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STATEMENT OF RESEARCH

The research presented in this scholarly project report was performed in the laboratories of Johannes Schlondorff, M.D., Ph.D., Assistant Professor at Harvard Medical School, and Martin Pollak, M.D., Professor at Harvard Medical School. The study was conducted under the guidance of Dr. Moumita Barua, Dr. Schlondorff, and Dr. Pollak full-time over the summer of 2012 and part-time during the 2011-2012 and 2012-2013 academic years.

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ABSTRACT

Studies of rare monogenetic forms of the glomerular kidney disease focal segmental glomerulosclerosis (FSGS) have highlighted the importance of the podocyte in glomerular filtration. Recent reports have also demonstrated that the integral membrane protein podocalyxin (PODXL) is essential for the proper functioning of podocytes, possibly through maintaining the patency of the filtration slits by virtue of charge repulsion. Through whole exome sequencing, our group has recently identified rare co-segregating variants in PODXL in several families with autosomal dominant FSGS. One of the private variants, p.L442R, changes a highly-conserved non-polar residue into a charged residue within the protein’s transmembrane domain. Using biochemical and cell-based assays, we demonstrate that the private variant enhances dimerization of the protein. However, the variant does not alter PODXL protein stability, subcellular localization, glycosylation, or interaction with known binding partner ezrin. Our data suggests that the variant is the most likely cause of disease in one family with autosomal dominant FSGS. However, the pathogenicity of the PODXL variant remains unclear, illustrating the challenges of confirming or refuting a rare mutation as disease causing.
INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is a histologically-defined form of glomerular kidney disease and is the most common glomerular cause of end-stage renal disease in adults. Because treatment is difficult and has limited success, patients frequently progress to end-stage renal disease. Over the past decade, studies of genetic forms of FSGS have proven instrumental in demonstrating that podocytes, the highly specialized and terminally-differentiated epithelial cells surrounding the glomerular capillaries, play a critical role in glomerular filtration. Podocyte injury is almost universally seen in proteinuric kidney diseases (3). A substantial body of evidence has also demonstrated that the integral membrane protein podocalyxin (PODXL) is required for the proper function of podocytes as glomerular filters (4, 5). This protein is thought to act as an anti-adhesin that maintains the patency of the filtration slits between adjoining podocytes by virtue of charge repulsion (6, 7). Through whole exome sequencing, our group has recently identified rare co-segregating variants in PODXL in several families with a presumed autosomal dominant form of FSGS (1). The overall hypothesis of this scholarly project is that the rare variants contribute to the development of FSGS by disrupting PODXL function. The goal of this project is to characterize the mechanism by which the FSGS-associated variants result in disease.

PODXL: a member of the CD34 subfamily of sialomucins

PODXL is classically grouped into the CD34 subfamily of sialomucins based on domain structure, sequence homology, and alternative splicing pattern. Similar to the topological features of other CD34 family members, PODXL has a large extracellular domain that undergoes heavy
O-linked and N-linked glycosylation, sialylation, and sulfation (9). PODXL also possesses a highly evolutionarily conserved single-pass transmembrane domain and a cytoplasmic tail with binding domains for the actin-binding protein ezrin and the PDZ adaptor proteins NHERF1 and NHERF2 (8).

**PODXL function and downstream targets**

*PODXL’s anti-adhesive properties*

The sulfate and sialic acid moieties on PODXL’s extracellular domain confer on PODXL a highly negatively charge (5, 10). Through charge-based repulsion, these motifs may help PODXL function as an anti-adhesive molecule (6, 7, 11-13). PODXL’s anti-adhesive effects have been observed in Chinese hamster ovary (CHO) cells, in which increased PODXL expression prevents cell aggregation, and in Madine-Darby canine kidney (MDCK) cells, in which PODXL decreases the electrical tightness of epithelial cell monolayers (6, 7). Furthermore, in podocytes, the appearance of PODXL coincides with the opening of intercellular spaces (6), whereas loss of PODXL’s negative charge with sialidase or protamine sulfate is associated with the closure of these spaces (12, 13). Collectively, these reports suggest that PODXL inhibits cell-cell adhesion through charge repulsion.

