B. P., and Powell, D. R. (2001) *Mol. Cell Biol.* **21,** 4829–4836
8. Shih, N. Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M. L., Kanagawa, O., Miner, J. H., and Shaw, A. S. (1999) *Science* **286,** 312–315
9. Winn, M. P., Conlon, P. J., Lynn, K. L., Farrington, M. K., Creazzo, T., Hawkins, A. F., Daskalakis, N., Kwan, S. Y., Ebersviller, S., Burchette, J. L., Pericak-Vance, M. A., Howell, D. N., Vance, J. M., and Rosenberg, P. B. (2005) *Science* **308,** 1801–1804
10. Reiser, J., Polu, K. R., Moller, C. C., Kenlan, P., Altintas, M. M., Wei, C., Faul, C., Herbert, S., Villegas, I., Avila-Casado, C., McGee, M., Sugimoto, H., Brown, D., Kalluri, R., Mundel, P., Smith, P. L., Clapham, D. E., and Pollak, M. R. (2005) *Nat. Genet.* **37,** 739–744
11. Tsukaguchi, H., Sudhakar, A., Le, T. C., Nguyen, T., Yao, J., Schwimmer, J. A., Schachter, A. D., Poch, E., Abreu, P. F., Appel, G. B., Pereira, A. B., Kalluri, R., and Pollak, M. R. (2002) *J. Clin. Investig.* **110,** 1659–1666
12. Kaplan, J. M., Kim, S. H., North, K. N., Rennke, H., Correia, L. A., Tong, H. Q., Mathis, B. J., Rodriguez-Perez, J. C., Allen, P. G., Beggs, A. H., and Pollak, M. R. (2000) *Nat. Genet.* **24,** 251–256
13. Kos, C. H., Le, T. C., Sinha, S., Henderson, J. M., Kim, S. H., Sugimoto, H., Kalluri, R., Gerszten, R. E., and Pollak, M. R. (2003) *J. Clin. Invest.* **111,** 1683–1690
14. Yao, J., Le, T. C., Kos, C. H., Henderson, J. M., Allen, P. G., Denker, B. M., and Pollak, M. R. (2004) *PLoS Biol.* **2,** E167
15. Weins, A., Kenlan, P., Herbert, S., Le, T. C., Villegas, I., Kaplan, B. S., Appel, G. B., and Pollak, M. R. (2005) *J. Am. Soc. Nephrol.* **16,** 3694–3701
16. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) *J. Cell Biol.* **111,** 721–729
17. Zamir, E., and Geiger, B. (2001) *J. Cell Sci.* **114,** 3577–3579
18. Michaud, J. L., Lemieux, L. I., Dube, M., Vanderhyden, B. C., Robertson, S. J., and Kennedy, C. R. (2003) *J. Am. Soc. Nephrol.* **14,** 1200–1211
19. Bijian, K., and Cybulsky, A. V. (2005) *Contrib. Nephrol.* **148,** 8–20
20. Kim, Y. H., Goyal, M., Kurnit, D., Wharram, B., Wiggins, J., Holzman, L., Kershaw, D., and Wiggins, R. (2001) *Kidney Int.* **60,** 957–968
21. Petermann, A. T., Pippin, J., Krofft, R., Blonski, M., Griffin, S., Durvasula, R., and Shankland, S. J. (2004) *Nephron Exp. Nephrol.* **98,** e114–123
22. Wharram, B. L., Goyal, M., Wiggins, J. E., Sanden, S. K., Hussain, S., Filipiak, W. E., Saunders, T. L., Dysko, R. C., Kohno, K., Holzman, L. B., and Wiggins, R. C. (2005) *J. Am. Soc. Nephrol.* **16,** 2941–2952
23. Pagtalunan, M. E., Miller, P. L., Jumping-Eagle, S., Nelson, R. G., Myers, B. D., Rennke, H. G., Coplon, N. S., Sun, L., and Meyer, T. W. (1997) *J. Clin. Invest.* **99,** 342–348
24. Shirato, I., Hishiki, T., and Tomino, Y. (2001) *Contrib. Nephrol.* 69–73
25. Patari, A., Forsblom, C., Havana, M., Taipale, H., Groop, P. H., and Holthofer, H. (2003) *Diabetes* **52,** 2969–2974
26. White, K. E., and Bilous, R. O. B. (2004) *Nephrol. Dial. Transplant.* **19,** 1437–1440
27. Sanden, S. K., Wiggins, J. E., Goyal, M., Riggs, L. K., and Wiggins, R. C. (2003) *J. Am. Soc. Nephrol.* **14,** 2484–2493
28. Hamano, Y., Grunkemeyer, J. A., Sudhakar, A., Zeisberg, M., Cosgrove, D., Morello, R., Lee, B., Sugimoto, H., and Kalluri, R. (2002) *J. Biol. Chem.* **277,** 31154–31162
29. Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) *EMBO J.* **18,** 2040–2048
30. Kelly, K. J., Plotkin, Z., Vulgamott, S. L., and Dagher, P. C. (2003) *J. Am. Soc. Nephrol.* **14,** 128–138
