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Abstracts

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Subtheme I: Glomerular Crosstalk, Soluble Factors & Metabolism

I1

Proteostasis Regulators Ameliorate Podocyte Injury Associated with Mutant α-Actinin-4

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Introduction: Mutations in α-actinin-4 are associated with focal segmental sclerosis (FSGS) in humans. Previously, we demonstrated that α-actinin-4 K256E undergoes aggregation in cultured glomerular epithelial cells (GECs). There is associated proteotoxicity, evidenced by induction of endoplasmic reticulum (ER) stress. By analogy, ER stress occurs in mice with FSGS induced by expression of an α-actinin-4 K256E transgene in podocytes. In the present study, we assessed if modulation of proteostasis can ameliorate α-actinin-4 K256E-induced proteotoxicity.

Methods: To modulate proteostasis, we employed 4-phenylbutyric acid (4-PBA), a compound that improves ER protein folding and reduces ER stress, as well as celastrol, a drug that increases expression of ER and cytosolic chaperones, including Grp78 and Hsp70, respectively. We also expressed constitutively-active HSF1 (ΔHSF1), which induces expression of Hsp70.

Results: After transfection and subsequent addition of cycloheximide (to block protein synthesis), α-actinin-4 wild type (WT) was stable for at least 48 h, while α-actinin-4 K256E declined by ~50% at 48 h. α-actinin-4 K256E, but not WT, formed aggregates in GECs, and disrupted ER-associated degradation. 4-PBA, celastrol and ΔHSF1 reduced aggregation of α-actinin-4 K256E. Celastrol and ΔHSF1 also retarded degradation of the K256E mutant, but had no effect on the WT. Ubiquitination of α-actinin-4 K256E was greater, compared with WT; however, ubiquitination was not directly affected by celastrol or ΔHSF1. α-actinin-4 K256E did not independently increase Hsp70 expression. In α-actinin-4 K256E transgenic mice, treatment with 4-PBA in the drinking water over 10 weeks significantly reduced albuminuria, and glomerular expression of the proapoptotic ER stress protein, CHOP.

Conclusion: α-actinin-4 K256E formed aggregates in cultured cells and disrupted ER function. Compounds that improve protein folding or enhance chaperone expression reduced aggregation. Improving protein folding in the ER ameliorated albuminuria in α-actinin-4 K256E-associated FSGS. Small molecule proteostasis regulators may represent a novel therapeutic approach to glomerular injury.

I2

Lack of Protein Fatty-Acylation Can Cause Focal Segmental Glomerulosclerosis (FSGS)

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Introduction: Protein fatty-acylation is the covalent attachment of fatty acids to proteins. Protein N-myristoylation represents one type of protein fatty-acylation and refers to the attachment of myristic acid to the N-terminal glycine. This modification is catalyzed by N-myristoyltransferases (Nmt1 and Nmt2) and promotes protein-protein and protein-membrane interactions. Our aim was to elucidate the role of protein N-myristoylation for the morphology and function of podocytes.

Methods: LoxP-sites were introduced into the Nmt1 and Nmt2 loci by homologous recombination in embryonic stem cells and podocyte-specific deletion was achieved by expressing Cre under the control of podocine promoter (Pod^{Cre}). At day 1, 17, 35 and 70 after birth, morphological analysis (conventional and electron microscopy) and functional tests (urine and serum) were performed.

Results: Mice with podocyte-specific deletion of both Nmt1 and Nmt2 ($Pod^{Cre}/Nmt1^{flox/flox}/Nmt2^{flox/flox}$) did not show proteinuria at day 1 after birth. However, starting at day 17, they developed a FSGS accompanied by an increasing proteinuria. At day 35, these changes progressed to a diffuse segmental and global glomerulosclerosis with an overt nephrotic syndrome leading to death before day 70 in most of the $Pod^{Cre}/Nmt1^{flox/flox}/Nmt2^{flox/flox}$ mice. Upon electron microscopy, podocytes lacking NMT activity showed a fusion of foot processes. In contrast, the glomerular scaring and proteinuria were significantly milder or absent in mice lacking only Nmt1 or Nmt2 respectively. We could show that cytoskeleton-associated proteins such as MARCKS (myristoylated alanine-rich C kinase substrate) need myristoylation activity for their function.

Conclusions: These results show that protein N-myristoylation is essential for a proper localization and function of proteins in

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podocytes. Investigations are currently done to determine which metabolic and immunologic diseases interfere with protein N-myristoylation in podocytes. We propose that metabolic alterations may potentially initiate the most frequent form of FSGS, which is associated with degenerative and immune complex diseases.

I3

Modulation by Angiotensin II of Glucose Transport in Podocytes

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Introduction: Angiotensin II has been implicated as a mediator of podocyte abnormalities in diabetes. Podocyte dysfunction arises from excessive glucose uptake through their differentiated GLUT transporter system. In other cell types, Ang II has been shown to modulate glucose transport by influencing GLUT1 expression. Crosstalk between AngII and insulin signaling is considered to be involved in diabetic complications. In our study we have examined the effect of Ang II on basal and insulin-dependent glucose transport in podocytes.

Methods: Immortalized mouse podocytes overexpressing human AT1 receptor were cultured in normal-(NG, 5.6 mM) or high-(HG, 30 mM) glucose media for 5 days. The cells were incubated with AngII and/or insulin for different time periods and glucose uptake was evaluated using 3H-deoxy-D glucose. Specific kinase and AT1 inhibitors were used to assess which signaling pathways were involved in AngII effects. Insulin receptor (IR) and GLUT1 expression were checked using RT-PCR, Western blotting and flow cytometry.

Results: In NG cells, glucose transport increased by 50% after 30 min and was doubled after 24 hours exposition to AngII. In HG group, AngII stimulated glucose uptake by 30% after 3 and 24 hours. The effect was mediated by AT1 receptor, and by PKC and PI3K kinases. AngII inhibited insulin-dependent glucose uptake in NG cells and had no effect on insulin action in HG group. Nevertheless, both AngII and high glucose increased the IR expression. HG downregulated GLUT1 mRNA by 15% in Control and Ang groups. AngII elevated GLUT1 in NG and HG cells by 9% and 15% respectively.

Conclusions: Ang II stimulated the basal glucose uptake into podocytes in a glucose- and time-dependent manner. The effect may be due to upregulation of GLUT1 transporter. Inhibition by AngII of insulin-dependent glucose transport despite increased IR expression suggests that AngII may interfere with IR signaling.

I4

Focal Segmental Glomerulosclerosis by Polymorphisms in Slit Diaphragm Genes Aggravating Kidney Damage in Children with Glomerular Basement Membrane Disease

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Introduction: Focal segmental glomerulosclerosis (FSGS) is the most common primary glomerular disorder causing end-stage renal disease. Homozygous mutations in glomerular basement membrane or slit diaphragm-genes cause early renal failure. Heterozygous carriers develop renal symptoms late, if at all. Most cases of FSGS are idiopathic. Here, we describe three families with FSGS, which were unexpectedly diagnosed in toddlers with Alport syndrome and in adolescent Alport-carriers with renal failure.

Methods: Written informed consent was given by the families. Genomic DNA was extracted from peripheral blood leukocytes. The coding regions of COL4A3, COL4A4, COL4A5, Nphs1, and Nphs2 were analyzed. Kidney biopsies were analyzed by light microscopy, immunohistochemistry, and electron microscopy.

Results: In addition to mutations in Alport syndrome-related genes, nephrin- and podocin-polymorphisms aggravated kidney damage, including severe FSGS with GBM-ruptures in a toddler and unusually early renal failure in heterozygous girls. In case 1, a X-chromosomal COL4A5 de novo glycine-mutation (p.W1538X (TGG>TGA), c.4614 G>A) was discovered in a two-year-old Lithuanian boy, typically associated with a rather mild phenotype. The severe FSGS and GBM-ruptures led to the exploration of slit diaphragm genes. A polymorphism (p.R408Q (CGG>CAG), c.1223G>A) in Nphs1 was found and homozygous silent polymorphisms in Nphs1 (p.S1105S (TCG>TCA), c.102A>G) and Nphs2 (p.G34G (GGA>GGG), c.3315G>A). Similarily, in case 2 and 3, the histological picture of FSGS was caused by heterozygous COL4A5-mutations aggravated by Nphs2-polymorphisms.

Conclusions: Our findings demonstrate that after thorough clinical evaluation, the molecular genetics of different players in the glomerular filtration barrier can be used to evaluate FSGS. The analysis of genes involved in the organization of podocyte architecture, the GBM, and the slit diaphragm will further our understanding of the histological picture of FSGS. Our increasing knowledge of genes, arising from next-generation sequencing, will help to personalize the diagnosis and prognosis of and therapy for FSGS.

I5

Role of mTORC1 for FSGS Progression

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Introduction: Mammalian Target of Rapamycin (mTOR) signaling is involved in a variety of cellular functions including growth, survival and metabolism. Recently, mTORC1 activation has been identified as underlying cause for several podocytopathies such as diabetic nephropathy. However, clinical FSGS trials administrating the mTOR inhibitor rapamycin led to conflicting results ranging from complete remission to severe deterioration of kidney function.

Methods: Here we combined genetic titration of mTORC1 levels in murine glomerular disease models, pharmacologic studies and human sample analysis to unravel the role of mTOR in focal segmental glomerulosclerosis (FSGS).

Results: mTORC1 target genes were significantly induced in both, human FSGS cohorts and murine Adriamycin nephropathy models. Curtailing mTORC1 signaling by genetically reducing the Raptor copy number in podocytes prevented glomerulosclerosis and significantly ameliorated the progression of murine FSGS models. Unexpectedly, however, induced deletion of both Raptor alleles dramatically accelerated the progression of murine FSGS models indicating a non-linear mTORC1 gene dosage effect. In agreement, rapamycin dose dependently affected the course of murine FSGS models with lower doses being beneficial and high doses aggravating the disease.

Conclusions: Thus, these data reveal a non-linear involvement of mTORC1 in FSGS progression pointing towards the difficulty of manipulating mTOR as therapeutic intervention. Furthermore, these data highlight a role for an individualized medicine approach by correlating kinase activities to potential treatment dosages of glomerular diseases.

I6

Unraveling the Mechanism of Action of Glucocorticoids in Glomerulonephritis

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Introduction: Glucocorticoids are commonly used for the treatment of glomerulonephritis. Particularly in the most severe form, crescentic glomerulonephritis (CGN), high-dose steroids are first-line therapy. To date, the mechanisms or target cells of glucocorticoid actions remain incompletely understood. The present study investigated whether glucocorticoids – beside their immunosuppressive effects – directly act on intrinsic glomerular epithelial cells in CGN.