*Relation to the actin cytoskeleton*

In addition to functioning as an anti-adhesive, PODXL modulates cell architecture by associating with actin. PODXL is part of a large apical complex containing several actin-associated proteins, including the actin-binding protein ezrin (19), the cytoplasmic adaptor proteins NHERF1/2 (17, 18), and the membrane-spanning chloride channel CLIC5A (14-16),
which has also been reported to function as a soluble actin-regulating protein. PODXL binds both directly and indirectly to ezrin through NHERF1/2 (17, 19). Activation of ezrin and NHERF by PODXL leads to activation of the actin-regulating GTPase, RhoA (18), microvillus formation (7) and an altered distribution of adherens junction and tight junction proteins (11) in kidney epithelial cells. Removal of PODXL’s negative charge by sialidase or protamine sulfate treatment prevents PODXL from interacting with the ezrin complex or the actin cytoskeleton, leading to perturbed cell morphology (21).

Effects on other downstream targets

PODXL also influences other signaling pathways. By activating ezrin, PODXL increases the expression of matrix metalloproteinases and enhances the activity of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (22). PODXL activates integrins and enhances cell adherence to extracellular matrix components, showing that PODXL also has pro-adhesive properties (23). Finally, PODXL’s ectodomain may function as an autocrine or paracrine signaling molecule, as demonstrated by experiments showing that soluble ectodomain fragments of PODXL inhibit cell adherence (23).

Physiologic and pathologic roles of PODXL

PODXL in the glomerulus

PODXL is highly expressed in podocytes (4), the visceral epithelial cells of the glomerular capillaries. These cells are an important component of the glomerular filtration barrier, a three-layered sieve comprised of 1) the fenestrated endothelium of the glomerular capillaries, 2) a surrounding basement membrane, and 3) the podocytes that sit atop the
membrane in the urinary space (Figure 2, reviewed in (3) and (24)). Podocytes are highly specialized cells with intricate cytoarchitecture. Podocyte cell bodies extend outward to form cytoplasmic extensions called foot processes, which interdigitate with foot processes of adjacent podocytes. The intercellular spaces or filtration slits between foot processes are bridged by multi-protein gap junction complexes called slit diaphragms. Slit diaphragms act as the barrier’s primary size- and charge-selective filter, preventing the passage of protein but enabling smaller molecules like metabolic wastes to pass into the urinary filtrate.

PODXL is required for the proper function of podocytes as glomerular filters, likely by keeping adjacent foot processes separated by charge repulsion (11, 25). This function is supported by studies on developing podocytes, which found that PODXL’s initial appearance on the cell membrane occurs simultaneously with filtration slit opening (6). In knockout animal studies, PODXL-null mice are born anuric, lack foot processes and slit diaphragms, and die within the first day of life (25). Mice unable to synthesize O-glycans required for the appropriate glycosylation of PODXL experience glomerular lesions and proteinuria (26). Furthermore, neutralizing podocytes’ negative charge by perfusing rat kidneys with polycations or glycosidases results in foot process effacement, filtration slits closure, and massive proteinuria (12, 13). In humans, reduced PODXL expression has been observed in several proteinuric glomerular diseases, including minimal change disease, membranous glomerulopathy, and FSGS (27). Taken together, these studies suggest that abnormal PODXL activity represents a common mechanism in kidney injury.

PODXL in other tissues

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While initially identified in podocytes, PODXL is also expressed in hematopoietic progenitor cells, vascular endothelial cells, platelets, mesothelial cells lining the coelomic cavity, and a subset of neurons (reviewed in (28)). In the hematopoietic system, PODXL is highly expressed during development and helps new hematopoietic cells cross endothelial barriers so that they can migrate to distant organs (29). In mesothelial cells, PODXL may play a key role in retracting the gut from the umbilical cord during development, since nearly one-third of PODXL-null mice are born with gut herniation or omphalocele (25). In neurons, PODXL functions to promote neurite growth and axonal fasciculation (30). When expressed on the luminal surface of high endothelial venules, PODXL binds L-selectin, which facilitates leukocyte migration across capillary walls (37).

