Two novel mechanisms of steroid-resistant nephrotic syndrome

PhD Thesis

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1. Introduction

Steroid-resistant nephrotic syndrome (SRNS) is responsible for 6-7% of childhood chronic renal failure. The two main causes of SRNS are monogenic podocytopathies and immune-dysfunction. As monogenic podocytopathies do not respond to immunosuppressive therapy, nor reoccur after renal transplantation, so it is crucial to differentiate these two forms in the clinical practice. Podocytopathies result from mutations of the genes encoding the glomerular filtration barrier proteins (*NPHS2*, *WT1*, *NPHS1, MYO1E, TRPC6, INF2*). The most commonly affected genes encode proteins implicated in podocyte cell adhesion, structure, differentiation and function.

My first research project focused on the function of podocin, encoded by *NPHS2*, the most commonly implicated gene in SRNS. Biallelic *NPHS2* mutations typically cause end-stage renal disease up to the age of 10 years. Podocin is exclusively expressed in podocytes. As a member of the stomatin superfamily, it is membrane-anchored. Binding nephrin, it is part of the glomerular slit diaphragm, and also modulates signaling pathways of podocytes. The function of podocin is however not entirely understood.

Our research group has formerly shown, that the pathogenicity of the most frequent non-silent *NPHS2* variant, R229Q (MAF: 3,6% in Europe, rs61747728) depends on the trans-associated mutation: it is only pathogenic when associated to specific 3' mutations. As such, this is the first variant in human genetics with a trans-associated mutation-dependent pathogenicity. Based on structural modelling the research group showed that R229Q podocin, similarly to the wild-type, forms oligomers, but in the pathogenic associations this is altered. According to literature, podocin oligomerizes through both its N- and C-terminals. Furthermore, *in silico* experiments showed that R229Q podocin has an increased rigidity. The pathogenicity of the specific R229Q associations was therefore explained by an

altered structure. Cell culture experiments showed that podocin is not membrane-targeted in the pathogenic associations. These findings have a fundamental impact on the diagnosis, treatment and genetic counselling of *NPHS2*-related SRNS. If one parent carries a mutation in *NPHS2* with no dominant negative effect, and the other is heterozygous for R229Q, their child is not at risk of developing SRNS.

The second aim of my work was to explore the effect of a *DKC1* mutation identified in an undescribed syndrome. The role of *DKC1* has never been described in SRNS, but in bone marrow failure syndromes as dyskeratosis congenita (DC) and Hoyeraal-Hreidarsson (HH) syndrome. These disorders result from telomere attrition and are therefore considered to be telomeropathies. The classical mucocutaneous features of DC are nail dystrophy, dyskeratosis and leukoplakia. HH syndrome is a more severe phenotype characterised by in utero growth retardation, cerebellar hypoplasia and early lethality. The protein encoded by *DKC1*, dyskerin is the main catalytic member of the H/ACA small nucleolar ribonucleoprotein complex (snoRNP). Other members of this complex are NOP10, NHP2, GAR1, the guide and the substrate RNA. The two main roles of this snoRNP complex are telomere maintenance and pseudouridylation. Telomere attrition is known to be causal in DC and HH syndrome. Pseudouridylation is the most abundant posttranscriptional RNA modification, affecting rRNA, tRNA, snoRNA and also mRNA. It is known to be important for the stabilisation of RNA, but its defect has not been described causal in human disorders. Most important targets of dyskerin are snoRNA and rRNA. *DKC1* mutations implicated in telomeropathies are located in the 5’ and 3’ regions of the gene. Mutations of the pseudouridylation domain have been described only in two cases, both causal in the severe HH syndrome.
We formerly identified a large family with an unknown form of SRNS associated with cataracts, hearing impairment and enterocolitis. The index case was a severely affected male who died

at the age of seven years. All the affected males died in early childhood. Females had a normal lifespan, and developed cataracts and hearing impairment in the second decade of life. The sex related difference of severity suggested an X-linked inheritance.

