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Rho GTPase Regulatory Proteins Contribute to Podocyte Morphology and Function

Abstract

Podocytes are a critical cellular component of the glomerular filtration barrier, whose strict permselectivity prohibits the passage of large proteins and charged species into the urine. Phenotypic variability or injury of these highly specialized cells can lead to proteinuria and has been linked with altered activity of Rho GTPases, which are strongly associated with the actin cytoskeleton. Notable regulators of these intracellular molecular switches are called guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). In this study, the roles of several GEFs in podocyte morphology and activity were investigated, including ECT2, ARHGEF2, ARHGEF26, and ARHGEF40. Results from RhoA and Rac1 G-LISA Activation Assays indicated that the absence of ARHGEF40 impairs epidermal growth factor (EGF)-stimulated RhoA and Rac1 activation, whereas knockout of ARHGEF2 and ARHGEF26 may selectively diminish RhoA activation. Furthermore, filopodia formation was hindered for the ARHGEF40 knockout. There are a number of additional investigations underway to understand Rho GTPase regulatory proteins, including the elimination of new sets of GEFs and GAPs *in vivo*. It is hopeful that these studies can provide insights into potential novel therapeutic strategies for proteinuria.

Introduction

Proteinuria is a commonly recognized manifestation and possible contributor to renal disease. This phenomenon, the leakage of proteins into the urine, is a result of kidney dysfunction in the glomerulus. The glomerulus is responsible for the filtration of blood and is composed of a network of capillaries contained within a structure called the Bowman's capsule, surrounded by a three-layered glomerular filtration barrier. After exiting the capillary endothelium, the filtrate passes through the glomerular endothelium, basement membrane, and the filtration slits established by podocytes and various proteins[1]. These layers prevent the passage of high molecular weight and negatively charged species into the urinary space. The origin of proteinuria can be described as the loss of selective permeability of the glomerular filtration barrier to plasma proteins in the kidney[2].

Podocytes are an essential component of the glomerular filtration barrier. They are highly differentiated epithelial cells with a polarized organization consisting of a cell body and a series of extensions called foot processes[3]. The basal side is fused to the glomerular basement membrane and the foot processes of neighbouring podocytes form an interdigitating structure called the slit diaphragm. This specialized cell-cell contact point contributes to the size selectivity of the glomerular filtration barrier[4]. Phenotypic variability or injury of podocytes can result in alterations of the slit diaphragm framework and ultimately the disruption of the filtration barrier. Consequently, the loss of permselectivity allows the transglomerular leakage of proteins into the urine.

The specialized cellular morphology of podocytes is reliant on an extensive actin cytoskeletal architecture. Foot processes contain a cortical network of actin filaments and bundles which provide mechanical support, shape, and a foundation for cell motility[5]. An undesirable reorganization of the actin cytoskeleton leads to foot process effacement, characterized by a simplification of the interdigitation pattern and detachment from the glomerular basement membrane[6]. Since the integrity of the slit diaphragm and glomerular filtration barrier is largely dependent on foot process configuration, the regulation of the podocyte actin skeleton is crucial for maintaining kidney function.

The Rho family of small GTPases have been established as skillful regulators of the actin cytoskeleton and are therefore relevant in the understanding of podocyte dysfunction and proteinuria. They are a subdivision of the Ras superfamily of small GTPases and exist as molecular switches, binding to various effectors and regulating downstream signalling pathways. Rho GTPases alternate between two distinct conformational states: an active GTP-bound state, during which they recognize target proteins and generate dynamic responses, and an inactive GDP-bound state. While the conversion between conformations involves a simple hydrolysis reaction, the activity of Rho GTPases is carefully regulated for homeostatic signalling cascades. There are three classes of proteins that regulate the activity of Rho GTPases. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting the dissociation of bound GDP and facilitating the binding of GTP. Conversely, GTPase-activator proteins (GAPs) inactivate Rho GTPases by increasing their intrinsic activity, causing them to return to 'off' states after interacting with effectors. Guanine nucleotide dissociation inhibitors (GDIs) sequester the inactive GDP-bound Rho GTPases, preventing the exchange for GTP[7]. While there are only 20 members of the Rho GTPase family[8], the human genome encodes over 80 GEFs and over 60 GAPs[9,20].