PODXL also serves as a marker for a number of aggressive cancers associated with poor outcomes (reviewed in (28)). PODXL is upregulated in breast cancer tumors associated with shorter mean survival times (31), is mutated in more aggressive prostate cancers (32), and undergoes additional post-translational modifications in non-seminomatous germ cell tumors (33). Increased PODXL expression has been observed in hepatocellular carcinomas (34), colorectal cancer (35), and several leukemias (36). PODXL appears to alter the cytoskeletal properties of malignant cells, enhancing their motility and metastatic potential (28). PODXL’s role in a multitude of cell types and diseases underscores the importance of understanding PODXL and the mechanisms by which it is regulated.

**PODXL and focal segmental glomerulosclerosis**

We began investigating PODXL because of our interest in identifying genes involved in the etiology of FSGS, a type of kidney disease characterized histologically by scarring of parts of
some of the glomeruli and clinically by nephrotic-range proteinuria and progressive renal failure (39). FSGS affects over 20% of all dialysis patients in the United States (40) and is broadly classified as “primary” if no association with an underlying systemic condition is known or “secondary” if a cause is identified (reviewed in (41)). Patients with the primary form usually present with acute-onset nephrotic syndrome; this form is now the second leading cause of kidney failure in children (42). By contrast, patients with the secondary form often present with asymptomatic subnephrotic range proteinuria that is sometimes only incidentally detected. Secondary FSGS is a much more common form of renal injury and is induced by systemic disease, such as viral infection with HIV (43) and parvovirus B19 (44), reflux nephropathy (45), intravenous drug abuse (41), and hyperfiltration states like renal dysplasia (39), obesity (46), and sickle cell disease (47). Patients frequently relapse, and only 30-50% of FSGS cases respond to available treatments (49). Half of patients with FSGS progress to kidney failure between 6 and 8 years of diagnosis (48).

Studies of monogenetic forms of FSGS have advanced our understanding of the key structures and pathways that lead to proteinuria and kidney injury. Despite the discovery of nearly a dozen genes that contribute to FSGS, these genes do not offer a genetic explanation for all of the families in our group’s cohort of inherited FSGS. We therefore hypothesized that novel mutations in additional genes could be responsible for the disease. To test this possibility, we performed whole exome sequencing on two affected cousins, III(3) and III(4), from a family with presumed autosomal dominant inheritance and incomplete penetrance (family FG-HI, Figure 3) (clinical data previously reported in (1) and (50)). Members of this family presented with disease in their teenage and early adult years and progressed to end-stage renal disease between the second to sixth decade. Whole exome sequencing revealed co-segregating genetic
variants in three different genes: C6orf103, OR9A2, and PODXL (1). C6orf103 has an unknown function and OR9A2 is an olfactory receptor; neither stained strongly in human glomeruli (http://www.proteinatlas.org/ENSG00000118492/normal) (1). As only PODXL stained strongly in glomeruli (http://www.proteinatlas.org/ENSG00000128567/normal) and had a biologically plausible connection to FSGS, PODXL was selected for follow-up study. The index case PODXL variant, p.L442R, changed a nonpolar leucine to a charged arginine residue within the highly-conserved transmembrane portion of PODXL and was predicted to be damaging by PolyPhen-2 and SIFT software.

PODXL in 176 probands with autosomal dominant FSGS was subsequently sequenced to examine whether PODXL variants were present in other affected families. Four other individuals were discovered to have rare variants in PODXL: p.S214R, p.M484I, p.E492*, and p.K515R (Table 1). Using publically available exome sequencing data from the 1000 Genomes Project and Exome Sequencing Project, we found that three of the four variants were present in nominally normal patients. The remaining private variant, p.S214R, was present in a patient with another possibly causal gene that has been linked to kidney disease (M.D., G.G., M.P.; unpublished data).