31. Kelly, K. J., Plotkin, Z., and Dagher, P. C. (2001) *J. Clin. Invest.* **108,** 1291–1298
32. Schiffer, M., Mundel, P., Shaw, A. S., and Bottinger, E. P. (2004) *J. Biol. Chem.* **279,** 37004–37012
33. Yo, Y., Braun, M. C., Barisoni, L., Mobaraki, H., Lu, H., Shrivastav, S., Owens, J., and Kopp, J. B. (2003) *Nephron Exp. Nephrol.* **93,** e92–106
34. Matthews, B. D., Overby, D. R., Alenghat, F. J., Karavitis, J., Numaguchi, Y., Allen, P. G., and Ingber, D. E. (2004) *Biochem. Biophys. Res. Commun.* **313,** 758–764
35. Petermann, A. T., Krofft, R., Blonski, M., Hiromura, K., Vaughn, M., Pichler, R., Griffin, S., Wada, T., Pippin, J., Durvasula, R., and Shankland, S. J. (2003) *Kidney Int.* **64,** 1222–1231
36. Mundlos, S., Pelletier, J., Darveau, A., Bachmann, M., Winterpacht, A., and Zabel, B. (1993) *Development* **119,** 1329–1341
37. Bardeesy, N., Zabel, B., Schmitt, K., and Pelletier, J. (1994) *Genomics* **21,** 663–664
38. Mundel, P., Reiser, J., Zuniga Mejia Borja, A., Pavenstadt, H., Davidson, G. R., Kriz, W., and Zeller, R. (1997) *Exp. Cell Res.* **236,** 248–258
39. Demmer, L., Primack, W., Loik, V., Brown, R., Therville, N., and McElreavey, K. (1999) *J. Am. Soc. Nephrol.* **10,** 2215–2218
40. Patek, C. E., Fleming, S., Miles, C. G., Bellamy, C. O., Ladomery, M., Spraggon, L., Mullins, J., Hastie, N. D., and Hooper, M. L. (2003) *Hum. Mol. Genet.* **12,** 2379–2394
41. Schiffer, M., Bitzer, M., Roberts, I. S., Kopp, J. B., ten Dijke, P., Mundel, P., and Bottinger, E. P. (2001) *J. Clin. Investig.* **108,** 807–816
42. Weibel, E. R., and Gomez, D. M. (1962) *J. Appl. Physiol.* **17,** 343–348
43. Susztak, K., Raff, A. C., Schiffer, M., and Bottinger, E. P. (2006) *Diabetes* **55,** 225–233
44. Adler, S., and Chen, X. (1992) *Am. J. Physiol.* **262,** F770–F776
45. Ferletta, M., and Ekblom, P. (1999) *J. Cell Sci.* **112,** 1–10
46. Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) *J. Biol. Chem.* **273,** 27084–27090
47. Duffield, J. S., Ware, C. F., Ryffel, B., and Savill, J. (2001) *Am. J. Pathol.* **159,** 1397–1404
48. Duffield, J. S., Erwig, L. P., Wei, X., Liew, F. Y., Rees, A. J., and Savill, J. S. (2000) *J. Immunol.* **164,** 2110–2119
49. Sun, X. M., Snowden, R. T., Skilleter, D. N., Dinsdale, D., Ormerod, M. G., and Cohen, G. M. (1992) *Anal. Biochem.* **204,** 351–356
50. Wyllie, A. H., Beattie, G. J., and Hargreaves, A. D. (1981) *Histochem. J.* **13,** 681–692
51. Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980) *Int. Rev. Cytol.* **68,** 251–306
52. Adler, S. (1992) *Am. J. Physiol.* **262,** F697–F704
53. Krishnamurti, U., Chen, Y., Michael, A., Kim, Y., Fan, W. W., Wieslander, J., Brunmark, C., Rondeau, E., Sraer, J. D., Delarue, F., and Tsilibary, E. C. (1996) *Lab. Investig.* **74,** 650–657
54. Smoyer, W. E., Mundel, P., Gupta, A., and Welsh, M. J. (1997) *Am. J. Physiol.* **273,** F150–157
55. Kretzler, M. (2002) *Microsc. Res. Tech.* **57,** 247–253
56. Kagami, S., and Kondo, S. (2004) *J. Med. Investig.* **51,** 1–13
57. Pedchenko, V. K., Chetyrkin, S. V., Chuang, P., Ham, A. J., Saleem, M. A., Mathieson, P. W., Hudson, B. G., and Voziyan, P. A. (2005) *Diabetes* **54,** 2952–2960
58. Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C., and Jaenisch, R. (1996) *Development* **122,** 3537–3547
59. Mayer, G., Boileau, G., and Bendayan, M. (2003) *J. Cell Sci.* **116,** 1763–1773
60. Kreidberg, J. A., and Symons, J. M. (2000) *Am. J. Physiol. Renal Physiol.* **279,** F233–F242