Three prototypical Rho GTPases are RhoA, Rac1, and Cdc42, all of which likely play a role in the regulation of the actin skeleton, and consequently podocyte morphology. In particular, RhoA is implicated in the production of stress fibers, a contractile actomyosin structure of the cytoskeleton[12]. Rac1 is responsible for lamellipodia formation and extension, which are protrusions at the leading edge of migrating cells driven by a network of polymerizing actin filaments[11]. Finally, Cdc42 is known to contribute to the development of finger-like projections called filopodia, which emerge from lamellipodia. These also contain bundles of actin and may act as sensory probes during cell migration[10].

Past experimentation established the foundation of a protein-protein interaction network for Rho GTPases and their aforementioned regulatory proteins in podocytes. A proximity-based biotinylation assay (BioID) using baits of RhoA, Cdc42, and Rac1 coupled with proteomic analysis identified 20 GEFs as players in the Rho GTPase environment (Figure 1). From this preliminary interactome, four GEFs were chosen to further investigate the role of Rho GTPase regulatory proteins in cytoskeletal dynamics and podocyte biology: ECT2, ARHGEF2, ARHGEF26, and ARHGEF40.

Epithelial cell-transforming sequence 2 (ECT2) acts as a specific regulator of RhoA in podocytes. Past studies have implicated this gene in the proliferation and invasion of non-small cell lung cancer tumours[13], as well as the progression of gastric carcinogenesis[14]. Furthermore, gene analyses of two nephrotic syndrome patients suggest that a non-functioning ECT2 gene may lead to renal tubulointerstitial injury and eventual glomerular sclerosis[15].

Figure 1. A protein-protein interaction network for Rho GTPases in podocytes. Each regulatory protein (GEF) is shown to affiliate with at least one Rho GTPase. Eight are specific for RhoA, four are specific for Rac1, and two are specific for Cdc42. The scaled WD-score indicates the confidence of the interaction. Unpublished data from Takano Lab.

ARHGEF2, similarly to ECT2, interacts with RhoA. It has been reported that, in Madin-Darby canine kidney (MDCKII) cells, ARHGEF2 overexpression promotes the activation of RhoA and induces the formation of stress fibers as well as focal adhesions, leading to a slow rate of wound healing[16]. Similarly, in HeLa cells, upregulation of GEF-H1 (encoded by the ARHGEF2 gene) resulted in increased actomyosin contractility, increased cell adhesion, and decreased cell migration, due to downstream pathways involving RhoA[17].

ARHGEF26 is an extensive regulator of Rho GTPases, shown through the BioID analysis to interact with RhoA, Cdc42, and Rac1 in podocytes. However, the contribution of ARHGEF26 in cytoskeletal dynamics has not yet been defined. A study has shown that decreased levels of ARHGEF26 lead to increased invadopodia formation in cancer cells[18]. Resembling filopodia and lamellipodia, invadopodia are actin-rich membrane protrusion structures that participate in the degradation of extracellular matrix during metastatic cancer. ARHGEF40, also known as Solo, is of particular interest due to its association with Cdc42. While this affiliation is not characterized in podocytes, reports have described that ARHGEF40 knockdowns accelerate the migration of collective MDCK cells with visible finger-like projections[19]. Furthermore, ARHGEF40-depleted cells showed the absence and thinning of stress fibers[20]. In this study, light microscopy, G-LISA Small GTPase Activation Assays, and actin assembly assays were used to investigate the functional interactions of these regulators (ARHGEF2, ARHGEF26, ARHGEF40, ECT2) at the cellular and molecular level. These findings are pertinent in the elucidation of the mechanisms of podocyte injury and proteinuric renal disease, so may prove to be potential therapeutic targets.

Methods

Cell Culture and Transfection

All procedures using cell lines were performed using immortalized human podocytes. Conditions of culture include maintenance at 33 °C with 5% CO2 in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin (PS). Cell lines were transfected at a density of 350k cells per well in 6-well tissue culture plates. Using the backbone vector PX-459-V2 encoding the Cas9 protein, a cloned sgRNA sequence, and the Lipofectamine 3000 Reagent (ThermoFisher), a CRISPR sequence targeted for the knockout (KO) of each GEF was integrated. Furthermore, the plasmid contained a puromycin resistance gene as a selective marker. Following an incubation period (~18 hours) with the transfection reagents, cells were provided with antibiotic-free medium, then subjected to puromycin (2 µg/mL) treatment for 48 hours. After returning to normal RPMI-supplemented media, cells were kept under 33 °C incubation until confluent.