The casual gene was identified in collaboration with the Imagine Institute, Paris and the University of Köln. Linkage analyses revealed a 5 Mb large region on the long arm of chromosome X (Xq28, logarithm of the odds 3,01), assuming a germline mosaicism in the great-grandparental generation. Accordingly, through sequencing a haploidentical but unaffected family member from the grand-parental generation and the index male, we identified indeed a *de novo* point mutation in *DKC1* (c.6161G>A, p.Glu206Lys). We considered this mutation to be causative, because it was absent in the general population, predicted to be pathogenic, affected a highly conserved glutamic acid, and the mutation segregated with the disease.

British collaborating partners of ours (UCL Great Ormond Street) identified a novel homozygous mutation in *NOP10*, which encodes the amino acid change p.Thr16Met. This mutation affects the same pseudouridylation domain as well, and is similarly located in an evolutionary highly conserved region. It does not disrupt the connection between dyskerin and NOP10. The phenotype of the patients highly resembles those of the p.Glu206Lys family, and just slightly overlaps with the clinical signs of DC or HH-syndrome.

2. Aims

1. To explore the mechanism of podocin oligomerisation and the role of C-terminal helical regions.
2. To explore the effect of R229Q in heterooligomerisation by determining the FRET-efficiency in different heterooligomers.
3. To determine the effect of the novel *DKC1* Glu206Lys mutation on the localisation and interactions of dyskerin.
4. To generate a *dkc1* knock-in mutant zebrafish model.
5. To explore the role of telomere attrition and pseudouridylation defect in the pathophysiology of the novel phenotype.

3. Methods

Investigation of podocin oligomerisation

To explore the oligomerisation of podocin we used truncated podocin mutants and observed the changes in binding capacity in their heterooligomers with wild-type and R229Q podocin. The four truncated mutants were R286Tfs*17, A317Lfs*31, F344Lfs*4 and F344*podocin. The C-terminal tail (CTT) of podocin consists of three helices (H1-3), the TVV(337-339)-internalisation domain and linker regions. R286Tfs*17 is lacking all the three C-terminal helices, A317Lfs*31 contains one (H1), F344Lfs*4 and F344*podocin contain two (H1-H2) intact C-terminal helices and the TVV-domain. F344Lfs*4 and F344*podocin only differ from each other in the three-amino acid frameshift sequence (LTY). The R229Q- F344Lfs*4 is the only known pathogenic association of the investigated ones.

Fluorescence spectroscopy

Podocin-coding plasmids were provided by our collaboration partners, amplified in competent bacteria, purified with plasmid extraction kits, controlled via Sanger sequencing. Podocin variants were expressed in HEK293 cells, cultured at 37 °C in DMEM, high glucose with 10% FBS and 1% Penicillin-Streptomycin. Cells were transfected with Calcium-phosphate based method. Transfected cells were incubated for 48 h and lysed by 150mM NaCl, 20mM Tris, 1% Triton-X supplemented with 0.1% protease inhibitor. Lysis and upcoming procedures were performed on ice. Lysates were incubated with monoclonal anti-HA antibodies, subsequently with Protein G magnetic beads. Immunprecipitates were washed three times with lysis buffer. Podocin variants were eluted by competition with HA peptides. Concentration of the eluates was measured by spectrophotometry. Eluates were verified by Western blot analysis. Protein aliquots (0.4 nmol) were stained separately with 4 nmol of either Alexa Fluor 488 C5 Maleimide (donor dye) or Alexa Fluor

555 C2 Maleimide (acceptor dye) molecules and were incubated overnight at 4 °C. Differently stained podocin variants were subsequently mixed two by two for oligomerization, incubated for 2 h on RT and washed through PD SpinTrap G-25 filter column to discard the unbound HA peptides and fluorophores. Förster type Resonance Energy Transfer (FRET) was measured between two differently stained podocin variants in a final volume of 100 µl, containing each podocin with a concentration of 4 µM. The fluorescence lifetime of the donor dye (Alexa 488 C5) was measured, out of which the FRET efficiency was counted, which refers to the binding capacity between the homo-end heterooligomers. Experiments were repeated three times on samples obtained from at least two different expressions. Statistical analyses were performed with ANOVA and Tukey HSD post hoc tests using Statistica 13.2 software. Additional molecular dynamics simulations performed by the MTA ELTE Protein Research Group completed our insights in to the understanding of structural changes.

