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FSGS-associated α-actinin-4 (K256E) impairs cytoskeletal dynamics in podocytes

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Mutations in the *ACTN4* gene, encoding the actin crosslinking protein α-actinin-4, are associated with a familial form of focal segmental glomerulosclerosis (FSGS). Mice with podocyte-specific expression of K256E α-actinin-4 develop foot process effacement and glomerulosclerosis, highlighting the importance of the cytoskeleton in podocyte structure and function. K256E α-actinin-4 exhibits increased affinity for F-actin. However, the downstream effects of this aberrant binding on podocyte dynamics remain unclear. Wild-type and K256E α-actinin-4 were expressed in cultured podocytes via adenoviral infection to determine the effect of the mutation on α-actinin-4 subcellular localization and on cytoskeletal-dependent processes such as adhesion, spreading, migration, and formation of foot process-like peripheral projections. Wild-type α-actinin-4 was detected primarily in the Triton-soluble fraction of podocyte lysates and localized to membrane-associated cortical actin and focal adhesions, with some expression along stress fibers. Conversely, K256E α-actinin-4 was detected predominantly in the Triton-insoluble fraction, was excluded from cortical actin, and localized almost exclusively along stress fibers. Both wild-type and K256E α-actinin-4-expressing podocytes adhered equally to an extracellular matrix (collagen-I). However, podocytes expressing K256E α-actinin-4 showed a reduced ability to spread and migrate on collagen-I. Lastly, K256E α-actinin-4 expression reduced the mean number of actin-rich peripheral projections. Our data suggest that aberrant sequestering of K256E α-actinin-4 impairs podocyte spreading, motility, and reduces the number of peripheral projections. Such intrinsic cytoskeletal derangements may underlie initial podocyte damage and foot process effacement encountered in *ACTN4*-associated FSGS.

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Focal segmental glomerulosclerosis (FSGS) is a common glomerular lesion and a significant cause of end-stage renal disease.[1,2] Clinically, FSGS patients present with variable levels of proteinuria and a progressive loss of renal function. Pathologically, FSGS is characterized by segmental sclerosis in a proportion of glomeruli, the filtering units of the kidney.[3] Accumulating evidence suggests that defects in podocytes initiate processes leading to the degeneration of filtration integrity and the development of sclerotic lesions.[4–8]

Podocytes are terminally differentiated cells that line the outer aspects of the glomerular capillaries.[9] The highly ordered podocyte architecture consists of a cell body from which emerge major processes, which branch into foot processes that interdigitate with those of neighboring podocytes to provide the structural platform upon which a molecular sieve is formed. The foot processes are endowed with a microfilament-based contractile apparatus composed of actin, myosin-II, α-actinin, talin, paxillin, and vinculin,[10] and are anchored to the glomerular basement membrane via an $\alpha_3\beta_1$-integrin complex.[11,12] The intricate morphology of the podocyte, coupled to its exposure to distensile forces within the glomerular capillary render these cells susceptible to damage in many nephropathies, including FSGS.

The actin bundling α-actinins are members of the spectrin superfamily. Four isoforms have been described (α-actinin-1 to -4).[13] While α-actinins-2 and -3 are expressed at the Z-line of striated muscle, α-actinins-1 and -4 are more ubiquitously expressed. α-Actinin-4 is expressed in podocytes and is thought to play a key role in the maintenance of this cell's architecture.[14] The putative function of α-actinin-4, which exists as a head-to-tail homodimer, is to crosslink actin filaments through its N-terminal actin-binding domain comprised of two calponin homology domains. Increasing evidence suggests that α-actinin-4 may interact with a number of other proteins such as β_1-integrin,[13,15,16] synaptopodin,[17] vinculin,[18] and phosphatidylinositol 3-kinase.[19] In addition to an actin-binding domain, a number of other functional domains are found in the α-actinin-4 sequence – including two calcium-binding EF hands, a phosphoinositide-binding domain, as well as a focal adhesion kinase (FAK) tyrosine phosphorylation consensus sequence. Accordingly, phosphorylation by FAK,[20] binding of phosphoinositides,[21,22]

and sensitivity to intracellular calcium[23] may modulate the actin-binding properties and localization of α-actinin following various environmental stimuli.

Mutations in the *ACTN4* gene (K228E, T232I, and S235P) are associated with an autosomal-dominant form of FSGS.[24,25] We developed a mouse model of *ACTN4*-associated FSGS by expressing the murine correlate of the K228E mutation (K256E) in a podocyte-specific manner using the *mNPHS1* promoter.[26] These mice exhibit significant proteinuria and develop FSGS-like lesions, confirming that the disease originates in the podocyte. However, the mechanism by which mutations in α-actinin-4 dysregulate podocyte function is not fully understood. Abrogation of α-actinin-4 expression in mice yields severe glomerular disease.[27] Furthermore, recent studies by Yao *et al.*[28] suggest that the familial mutations promote α-actinin-4 aggregation and thereby target the protein for degradation via the proteasome pathway, resulting in a partial loss-of-function. In contrast, mutations in α-actinin-4 increase its affinity for filamentous actin (F-actin), suggesting a gain-of-function mechanism.[24,26] In support of the latter mechanism, the severity of the FSGS-like phenotype correlates directly with K256E α-actinin-4 levels in transgenic mice.[26] Thus, it remains unclear whether and how these two viewpoints can be reconciled.