Methods: For this purpose, the glucocorticoid receptor was inactivated specifically in all kidney epithelial cells in Pax8-Cre/$GR^{fl/fl}$ mice, including podocytes and parietal epithelial cells (PECs).

Results: GR inactivation did not impair renal development or function up to 12 months of age. Next, the nephrotoxic serum nephritis model (NTS) was induced in knockout and wild-type control mice. CGN was attenuated by high-dose prednisolone, as expected. Surprisingly, disease activity was also attenuated in renal epithelial GR knockout animals. To verify this, animals were treated with the GR antagonist mifepristone alone. CGN was attenuated more potently than when using prednisolone without obvious effects on the immune system. These findings were verified in a second, non-immunological model (Alport-mouse), where again progression of glomerulosclerosis by activated PECs was attenuated more efficiently by mifepristone than by high-dose prednisolone treatment. Direct actions of steroids on intrinsic renal cells were also verified in vitro using primary parietal epithelial cells.

Conclusions: In summary, glucocorticoids exert direct effects on activated glomerular epithelial cells in CGN, which appear to be therapeutically more important than immunosuppressive effects. Strikingly, genetic or pharmacological GR inactivation in glomerular epithelial cells is at least as effective as GR stimulation using high-dose steroids to treat glomerulonephritis.

I7

Glycosylation and Diabetic Kidney Disease – The Effect of Insulin on Glyco-Gene Expression of Podocytes

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Introduction: The developmental and functional status of the podocyte is reflected in the glycodecoration of podoproteins. Experimental neutralization of the podocyte glycocalyx associates with protein leakage and podocyte flattening also seen as increased glomerular permeability in diabetic kidney damage. Podocytes are insulin responsive and increase their glucose uptake when stimulated by insulin via its effect on glucose transporters GLUT1 and GLUT4.

Here we mapped the glyco-genes regulated in podocytes under insulin induced diabetes mimicking stress in an aim to identify the key genes involved in glomerular filtration barrier and podocytes in diabetic kidney disease.

Methods: Changes in glyco-gene expression were screened in a human conditionally immortalized podocyte cell line (kind gift from Prof Moin Saleem) after various insulin treatments (normal culture conditions, insulin starvation, differing insulin concentrations). The glyco-gene expression changes were screened using the GlycoV4 oligonucleotide array designed for the Consortium for Functional Glycomics at the Scripps Institute, La Jolla, CA (http://www.functionalglycomics.org/static/consortium/consortium.shtml). Selected genes and respective products were analyzed using real-time RT-PCR, Western blotting and immunocytochemistry.

Results: Differential expression was detected both in the insulin-starved and in the insulin-treated samples. Several glyco-genes that are not previously linked to human podocytes under diabetic/insulin stress were identified. These included glyco-genes from several categories including glycan-transferases, -degradation enzymes, growth factors and receptors. In total, 25 distint glyco-genes were found to be differentially expressed. From these, a set of 6 genes was selected for further analysis.

Conclusions: Diabetes mimicking stress represented by different concentrations of insulin in the culture media resulted in differential glyco-gene expression in podocytes. Changes in insulin concentration induced differential expression in 25 glyco-genes. The changes in 5 glycan-transferases and a glycan degradation associated proteins were confirmed using real-time RT-PCR, Western blotting and immunocytochemistry.

I8

Changes in the Mitochondrial Network and Bioenergetic Capacity of Human Podocytes

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Introduction: Changes in mitochondria or their functional capacity have been associated with several metabolic disease states, and mitochondrial dysfunction associates with podocyte injury. However, details of bioenergetics or the morphology and network structure of podocyte mitochondria have not been characterized.

Methods: Several mitochondrial parameters were determined from transformed human podocytes (AB 8/13) using high-resolution respirometry. Morphology was studied using fluorescence microscopy. Real-time quantitative PCR analysis was used to analyze the expression of key genes of mitochondrial biogenesis and metabolic adaptation, as well as to determine mtDNA/nuclear DNA ratio.

Results: During differentiation, cellular respiration increased from 50 pmols (s-1 10^{-6} cells) up to 131 pmols, and oligomycin-inhibited respiration increased from 7.2 pmols to 63.3 pmols. Uncoupled respiration showing the maximal capacity of the respiratory chain was 2.5 times higher in differentiated podocytes. Both routine respiration and uncoupled respiration were lower in differentiated podocytes. In undifferentiated cells, mitochondria showed fragmented perinuclear morphology which changed to filamentous networks during differentiation. Differentiation greatly induced the expression of PGC-1a, while the expression of the mitochondrial transcription factor A (TFAM) remained unchanged.

Conclusions: During differentiation the abundance and morphology of mitochondria are tuned to meet the cell-specific demands, while respiratory properties of mitochondria undergo only minor changes. The coactivator PGC-1a promotes mitochondrial biogenesis, but no appreciable changes are needed in the control of transcription and replication of the mitochondrial genome as judged by unchanged levels of TFAM. Our results also show that approximately 40% of the electron transport capacity is utilized in routine respiration in undifferentiated and differentiated cells, indicating large respiratory excess capacity. In undifferentiated cells, 37% of the total uncoupled electron transport capacity was activated to ATP production, while in differentiated podocytes this figure was only 19%. This demonstrates that non-mitochondrial respiration and other oxygen-consuming processes are markedly induced in differentiated podocytes.

I9

Differential Glycosylation Study in a Rat Model of Type 1 Diabetic Nephropathy

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Introduction: Diabetes mellitus is one of the most common chronic diseases worldwide and the number of patients continues to increase. The chronic elevation of blood glucose levels leads to damage of many target organs. Diabetic angiopathy of the capillaries in the kidney glomeruli is one of the causes of the development of diabetic nephropathy (DN). An estimated 30–40% of diabetic patients will develop progressive Chronic Kidney Disease (CKD).

The worsening epidemic of CKD has been identified as a major health-care socio-economic challenge throughout the world, with an urgent need to transfer the results of basic research to effective early diagnostics and better therapeutic strategies.

The aim of this work is to study weather the development of the diabetic nephropathy onset associates with changes in the glycosylation of kidney glycoproteins.

Methods: Kidney cortex specimens from the well established in vivo rat model of Streptozotocin-induced diabetes were studied. Detection of differential glycosylation patterns associated with progression of the disease was first carried out by the means of lectin assays. Candidate glycoproteins were isolated by affinity chromatography experiments, and identified by LC-MS analysis.

Results: Two plant lectins proved to efficiently reveal glycosylation differences between diabetic and healthy kidney cortexes. After chromatography isolation and MS identification, three protein candidates were selected as most interesting hits. The MS data was further validated by Western blot and histochemistry.

Conclusions: This study represents the first systematic approach towards a novel understanding of the glycobiology of diabetic nephropathy. We demonstrated the feasibility of detecting diabetes-induced changes in the glycosylation patterns of the kidney by lectin assays. Furthermore, it is expected that the protein candidates revealed will provide new candidate molecules for detailed studies.

I10

Determination of Podocyte VEGF-A Pre-mRNA Sequence Used by Serine-Rich Protein Kinase-1 (SRPK1) for Pro-Angiogenic Splicing

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Introduction: In podocytes, vascular endothelial growth factor (VEGF) is alternatively spliced to produce isoforms with different angiogenic activity[1]. SRPK1 (Serine-rich protein kinase-1), a regulator of pro-angiogenic (VEGF165) splicing phosphorylates serine-rich splicing factor-1 (SRSF1), which binds to VEGF pre-mRNA[2]. A previous study showed a lack of the anti-angiogenic isoform (VEGF165b) in glomeruli of Denys Drash Syndrome (DDS) patients[3], who have a mutation in the Wilms' tumor suppressor gene WT1. In normal podocytes WT1 binds to the promoter of SRPK1 repressing expression through a specific WT1 binding site. In WT1 mutant cells SRPK1-hyperphosphorylates SRSF1 and consequently promotes pro-angiogenic splicing of VEGF. We used minigene approaches to investigate the mechanisms required for SRSF1 binding to the VEGF transcript, when phosphorylated by SRPK1.

Methods: We generated wild type minigenes with appropriate splice sites for VEGF-A165b (anti angiogenic) and VEGF-A165 (angiogenic), consisting of exons 1-5, exon 7, intron 7 and exon 8, and minigenes with mutation at the exon 8a splice site (AG-TC). Expression of VEGF and VEGF165b was examined via ELISA, Immunofluorescence, and western blotting in differentiated conditionally immortalised podocyte cells (DCIPs).

Results: DCIPs, which expressed more VEGF165b (2.5±0.16 ng/mg) than DDS cells (0.77±0.09 ng/mg), whereas DDS podocytes expressed more VEGF165 (2.9±0.25 ng/mg) than DCIPs (0.39±0.069 ng/mg) as previously described. DCIP and DDS cells were transfected with minigenes. Transfection of the wild type minigene raised VEGF165b expression in DDS to the level of that in DCIP cells. Mutation of the exon 8a splice site resulted in a 60% decrease in VEGF165 expression in DDS, but a 62% increase in VEGF165 in DCIP. In contrast, VEGF165b was not altered in PCIPs, but increased by 40% in DDS.

Conclusions: One explanation of these results is that a repression of VEGF165 expression may be occurring in DCIPs by proteins binding to the Exon8a site, which is lifted by SRPK1 activity (i.e. in DDS).

I11

Molecular Characterization of Podocyte-Endothelial Cell Signaling Crosstalk in Glomerular Diseases

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Introduction: The molecular signaling mechanisms between glomerular cell types during initiation/progression of glomerulosclerosis remain poorly understood. We have recently demonstrated that inducible activation of podocyte-specific TGFβ signaling in transgenic mice (PodTbrI) was associated with Endothelin-1 (Edn1) release by podocytes, mediating mitochondrial oxidative stress (mtStress) and dysfunction selectively in adjacent endothelial cells via paracrine Edn1 receptor type A activation and endothelial dysfunction. Endothelial dysfunction feedback signals were required to promote foot process effacement and loss of adjacent podocytes. We hypothesize that glomerular endothelial mtStress-dependent endothelial dysfunction is required for podocyte injury/loss, underlying the podocyte depletion paradigm of segmental sclerosis. To examine the functional interdependence between dysfunctional glomerular endothelial cells and podocyte loss.