Investigation of the effect of the novel *DKC1* mutation

Generation of wild-type and mutant dyskerin and NOP10 encoding plasmids

cDNA was amplified from bacterial expression vectors, N-terminal Flag tag was added to dyskerin, C-terminal V5 tag to NOP10. Plasmids were amplified in competent bacteria, coding sequence was verified by Sanger sequencing. Site directed mutagenesis was performed on both wild-type plasmids to generate the E206K dyskerin and the T16M NOP10 mutant plasmids.

Pressure tuning fluorescence spectroscopy

Wild-type and mutant dyskerin and NOP10 proteins were expressed and total protein was harvested as described above. Lysates were incubated with monoclonal anti-flag or anti-V5/NOP10 antibodies and subsequently with Protein G beads. Dyskerin and NOP10

variants were eluted by competition with Flag and V5 peptides. Eluate concentration was measured by spectrophotometry and verified by Western blot analysis. Pressure tuning fluorescence spectroscopy was used to determine the dissociation constant of the NOP10-dyskerin complex (Kd) and the pKd (pKd = -log(Kd)). The method involves the use of a fluorescent dye (8-Anilinonaphthalene-1-sulfonic acid, ANS) which binds to the solvent-accessible, hydrophobic regions of proteins, hydrostatic pressure is then varied to induce dissociation. At the pressure where the complex dissociates, fluorescent intensity increases. As a result pKd was determined, which refers to the strength of binding. Additionally, directly related to the pKd, the interaction surface was determined. Structural heterogeneity was qualitatively assessed. Statistical difference between WT and mutant complexes was assessed via a Welch-test (Statistica 13.4). Experiments were repeated three times on proteins from at least two expressions. Fluorescent spectroscopy experiments were carried out at the Semmelweis University, Department of Biophysics and Radiation Biology.

Analysis of the subcellular localisation of dyskerin

A human immortalized podocyte cell line (AB8/13) provided by M. Saleem was cultured on type I collagen–coated coverslips and transfected with plasmids encoding Flag-tagged WT, p.Glu206Lys, and p.Ala353Val dyskerin using FuGENE HD and incubated for 48 hrs. Transfected podocytes were fixed in ice cold 4% PFA/100% ethanol, blocked with PBS-BSA 1%, incubated with rabbit anti-flag primary antibodies (1:500), followed by Alexa Fluor 647 conjugated goat anti-rabbit IgG secondary antibodies (1:200) and Alexa Fluor 555 phalloidin (1:200); all conducted at RT. Nuclei were stained with Hoechst. Confocal optical slices were captured using a 40× oil objective lens (Leica Microsystems), an optical slice thickness of 800 nm and an x-y pixel size of 86 nm (Leica SP8 confocal microscope, Necker Imaging Facility).

Generation of zebrafish mutants

CRISPR/Cas9 mediated mutagenesis of the *dkc1* gene was carried out in the WT tuebingen (tue) strain, targeted regions were in exon exons 7 and 11. Our aim was to generate the p.Glu206Lys and the p.Ala353Val mutants. Genotyping was conducted using allelespecific PCR, Sanger sequencing and fragment analysis. DNA was isolated from zebrafish embryos, larvae or fin clips via NaOH and 95°C, pH was equillibrated with Tris. DNA was purified in further steps with ethanol extraction. Primers were designed using Sequencher 4.9. Injections were performed, WT (tue) and mutant dkc1 and tp53 fish lines were maintained in the animal facility of ELTE, Department of Genetics.

