Final report

Background

We formerly described the first variant in human genetics with a mutation-dependent pathogenicity. The *NPHS2* R229Q variant is only pathogenic when trans-associated to specific 3' missense mutations on the other parental allele [1].
Mutations of *NPHS2* cause autosomal recessive steroid-resistant nephrotic syndrome [2]. *NPHS2* encodes podocin, a membrane-associated component of the slit diaphragm, that anchors nephrin to the cell membrane. The *NPHS2* R229Q variant is a frequent polymorphism with a MAF of 3.6% in Europe. It was shown to cause a late-onset glomerulopathy when associated to *NPHS2* mutations [3]. Our findings demonstrated that this is only true for a very specific subset of 3' mutations which cause amino acid substitution in the C-terminal region [1]. These C-terminal podocin mutants exert a dominant negative effect on R229Q podocin and retain it in intracellular compartments. Based on molecular modeling, we formerly suggested that the dominant negative effect is mediated by an altered oligomerization [1].

These observations raised several **questions in the clinical practice**:

1) Which R229Q associations are pathogenic? This question is of direct clinical relevance, as it determines the clinical classification (immune mediated vs. hereditary disorder) and thus the necessity of immunosuppressive therapy.
2) Are there other *NPHS2* variants with a mutation-dependent pathogenicity? The discovery of the mutation-dependent pathogenicity of R229Q was greatly facilitated by its high allele frequency, but rare variants may also be subject of interallelic interactions.
3) Based on our cell culture experiments, we considered the possibility of an interallelic complementation, a beneficial/complementary effect of two trans-associated pathogenic mutations, which could result in an unaffected phenotype.

Aim

We aimed to establish an *in vivo* model to answer these questions. This was expected to be the first animal model to study human interallelic interactions. As a large number of compound heterozygous knock-in animals are needed for this project, a vertebrate model was not realistic to choose. Along the same line, oligomerization may also be influenced by benign, non-conserved substitutions in the amino acid sequence, which supported the necessity of studying the *human* podocin itself.

We therefore aimed to generate a *Caenorhabditis elegans* model, deficient for the homologue gene of *NPHS2* (mec-2) and coexpressing differently (GFP- and mCherry-) tagged podocin pairs. MEC-2 shares 45% identity and 83% similarity over 275aa (72%) of podocin (383aa). Therefore, MEC-2 was formerly used to study the effect of podocin mutations [4]. MEC-2 is expressed in six neurons and is responsible for gentle-touch mechanosensation. We therefore expected that podocin can rescue the mechanosensation defect of *mec-2* mutants similarly to other homologous proteins with a lower ratio of identity [5, 6], and coexpressing different podocin variants will allow us to study the interactions of *human* podocin.

Implementation

We formerly obtained all the necessary equipment: incubators, fluorescent and standard stereomicroscopes from the MTA-SE Lendulet Research Grant (LP2015-11/2015). Here we applied for a gene gun (Biolistic PDS-1000/He Particle Delivery System) that is necessary for vector bombardment, i.e. transformation of the nematodes and a thermal cycler. Half of the OTKA grant was spent on the acquisition of the gene gun (even with 23% discount). The procurement procedure took a much longer

time than expected, we managed to establish the gene gun in 03/2018. We formerly obtained two mec-2 mutant strains (CB75 és CB3273). We verified their mutations by Sanger sequencing. Ágnes Regős went on maternity leave in 11/2017. The project has then been carried on primarily by Magdolna Keszthelyi, and from 05/2018 by Regina Légrádi (full-time researcher) and the biologist research student Tímea Köles (06/2018-).

1. Vector construction

We aimed to generate vectors encoding C-terminal GFP- or mCherry-tagged human podocin with codon optimization for *C. elegans*, under *mec-2* promoter (p*mec-2*::*NPHS2*(+)::GFP, p*mec-2*::*NPHS2*(+)::mCherry) and a selection marker. After several unsuccessful attempts of cloning by restriction enzyme digest and cold cloning, we finally generated the vector constructs with a NEBuilder Assembly method with Magdolna Keszthelyi. In order to select the transformed nematodes, we inserted *rol-6* and later *unc-119* genes in the vector. The introduction of the transformation was greatly helped by Prof. Tibor Vellai and János Barna (Genetic Department, ELTE).