Methods: Studies performed in mice with doxycycline treated PodTbr1 (0–14 days) and Type I diabetic DBA/2J and C57/Bl6 mice induced by multiple, low-dose streptozotocin (STZ) injections. Tissue was collected from diabetic and non-diabetic controls after 1, 3, 6, 12 weeks onset of hyperglycemia. In-vitro studies were performed with glomerular endothelial cells (mGEC) and podocytes.

Results: Endothelial damage by increased mtStress in PodTbrI mice and in diabetic nephropathy (DN) susceptible mice STZ-DBA/2J resulted in loss of Isolectin B4 staining associated with dysfunction and loss of fenestrae contrast to PodTbrI controls and diabetic STZ-C57/Bl6 mice with comparable (>400 mg/dl) blood glucose levels. One week after hyperglycemia uncoupled oxygen consumption was significantly reduced in STZ-DBA/2J glomeruli, but not in STZ-C57/Bl6 compared to controls. STZ-DBA/2J mice had increased mtDNA lesions, oxidative stress markers (3NT) and oxidative DNA damage (8-oxoG in mtDNA), exclusively in endothelial cells after 3 weeks of hyperglycemia. The endothelial dysfunction in turn was required for podocyte apoptosis as selective mitochondrial-targeted ROS scavenging prevented podocyte loss, ameliorated albuminuria, and glomerulosclerosis in STZ-DBA/2J mice. In vitro treatment of mGEC cultures with mitochondrial-targeted ROS scavenger prevented mtStress and fragmentation induced by incubation with diluted STZ-DBA/2J serum in the media.

Conclusions: Our studies extended our novel observations that endothelial dysfunction mediated by mtStress is required for podocyte defects, albuminuria and glomerulosclerosis in a model of podocyte-initiated segmental glomerulosclerosis (PodTbrI) and DN susceptible (DBA/2J) mice strains but not in DN-resistant C57/Bl6 mice without podocyte depletion.

I12

Trehalose, A mTOR Independent Autophagy Inducer, Alleviates Human Podocyte Injury After Puromycin Aminonucleoside Treatment

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Introduction: Glomerular diseases are commonly characterized by podocyte injury including apoptosis, detachment and actin cytoskeleton rearrangement. However, the strategies for preventing podocyte damage remains insufficient. Recently, autophagy has been regarded as a vital cytoprotective mechanism for podocyte homeostasis. Trehalose, a natural disaccharide, has shown to be a mTOR independent autophagy inducer. Its cytoprotective role has been demonstrated in neurodegenerative diseases. However, it remains unknown whether it may alleviate podocyte injury. In this study, investigated the effect of trehalose in puromycin aminonucleoside-treated podocytes which mimics minimal change nephrotic syndrome.

Methods: Human conditional immortalized podocytes were treated with PAN±Trehalose. Then autophagy was being investigated by immunofluorecence staining for LC3 puncta and western blotting for LC3, mTOR and its substrates. Podocyte apoptosis and necrosis was evaluated by flow cytometry and by measuring lactate dehydrogenase (LDH) activity respectively. We also performed the podocyte migration and adhesion assays to evaluate podocyte recovery. Furthermore, for studying the mechanism of trehalose induced autophagy, chloroquine (CQ) and wortmannin (WT) were used to inhibit autophagy.

Results: Trehalose induced podocyte autophagy in an mTOR independent manner and podocyte apoptosis was significantly decreased after trehalose treatment. Moreover, the inhibition of trehalose-triggered autophagy abolished its protective effect. Additionally, the disrupted actin cytoskeleton of podocytes was also partially reversed by trehalose, accompanying with the decreased mobility and less lamellipodias.

Conclusions: These results suggest that trehalose induced autophagy in human podocytes and demonstrated cytoprotective effects in alleviating podocyte injury. Further investigation is warranted to determine whether it may be a therapeutic candidate for glomerular diseases.

I13

Angiotensin II Induces Podocyte Apoptosis Through CD2AP/PI3-Kinase Pathway and Endoplasmic Reticulum Stress

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Introduction: Angiotensin II (Ang II) contributes to the pathological process of vascular structures, including renal glomeruli by hemodynamic and nonhemodynamic direct effects. On renal effects, Ang II plays an important role in the development of proteinuria and glomerulosclerosis by the modification of podocyte molecules and cell survival. In the present study, we investigated the effect of Ang II on podocyte apoptosis in relation to endoplasmic reticulum (ER) stress.

Methods: We cultured mouse podocytes with increasing doses of Ang II and evaluated apoptosis by FACS and TUNEL assay and observed ER stress markers by Western blotting.

Results: Ang II induced podocyte apoptosis in FACS and TUNEL assay in dose- and time-dependent manners and upregulated phospho-JNK, a proapoptotic signaling molecule, which was augmented by siRNA for CD2AP. Ang II also downregulated CD2AP, which was augmented by PI3-kinase inhibitor, LY294002, which meant that Ang II could induce podocyte apoptosis via CD2AP/PI3-kinase complex. In addition, Ang II increased GRP78, ATF6, and XBP-1, however, did not show apparent differences in phospho-PERK, phospho-eIF2, or ATF4.

Conclusions: These studies suggest that Ang II induce podocyte apoptosis by the mechanisms involving the suppression of CD2AP/PI3-kinase signaling and the induction of ER stress concerning ATF6/XBP-1 pathway rather than early ER stress response stage, which would contribute to the development of podocyte injury induced by Ang II.

I14

Interaction of Glomerular Basement Membrane Components with Slit Diaphragm Proteins

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Introduction: Podocin polymorphisms can aggravate the phenotype of heterozygous carriers of type IV collagen mutations (COL4A3/4/5). This points towards an interaction between the slit diaphragm (SD) and the glomerular basement membrane (GBM), which is essential for podocytes´ structure and function. In the present study, we investigated several SD-proteins using COL4A3 knockout mice with the type IV collagen disease Alport syndrome untreated, treated with ACE-inhibitor (ACEi) or Alport mice with loss of two collagen receptors, DDR1 and alpha2beta1-integrin (Triples).

Methods: Mice were investigated at different stages of disease. SD- and GBM-morphology were determined by electron microscopy. Relative expression of podocin and nephrin was determined by real-time qPCR and Western blotting. Protein localization was visualized by immunogoldhistochemistry.

Results: ACEis as well as loss of collagen receptors prolonged kidney function and morphology to a certain extent. Nephrin expression did not differ between untreated and treated Alport mice. In contrast, podocin expression decreased in untreated Alport mice and could not be restored by ACEi-therapy. Loss of the collagen receptors resulted in significantly higher podocin expression. In immunogold-staining, quantity of nephrin and podocin in presymptomatic stages of Alport mice were comparable to wild-type, but podocin accumulated in the areas of podocyte effacement. The same accumulation was found after loss of collagen receptors in the Triples at later stages of disease.

Conclusions: In conclusion, ACEi therapy as well as loss of collagen receptors delay renal failure in a similar manner, but influence different therapeutic targets. Triples, for example, maintain podocin expression, but the protein loses its typical localization. Further studies about the interaction of GBM and SD will help to understand these processes. Knowledge about the connection of GBMand SD-proteins will lead to a better understanding of the podocyte behavior in glomerular diseases and to targeted therapies.

I15

Notch1 Dependant Regulation of Snail1 Abrogates Podocyte Dedifferentiation

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Introduction: Notch proteins are markedly upregulated in glomeruli during diabetic nephropathy. Global inhibition of Notch signaling in podocytes inhibits glomerulosclerosis. In podocytes, the expression of the four Notch receptors (N1-4) and five ligands Jagged (Jag1&2) and Delta-Like (DLL1, 3, 4), shows that they are not functionally redundant. Our aim is to determine which specific members of the Notch signaling pathway engage during diabetic podocytopathy.

Methods: In vivo, either Notch1 or -2 was ablated from podocytes using Notch floxed mice crossed with podocin Cre ($NPHS2^{cre}Notch^{F/F}$). Nephrectomy at week 3 followed by streptozotocin (STZ) injection at 4 weeks induced diabetic conditions. Kidneys were examined by histology and for albumin:creatinine at 24 weeks. As an in vitro proxy for fibrosis, immortalized podocytes and primary podocytes isolated from $NPHS2^{cre}Notch^{F/F}$ glomeruli were treated with TGF-β1. Podocyte samples were analyzed for Notch receptor-dependant responses by RT-qPCR, immunoblotting and immunofluorescence.

Results: In vivo, diabetic $NPHS2^{cre}Notch1^{F/F}$ mice (n = 16 NT, n = 15 STZ) had high blood glucose but albumin:creatinine levels similar to untreated wild-type controls (n = 9 NT, n = 8 STZ). Glomerulosclerosis (PAS) is largely inhibited in $NPHS2^{cre}Notch1^{F/F}$

mice. STZ NPHS2^{cre}Notch2$^{F/F}$ mice do not differ from STZ treated wild-type mice. Together, these results demonstrate podocyte protection via specific Notch1 ablation. In vitro, TGF-β1 stimulation of immortalized and primary mouse podocytes increases levels of Notch1, Jagged-1 and DLL4, but not Notch2. NPHS2^{cre}Notch1$^{F/F}$ podocytes are protected from loss of podocin following TGF-β1 stimulation and have significantly reduced levels of ligand DLL4. Importantly, Snail1, a master regulator of dedifferentiation, is significantly upregulated by TGF-β1 in wild-type, but not NPHS2^{cre}Notch1$^{F/F}$ podocytes. Immunohistochemistry confirmed upregulation of Snail1 protein expression in WT STZ diabetic mice but not in NPHS2^{cre}Notch1$^{F/F}$ STZ mice.

Conclusions: In podocytes, specific engagement of Jagged-1, DLL4 and Notch1 mediate upregulation of Snail1, indicating Notch1 dependant podocyte dedifferentiation during diabetic podocytopathy.

I16

Expression and Functional Characterization of Complement Factor H (CFH) and Factor H Related Proteins (CFHRs) on Human Podocytes

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Introduction: CFH/CHFR are major regulators of the complement alternative pathway (AP). Dysfunction in these proteins are associated with a number of diseases including atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathies (C3G). CFH/CFHRs are mainly produced within the liver, but besides, renal synthesis has been demonstrated in endothelial cells, mesangial cells and rodent podocytes. Here, we hypothesize that CFH and CFHRs are synthesized by human immortalized podocytes and can regulate local AP activity in the kidney.