To address this issue, we assessed the functional consequences of an FSGS-associated mutation (K256E) in α-actinin-4 at the cellular level. We now report the intracellular mislocalization of K256E α-actinin-4 in mouse podocytes, which undermines the processes of cell spreading and migration, and impairs the formation of actin-rich peripheral projections. Our data suggest that such defects in key cytoskeletal-associated processes may compromise the podocyte's ability to cope with the demands of the glomerular environment, maintain foot processes structure, and thereby initiate the progression towards sclerosis.

RESULTS

Mislocalization of K256E α-actinin-4 in cultured podocytes

Mutations in α-actinin-4 increase its affinity for F-actin *in vitro*.[24,26] However, the subcellular localization of mutant α-actinin-4 is not clearly defined. We therefore generated adenoviral constructs with hemagglutinin (HA)-tagged wild-type or K256E α-actinin-4 and transduced a conditionally immortalized mouse podocyte cell line. Podocytes were infected with a range of virus to determine a concentration yielding efficient expression (Figure 1a). For all subsequent experiments, infections were performed with a multiplicity of infection (MOI) of 25 and incubated for 72 h. There was no apparent degradation of heterologously expressed K256E α-actinin-4 during this timeframe as its expression paralleled that of the wild-type protein (Figure 1a). As shown in Figure 1b, wild-type α-actinin-4 localized predominantly with cortical actin. The wild-type protein was also distributed along stress fibers and at focal adhesions, as identified by vinculin co-immunofluorescence (Figure 1c). Conversely, K256E α-actinin-4 was absent from the cell periphery, but was preferentially associated with stress fibers (Figure 1b) and focal adhesions (Figure 1c).

Differential association of K256E α-actinin-4 with intracellular actin pools

We next performed cellular fractionation experiments to determine the association of wild-type and K256E α-actinin-4 with various intracellular actin pools. Podocytes expressing wild-type or K256E α-actinin-4 were lysed in Triton X-100-containing buffer and subject to differential centrifugation. As shown in Figure 2a and b, only $16.0 \pm 2.5\%$ of the wild-type α-actinin-4 associated with large cytoskeletal structures (Triton X-100 insoluble (TI) fraction), whereas $71.4 \pm 5.5\%$ of the protein remained soluble (Triton X-100 soluble (TS):S fraction). Conversely, $81.0 \pm 9.6\%$ of the K256E α-actinin-4 was associated with large cytoskeletal structures (TI fraction), and only $4.9 \pm 3.4\%$ of the mutant protein remained soluble (TS:S fraction). Expression of both wild-type and K256E α-actinin-4 was similar, as evidenced by the input. Furthermore, neither the expression of wild-type nor K256E α-actinin-4 altered total actin levels. These data reveal a differential association of wild-type versus K256E α-actinin-4, with K256E α-actinin-4 sequestered to large cytoskeletal structures such as actin bundles, whereas wild-type α-actinin-4 remains predominantly soluble.

Effect of K256E α-actinin-4 expression on cell adhesion, spreading, and migration

The inappropriate association of K256E α-actinin-4 with the actin cytoskeleton suggested that it may negatively affect cytoskeletal dynamics. We therefore determined its effect on cytoskeletal-dependent processes, such as cell adhesion, spreading, and migration. Since α-actinin-4 is associated with focal adhesions, we hypothesized that the mutant protein may negatively affect the ability of cells to adhere to an extracellular matrix. Adhesion assays were performed using podocytes expressing green fluorescent protein (GFP) alone (control), wild-type α-actinin-4, or K256E α-actinin-4 measuring their ability to bind to collagen-I-coated wells (Figure 3). The number of adherent cells was quantified at measuring various time points (3–24 h). Irrespective of the time allowed for adhesion, there was no difference in adhesion between wild-type and K256E α-actinin-4-expressing podocytes, suggesting that cell–matrix interactions are not adversely affected by K256E α-actinin-4.

We next performed a replating assay to assess the ability of cells expressing either wild-type or K256E α-actinin-4 to efficiently spread on an extracellular matrix (collagen-I). Podocytes expressing GFP alone (control), wild-type, or K256E α-actinin-4 were harvested and replated onto collagen-I-coated glass coverslips. Adherent cells were fixed after 3 or 6 h and visualized by immunofluorescence. Within 3 h of replating, a significant number of podocytes had adhered to the substratum and had begun to spread. For each condition, we observed no differences in the total number of

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