**Objectives**

The goal of this scholarly project was to investigate the effect of rare PODXL variants on PODXL protein function. Using biochemical and cell-based experiments, we found that only the index case variant had a differential effect on the protein’s biochemical properties. This variant was thus the most likely cause of glomerular disease in a single family with FSGS. However, the
effect of this biochemical change on the protein’s function remains unknown, pointing to the difficulty of reaching a definitive conclusion on a rare variant’s pathogenicity.
MATERIALS AND METHODS

Antibodies

The following antibodies were obtained: mouse monoclonal anti-PODXL 3D3 (Santa Cruz Biotechnology), rabbit polyclonal anti-GFP FL (Santa Cruz), peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz), peroxidase-conjugated goat anti-mouse antibody (Cell Signaling), and protein G-agarose (Thermo Scientific).

Plasmids and site-directed mutagenesis

Myc-DDK-tagged ORF clone of human PODXL, transcript variant 2, in pCMV6-Entry vector was purchased from Origene. To generate PODXL without the myc and DDK tags, the Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent Technologist, Santa Clara, CA, USA) was used to introduce a stop codon immediately after the PODXL ORF. The same kit was also used to introduce the desired mutations into PODXL. Briefly, using the pCMV6-PODXL vector as a template for mutagenesis, 100 ng of the vector was incubated with mutagenesis primers and mutagenesis enzyme mix according to the manufacturer’s protocol. To verify the sequence of the PODXL gene for each mutation, the entire coding region of the gene was sequenced. Vectors were purified for transfection using the Qiagen Plasmid Maxi kit.

GFP-tagged ezrin in pEGFP-N2 was provided by Anthony Bretscher (Weill Institute of Cell and Molecular Biology, Cornell University, Ithaca, NY).

Cell culture and transfection
MDCK Tet-Off cells (Clontech) were maintained at subconfluence in Dulbecco’s modified Eagle’s medium supplemented with 5% tetracycline-free heat-inactivated fetal bovine serum, 100 μg/ml G418 disulfate salt solution, and penicillin G, streptomycin, and amphotericin B at 37°C in 5% CO₂. Cells were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol and processed 16-24 h after transfection.

**Cell surface biotinylation**

Following transient transfection, cells were washed in ice-cold PBS and subsequently incubated in ice-cold PBS containing 0.5 mg/ml of Sulfo-NHS-Biotin at 4°C for 1 h (Thermo Scientific). The biotin solution was removed and the cells were incubated in PBS supplemented with 10mM glycine at 4°C for 10 min to quench the reaction. Cells were lysed in ice-cold TBS with 1% (vol/vol) NP-40 lysis buffer supplemented with Complete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Cell lysates were cleared by centrifugation for 10 minutes at 14,000 rpm at 4°C. An aliquot of the cleared lysate was set aside and mixed with sample loading buffer for Western blot analysis. The remaining lysate was mixed with 20 μl of a 50% slurry of streptavidin beads (Pierce) in lysis buffer and incubated 4°C for 2 h. The samples were rocked gently at 4°C for 2 h, spun down at 5000 rpm, and washed three times with 800 μl of lysis buffer. To elute the bound protein, the beads were mixed with sample loading buffer and boiled for 5 minutes at 95°C. Total PODXL and biotinylated PODXL was visualized by Western blot, and relative band intensities were analyzed using FluorChem Q software (ProteinSimple).
**Deglycosylation**

Cell lysates were mock treated or treated with peptide N-glycosidase F, neuraminidase, or O-glycosidase and neuraminidase (PNGase F; O-Glycosidase and Neuraminidase Bundle; New England Biolabs) according to the manufacturer’s recommendations. After treatment, lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

**Immunofluorescence confocal microscopy**

Transfected cells grown on collagen I-coated glass coverslips (BD) were fixed in 2% paraformaldehyde and permeabilized in Triton X-100 in PBS, and incubated sequentially with primary antibodies followed by Alexa 488 goat anti-rabbit or Dylight 594 goat anti-mouse antibodies. FITC Phalloidin and Hoechst 33342 were used to visualize actin filaments and nuclei. Cells were mounted with Fluoromount-G (Southern Biotech), and confocal images were taken using a Zeiss LSM510 upright confocal microscope, with a 63x objective and 0.1 µm optical sections. Images were analyzed with Zeiss software.