Methods: Expression of CFH/CFHR in human liver, podocytes und endothelial cells (HUVEC) was analyzed by RT-PCR. Induction of expression was investigated after stimulation of cells with pro-inflammatory cytokines and subsequent qRT-PCR. Cellular binding of CFH has been analyzed by flow cytometry and co-factor activity of surface bound CFH has been assessed by the detection of specific C3b cleavage products after incubation with CFH and subsequent co-factor assay.

Results: All CFH/CFHR transcripts were detected in liver. CFH and CFHR3 were strongly expressed in podocytes and HUVECs, while CFHR5 expression was weakly detectable in podocytes. Stimulation of podocytes increased the expression of CFH, CFHR3, CFHR5 to a moderate level. CFH directly binds to the surface of podocytes and thereby mediates cellular co-factor activity.

Conclusions: Preliminary results suggest that podocytes express CFH/CFHRs at different levels and transcription may be induced by pro-inflammatory cytokines. CFH binding on podocytes mediates cellular co-factor activity and thereby has the potential to locally de-regulate complement activity. Further work to define the role of CFH and CFHR on glomerular cells, both in healthy and in disease states is under progress. Given the dysregulation of the AP seen in the development of complement mediated renal disorders, uncovering the local role and synthesis of these proteins may help to understand their pathophysiological process in these diseases.

I17

Global Analysis of Insulin Action in Glomerular Podocytes

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Introduction: Insulin signaling contributes to multiple cellular activities such as protein and lipid synthesis, cell proliferation and survival. Insulin resistance describes the inability of cells to activate insulin signaling pathways effectively and is associated with disease states including diabetic nephropathy. Recent studies have shown that glomerular podocytes are direct targets for insulin action and that insulin resistance can be induced by the saturated fatty acid, palmitate. Using proteomics we aim to isolate insulin receptor (IR) complexes from podocytes under normal and resistant conditions and to identify signaling hubs that could be targeted for therapeutic purposes.

Methods: Immortalised human and murine podocytes were stimulated with insulin (100 nM) and palmitate (750 μM) and insulin signaling assessed by western blotting. Whole cell-IR protein complexes were isolated from podocytes by immunoprecipitation for analysis by label-free mass spectrometry (MS). The ability to isolate plasma membrane-IR complexes was also examined using methodology previously established for isolation of adhesion receptor complexes.

Results: Human and murine podocytes responded to insulin with phosphorylation of Akt and MAPK1/2 and palmitate abolished this response. Conditions for isolating whole cell-IR and plasma membrane-IR by were optimised using antibodies raised to IR-alpha (extracellular) and IR-beta (intracellular) subunits and known IR interactors were detected by western blotting. An in silico IR interaction network was created from published interaction datasets for comparison with in vivo IR protein complexes isolated from podocytes and analysed by MS.

Conclusions: In this investigation we have developed an experimental template for the global analysis of insulin action using proteomics. In these studies may provide further insight into insulin signaling in podocytes and enhance our understanding of mechanisms leading to chronic kidney disease in insulin resistant states.

I18

Oxidative Stress by Palmitate Mediates ER Calcium Depletion and Mitochondrial Dysfunction in Mouse Podocytes

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Introduction: Podocytes play a major role in glomerular filtration barrier and participate in pathogenic processes of diabetic nephropathy. Diabetic patients have elevated plasma levels of saturated free fatty acids (FFA) that induce ER stress and apoptosis. We investigated the cytotoxic mechanism of palmitate, the predominant saturated FFA, in immortalized mouse podocytes.

Methods: Cytotoxicity was assessed by MTT, nuclear staining, and ELISA for apoptotic DNA fragments. Confocal microscopy was used for fluorescence imaging of organellar structure, measurements of reactive oxygen species (ROS) with DCF and mitoSox as well as ER Ca^{2+} after D1ER transfection. Mitochondrial membrane potential was estimated with JC-1 or TMRM, and ATP production was monitored with a bioluminescence method. Hydrolysis of phosphatidylinositol-4,5 bisphosphate (PIP_2) was visualized by overexpressing pleckstrin homology (PH) domain of phospholipase-Cδ (PLCδ)-green fluorescent protein fusion protein.

Results: Incubation with palmitate dose-dependently increased cytosolic and mitochondrial ROS production, mitochondrial depolarisation, reduced ATP synthesis, and caused apoptotic cell death. Palmitate not only evoked mitochondrial fragmentation but also a marked ER lumen dilation. Consistently, palmitate upregulated ER stress proteins such as GRP78/Bip, spliced xbp1, and CHOP. Palmitate depleted the luminal Ca^{2+} level in ER and abolished the cyclopiazonic acid-induced cytosolic Ca^{2+} increase. Palmitate-induced ER Ca^{2+} depletion and cytotoxicity were blocked by a selective inhibitor of the fatty acid transporter (FAT/CD36). Loss of the ER Ca^{2+} pool induced by palmitate was recovered by the PLC inhibitor edelfosine, which was supported by the cytosolic distribution of the PH domain of PLCδ due to inositol 1,4,5-trisphosphate (IP_3) generation in palmitate-treated podocytes. Remarkably, the mitochondrial antioxidant mitoTEMPO inhibited palmitate-induced PIP_2 hydrolysis, ER Ca^{2+} depletion, and cytotoxicity.

Conclusions: These data suggest that oxidative stress by palmitate leads to mitochondrial dysfunction and ER Ca^{2+} depletion through FAT/CD36 and PLC signaling in mouse podocytes.

I19

Activation of ERK1/2-mTORC1 by TGF-β1 Mediates Oxidative Stress and Apoptosis in Mouse Podocytes

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Introduction: TGF-β, a pleiotropic cytokine, accumulates in injured kidney tissue of chronic renal diseases. Previously, we have reported that TGF-β1-induced activation of Smad2/3 selectively upregulates mitochondrial Nox4 which plays an important role in podocyte apoptosis. Here, we investigated the pathogenic role of mammalian target of rapamycin complex1 (mTORC1) activation in TGF-β1-induced apoptosis through Nox4-mediated oxidative stress in mouse podocytes.

Methods: The levels of mRNA and protein were measured by real time PCR and Western blotting, respectively. ROS generation was checked with DCF using confocal microscopy. Enzymatic activity of total Nox was measured by NADPH-dependent superoxide production using lucigenin chemiluminescence. JC-1 was used to measure mitochondrial membrane potential.

Results: TGF-β1 treatment markedly increased phosphorylations of mTOR and its downstream target p70S6 kinase. Inhibition of mTORC1 by low dose of rapamycin (10 nM) protected podocytes from apoptosis through attenuation of total Nox activity, ROS generation, loss of mitochondrial membrane potential, and caspase-3 activation induced by TGF-β1. Treatment with SB431542, a TGF-β receptor-I inhibitor, completely blunted phosphorylation of ERK1/2, mTOR and p70S6 kinase. ERK1/2 inhibitors (U0126 and PD184352) abolished TGF-β1-induced mTOR and p70S6 kinase phosphorylation. Interestingly, inhibition of neither ERK1/2 nor mTOR reduced the TGF-β1-stimulated increase of Nox4 mRNA level, but inhibited total Nox activity, ROS generation, and apoptosis induced by TGF-β1.

Conclusions: Our data suggest that ERK1/2-mTOR axis mediates TGF-β1-induced total Nox activity which is independent of transcriptional regulation of Nox4 by Smad2/3. Activation of this pathway plays a crucial role in oxidative stress-induced mitochondrial dysfunction leading to podocyte apoptosis.

I20

mTOR-Independent Control of Autophagy in Podocytes

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Introduction: Autophagy emerged as a key mechanism to eliminate unwanted cytoplasmic materials, thereby preventing cellular damage and stress to safeguard long-lived podocytes. The

serine/threonine kinase mTOR (mammalian target of rapamycin) is a central regulator of cell growth and metabolism and usually inhibits autophagy. Podocytes however show high levels of basal autophagy in the presence of mTOR activation suggesting and unexpected uncoupling of mTOR and autophagy in podocytes. We did set up complementary mouse models to study the functional interplay of mTOR signaling and autophagy in podocytes.

Methods: Autophagy levels were monitored *in vivo* by crossing *GPF-LC3* reporter mice to models of mTOR hyperactivation (*Tsc1* PcKO) and mTOR loss of function (*Raptor* PcKO). In addition, podocyte-specific *Raptor* and *Tsc1* KO mice were crossed to a *Tomato/eGFP* reporter line to efficiently isolate podocytes for primary cell culture studies.

Results: In contrast to other known cellular systems podocytes did exhibit high basal autophagy rates independently of the mTOR activation status. Strikingly, there was no difference in LC3 conversion in vivo and no difference of the GFP-LC3 signal between *Raptor* and *Tsc1* PcKO mice. Unexpectedly, Beclin 1 expression was inversely regulated by mTOR activation. Isolated primary podocytes showed typical features of mTOR activation (proliferation, hypertrophy) and mTOR suppression, respectively, but autophagy remained unaffected even in case of starvation suggesting that mTOR signaling influences the repertoire of expressed autophagy-related proteins but did not regulate the autophagy-response towards metabolic stimuli.

Conclusion: mTOR and autophagy are key regulators of podocyte function and maintenance. Our data highlight a specific mTOR-autophagy regulatory cascade, which allows to operate mTOR activity and high basal autophagy rates simultaneously in podocytes.

I21

An in Vitro Investigation of Factors That Modulate Podocyte Insulin Sensitivity

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Introduction: Insulin resistance is associated with albuminuria and there is increasing evidence that podocyte insulin responses are disrupted in situations of diabetes. We have previously demonstrated that podocytes are insulin responsive in vitro and the specific deletion of the insulin receptor (IR) in vivo results in a glomerular phenotype. This study aims to further investigate how factors associated with the metabolic syndrome and insulin resistance influence podocyte insulin signalling.

Methods: Conditionally immortalised mouse podocytes were exposed to TNF-alpha & IL-6 (0.1 ng/ml, 1 ng/ml), high insulin (10 nM/100 nM) and high glucose (25 mM) levels in vitro, prior to insulin stimulation. Alterations in insulin signalling cascades were determined via western blotting and cellular glucose uptake assays. Focused insulin signalling PCR arrays were performed to determine changes at the mRNA level.