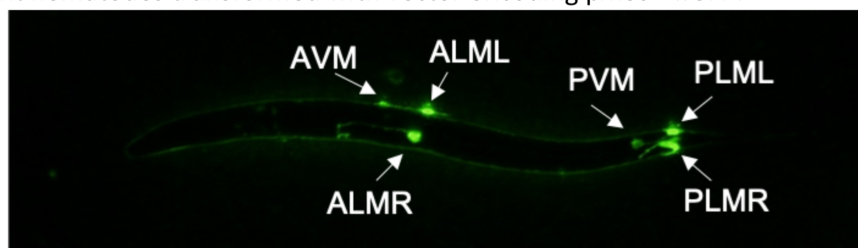
2. Transformation of the nematodes

Transformation of the nematodes occurs in an extremely low proportion (0.01%-0.001%). Effective selection is therefore of utmost importance. For this purpose, we first integrated the *rol-6* gene in the vector, which causes a dominant roller phenotype. This selection method was however unsuccessful: the phenotype was impossible to recognize in such a small proportion of the nematodes even after several days of hunting. Therefore, we replaced the selection marker *rol-6* with *unc-119*, and crossed *unc-119* and *mec-2* nonsense (p.W119*) mutant strains to generate double mutants. The *unc-119* mutants are easily recognized based on their recessive uncoordinated movement phenotype, and naturally selected based on their dauer formation defect. We transformed *mec-2* and *unc-119* double mutant strains in 08/2018, and selected those that are properly transformed, i.e. capable of dauer formation and have a rescued, coordinated movement. We successfully selected the transformed nematodes, some of which also integrated the vector into chromosomes based on the transmission rate of the coordinated movement phenotype/GFP expression. Despite the successful transformation, no podocin-GFP expression was visible in the nematodes with coordinated movement.

3. Validation of the *mec-2* promoter

To overcome the lack of podocin expression, we first validated the *mec-2* promoter. We received the promoter-encoding vector from the lab of the Nobel Laurate Martin Chalfie (Columbia University). To validate its effect, we removed the podocin encoding cDNA and aimed to express only GFP under the *mec-2* promoter. The GFP was perfectly visible in the six target neurons after transformation with p*mec-2*::GFP encoding vector, indicating the proper function of the promoter (Fig. 1).

Figure 1. GFP expression in the six neurons (AVM, ALMR, ALML, PVM, PLMR and PLML) in *mec-2* and *unc-119* mutant nematodes transformed with vector encoding p*mec-2*::GFP.



4. Expression of podocin-GFP

Despite the successful expression of GFP in the target neurons, it was challenging to express the GFP-tagged podocin. To improve its expression, we first inserted the generally used *unc-54* 3'UTR, then made the linker region longer between the podocin and the GFP, none of which yielded any result. We

then changed the Kozak sequence without any success either: found no *NPHS2* expression by qRT-PCR. We then redesigned a *C. elegans*-optimized *NPHS2* cDNA which also contained introns. This finally allowed us to achieve both podocin-GFP and podocin-mCherry expression which were well visible in the six target neurons.

5. Validation of the rescue effect

We next tested the rescue effect of human podocin-GFP on the gentle-touch sensation defect of *mec-2* mutants together with positive (wild type) and (negative) (non-transformed mutant) controls. In three independent experiments performed by three blinded examiners each, we observed no rescue. We therefore generated three vectors: the first encoding wild type MEC-2-GFP, and two encoding fusion hybrid proteins consisting of the non-conserved N- and C-terminal parts of MEC-2 and the conserved regions of podocin, including the entire oligomerization region (our region of interest) with a different extent in its C-terminal region. The second construct thus contains the 161-350 residues, the third the 161-375 residues of podocin (total: 383aa). Despite its perfectly visible expression in the six target neurons, we found no rescue with the wild type MEC-2-GFP in blinded experiments. This unexpected observation suggested that either its extrachromosomal expression (variable expression level) or the GFP tag prevents its proper functioning. We have not achieved chromosomal integration in any of the podocin- or MEC-2- expressing strains. Therefore, in order to properly assess the effect of podocin or the fusion proteins, we first aimed to rescue the phenotype with MEC-2.