**Immunoprecipitation assay**

Transfected cells were washed once with PBS and lysed in 700 µl of ice-cold TBS with 1% (vol/vol) NP-40 lysis buffer supplemented with Complete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Cell lysates were cleared by centrifugation for 10 minutes at 14,000 rpm at 4°C. An aliquot of the cleared lysate was set aside and mixed with sample loading buffer for Western blot analysis. The remaining cleared lysates were mixed with 20 µl of a 50% slurry of antibody-immobilized agarose beads in lysis buffer and rocked
gently at 4°C for 2 h. Samples were spun down at 5000 rpm and washed three times with 700 µl of lysis buffer. To prepare the samples for Western blot analysis, the beads were mixed with sample loading buffer containing 2-mercaptoethanol and boiled for 5 minutes at 95°C.

**Western blot analysis**

Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The membranes were stained with Ponceau S, blocked on 5% bovine serum albumin in TBS with 0.05% Tween 20 (TBST) for 30 min, and incubated with primary antibody for 1 h at room temperature. The blots were then rinsed three times with TBST and incubated with 1:2500 secondary antibody conjugated to horseradish peroxidase in TBST. Following three 5 min washes, the blots were detected with SuperSignal West Dura Chemiluminescent Substrate (Pierce). Relative band intensities were analyzed using FluorChem Q software (ProteinSimple).
RESULTS

PODXL protein stability unaltered by disease-associated mutations

Decreased PODXL activity is associated with podocyte foot process effacement, a hallmark of glomerular injury, and human proteinuric glomerulopathies (12, 13). We therefore hypothesized that the disease-associated variants in PODXL lead to FSGS by impairing the stability of the protein. To test this possibility, several control variants were selected from the Exome Sequencing Project, which presumably represent non-proteinuric controls (Table 1). The control variants and FSGS-associated variants were introduced into PODXL expression plasmids with site-directed mutagenesis. Western blot analysis of transiently transfected Madin-Darby canine kidney (MDCK) cells failed to detect any significant differences in the quantity of protein expressed between the suspected disease-causing variants, control variants, and wild-type (Figure 4), suggesting that the disease-associated variants do not alter PODXL protein expression.

Enhanced PODXL dimerization by the p.L442R variant

Although the disease-associated variants did not affect the quantity of protein expressed, p.L442R PODXL produced a higher-molecular weight aggregate in addition to a normally sized product. By SDS-PAGE, p.L442R PODXL expressed in MDCK cells is detected as a ~330 kDa upper band and an ~165 kDa lower band (Figure 4). By contrast, the remaining variants and wild-type were predominately detected as an ~165 kDa lower band under the same experimental conditions. Given its molecular weight, this upper band likely represents a dimer. However, we
cannot rule out the possibility that this upper band is an aggregate of PODXL protein with another unrelated protein.

As PODXL undergoes significant post-translational glycosylation of its extracellular domain (9), we hypothesized that the higher molecular weight band represents differentially glycosylated PODXL. Cell lysates were treated with either PNGase F to remove N-glycosylations, neuraminidase to remove sialic acid motifs, or a combination of O-glycosidase and neuraminidase to remove O-glycosylations. Western blot analysis showed no significant differences in the glycosylation pattern of p.L442R PODXL (Figure 6).

To investigate the effect of different lysis buffers on the higher-molecular weight band, p.L442R and wild-type PODXL were lysed in either mild lysis buffer with mild non-ionic detergent (TBS + 1% NP-40) or lysis buffer with ionic detergent (RIPA). Lysis in RIPA blocked the formation of the upper band, suggesting that the higher molecular weight band is an aggregate of PODXL formed during lysis with a mild non-ionic detergent.