Results: Exposure of podocytes to a combination of TNF-alpha, IL-6, high insulin and high glucose blocks the insulin-stimulated glucose uptake response. Individually, these factors exert their effects via different mechanisms. Exposure of cells to TNF-alpha and IL-6 reduces insulin stimulated glucose uptake by 74.08±9.22%, yet Western blotting demonstrates no significant reduction in IR protein levels and insulin-stimulated phosphorylation of IGF-IRβ (Tyr1131)/IRβ (Tyr1146) and Akt (Ser473, Thr308). Results obtained using focused insulin signalling PCR arrays are currently being validated. In contrast, high insulin exposure reduces IR protein levels and insulin-stimulated phosphorylation of Akt (Ser473, Thr308); yet insulin-stimulated glucose uptake remains unchanged. Preliminary results indicate that high glucose exposure also reduces insulin-stimulated Akt phosphorylation (Ser473, Thr308) in podocytes, yet IR levels and insulin stimulated glucose uptake are not significantly changed.

Conclusions: Exposure of podocytes in vitro to factors associated with insulin resistance disrupts insulin signalling. Investigating how factors associated with the metabolic syndrome contribute to podocyte insulin insensitivity will enhance our understanding of the causes of glomerular disease in settings of insulin resistance, potentially identifying new therapeutic targets.

I22

High Glucose Induces Insulin Resistance in Rat Cultured Podocytes via AMPK-SIRT1 Pathway

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Introduction: Podocytes insulin sensitivity is critical for glomerular function and the loss of correct insulin signaling in consequence leads to alterations and disorders featuring diabetic nephropathy (DN). The energy sensing pathways, crucial for metabolic control, like AMP-dependent protein kinase (AMPK), and most recently class III protein deacetylase SIRT1 have been shown to play an important role in insulin resistance. The aim of this study was determination of the role of AMP-dependent protein kinase (AMPK) and class III protein deacetylase (sirtuin) SIRT1 in insulin resistance of podocytes exposed to high glucose concentration.

Methods: Experiments were performed in primary rat podocytes cultured with normal (NG, 11.1 mM) or high (HG, 30 mM) glucose concentrations for 5 days. Immunodetection methods were used to detect AMPK (total and AMPK P-Thr^{172}) and SIRT1 proteins. Insulin-stimulated changes in glucose uptake were used to detect insulin resistance. SIRT1 activity was modified by siRNA SIRT1 or resveratrol (RSV, activator).

Results: A high glucose concentration decreased the phosphorylation of AMPK by approximately 20% ($P < 0.05$) and abolished insulin-stimulated glucose uptake into podocytes. Knockdown of SIRT1 protein expression in NG-cultured podocytes decreased AMPK phosphorylation by about 30% (1.00±0.07 vs. 1.31±0.03, $P < 0.05$) and abolished insulin-stimulated glucose uptake. The stimulating effect of RSV on AMPK phosphorylation was observed

in cells cultured in NG or HG medium, by about 50% (1.30±0.05 vs. 0.86±0.07, P < 0.05) and 65% (1.18±0.05 vs. 0.72±0.05, P < 0.05), respectively. Glucose uptake was also increased in HG-cultured podocytes treated with RSV Additional effect of insulin action was not observed.

Conclusions: We found that impairment of insulin induction of glucose uptake into podocytes cultivated in the presence of high glucose concentrations for long periods of time is associated with decreased AMPK phosphorylation in an SIRT1-dependent manner.

I23

1,25-Dihydroxyvitamin D_3 Deficiency Induces Reversible Podocyte Injury and Proteinuria

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Introduction: Vitamin D plays an important role in renal (patho)physiology. In patients with glomerular disease and animal models thereof, 1,25-dihydroxyvitamin D_3 (1,25-D_3) treatment reduces proteinuria. Conversely, renal insufficiency leads to 1,25-D_3 deficiency, due to loss of renal 1α-hydroxylase (1α-OHase) activity. Importantly, a correlation between decreased vitamin D levels and increased prevalence of albuminuria was demonstrated. Therefore, we hypothesized that 1,25-D_3 deficiency itself can cause podocyte injury.

Methods: Our hypothesis was tested in 1,25-D_3-deficient 1α-OHase knockout (KO) mice and vitamin D-deficient rats. The effect of vitamin D analogs was evaluated in injured cultured podocytes. 1,25-D_3-deficient animals were supplemented with 1,25-D_3 or paricalcitol (1,25-D_2). Serum and urine analyses, light and electron microscopy (EM) of kidney sections was performed.

Results: The 1,25-D_3-deficient animals developed non-immune-mediated glomerular injury. 1,25-D_3 deficiency was accompanied by proteinuria and partial podocyte foot process effacement. Expression of functionally relevant slit diaphragm-associated proteins was altered, with e.g. reduced nephrin and podocin, and increased TRPC6 and desmin expression. Minor glomerular basement membrane changes were accompanied by altered expression of collagen IV chains, possibly secondary to podocyte injury. In addition, tubulointerstitial injury occurred. 1α- and/or 25-hydroxylated vitamin D_3 analogs and 1,25-D_2 reduced podocyte injury in vitro. Supplementation with 1,25-D_3 or 1,25-D_2 prevented podocyte effacement, and reversed glomerular and tubulointerstitial damage in 1,25-D_3-deficient animals. A low dose of 1,25-D_3, which did not normalize serum calcium, PTH, FGF23 and phosphate, also corrected glomerular injury, while restoration of serum calcium with a calcium-rich diet did not. Thus, podocyte and glomerular injury was independent of concomitant alterations in calcium/phosphorus balance.

Conclusions: This study demonstrates that 1,25-D_3 deficiency directly causes reversible podocyte/glomerular and tubulointerstitial injury in rodents. These data underscore a possible need for early vitamin D supplementation in patients with glomerular disease and CKD, which could inhibit or even reverse glomerular and tubulointerstitial injury.

I24

Hyperglycemia and Insulin Signaling in Podocytes Modulates Endoplasmic Reticulum Stress in Diabetic Nephropathy

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Introduction: Endoplasmic reticulum stress (ER-Stress) is associated with insulin resistance in diabetes and with diabetes induced complications.

Methods: To systematically characterize the compensatory stress signaling cascades triggered by ER perturbations we have employed mouse models of db/db and streptozotocin (STZ)-induced diabetic nephropathy (DN) as well as in vitro models.

Results: Here we show that in both mouse models of db/db and STZ-induced DN hyperglycemia selectively impairs nuclear translocation of the highly conserved transcription factor spliced X-box binding protein-1 (sXBP1). This results in severe ER-stress associated with increased nuclear levels of the active form of activating transcription factor-6 (ATF6), but not ATF4. ATF6 mediates transcriptional activation of the pro-apoptotic/pro-inflammatory transcription factor CHOP. In diabetic (STZ) mice doxycycline mediated inducible expression of ATF6 or genetic deletion of XBP1 ($XBP1^{flox/flox}$ x $Pod^{Cre/Cre}$) specifically in podocytes aggravates DN. In agreement with these in vivo observations hyperglycemia impaired nuclear translocation of sXBP1 in both podocytes and endothelial cells in vitro, resulting in increased expression and nuclear translocation of ATF6 and CHOP but not ATF4. Treatment with chemical chaperone tauroursodeoxycholic acid (TUDCA), which enhances the adaptive ER-response, restores ER-homeostasis and protects against DN in wild type and in ($XBP1^{flox/flox}$ x Pod^{Cre}) mice. Insulin signalling is required for normal podocyte function and insulin regulates XBP-1 in hepatocytes. Hence, we next determined whether insulin modulates the ER-function in podocytes. Mice with podocyte specific homozygous deletion of insulin receptor ($IR^{-/-}$) display ER-stress and proteinuria. In ongoing studies we are evaluating the functional relevance of hyperglycemia and ER-stress for podocyte dysfunction.

Conclusions: These studies identify a crucial role of XBP1 in regulating DN-associated ER-stress. The current data imply a

function of insulin in maintaining podocyte function through regulating ER-homeostasis. These findings provide insight into potential new therapeutic targets for DN in humans.

I25

Soluble Urokinase Receptor (Supar) Injection Does Not Influence LPS-Induced Albuminuria in Mice with or without Vitronectin

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Introduction: The soluble form of urokinase receptor (suPAR) has been associated with numerous inflammatory or tumoral processes. SuPAR has also been suggested as a potential circulating factor that may explain podocyte dedifferentiation and proteinuria recurrence in a Focal segmental glomerulosclerosis (FSGSR) mouse model. It has been suggested that action of suPAR on podocytes is in part vitronectin-dependent. We have already demonstrated that vitronectin is a circulating protein that localizes fibrinolytic activity in glomerular extracellular matrices. In the current model of LPS-induced proteinuria, vitronectin is thought to promote upar/beta3 complexes formation and ß3 integrin activation on podocytes. We hypothesized here, that glomerular vitronectin deposits would bolster deleterious uPar/SuPAR/ß3 integrin activities, thus majoring albuminuria.

Methods: An LPS-induced proteinuria was induced in either Vitronectin KO or wild type (WT) C57BL6J animals. In addition, a massive dose of suPAR (50 µg) was intravenously injected to C57BL6/J mice and Vitronectin KO mice 24 h after the LPS insult. Albuminuria and glomerular suPar deposits were evaluated 24 h later.

Results: Following the LPS injection both strains develop a significant albuminuria. Albuminuria was virtually identical comparing both LPS-induced groups (Relative Unit 4,022±1,288 Vitronectin KO vs 4,719±2,485 WT mice, P = 0.7). Additionally, following a recombinant suPAR injection, and despite its deposit on glomerular structures, both strains develop similar albuminuria.

Conclusions: In conclusion we found that the injection of recombinant suPAR was not associated with an increase in proteinuria in absence or presence of vitronectin. This accords with our findings in a forthcoming article (Cathelin et al, JASN 2014 in press) in which we showed that the injection of different recombinant forms of suPAR in WT animals did not induce any proteinuria. In hindsight our results speak against a role for vitronectin/suPAR complexes in the LPS-induced albuminuria model.

I26

Semaphorin3a Promotes Advanced Diabetic Nephropathy

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Introduction: The onset of diabetic nephropathy (DN) is highlighted by glomerular filtration barrier abnormalities. Identification of pathogenic factors and targetable pathways driving DN is crucial to develop novel therapies and improve the disease outcome. Semaphorin3a (sema3a) is a guidance protein secreted by podocytes required for normal kidney development. However, excess sema3a disrupts the glomerular filtration barrier. We observed sema3a upregulation in diabetic mouse kidneys. This study aimed to define whether excess sema3a has a pathogenic role in diabetic nephropathy and to identify the molecular mechanisms involved.