CRISPR/Cas9 Knockout System

The CRISPR-Cas9 system is reliant on two major components: a guide RNA sequence (Table 1) and a CRISPR-associated nuclease (Cas9). For the GEF KO cell lines, a single guide CRISPR strategy was implemented, in which the complementary sequence to the target DNA and the tracrRNA (important for target recognition) are fused together. This allowed the CRISPR/Cas9 system to generate inactivating mutations in the protein-coding genes by creating frameshift insertion-deletions (indels) in exonic sequences. Notably, an additional scrambled sequence was created that does not recognize the human genome, so the Cas9 protein would not perform an incision/excision.

ECT2 guide	5' TATTAACATCCACTACTGGG 3'
ARHGEF2 (assembly 2) guide	5' AAGAGAAACGGACTGCAAGG 3'
ARHGEF26 guide	5' GAGTGAGGTCGATAACGACG 3'
ARHGEF40 (assembly 1) guide	5' GGTGGAGAGGACTTATCGGG 3'
Scrambled guide	5' GCACTACCAGAGCTAACTCA 3'

Table 1. sgRNA sequences used in conjunction with the CRISPR-Cas9 system to achieve gene knockouts of four different GEFs in human podocytes.

The validation of CRISPR/Cas9 editing was accomplished using Tracking of Indels by Decomposition (TIDE) analyses, which were preceded by genomic DNA (gDNA) extraction of KO cell lines and polymerase chain reaction (PCR) to amplify the targeted region. The demonstration of the presence of an indel mutation at the guide RNA cut side is sufficient evidence for the gene knockout[21].

Human podocyte GEF KO cell lines were maintained in 6 cm tissue culture plates and allowed to reach full confluency before trypsinization and preparation for gDNA extraction. The extraction was performed according to the Qiagen Genomic DNA Handbook and the Qiagen Blood & Cell Culture DNA Kit. PCR primers (Table 2) were designed to amplify a 400 to 700 base-pair region around the target cut site. The genomic DNA was amplified using the Q5 High-Fidelity PCR Kit (New England Biolabs: Product No. M0491S) according to the manufacturer's instructions. The amplified DNA for each GEF KO cell line, grouped with a scrambled sample, was sent for conventional Sanger sequencing by Genome Québec. The resulting chromatograms were input into the TIDE Analysis software[22]. Comparing the scrambled DNA and potential knockout DNA produced information about the quality of sequence data, verification of the expected cut site, relative abundance of aberrant nucleotides over the sequence trace, and overall gene editing efficiency.

Rho GTPase Activity Assay (G-LISA)

The intracellular amounts of Rac1-GTP and RhoA-GTP (active forms) in each GEF KO human podocyte cell line were determined using the G-protein linked immunosorbent assay (G-LISA) (Cytoskeleton Inc.). Kit and lysate preparation were performed per the manufacturer's protocol.

Transcript	Primer Pair
ECT2	5' ACTTACTTTTGCCCAATGACCA 3' (forward)
	5' GCTAGACCGCCCTCACATAC 3' (reverse)
ARHGEF2 (assembly 2)	5' TACCAACACTCCCGAAATGC 3' (forward)
	5' CACCCTGATCCCCTTAACCA 3' (reverse)
ARHGEF26 (assembly 3)	5' TTCCGCTTAGTGAATGGCGT 3' (forward)
	5' GCTCCTCGAGAATCCTTCCG 3' (reverse)
ARHGEF40 (assembly 1)	5' GGGATCTGTAGCCTGGTCCT 3' (forward)
	5' GCAGAATAGTGATGCACGGC 3' (reverse)
Scrambled	5' CACCGCACTACCAGAGCTAACTCA 3' (forward)
	5' aaacTGAGTTAGCTCTGGTAGTGC 3' (reverse)

Table 2. PCR Primers designed for each GEF genomic sequence targeted by sgRNA.

Cell Morphology Assessment

A basal cell morphology assessment was completed using fluorescence staining and confocal microscopy. The condition groups included ECT2 KO, ARHGEF2 KO, ARHGEF26 KO, ARHGEF40 KO, and scrambled. Coverslips were prepared in a 12-well plate. All steps were performed at room temperature unless otherwise stated. Wells were filled with ethanol for 10 minutes, washed with PBS, and exposed to a 1/200 dilution of collagen type I (Sigma) in PBS for 1 hour at 37 °C. A final wash with PBS was performed before adding 1 mL of RPMI and 60k human podocyte cells from a pre-existing culture line. Podocytes were serum-starved in RPMI containing 1% FBS for 18 hours before experimentation.