**Subcellular trafficking and localization of PODXL unaffected by p.L442R variant**

In podocytes, PODXL is localized to the apical surface of podocyte foot processes above the level of the slit diaphragm, which enables PODXL to keep open the filtration slits between adjacent foot processes (11, 25). In MDCK cells, PODXL is also predominately located at the apical surface, although some protein is also distributed in cytoplasmic vesicles, endoplasmic reticulum, and Golgi complexes (51). To test whether the p.L442R variant alters subcellular trafficking of the protein, confocal immunofluorescence microscopy and cell surface biotinylation experiments were performed. Similar staining patterns between wild-type and p.L442R PODXL were observed on confocal microscopy, with punctate staining on the apical
aspect of the MDCK cells. Biotinylation experiments to quantify protein expression on the cell surface were not significantly different between wild-type and p.L442R PODXL on Western blot analysis.

**Interaction of PODXL with ezrin unaffected by p.L442R variant**

Previous studies have identified mutations in several actin-associated FSGS genes, including the actin-bundling protein alpha-actinin 4 (52, 53), actin-regulating protein INF2 (54), and cation channel TRPC6 (55). These studies suggest that the integrity of the podocyte actin cytoskeleton is essential to podocyte function. Thus, we were interested in whether the p.L442R variant disrupts PODXL’s interaction with its usual binding partner ezrin, which links PODXL to the actin network. To assess this possibility, confocal immunofluorescence microscopy was performed on MDCK cells co-transfected with PODXL and ezrin. Staining showed approximately equivalent partial co-localization of PODXL with ezrin for both wild-type and variant PODXL. This result suggests that the p.L442R variant does not impair the PODXL-ezrin interaction.
DISCUSSION

Whole exome sequencing of two affected cousins from a family with autosomal dominant FSGS revealed a previously-unreported private variant in PODXL, a gene with known links to podocyte biology and nephrotic syndrome. PODXL is widely believed to act as an anti-adhesin that keeps the slit membranes between adjoining podocyte foot processes open for filtration by virtue of charged-based repulsion (6, 7). This notion is supported by knock-out animal studies showing foot process effacement and massive proteinuria (25) and by human observational studies showing reduced PODXL expression in several glomerular diseases (27). Given the biologic plausibility and its critical role in the podocyte, PODXL was a highly attractive candidate for further study. We therefore sequenced PODXL in 176 additional probands, identifying one other private variant in PODXL.

This scholarly project investigated whether the two private PODXL variants had an effect on podocyte function. Using biochemical and cell biological assays, we demonstrate that compared to wild-type and the other rare variants, only the index case variant, p.L442R, had an differential effect on PODXL. This variant produced a ~330 kDa band in addition to a normally-sized ~165 kDa band on Western blotting. This finding indicated that the variant induces the formation of PODXL dimers. That the variant localizes to the transmembrane portion of the protein further suggests that the transmembrane domain is involved in the higher order aggregation of PODXL monomers. However, we cannot exclude that the higher molecular weight band represents an aggregate of PODXL protein with other unrelated proteins or that the aggregate was produced only after cell lysis.
Our working hypothesis was that enhanced PODXL dimerization caused podocyte dysfunction by 1) reducing PODXL’s negative surface charge, or 2) altering PODXL’s interactions with its actin-associated binding partners. Our subsequent experiments did not bear out these hypotheses. Biochemical and cell biological assays showed that the variant does not affect the protein’s stability or expression levels, subcellular localization or trafficking to the apical surface, glycosylation pattern, or ability to bind with its normal interactor ezrin, an actin-binding protein. Thus, the effect of increased dimerization on PODXL function remains unclear.

While the variant did not impair PODXL’s ability to co-localize with ezrin at the apical surface, altered multimerization of PODXL protein could still potentially affect PODXL’s association with its other binding partners. These partners belong to a large apical multi-protein complex and include not only ezrin (19) but also cytoplasmic adaptor proteins NHERF1/2 (17) and actin-regulating protein CLIC5A (14-16). NHERF1/2 has been proposed to act as an aggregating protein that mediates PODXL dimerization and integrates PODXL dimers into clusters of lipid rafts called apical sorting platforms (56, 57). Activation of NHERF1/2 by PODXL has been shown to result in phosphorylation and activation of ezrin, activation of RhoA, redistribution of actin, the formation of microvilli, and an altered arrangement of adherens junctions and tight junctions in kidney epithelial cells (18). We propose several additional experiments to parse out the variant’s effect on 1) PODXL’s interaction with NHERF1/2 and CLIC5A, 2) the ability of PODXL to sort into lipid rafts, 3) the levels of activated NHERF1/2, ezrin, and RhoA, and 4) PODXL’s association with actin filaments.