Flow-Fluorescence in-situ hybridization (FISH)

Zebrafish cells were isolated from 4-5 dpf wild-type and mutant zebrafish larvae, ten per sample, following previously described protocols (Monique Anchelin et al., 2013). Cells were stained using the 'Telomere PNA Kit/FITC for Flow Cytometry'. Cells were analysed on a BD FACSAria flowcytometer. Following the gating of viable lymphocytes in the G0/1-phase, mean fluorescence intensity was established in samples hybridized with the FITC-conjugated telomere specific PNA probe. Telomere length was determined by the subtraction of cell autofluorescence from the fluorescence of telomere specific stained samples. Telomere length of wild type and mutant zebrafish was compared by Mann-Whitney U test (Statistica 13.4).

Western blot (dkc1, p53)

We performed Western blot experiments on lysates of six-six, wild-type and *dkc1* null mutant 2., 4., 5 dpf zebrafish larvae following previously described protocols (Link et al., 2006). Primary antibodies were against dyskerin and p53. Gamma or alpha tubulin was used as loading control. Densitometry was performed using ImageJ software.

X-inactivation measurements

RNA was isolated from peripheral blood, skin and fibroblasts and was reverse transcribed. The X-inactivation ratio was assessed by allele-specific qPCR using a Taqman probe (LightCycler). Dilution series of Flag-tagged WT and E206K *DKC1* plasmids were used for standardization. All measurements were repeated three times.

Analysis of pseudouridylation and the 28S/18S rRNA ratio

Immunonorthern blotting: Total RNA was isolated from n=12 4.5- 5 dpf, wild-type and null mutant zebrafish larvae by TRIzol Reagent and isolated on 1,5 % denaturing agarose gel. 3 μg RNA was loaded per well. Gel image was taken by Molecular Imaging Gel Doc System. Gel blotting was performed o/n onto a nylon membrane. Blotted RNA was cross-linked under UV. Nylon membranes were blocked with 5% non-fat dry milk and subsequently incubated with primary antibodies against pseudouridine (D-347-3, MBL, dil. 1:500). As a seconder antibody HRP-conjugated anti-mouse IgG was used. Bands were visulaised by chemiluminescence on Molecular Imager VersaDoc MP 5000 System. Densitometry was performed using ImageJ software. Statistica 13.2 software was used for statistical analysis by Mann-Whitney U test. The Norther blotting was carried out in the Agricultural Biotechnology Institute.
RNA quality assessment: Samples were prepared as described for the immunonorthern blotting. RNA quality was assessed on Agilent 2100 Bioanalyzer using the RNA 6000 Pico kit according to the manufacturers' instructions. The size of the 18S and 28S peaks on the electropherogram was compared in mutant and wild type zebrafish larvae by independent t-test using the Statistica software version13.2.

4. Results

Podocin oligomerisation

We found an approximately 20% FRET efficiency in the wild-type and the R229Q homooligomers supporting the in vitro formation of strong oligomers. Heterooligomers of wild-type and R229Q podocin variants showed an even higher FRET efficiency. In contrast, the FRET efficiency measured between R286Tfs*17 podocin and either the wild-type or R229Q podocin did not exceed that of the negative control indicating that podocin is unable to dimerize if all three C-terminal helical regions are disrupted. These results show the exclusive role of the CTT in the oligomerization. The R229Q, A317Lfs*31, F344* or F344Lfs*4 variants markedly influenced the FRET efficiency, indicating the significant effect of R229Q and the three helical regions on the oligomer conformation. The frameshift sequence of the dominant negative F344Lfs*4 podocin changed the FRET efficiency in the pathogenic R229Q-F344Lfs*4 differently than in the benign WT-F344Lfs*4 heterooligomer. The three amino acid encoded by the frameshift sequence weakened the binding capacity in the R229Q-F344Lfs*4 heterooligomer.