To achieve a fixed cell-staining environment, each well containing cells and a coverslip were washed with PBS, then 500 µL of a 4% paraformaldehyde (PFA) in PBS solution was added for 15 minutes. After washing once again with PBS, permeabilization was accomplished by exposing the wells to 500 µL of a 0.5% triton (Sigma-Aldrich) in PBS solution for 5 minutes. Two dyes were used to visualize the target structures of podocytes: PromoFluor-488-Phalloidin (PromoKine, Cedarlane) in a 1/100 dilution and DAPI (ThermoFisher) in a 1/1000 dilution. The former detects polymerized actin in the cytoskeleton and the latter stains nuclei. The wells were simultaneously incubated with phalloidin and DAPI for 20 minutes. Finally, the coverslips were mounted on a slide using Aqua Mount (Epredia, ThermoFisher) and kept at 4 °C until microscopy was performed on a Zeiss LSM780 Laser Scanning Confocal Microscope at 20x magnification.

Micrographs were analyzed on ImageJ using only the phalloidin overlay. Individual podocytes were identified and the relevant metrics of inquiry included cell area, cell perimeter, cell aspect ratio, and integrated density. The aspect ratio was calculated as a fraction of the minor axis (shortest distance between cell boundaries crossing the center) to the major axis (longest distance between cell boundaries).

Filopodia Assay

In a 12-well plate, 75k cells were plated for each human podocyte GEF KO cohort and treated with epidermal growth factor (EGF) at a concentration of 100 ng/mL in order to stimulate Rho GTPase activity. These wells contained a modified liquid medium: RPMI1640 with 1% FBS. The plate was placed in a 37 °C incubator, which promotes cell differentiation, and snapshots were taken using the IncuCyte S3 (Essen Bioscience) instrument and software every 2 hours over a 24-hour period.

Analysis of filopodia involved the manual counting of characteristic sharp and thin projections from podocytes, followed by normalization to cell confluency. Photographs from the 10-hour time point were chosen, as EGF stimulation was in effect, but not the sole player in filopodia formation. Rather, phenotypic variability in cell shape and the degree of Rho GTPase responses to stimulation would be more potent contributors. For each well containing either a GEF KO, scrambled, or parental cohort, sixteen snapshots were analyzed to ensure precision.

Results

Knockout Validation

TIDE determined the indel spectrum plot for each treated pool, which explains the composite sequence trace in the sample in comparison to the control (scrambled). Furthermore, TIDE provided an aberrant sequence signal plot, depicting the percentage of irregularity along the sequence trace of the control and experimental samples. As shown in Figure 2, the CRISPR/Cas9 system generated a considerable amount of indels in each GEF group, indicating a high degree of gene disruption and knockout efficiency.

Rho GPTase Activity

The G-LISA colorimetric assays provided variable evidence for the effects of Rho GTPase GEFs on stimulation from EGF (Figure 3). As a baseline control, the parental podocyte cell line showed an approximate 27% increase in activated RhoA (GTP-RhoA) and an approximate 37% increase

in activated Rac1 after treatment with EGF. In the ECT2 KO, there was an ~20% increase in GTP-RhoA and ~53% increase in GTP-Rac1 following EGF treatment. Under the same conditions, ARHGEF2 KO tended to increase to a similar degree in GTP-Rac1 (~27%); however, a difference in GTP-RhoA was not apparent after treatment. A similar tendency was observed for the ARHGEF26 KO; a ~19% increase in GTP-Rac1, but no

Effect of Knockout on Cell Morphology

For basal morphological analysis using fluorescence staining, phalloidin was employed to measure cell area, perimeter, and to label actin filaments in podocytes. DAPI served to confirm the viability of nuclei and cell life (Figure 4). There was no significant effect of the knockout GEFs on cell