Methods: SEMA3A expression was examined by immunohistochemistry in renal biopsies from diabetic patients. Diabetic nephropathy phenotypes from inducible, podocyte-specific Sema3a gain-offunction mice (Sema3a+) made diabetic with streptozotocin, given doxycycline or standard chow for 12–16 weeks were studied. In addition, induced Sema3a+ diabetic mice were treated with a sema3a inhibitor for 4 weeks. Sema3a signaling was examined in a Sema3a+ podocyte cell line by co-immunoprecipitation, siRNA and cell assays.

Results: We report that sema3a is upregulated in human diabetic nephropathy. We demonstrate that sema3a is pathogenic in diabetic mice, promoting advanced diabetic nodular glomerulosclerosis, leading to massive proteinuria and renal failure, mimicking human advanced DN. In the context of diabetes excess sema3a induces laminin and collagen IV accumulation in Kimmelstiel-Wilson-like glomerular nodules, causes diffuse podocyte foot process effacement and F-actin collapse via nephrin, (v)3 integrin and MICAL1 interactions with plexinA1. We identify MICAL1 in podocytes and show that MICAL1 knockdown or sema3a binding inhibition abrogates sema3a-induced F-actin collapse in podocytes. Moreover, sema3a inhibition in vivo attenuates diabetic nephropathy.

Conclusions: We conclude that excess sema3a plays a pathogenic role in diabetic nephropathy in mice. MICAL1 mediates sema3a-plexinA1 signals in podocytes leading to F-actin collapse. Collectively, our findings indicate that excess sema3a promotes severe diabetic nephropathy and identify novel potential therapeutic targets.

I27

Morphological Analysis and Dysfunction of Mitochondria During Podocyte Apoptosis Induced by Adriamycin

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Introduction: The mechanism of podocyte apoptosis has not been fully elucidated. Several mitochondrial genes have been revealed to be the causes of focal segmental glomerulosclerosis. Abnormal morphology of mitochondria was also observed occasionally in some patients with mitochondrial nephropathy. However, the role of mitochondria dysfunction and morphological change during podocyte apoptosis is not clear.

Methods: Cultured mouse podocyte was treated by adriamycin at concentrations of 0.25, 0.5, 1.0 μg/ml for 24 hours, separately. Podocyte was also treated by adriamycin at a concentration of 0.5 μg/ml for 6, 12, and 24 hours, separately. The apoptosis and the mitochondrial transmembrance potential were measured. Electron microscopy specimen was prepared and morphological analysis of mitochondria was performed. For each group, 5 to 15 mitochondria in 40 cells were randomly analyzed, three independently experiments were performed.

Results: With the increase of the concentration of adriamycin, the mitochondria transmembrance potential of podocyte decreased, and podocyte apoptosis rate increased significantly. With the increase of adriamycin concentration, the mitochondria became smaller and irregular. The average area of mitochondria after treatment of adriamycin 0.25 μg/ml (235072.9±94208.1), 0.5 μg/ml (207288.5±73568.4), 1.0 μg/ml (180068.4±94572.0) decreased significantly compared with control (322955.4±100711.3, $P < 0.01$). After adriamycin 1.0 μg/ml for 24 hours, the circumference of mitochondria decreased significantly compared with control (1666.0±396.9 vs. 2081.3±462.9, $P < 0.01$). The mitochondria density increased significantly after adriamycin 0.5 μg/ml 6 hour treatment and decreased after 12 hours (0.054 vs. 0.012, $P < 0.05$).

Conclusions: The mitochondrial transmembrance potential decreased during apoptosis of podocyte induced by adriamycin. With the increase of apoptosis rate, the mitochondria became smaller and irregular. At early stage of adriamycin treatment, the density increased at 6 hours time point and decreased at 12 hours. Our results show that mitochondria dysfunction and mitochondria morphological change might contribute to the apoptosis of podocyte. Mitochondria fission might be involved in the podocyte apoptosis.

I28

Testosterone Is the Driving Force for Glomerular Damage Induced by Podocyte Specific Angiotensin II Type 1 Teceptor (AT1R) Overexpression

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Introduction: Transgenic rats Neph-hAT1R (TGR) overexpressing the AT1R in podocytes develop albuminuria and glomerulosclerosis in males, but not in females. This study aimed at delineating whether androgens or lack of estrogen contribute to disease progression.

Methods: 5-week-old male TGR were subjected to the androgen receptor antagonist Flutamid (30 mg/kg/d) (TGR-Fl). 4-week-old female TGR and wild-type littermates (WT) were ovariectomized (Ov), followed by testosterone treatment (1mg/kg/d) in half of them (OvT). Sham-treated TGR and WT served as controls (C). Urinary (u) albumin excretion was monitored in 4-week intervals up to an age of 35 weeks. Glomerular tuft area was morphometrically measured and the glomerular tuft volume (GTV) was calculated using Weibel formular. Gene expression levels were determined by LC-RT-PCR on RNA from isolated glomeruli using both, GAPDH and WT-1, for standardization.

Results: Flutamid treatment markedly reduced u-albumin and glomerular damage in TGR-Fl (6 mg/24 h) compared to TGR-C (129 mg/24 h) and retarded renal and glomerular hypertrophy. While Ov did not affect glomerular structure and function at all, testosteron induced a dramatic increase in u-albumin and glomerular damage in TGR-OvT (TGR-OvT: 87.4 mg/24 h vs. TGR-Ov: 3.3 mg/24 h and TGR-C: 1.0 mg/24 h) but not in WT-OvT. Both WT-OvT and TGR-OvT responded to testosterone with enhanced renal growth, but only TGR-OvT exhibited increased glomerular tuft volume relative to controls. Further, we found that glomerular tuft volume increase preceded the initiation of albuminuria in male TGR and were associated with decreased ACE2 and increased angiotensinogen expression in glomeruli of male TGR vs. WT rats. Testosteron stimulated and flutamid suppressed the angiotensinogen expression.

Discussions: AT1R overexpression in podocytes needs testosterone as a co-factor to induce albuminuria and glomerulosclerosis. Testosteron increases glomerular Ang II, wich by acting via overexpressed AT1 receptors on podocytes cause glomerular hypertrophy leading to leakage of the filtration barrier and glomerulosclerosis.

I29

$VEGF_{165}b$ Protects Against Albuminuria in the Podocyte-Specific VEGF KO Model of Glomerular Disease

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Introduction: Vascular endothelial growth factor (VEGF) is differentially spliced to give two functionally different isoform families, the pro-angiogenic $VEGF_{xxx}$ and the anti-angiogenic $VEGF_{xxx}b$. $VEGF_{165}b$ has been shown to decrease glomerular water permeability (L_pA/V_i) in vivo, be cyto-protective on podocytes in culture, and protects against albuminuria in diabetic rodent models. This study aimed to investigate whether constitutive overexpression of $VEGF_{165}b$ under a nephrin promoter is able to rescue the injury phenotype seen in the inducible podocyte-specific VEGF KO mouse. The mechanism of action of $VEGF_{165}b$ within the glomeruli was also investigated.

Methods: Podocyte-specific VEGF KO was induced via doxycycline administration in the drinking water for 10–14 weeks in WT, VEGF KO and VEGF KO x neph-$VEGF_{165}b$ mice. Successful KO was confirmed by in situ hybridization. Urinary albumin:creatinine ratio (uACR) was measured every two weeks and glomerular L_pA/V_i was assessed using an oncometric assay described by Salmon (2006) on weeks 10 and 14. The action of $VEGF_{165}b$ upon VEGFR-2 was assessed using immunofluorescence and western blotting in glomeruli in vivo and glomerular endothelial cells (GEnCs) in vitro.

Results: 10 weeks after VEGF KO induction, a 40% reduction in VEGF mRNA is observed in glomeruli. VEGF KO mice became albuminuric at week 12, compared to WT littermates, and this is rescued in VEGF KO x neph-$VEGF_{165}b$ mice. Glomerular L_pA/V_i is also significantly elevated in VEGF KO mice, which is rescued by $VEGF_{165}b$ at 10 weeks post-induction. In GEnCs $VEGF_{165}b$ increases the expression and phosphorylation of VEGFR-2.

Conclusions: Podocyte $VEGF_{165}b$ expression rescues the podocyte-specific VEGF KO model of glomerular disease, suggesting that $VEGF_{165}b$ plays a protective role and that manipulation of VEGF isoform ratio may have therapeutic potential. $VEGF_{165}b$ seems to act through VEGFR-2 probably as a weak agonist to the receptor.

I30

Glycogen Synthase Kinase 3 Is Critical for Podocyte Development and Survival

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Introduction: Glycogen synthase kinase 3 (GSK3) is a multifunctional serine/threonine kinase existing as two distinct but related isoforms (α and β). There is evidence that the GSK3 isoforms have different functions: GSK3β null mice die during late embryogenesis whereas total GSK3 α knockout mice are viable and interestingly, exhibit enhanced insulin sensitivity. A number of cell-specific GSK3 knockout mouse models have been described and indicate that the functions of the GSK3 isoforms are cell-type dependent. This study aims to aims to elucidate the role of GSK3 in the podocyte.

Methods: In order to investigate the role of the GSK3 isoforms in podocyte development, we have used Cre/LoxP technology to generate mice with podocyte-specific ablation of GSK3 α and/or GSK3β.

Results: Using an in vitro and in vivo approach we have shown that in the glomerulus, GSK3 is preferentially expressed in the podocyte and that both of its isoforms are phosphorylated and thus deactivated in response to insulin. Mice lacking either GSK3α or GSK3β specifically in the podocyte are viable with normal life span (up to 2 years) and normal renal histology indicating a degree of redundancy between the isoforms in this cell type. In contrast, mice null for both GSK3 isoforms, although born at normal Mendelian frequency, die predictably at 12–16 days at which time they are albuminuric. Kidneys from podocyte-specific GSK3α/β null mice appear larger and are around 30% heavier than those of littermate controls with grossly abnormal, collapsed glomeruli on histological analysis and evidence of large protein casts in the tubules. Western blot and immunofluorescence analysis indicates an increase in wnt signalling in podocyte-specific GSK3α/β null mice relative to controls.

Conclusions: This work reveals a fundamental role of GSK3 in the podocyte and its importance in glomerular development and survival.

I31

The Expression Profile of Complement Components in Podocytes

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Introduction: Complements are a series of serum glycoproteins with the enzymatic activity. The majority of complements are produced by hepatic cells. In recent years, many studies showed extrahepatic tissues, such as the kidney, brain, blood vessels, lungs, intestines, joints and skin could synthesize small amounts of complement components, which exhibited important biological significances. Among these extrahepatic tissues, some studies reported that the kidney was one of the main sources for the synthesis of complements. However, the expression profile or extent of complement components in podocytes is unknown. The present study aimed to determine the profile of complement components expressed in podocytes under physiologic conditions as well as in podocyte injury induced by multiple stimuli factors.