Effect of the novel *DKC1* mutation

Changes in the dissociation constant

Pressure tuning fluorescent spectroscopy revealed a change in the dissociation constant of the E206K dyskerin-WT NOP10 and WT dyskerin-T16M NOP10 associations compared to the wild-type associations. Mutant complexes altered the pKd, the interaction surface and the structural heterogeneity. Additional molecular dynamics simulations showed that both mutations interrupt H-bonds in the pseudouridylation catalytic domain and affect the structure of the pseudouridylation site: the interaction between the substrate uridin and the catalytic aspartic acid got disrupted.

Localisation of E206K dyskerin

The novel mutation does not alter the localisation of dyskerin, the nuclear localisation is similar to that of the wild-type and the A353V mutant.

Generation of zebrafish mutants

No homologous recombination was achieved while mutagenesis. Therefore, we only succeeded to generate null mutants with different indels, but no knock-in mutants. We studied two null mutant strains with indels in the 7. and 11. exons. Both homozygous null *dkc1* mutants died at 5 dpf, with a phenotype resembling the human phenotype: opaque lenses (cataracts), microphthalmia, abnormal eye and tectum structure, elevated cell-cycle marker (ccD1, PH3) levels, hypoplastic inner ears, gut and pronephros (low Wt1-positive cell count), an abnormal corpus pineale structure, haemopoetic defect (gata1, rag1) and a defective jaw cartilage development.

Telomere length in zebrafish mutants

We observed no telomere shortening in the homozygous null *dkc1* mutant zebrafish larvae at 5 dpf.

Western blot analysis results

Dyskerin levels were reduced in 4 and 5 dpf null mutant zebrafish larvae, in contrast p53 levels increased at that stage. The (*dkc1-/-*) phenotype could not be rescued on a p53-null background (*dkc1* -/- and *p53* -/-), indicating that the major phenotypic features observed upon loss of dyskerin function are not mediated by p53.

X-inactivation measurement results

We found a highly skewed X-inactivation in patient peripheral blood mononuclear cells, skin and fibroblast, explaining the severe phenotype of the girl who developed nephrotic syndrome. In accordance with the survival advantage of the cells expressing the WT allele in *DKC1*-linked DC, her X-inactivation in leukocytes tended to be skewed towards the mutant allele by the second decade of life.

Ribosomal RNA pseudouridylation in mutant zebrafish larvae

The pseudouridylation of 18S rRNA is reduced in *dkc1* null mutant zebrafish larvae at 5 dpf as determined by immunonorthern blot.

RNA integrity measurement results

In parallel with previous findings, the abundance of processed 18S rRNA was low: the 28S/18S ratio increased in *dkc1* null mutant zebrafish larvae.

5. Theses

1. Podocin oligomerises exclusively through the C-terminal region.
2. All three C-terminal helical regions affect the oligomerisation.
3. The frameshift sequence responsible for the dominant negative effect of the F344Lfs*4 podocin induces a change in the oligomer conformation. It may explain its dominant negative effect.
4. *DKC1* E206K causes a novel phenotype with cataracts, hearing impairment, nephrotic syndrome, enterocolitis and early lethality in males and cataracts and hearing impairment in females.
5. Dyskerin E206K results in an altered interaction with NOP10.
6. Dyskerin E206 does not alter the subcellular localisation of dyskerin.
7. The severe phenotype of a heterozygous girl is secondary to a skewed X-inactivation. Peripheral blood mononuclear cells expressing the mutant allele undergo natural selection.
8. Null *dkc1* mutant zebrafish phenocopies the human disorder with no telomere attrition but a pseudouridylation defect of the 18S rRNA.
9. The novel phenotype results at least partly from pseudouridylation defect of the rRNA and not from telomere attrition.

6. List of publications

Publications related to the thesis

Stráner P[1], Balogh E[1], Schay G, Arrondel C, Mikó Á, L'Auné G, Benmerah A, Perczel A, Menyhárd D K, Antignac C, Mollet G[2], Tory K[2]
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Proc Natl Acad Sci USA, in press. IF(2018): 9,5804

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