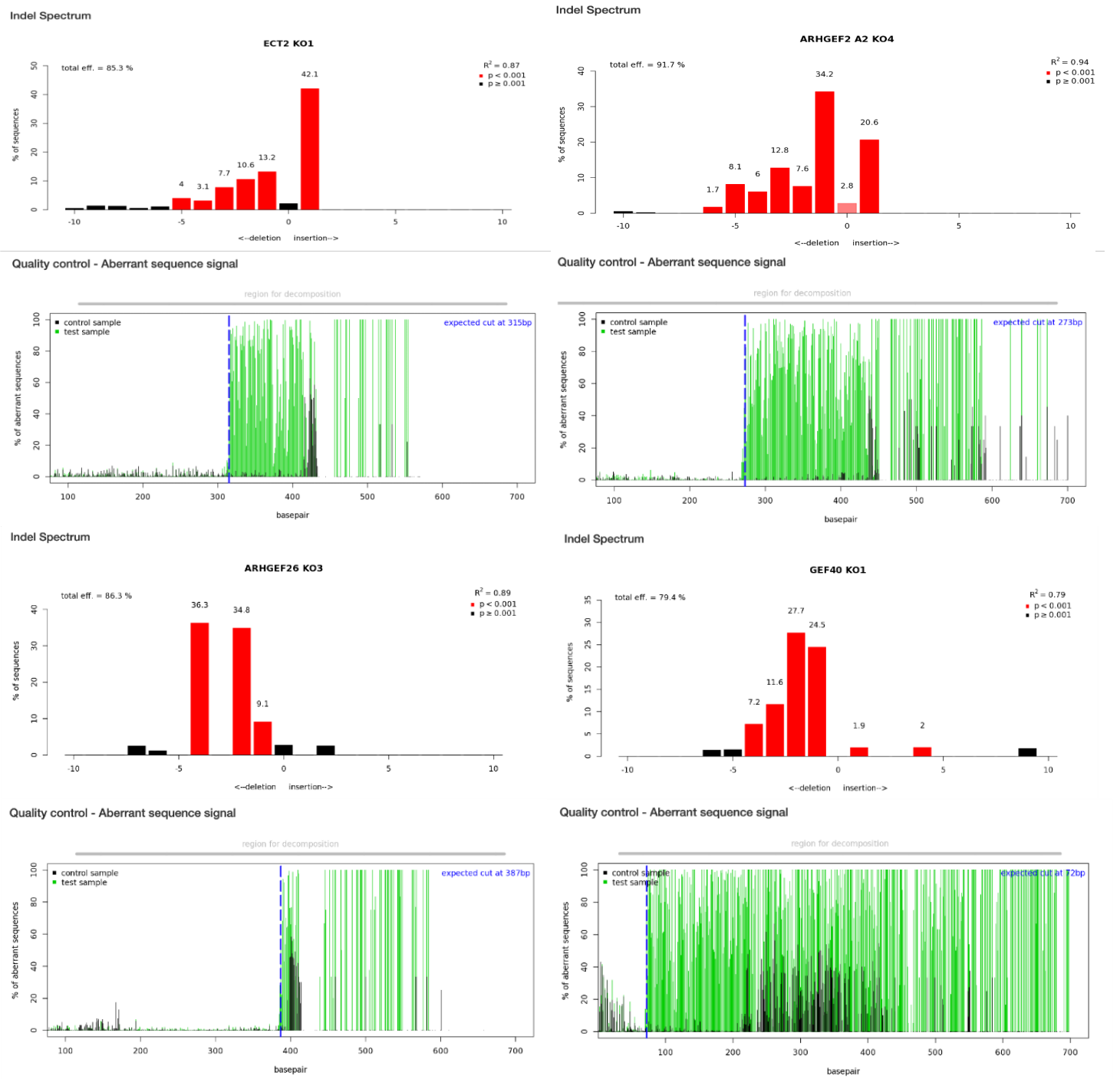

Figure 2. TIDE Analyses for each GEF knockout in human podocytes. The indel spectrum indicates the type of insertion/deletion and efficiency of gene editing. The aberrant sequence signal plot describes the amount of deviation in the genome sequence of knockout pools to the scrambled, unedited pool.

change in GTP-RhoA. Finally, the ARHGEF40 KO1 group showed neither an increase nor decrease of GTP-RhoA or GTP-Rac1 following EGF treatment. This suggests that the absence of ARHGEF40 impairs EGF-stimulated RhoA and Rac1 activation, whereas knockout of ARHGEF2 and ARHGEF26 may selectively impair RhoA activation.

surface area or cell aspect ratio (shape elongation) in human podocytes. However, an ARHGEF2 knockout resulted in a decrease in intensity of phalloidin staining, as shown in Figure 5. This indicates a decreased presence of actin filaments and provides more evidence that ARHGEF2 plays a role in the development and maintenance of the actin skeleton via RhoA activation.