6. Attempts to achieve a rescue with MEC-2

To rescue the recessive gentle touch sensation defect of the *mec-2* mutants with the reexpression of the wild type MEC-2, we aimed to achieve 1) self-cleaving of MEC-2 and GFP and 2) chromosomal integration.

1. To achieve self-cleaving of MEC-2 and GFP, we inserted a T2A peptide-encoding sequence between *mec-2* and the GFP/mCherry coding sequence. This sequence allows the spontaneous self-cleavage of the protein to separate MEC-2 and GFP by ribosomal skipping. We successfully transformed a few nematodes, but achieved only a mosaic expression.

2. Generation of transgenic strains, i.e. chromosomal integration of a single vector copy would allow to normalize the expression level and to avoid mosaicism, that is often observed in nematodes with extrachromosomal expression. To achieve both goals, we aimed to implement the recently described MosSCI (mos1-mediated single copy insertion) technique. We acquired three mutant lines carrying the Mos1 transposon on chromosomes I, II and IV (*unc-119* is located on chr III and *mec-2* on chr X). We have already generated *mec-2* and *unc-119* mutants with MosI transposon site on chr I. We also acquired a vector encoding the transposase and three vectors with the corresponding flanking regions of the target transposon sites. We are currently constructing the MEC-2 – T2A – GFP encoding cDNA. We are going to start the transformation experiments in a few weeks and thus aim to generate transgenic lines stably expressing GFP-tagged MEC-2 from chromosome II. Once the rescue with the wild type MEC-2 is achieved, this approach will allow us to establish stable lines coexpressing mCherry-tagged podocin (or fusion protein) variants from chr I and GFP-tagged podocin or fusion protein variants from chr II. We expect that these transgenic lines will allow us to properly study the rescue effect of podocin/fusion protein variants and thus the interallelic interactions of the podocin mutations. All mutations of interest are located within the conserved region of podocin, and are thus possible to investigate also by the fusion protein.

We published a Hungarian article entitled "A nematode in the service of medicine: *Caenorhabditis elegans*" in Gyermekgyógyászat (Pediatrics) [7], and presented our efforts at several national congresses:

- Keszthelyi TM, Regős Á, Légrádi R, Schnabel K, Seidl D, Mikó Á, Tory K: Egy *Caenorhabditis elegans* modell létrehozása és validálása a humán podocin interakcióinak vizsgálatához (2018), Magyar Gyermeknephrologiai Egyesület 2018. évi Kongresszusa, Pécs, 12-13/10/2018
- Keszthelyi TM, Légrádi R, Schnabel K, Köles T, Seidl D, Mikó Á, Regős Á, Tory K: Egy caenorhabditis elegans modell létrehozása és validálása a humán podocin interakcióinak vizsgálatához, Remedicon 49. Membrán-transzport Konferencia, Sümeg, 14-17/05/2019
- Schnabel K: A podocin interallelikus interakcióinak vizsgálata *Caenorhabditis elegans* modellen – betekintés egy *in vivo* modell létrehozásának küzdelmeibe (TDK presentation, Semmelweis University, 2019. TDK Conference, I. prize, nominated for OTDK participation)

Related works

Clinical guideline to assess the pathogenicity of *NPHS2* R229Q

We established a clinical guideline to help the assessment of the mutation-dependent pathogenicity of *NPHS2* R229Q and published it in Human Mutation (D1) [8]. In order to differentiate benign and pathogenic R229Q associations we combined the population-genetic, biochemical, genetic and cell biological aspects. I also wrote an editorial letter [9] in response to another genetic counseling guide about R229Q published in American Journal of Kidney Diseases [10] as it was false at several points.