Methods: The expression profile of complement components in the primary culture of podocytes and in the cell line of mouse podocytes was detected and analyzed by conventional reverse transcription polymerase chain reaction (RT-PCR) for mRNA expression. The effect of puromycin aminonucleoside (PAN, 50 μg/ml), angiotensin II (Ang II, 10^{-5} mol), interleukin-6 (IL-6, 100 ng/ml) or transforming growth factor-β (TGF-β, 10 ng/ml) on the expression of complement genes and proteins was tested in vitro in the cell line of mouse podocytes and quantified by real-time PCR and isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomic analysis, respectively.

Results: In this study, there were 17 over 33 complement components mRNA expression in the primary culture of podocytes. Among these expression complement components, including complement C1qb, complement component 2 (C2), complement component 3 (C3), Complement factor D, complement factor P, mannose-binding lectin 2 (MBL2), Ficolin 1(Fcn1), mannose-binding lectin (MBL)-associated serine protease (MASP)-1, complement component 5 (C5), complement component 7 (C7), complement factor H, decay-accelerating factor (DAF), CD 59, Serping 1, C3a receptor (C3aR), C5a receptor (C5aR), C1q receptor (C1qR), complement receptor 1-related protein y (Crry), which were belonged to the complement inherent components, complement regulation factors and complement receptors, respectively. The analysis of the effect of multiple stimuli factors on the expression of complement genes and proteins is still undergoing.

Conclusions: A variety of complement components was expressed in podocytes with the primary culture. Podocyte complement gene expression could be affected by multiple factors, such as PAN, Ang II, IL-6 and TGF-β.

I32

Insulin Increases Glomerular Filtration Barrier Permeability Through Activation of BKca Channels; Role of the PKGIα in Mobilization of BKca in Podocytes

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Introduction: Podocytes are highly specialized cells that cover the glomerular capillaries and are a key part of the glomerular filtration barrier. Podocytes are uniquely sensitive to insulin, they have demonstrated similarities to skeletal muscle and fat cells with respect to insulin stimulated glucose uptake kinetics and the expression of glucose transporters. Insulin signaling is regulated by oxidative stress and intracellular energy levels. We demonstrated recently that insulin increases activation of protein kinase G type Iα (PKGIα) subunits, leading to podocyte dysfunction. Here we investigated whether BKca is involved in insulin-dependent regulation of filtration barrier permeability in PKGIα-dependent manner.

Methods: We assessed changes in insulin-induced glomerular permeability by measuring glomerular capillary permeability to albumin in isolated glomeruli from Wistar rats and transmembrane albumin flux in cultured rat podocytes. Expression of BKca subunits was detected by RT-PCR. Expression of BKca, PKGIα and upstream proteins was confirmed in the podocytes using Western blotting and immunofluorescence. The BKca-PKGIα interaction was confirmed by coimmunoprecipitation.

Results: The RT-PCR showed the presence of mRNAs encoding the pore forming α subumit and four accessory β subunits of BKca in primary cultured rat podocyte. The BKca inhibitor iberiotoxin (ibTX) abolished insulin-dependent glomerular albumin permeability in Wistar rats and PKGI-dependent transmembrane albumin flux in cultured podocytes. We confimed the role of BKca in insulin-evoked increases in albumin permeability in podocytes with BKca siRNA. Moreover, the ibTX abolished insulin induce changes in the phosphorylation of the PKG target proteins MYPT1 and RhoA, and disruption of the actin cytoskeleton.

Conclusions: The results indicate that insulin increases filtration barrier permeability through activation of BKca channels via protein kinase G type I in cultured rat podocytes. The experimental results suggest a molecular mechanism that could explain podocyte injury and proteinuria in diabetes.

I33

Complement Activation Profile in Patients with Primary Focal Segmental Glomerulosclerosis

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Introduction: Studies on Adriamycin-induced mice model suggested that activation of complement and IgM deposition contribute to the glomerular injury of primary focal segmental glomerulosclerosis (FSGS). We examined the plasma and urinary complement profile of patients with primary FSGS, aiming to investigate the pathogenic role of complement activation in human FSGS.

Methods: Plasma samples from 72 patients and urine samples from 62 of the 72 patients with renal biopsy-proven primary FSGS were collected at the day of renal biopsy. The plasma and urinary levels of C3a, C5a, soluble C5b-9, Bb, C4d, C1q and MBL were determined using commercial available ELISA assays.

Results: Plasma and urinary levels of C3a, C5a and soluble C5b-9 were significantly elevated in patients with primary FSGS compared with normal controls [C3a: (535.24, 217.84–969.76) ng/ml vs. (87.4, 55.8–126.31) ng/ml, $P < 0.001$, (7.76, 0.43–69.61) ng/mgCr vs. (0.01, 0.00–0.01) ng/mgCr, $P < 0.001$; C5a: (9.95, 6.60–15.93) ng/ml vs. (6.42, 2.68–10.88) ng/ml, $P < 0.001$, (16.15, 0.91–160.62) ng/mgCr vs. (0.03, 0.01–0.05) ng/mgCr, $P < 0.001$; soluble C5b-9: (381.61, 291.33–522.19) ng/ml vs. (303.98, 240.24–448.45) ng/ml, $P = 0.011$, (43.69, 5.68–220.95) ng/mgCr vs. (0.64, 0.07–1.33) ng/mgCr, $P < 0.001$]. Plasma C4d levels were significantly elevated [(14.56, 11.02–19.80) ug/ml vs. (1.61, 1.28–2.40) ug/ml, $P < 0.001$]. Plasma, but not urinary, C1q levels were significantly decreased [(77.65, 65.24–90.62) ug/ml vs. (89.38, 83.27–104.93) ug/ml, $P = 0.006$]. Both plasma and urinary MBL levels were in normal range. Urinary, but not plasma, Bb levels were significantly elevated [(0.11, 0.05–0.67) ug/mgCr vs. (0.01, 0.00–0.02) ug/mgCr, $P < 0.001$]. Plasma and urinary soluble C5b-9, and plasma C1q levels were correlated with the amount of urinary protein (soluble C5b-9: $r = 0.276$, $P = 0.020$; $r = 0.401$, $P = 0.003$; C1q: $r = -0.283$, $P = 0.018$). Plasma C5a levels were positively correlated with the proportion of segmental sclerotic glomeruli ($r = 0.261$, $P = 0.033$).

Conclusions: Complement was activated, possibly through classical pathway in primary FSGS. Complement activation products were associated with disease severity.

I34

Urinary Soluble Urokinase Receptor Levels Are Elevated and Potentially Pathogenic in Patients with Primary Focal Segmental Glomerulosclerosis

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Introduction: Recent studies proposed soluble urokinase receptor (suPAR) might be a causative circulating factor in the pathogenesis of primary focal segmental glomerulosclerosis (FSGS) but with controversy. In this study, we examined the levels and clinical significance of urinary suPAR in patients with primary FSGS. The activation effects of urinary suPAR on human podocytes were further investigated.

Methods: Sixty-two patients with biopsy-proven primary FSGS were enrolled, together with disease and normal controls. Urinary suPAR levels were measured using commercial available ELISA and adjusted by urinary creatinine. Podocyte motility and activation of integrin β3 were examined on human podocytes with urinary suPAR.

Results: The urinary suPAR level of patients with primary FSGS (500.56, 262.78–1059.44 pg/μmol Cr) was significantly higher than that of patients with minimal change disease (307.86, 216.54–480.18 pg/μmol Cr, $P = 0.033$), membranous nephropathy (250.23, 170.37–357.59 pg/μmol Cr, $P < 0.001$), secondary FSGS (220.45, 149.38–335.54 pg/μmol Cr, $P < 0.001$) and normal subjects (183.59, 103.92–228.78 pg/μmol Cr, $P < 0.001$). The urinary suPAR level of patients with cellular variant was significantly higher than that of tip variant. The urinary suPAR level in patients with primary FSGS was positively correlated with 24-hr urine protein ($r = 0.287$, $P = 0.024$). During follow up, the urinary suPAR level of patients with complete remission decreased significantly ($P = 0.017$). The AP5 signal of integrin activation could be strongly induced along the cell membrane of human podocytes incubated with the urine of FSGS patients, but not with disease and normal controls. The urine from FSGS patients promoted human podocyte wound closure significantly (0.57±0.10 vs. 0.24±0.04, $P = 0.001$). Anti-uPAR antibodies could inhibit the AP5 signal and podocyte motility induced by urine of patients with primary FSGS.

Conclusions: Urinary suPAR was elevated in primary FSGS and associated with disease severity, which might involve in the pathogenesis of FSGS through activation of podocytes.

I35

Apolipoprotein L1-B3 Isoform Is a Novel Splice Variant That Localizes to Mitochondria

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Introduction: Apolipoprotein L1 (APOL1) is a component of the innate immune system and a trypanosomal lytic factor. The APOL1 gene variants, present in individuals of recent African de-

scent, are strongly associated with risk for glomerular disease, although the mechanisms are unknown. ApoL1 has numerous RNA splice variants, summarized as A, B and C variants. The A isoform is encoded by exons 1 and 3-7 and has been studied most extensively. Little is known about the B isoform, encoded by exon 1-7, and the C isoform, encoded by exons 1, 3, and 5-7.

Methods: We examined mRNA expression by TA cloning and RT-PCR and protein localization using immunofluorescence of Flag-tagged vector transfection using cultured HeLa cells and immortalized human podocytes. Since only APOL1-B isoforms contain exon 2, we raised a rabbit antiserum specific for APOL1-B peptide by immunizing with exon 2 sequences. Mitochondrial fractions were isolated by a detergent-based method.

Results: Seven APOL1 splicing variants were identified in podocytes. Three variants contained exon 2: APOL1-B1, B2 and B3 RNA. APOL1-B1 and B2 RNA contained exon 1-7, with B2 having an extended exon 3. APOL1 B3 lacked exon 4, which contains the signal sequence; this isoform had not previously been reported. Using RT-PCR, normal human kidney expressed 4 APOL1 splicing variants by RT-PCR: APOL1-A, B1, B3 and C. Transfected Flag-tagged APOL1-B3 was detected by both APOL1-B Ab and anti-Flag Ab. APOL1-B isoforms localized to mitochondria in serum-starved immortalized human podocytes. Specifically, transfected APOL1-B3 localized to mitochondria in HeLa cells. Finally, detergent-isolated mitochondrial fractions were shown to contain APOL1-B variants and transfected APOL1-B3.