Gene Target	Percentage Total Efficiency
ECT2	85.3
ARHGEF2 (assembly 2)	91.7
ARHGEF26	86.3
ARHGEF40 (assembly 1)	79.4

Table 3.TIDE determined the percentage of sequences in each transfected podocyte condition that carried an indel. A high degree of efficiency indicates the success of gene disruption (knockout) for the Rho GTPase GEF.

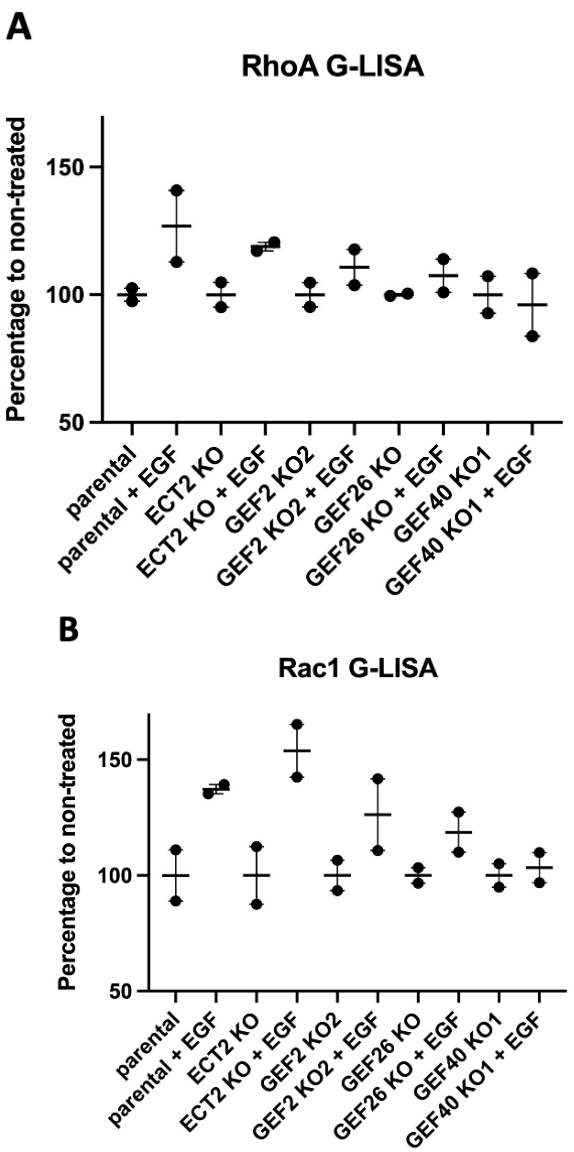

Figure 3.G-LISA experiments measure the activation of specific Rho GTPases using a colorimetric assay. When cells are treated with EGF, an increase in Rho GTPase activity is expected. The effects of GEF knockouts with respect to this activation rate are depicted as the percentage change to non-treated cells. Note that n=1 and each set of points represent duplicates. (A) The activity of RhoA in a parental human podocyte line, compared to podocytes with knockouts of each GEF (ECT2, ARHGEF2, ARHGEF26, ARHGEF40). Cells treated with EGF help to decipher the inhibition or facilitation of each GEF in Rho GTPase activation. (B) The activity of Rac1 in each of the same knockout and treatment groups.

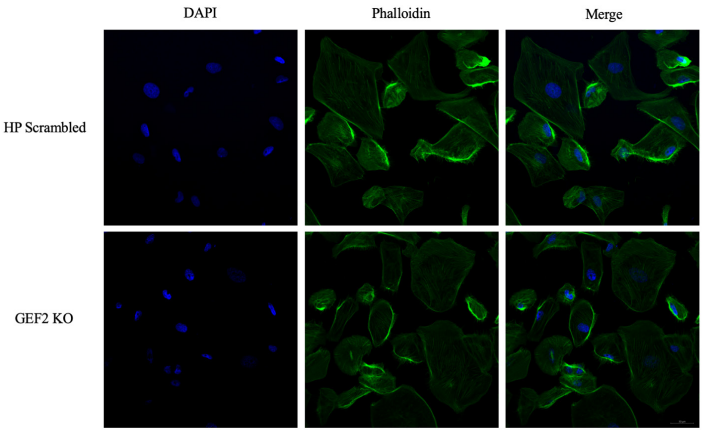

Figure 4. Representative images of the fluorescence staining for DAPI (blue) and Phalloidin (green) in human podocytes.