More recent reports have also highlighted the process of ectodomain shedding as an important mechanism by which PODXL affects cell adhesion, cell-cell interactions, and downstream signaling pathways (23, 58, 59). Through cleavage by several types of
metalloproteinases, PODXL is released into the extracellular space as a soluble ectodomain fragment. This cleaved fragment enters the extracellular space and act as an autocrine or paracrine signaling molecule. These ectodomain shedding events are often followed by intramembrane cleavage to release the cytoplasmic domain from the membrane, though this has not yet been documented for PODXL. Given the location of the p.L442R variant in the transmembrane domain of PODXL and the fact that the variant resides within a sequence that undergoes proteolysis by matrix metalloproteinase-14 (MMP-14) (58), the variant might alter the proteolytic processing of PODXL. While we did not observe any differences in proteolysis with variant PODXL on Western blot analysis, whether MMP-14 is endogenously expressed in MDCK cell is unclear. Future experiments should explore this issue.

We are aware of the limitations of our study. We performed our cell-based experiments on Madine Darby canine kidney (MDCK) cells, which derive from the collecting duct or distal tubule of the nephron. These cell lines express endogenous PODXL but may handle PODXL differently than podocytes. Nevertheless, many groups have employed MDCK cells as a proxy in studying PODXL, since MDCK cells display columnar morphology and represent a paradigm cell line for investigating apical versus basolateral membrane trafficking (18, 20, 57). While it would be ideal to study PODXL in podocytes, we were unable to obtain efficient expression of PODXL in cultured podocytes. A second limitation is that the statistical support for PODXL as a disease-causing gene was not strong. The burden of the variants in our probands with FSGS was not significantly different from the frequency of rare variants in the general population. The p.L442R variant was also identified in a family too small for conclusive genetic results.

In summary, we have identified a variant in PODXL as the most likely cause of disease in one family with autosomal dominant FSGS. Our conclusion is based on suggestive but not
definitive genetic data and in vitro experiments demonstrating enhanced dimerization of variant PODXL. To our knowledge, this is the first time that a variant in the PODXL gene has been reported in patients with FSGS. This is despite many previous animal and human studies showing links between PODXL and glomerular disease. Uncovering a causal link between a PODXL mutation and FSGS lends credence to the argument that PODXL is a potential target molecule in the treatment of some forms of proteinuric kidney disease. The second conclusion we draw from the data is the difficulty of using biochemical studies to definitively validate or refute a candidate gene as disease causing. While we have demonstrated that the variant produces a biochemical change in PODXL, its effect on PODXL function remains unknown despite numerous biochemical studies. We believe that these challenges will arise more frequently as whole exome sequencing is increasingly performed on smaller previously uninformative autosomal dominant pedigrees in the search for disease-causing genes.
Table 1: PODXL rare variants discovered in the FSGS cohort and selected PODXL rare variants from the ESP.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Mutation type</th>
<th>Location</th>
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Abbreviations: 1KG, 1000 Genomes Project; ESP, Exome Sequencing Project; FSGS, focal segmental glomerulosclerosis; NA, not applicable; PODXL, podocalyxin; TM, transmembrane

The number of alleles with the indicated variant found in the 1KG and ESP are indicated in columns 7 and 8. At the time of reference, sequence information for 2200 and 10,800 haplotypes was available in the 1KG and ESP, respectively. All of these variants were introduced by mutagenesis in plasmids harboring the PODXL gene for further experiments. Amino acid changes are indicated using NCBI accession number NM_005397. DNA coordinates are indicated with reference to human Hg19. *in-frame deletion (previously published in (1))
Figure 1: PODXL protein structure and binding partners. PODXL possesses a heavily glycosylated extracellular domain covered by negatively-charged sialic acid and sulfate moieties. The single-pass transmembrane domain and intracellular domain are highly conserved.