The role of the C-terminal in the podocin oligomerization at the molecular and cellular level

We completed and published our work about the role of C-terminal oligomerization in interallelic interactions of podocin in collaboration with the MTA-ELTE Protein Modeling Research Group, the Biophysical Department of the Semmelweis University and the Laboratory of Hereditary Kidney Diseases of Institute Imagine, Paris [11]. We studied the podocin oligomerization through FRET analysis of podocin heterooligomers and the podocin localization in cultured podocytes and showed that

- the podocin oligomerizes exclusively in its C-terminal part
- truncation of all three C-terminal helical regions strongly influenced the oligomerization
- besides R229Q podocin, several other podocin variants influenced the localization of coexpressed podocin variants, indicating that interallelic interactions of *NPHS2* are not restricted to the R229Q variant
- a truncation distal to the P343 residue induces internalization
- this internalization can be prevented if the coexpressed podocin is membranous and possess an intact C-terminal part

Effect of podocin oligomerization on the nephrin oligomerization

In collaboration with Dóra Karancsiné Menyhárd (MTA-ELTE Protein Modeling Research Group), we hypothesized that podocin oligomerization may influence the nephrin oligomerization in the glomerular slit diaphragm. To explore the effect of podocin oligomerization on the oligomerization of nephrin, YPet- and mRuby-tagged wild-type nephrin and podocin variants with different oligomer-forming capacity were transiently expressed in HEK293 cells. We very recently found a significant reduction in the lifetime of the donor YPet fluorescence in the presence of wt podocin, but not with the monomer-forming R286Tfs*17, indicating that podocin oligomerization reduces the distance between neighboring nephrin molecules. Thus, podocin oligomerization significantly decreases the shortest dimension of the glomerular pore. We thus suggest that a major function of podocin is the regulation of the glomerular pore size.
We currently aim to explore the effect of pathogenic R229Q and non-pathogenic R229Q-associations on the glomerular pore size.

Two more non podocin-related works were published with the partial support of the KH125566 grant:

Phenocopies in autosomal recessive polycystic kidney disease (ARPKD)

In a collaborative work with the University of Debrecen and the University of Szeged, we studied the genetics of ARPKD. This genetically highly homologous nephropathy is caused by *PKHD1* point mutations in 80-85% of the cases. The remaining 15-20% are genetically unresolved. We aimed to understand whether the negative cases result from *PKHD1* copy number variations or second locus mutations. In a cohort of 36 patients with clinical ARPKD we showed that all eight negative cases developed as a result of second-locus mutations, and were thus phenocopies [12]. This was the first ARPKD study which aimed to achieve a genetic diagnosis in all patients.

Severe clinical presentation of Vici syndrome secondary to EPG5 c.1007A > G mutation

We aimed to understand the reason for the severe clinical presentation of Vici syndrome in a sibling pair with the *EPG5* c.1007A > G mutation, who died in infancy. The c.1007A>G (p.Q336R) mutation, affecting the penultimate nucleotide and the splicing of exon 2 is the most common mutation of *EPG5* and is typically associated with a less devastating prognosis with a median survival of 78 months. The less severe course related to c.1007A > G was formerly explained by the preserved canonical splicing in 25% of the transcripts. In contrast, we found the messenger RNA encoded by the c.1007A > G allele to be completely absent, explaining the severe course of the disease. This family provided another example of phenotypic variability related to a differential splicing [13].

References

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12. Szabo, T., et al., *Comprehensive genetic testing in children with a clinical diagnosis of ARPKD identifies phenocopies.* Pediatr Nephrol, 2018. **33**(10): p. 1713-1721.
13. Vojcek, E., et al., *EPG5 c.1007A > G mutation in a sibling pair with rapidly progressing Vici syndrome.* Ann Hum Genet, 2019.