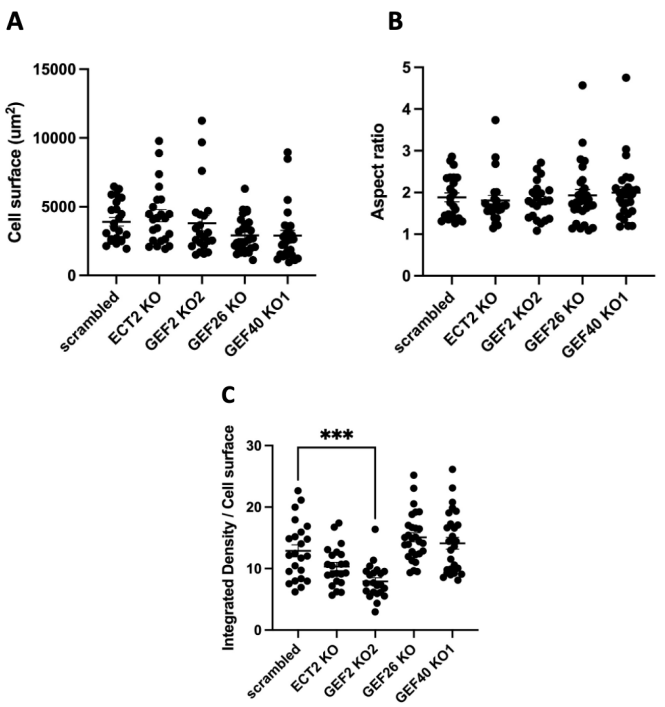

Figure5. Morphological analyses of human podocytes for GEF knockouts from fluorescence staining, n>20 for each group. (A) Cell surface area showed no significant change between cohorts. (B) Fit ellipse showed no significant differences. (C) Density of phalloidin staining normalized to cell surface area. A noticeable decrease was present in the ARHGEF2 KO2 group, compared to the scrambled control.

The filopodia assay indicated that an ARHGEF40 knockout impairs filopodia formation in human podocytes. As shown in Figure 6, the number of cellular projections detected, when normalized to cell confluence, was decreased by approximately 40%. Such an effect indicates that ARHGEF40 may be a facilitator of cell migration and sensory activities. This result was not observed for any other knockout (ECT2, ARHGEF2, ARHGEF26).

Discussion

While it is well-known that Rho GTPase proteins play a critical role in the maintenance of the actin cellular framework and podocyte function, the underlying mechanisms of their regulatory proteins' behaviour have yet to be fully understood. This is likely due to the complex interaction networks that comprise cytoskeletal dynamics and the difficulty in targeting distinct signalling pathways. In this study, an attempt was made at deciphering the ways in which four guanine nucleotide exchange factors of Rho GTPases

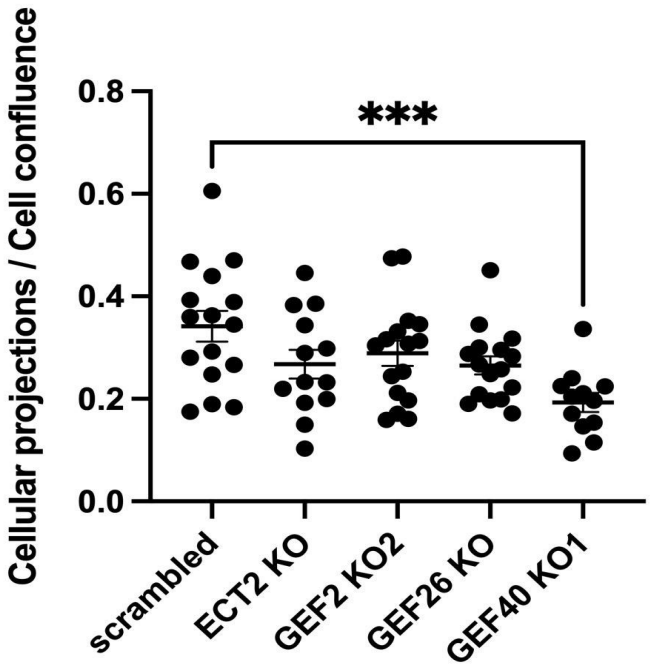

Figure 6. Filopodia formation in human podocytes 10 hours after treatment with EGF. The number of cellular projections in n=16 photographs of each treatment group were counted and normalized to the cell confluence.