White boxes, mucin domain; horizontal bars with or without arrows, O-linked glycosylations; arrows, sialic acid moieties on O-linked glycosylations; black circles, N-linked glycosylations; black boxes, disulfide-bonded globular domain; white circles, potential phosphorylation sites for protein kinase C and casein kinase II; DTHL, potential PDZ protein interaction domain. Figure adapted from (25).
Figure 2: Schematic representation of a glomerular capillary loop. (a) The glomerular filtration barrier is comprised of the fenestrated endothelium of the glomerular capillaries, a surrounding basement membrane, and the podocytes that sit atop the membrane in the urinary space. (b) Enlarged view of the glomerular filtration barrier. The gaps between podocyte foot processes are bridged by multi-protein slit diaphragms, which serve as the primary size- and charge-selective filter of the barrier. Figure adapted from (60).
Figure 3: Pedigree of family FG-HI, sequencing, and multisequence alignment. (a) Pedigree for family FG-HI. Affected individuals are indicated in gray. Individuals who are heterozygous for the variant PODXL p.L442R are denoted by ‘+’, whereas those without the mutation are denoted by ‘−’. Individuals without a notation were not tested because no sample was available. (b) Next generation sequencing reads across PODXL aligned to the reference genome in Integrative Genome Viewer. The antisense strand is indicated as reference. (c) Sanger sequencing confirming the PODXL variant in all affected individuals where DNA was available. The sense strand is indicated. (d) Multisequence alignment showing conservation of the affected amino acid residues. P.L442R is indicated with a ‘*’. Previously published in (1).
Figure 4: FSGS-associated genetic variants do not alter the stability of PODXL protein. MDCK cells were co-transfected with GFP and equal amounts of either wildtype PODXL plasmid, PODXL plasmid containing an FSGS-associated variant (p.L442R to p.K515R), or PODXL plasmid containing a control variant from the Exome Sequencing Project (p.G112S to p.M490V). After 24 h, the cells were lysed, and the lysates were immunoblotted for PODXL (upper panel) or GFP (lower panel). * indicates amino acid is deleted. Previously published in (1).
Figure 5: The FSGS-associated p.L442R variant does not alter the subcellular localization of PODXL. MDCK cells transfected with PODXL wildtype or p.L442R plasmid. PODXL was labeled with an anti-PODXL antibody (red), F-actin was labeled with phallodin (green), and nuclei were labeled with Hoechst 33342 (blue). (a) Horizontal slices of confocal stacks. (b) Vertical slices of confocal stacks. (c) Transfected MDCK cells were surface biotinylated, and cell surface proteins were pulled down using streptavidin beads. Total PODXL and biotinylated PODXL were visualized by Western blot. Previously published in (1).
Figure 6: The FSGS-associated p.L442R variant induces the formation of PODXL dimers.

(a) MDCK cells transfected with either wildtype or p.L442R PODXL plasmid were mock treated or treated with either PNGase F, neuraminidase, or a combination of O-glycosidase and neuraminidase. No differences were observed in the before or after deglycosylation patterns in both PODXL wildtype and p.L442R mutant on Western blotting. (b) MDCK cells were transfected with either PODXL wildtype plasmid or PODXL p.L442R plasmid. The cells were lysed after 24 h in either mild lysis buffer (TBS + 1% NP-40) or lysis buffer with ionic detergent (RIPA). PODXL was detected using an anti-PODXL antibody. Arrows indicates upper form. Previously published in (1).
Figure 7: The FSGS-associated L442R variant does not alter co-localization of PODXL with ezrin at the apical surface. MDCK cells co-transfected with ezrin and either wildtype or p.L442R PODXL plasmid were fixed. PODXL was labeled with an anti-PODXL antibody (red) and GFP-tagged ezrin was labeled with GFP (green). Scale bar: 20 µm. Previously published in (1).
REFERENCES