61. Goode, N. P., Shires, M., Khan, T. N., and Mooney, A. F. (2004) *Nephrol. Dial. Transplant.* **19,** 844–851
62. Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V., and Wang, Y. L. (2001) *J. Cell Biol.* **153,** 881–888
63. Bois, P. R., Borgon, R. A., Vonrhein, C., and Izard, T. (2005) *Mol. Cell Biol.* **25,** 6112–6122
64. Mulrooney, J., Foley, K., Vineberg, S., Barreuther, M., and Grabel, L. (2000) *Exp. Cell Res.* **258,** 332–341
65. Mulrooney, J. P., Hong, T., and Grabel, L. B. (2001) *J. Cell Sci.* **114,** 2525–2533
66. Matsusaka, T., Xin, J., Niwa, S., Kobayashi, K., Akatsuka, A., Hashizume, H., Wang, Q. C., Pastan, I., Fogo, A. B., and Ichikawa, I. (2005) *J. Am. Soc.*

Nephrol. **16,** 1013–1023
67. Wolf, G., and Stahl, R. A. (2003) *Lancet* **362,** 1746–1748
68. Marshall, C. B., and Shankland, S. J. (2006) *Nephron Exp. Nephrol.* **102,** e39–e48
69. Durvasula, R. V., Petermann, A. T., Hiromura, K., Blonski, M., Pippin, J., Mundel, P., Pichler, R., Griffin, S., Couser, W. G., and Shankland, S. J. (2004) *Kidney Int.* **65,** 30–39
70. Lai, X. X., Ding, G. H., Huang, C. X., Shi, M., and Chen, C. (2004) *Beijing Da Xue Xue Bao* **36,** 131–134
71. Otey, C. A., and Carpen, O. (2004) *Cell Motil Cytoskeleton* **58,** 104–111
72. Barreuther, M. F., and Grabel, L. B. (1996) *Exp. Cell Res.* **222,** 10–15
73. Sakai, T., Zhang, Q., Fassler, R., and Mosher, D. F. (1998) *J. Cell Biol.* **141,** 527–538
74. Wennerberg, K., Fassler, R., Warmegard, B., and Johansson, S. (1998) *J. Cell Sci.* **111,** 1117–1126
75. Wennerberg, K., Armulik, A., Sakai, T., Karlsson, M., Fassler, R., Schaefer, E. M., Mosher, D. F., and Johansson, S. (2000) *Mol. Cell Biol.* **20,** 5758–5765
76. Attwell, S., Roskelley, C., and Dedhar, S. (2000) *Oncogene* **19,** 3811–3815
77. Attwell, S., Mills, J., Troussard, A., Wu, C., and Dedhar, S. (2003) *Mol. Biol. Cell* **14,** 4813–4825
78. Nilsson, S., Kaniowska, D., Brakebusch, C., Fassler, R., and Johansson, S. (2006) *Exp. Cell Res.* **312,** 844–853
79. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* **379,** 91–96
80. Wu, C., and Dedhar, S. (2001) *J. Cell Biol.* **155,** 505–510
81. Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., and Ishigatsubo, Y. (2001) *J. Cell Biol.* **153,** 1251–1264
82. Yamaji, S., Suzuki, A., Kanamori, H., Mishima, W., Yoshimi, R., Takasaki, H., Takabayashi, M., Fujimaki, K., Fujisawa, S., Ohno, S., and Ishigatsubo, Y. (2004) *J. Cell Biol.* **165,** 539–551
83. Kelly, D. F., and Taylor, K. A. (2005) *J. Struct. Biol.* **149,** 290–302
84. Ezzell, R. M., Goldmann, W. H., Wang, N., Parasharama, N., and Ingber, D. E. (1997) *Exp. Cell Res.* **231,** 14–26
85. Rajfur, Z., Roy, P., Otey, C., Romer, L., and Jacobson, K. (2002) *Nat. Cell Biol.* **4,** 286–293
86. Zhang, W., and Gunst, S. J. (2006) *J. Physiol.* **572,** 659–676
87. Chin, A. C., Vergnolle, N., MacNaughton, W. K., Wallace, J. L., Hollenberg, M. D., and Buret, A. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11104–11109
88. Rehn, M., Veikkola, T., Kukk-Valdre, E., Nakamura, H., Ilmonen, M., Lombardo, C., Pihlajaniemi, T., Alitalo, K., and Vuori, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1024–1029
89. Henriquez, J. P., Casar, J. C., Fuentealba, L., Carey, D. J., and Brandan, E. (2002) *J. Cell Sci.* **115,** 2041–2051
90. Reiser, J., Oh, J., Shirato, I., Asanuma, K., Hug, A., Mundel, T. M., Honey, K., Ishidoh, K., Kominami, E., Kreidberg, J. A., Tomino, Y., and Mundel, P. (2004) *J. Biol. Chem.* **279,** 34827–34832