(ECT2, ARHGEF2, ARHGEF26, and ARHGEF40) affect podocyte biology. Such information could provide valuable insights on the progression of foot process effacement, glomerular filtration barrier dysfunction, and proteinuria.

The G-LISA experiments provided preliminary clues into how GEFs allow Rho GTPases to be stimulated and activated by EGF. While a tendency to maintain activation with EGF treatment was detected for most GEF KO pools, the reliability of these results are limited by the lack of substantial sample sizes. Knockout of each GEF appears to have an impact on distinct Rho GTPases in duplicates; however, further experimentation will be performed to confirm the results.

The morphological analysis using fluorescence staining did not administer conclusive evidence of the role of particular GEFs on podocyte architecture. With no visible changes in cell surface area and shape (elongation), it is possible that there are more potent contributors to these features of podocytes than the Rho GTPase regulatory proteins. Furthermore, there may be multifactorial cooperation between complexes that contribute to podocyte appearance. However, since this was an elementary basal phenotype screening, the logical next step is to ascertain any morphological differences in these knockout groups following stimulation with a substance such as EGF.

The decrease in integrated density of phalloidin staining in the ARHGEF2 knockout cohort is an intriguing outcome. As previously mentioned, phalloidin detects actin filaments/stress fibers in the cytoskeleton. Since it was established that RhoA participates in the formation of stress fibers, and ARHGEF2 is a regulator of RhoA, it is sensible that the absence of ARHGEF2 would alter the architecture of these thread-like structures within podocytes. To validate this outcome, the cell morphology assessment investigations are being repeated on differentiated podocytes. This will provide further insight the true *in vivo* phenotypic state of these cells.

The filopodia assay produced robust evidence for the role of ARHGEF40 in cell migration and sensory performance. It is expected that the absence of ARHGEF40 would lead to decreased activation of Cdc42, consequently impairing the formation of filopodia. This was confirmed by a significant 40% decrease in the number of fibers projecting from podocytes after stimulation by EGF. This phenomenon suggests that ARHGEF40 affects the actin cytoskeleton via interactions with Rho GTPases (Cdc42), and its elimination from the kidney could generate considerable functional modifications.

One unforeseen complication arose in the TIDE analysis of ARHGEF40. The software gave an alert indicating there was no good alignment found between the control (scrambled) and test (ARHGEF40 KO) samples. This means that the alignment window may have been too small or the sequence read was of poor quality and could negatively skew the TIDE estimation. To ameliorate this, the left boundary of the alignment window was set 10 base pairs lower. In doing so, the control and test samples appeared better aligned and the TIDE analysis continued. To ensure accuracy of these results, the protocols for gDNA extraction, PCR, and sequencing could be repeated.

As previously mentioned, there is much work to be done to understand the mechanisms of Rho GTPase regulatory proteins. The potential future directions of these studies are manifold and are a significant undertaking. Firstly, a large portion of the Rho GTPase interactome discovered in podocytes has yet to be analyzed in this manner. Fortunately, the same methods of gene knockouts and analyses for ~20 other GEFs, as well as some GAPs, are in progress.

Secondly, it may be advantageous to explore other functional assays involved in actin dynamics. They may provide additional insights into the alterations in cell behaviour induced by the absence of regulatory proteins. Finally, on a broader scale, the investigation of renal phenotypes in systemic or conditional gene knockout mice could produce compelling evidence for the role of these proteins.

Conclusion

Collectively, the present study attempts to provide further rationale for the relevance of Rho GTPase regulatory proteins in podocyte architecture and function. In this way, an understanding of the mechanisms of the glomerular filtration barrier and its associated pathological variations are improved. Together with previous work into the Rho GTPase interactome in podocytes, there is clearly much to be determined about such crosstalk and signalling. Further experimentation should reveal concrete evidence for the impact of GEFs and GAPs on actin cytoskeletal dynamics, cell migration, and Rho GTPase activity. There are also expanding opportunities using these Rho GTPase regulatory proteins for the identification and development of novel therapeutic agents against proteinuria.

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