Conclusions: APOL1-B variants were expressed in cultured human podocytes. APOL1-B1 and B2 contain the signal peptide and are likely secreted. Transfected APOL1-B3, a novel variant, lacks the signal peptide and localized to mitochondria, where its function remains to be determined.

I36
Global Analysis of Glucocorticoid Action in Human Podocytes

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Introduction: Children presenting with nephrotic syndrome (NS) typically receive an empiric course of glucocorticoid (Gc) therapy. Those who do not respond to Gc treatment are at high risk of developing end stage renal disease. Although podocyte effacement is a key feature of NS, the clinically-relevant, podocyte-specific mechanisms of action of glucocorticoids in NS have not been determined. We aim to elucidate relevant mechanistic pathways to guide novel therapeutic targeting using a selection of in vitro and in vivo methods.

Methods: The transcriptional profile of Gc-treated and Gc-naïve human podocytes were compared using the Affymetrix U133 Plus 2.0 Array. Comparing whole genome expression data from the two cell groups allowed the creation of a list of Gc-regulated transcripts. This list underwent enrichment analysis for gene ontology (GO) terms and experimental validation with live cell imaging. In vivo data is currently being generated through the use of mice with a podocyte-specific deletion of the glucocorticoid receptor (GR).

Results: 606 Gc-regulated transcripts were identified (fold change with treatment ±1.5, q value ≤0.05). A highly enriched GO term was cell motility. Live cell imaging showed that Gc exposure significantly reduced podocyte speed (0.0053 μm/sec in untreated cells Vs. 0.0034 μm/sec in treated cells, p-value <0.05), and this difference was evident 2 hours after Gc-exposure. Preliminary in vivo data shows that GR deficient mice have no significant proteinuria at 8 weeks of age.

Conclusions: Accumulating evidence suggests podocyte motility is a determinant of glomerular filtration barrier function. Transcriptional profiling of human podocytes suggests Gc exposure has effects on podocyte motility, and we have validated this using live cell imaging. Future work will focus on defining a podocyte GR-cistrome through ChIP-Seq and challenging GR deficient mice to a proteinuria-inducing agent to determine the key target cell of Gc action in NS.

I37
Insulin Modulates the Expression and Activity of Autophagic Proteins in Rat Podocytes

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Introduction: Autophagy is proposed to be an essential defense mechanism in podocytes and is suggested to play a key role for their survival under stress conditions. The impairment of podocyte function and their loss takes part in pathogenesis of diabetic nephropathy and leads to renal failure. To shed light on the autophagy role in this complication, we investigate how autophagic pathways are regulated by a (patho)physiological factor, such as insulin.

Methods: All experiments were conducted on primary culture of rat podocytes. Insulin effects on autophagy were investigated in cells cultured for 60 minutes, 3 or 5 days in medium supplemented with 300 nM insulin. The expression level of proteins involved in autophagy (LC3, beclin1, Atg5-12, ULK1, AMP-activated protein kinase (AMPK), class III phosphatidylinositol 3-kinase (PI3KC3), JNK) was analysed by immunodetection. Changes in AMPK and JNK activity were determined by immunoblotting against AMPKα P-Thr^{172} and JNK P-Thr^{183}/P-Tyr^{185}. PI3KC3 activity was analysed by phosphatidylinositol-3-phosphate quantification (ELISA).

Results: The increase of LC3-II expression was observed in podocytes after 60 minutes (by 55%, $p < 0.05$) and after 3 days (by 24%, $p < 0.05$) incubation with insulin. Atg5-12 complexes expres-

sion was also augmented after 3 days (117% of control, $p < 0.05$). The expression of beclin1 in podocytes showed slightly upward tendency dependent on time of incubation, though there was no effect of insulin on PI3KC3 expression, nor its activity. Insulin increased JNK expression after 5 days of incubation (by 43%, $p < 0.05$), as well as P-JNK/JNK and P-AMPK/AMPK ratios after 3 days.

Conclusions: Insulin regulates the expression and activity of proteins involved in autophagy in podocytes. It seems plausible, that insulin stimulates the processes of autophagosome maturation and closure, which may be activated independently of PI3KC3-beclin1 complex.

I38

The Role of Tankyrases in the Regulation of Glucose Metabolism in Zebrafish Larvae

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Introduction: Tankyrase 1 and 2 are poly(ADP-ribose) polymerases that transfer ADP-ribose groups to target proteins and thereby affect various cellular processes, such as WNT/β-catenin signaling and insulin-mediated glucose uptake. We found that tankyrases interact with the key podocyte protein CD2AP in the glomeruli. Here, we aimed to define the functional role of tankyrases in vivo using zebrafish as a model.

Methods: We explored the expression pattern of tankyrases by whole-mount in situ hybridization and RT-PCR. We knocked down tankyrases and inhibited their activity by exploiting morpholino antisense oligonucleotides and a tankyrase inhibitor, XAV 939, respectively. The expression of genes regulating glucose homeostasis and hormone secretion was accessed by qRT-PCR, immunofluorescence, and in situ hybridization.

Results: We identified two paralogous tankyrase genes in zebrafish, TNKS1a and TNKS1b, which have higher homology to human TNKS1 than TNKS2. Both genes were strongly expressed in the brain. Notably, TNKS1b was prominent in the hypothalamus and pituitary. Disruption of TNKS1a did not induce mortality or any obvious changes in the phenotype, whereas knockdown of TNKS1b or administration of XAV939 resulted in reduced linear growth without altering the motility. Furthermore, downregulation of TNKS1b suppressed fasting-inducible gluconeogenic gene, pck1, and stimulated insulin and glucose transporter expression, suggesting the involvement of tankyrase in the regulation of glucose homeostasis. Moreover, downregulation of TNKS1b supressed the expression of pro-opiomelanocortin (pomc), and induced loss of pomc-positive neurons with subsequent decline of α-melanocyte stimulating hormone (α-MSH) at pars intermediate. Interestingly, we observed reduced expression of melanocortin receptor 3 (MC3R) and enhanced expression of MC4R when TNKS1b expression was downregulated. This suggests that tankyrase, via α-MSH, distinctly regulates the melanocortin signaling pathway involved in insulin secretion, glucose homeostasis, and energy expenditure.

Conclusions: Our data suggest that tankyrases may be involved in the regulation of glucose homeostasis via the central melanocortin system.

I39

Podocyte-Targeted Therapies for Diabetic Nephropathy

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Introduction: Podocytes are essential constituents of the glomerular filtration barrier. They interdigitate specialized actin-rich foot structures to create the slit-diaphragm; a multi-protein complex of cell-cell adhesion molecules linked to the actin cytoskeleton. Diabetic nephropathy, the leading diagnosis in end-stage renal disease, is recognized to involve injury or loss of podocytes with the resultant onset of proteinuria. Evotec and AstraZeneca, in a collaborative venture, have used high content imaging techniques to identify small molecules capable of protecting human conditionally immortalized podocytes with the aim to identify new targets and develop new therapies for treatment of diabetic nephropathy.

Methods: The primary screening assay employed measured apoptosis in human conditionally immortalized podocytes treated with a combination of palmitate and high glucose. A secondary assay which measured changes in the podocyte actin cytoskeleton was used to confirm compound activity. In total, approximately 120,000 compounds from a variety of small molecule collections were tested in this work flow. Included in these collections were a number of known drugs plus well-developed lead-like compounds with established pharmacology.

Results: Within this collection a novel candidate drug molecule/target class has been identified as being protective against podocyte injury in vitro, and protective against glomerular injury ex vivo. Experiments are now being performed to assess protection in vivo using the STZ model of diabetic nephropathy. Additional targets are being identified by entering suitable screening hits into Evotec's Cellular Target Profiling workflow. Here, hit molecules, first optimized for physicochemical properties, are used in combination with quantitative chemoproteomics to identify specific binding partners.

Conclusions: Using these multiple strategies Evotec and AstraZeneca hope to deliver podocyte-targeted therapies for treatment of Diabetic Nephropathy.

I40

Diabetic E1-DN Mice Present Structural Changes in the Glomeruli Characteristic of Diabetic Nephropathy

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Introduction: Currently available animal models for diabetic nephropathy recapitulate only partially the features of the human disease. The transgenic E1-DN mice express a kinase-negative EGF-R in their pancreatic islets and are diabetic due to impaired postnatal growth of β-cells. The mice survive without insulin treatment, and can be used to investigate the effects of long term exposure to hyperglycaemia. Here, we characterize the renal phenotype of E1-DN mice, focusing on glomerular injury.

Methods: The transgenic mice in FVB background were generated previously. Mice were followed up to 57 weeks (wks). Blood glucose and albumin excretion were measured. Homozygous male mice were selected for further analyses based on albuminuria. Histological stainings and electron microscopy (EM) were used to characterize the morphological changes of the glomeruli.

Results: Homozygous E1-DN mice showed increased albumin excretion rate at 10 wks, and the albuminuria increased over time. Morphometric analysis using PAS-stained histological sections or EM indicated mesangial expansion, and EM also revealed thickening of the glomerular basement membrane and foot process widening in the homozygous E1-DN mice. An expert pathologist blinded of the genotypes characterized focal glomerular sclerosis in the most albuminuric mice at 50 wks. Confocal microscopy analysis revealed decreased expression of nephrin in the glomeruli of homozygous E1-DN mice at 50 wks. Immunohistochemistry for cleaved caspase-3 pinpointed increased glomerular apoptosis already at 20 wks, and co-staining with nephrin indicated that apoptotic cells were podocytes.

Conclusions: Hyperglycaemic E1-DN mice develop substantial albuminuria. The structural changes in the glomeruli of the homozygous mice include mesangial expansion, thickening of the glomerular basement membrane and podocyte foot process widening, resembling the changes observed in human diabetic nephropathy. Nephrin expression is reduced and apoptosis increased in podocytes. Collectively, our data indicate that E1-DN mice are a valuable model to study diabetic nephropathy.

I41

Regulation of Polyol Pathway Activity in Podocytes

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Introduction: The polyol pathway (PP), an alternative route of glucose metabolism implicated in diabetic complications comprises two enzymes: aldose reductase (AR) and sorbitol dehydrogenase (SDH). While AR is widely expressed, its co-expression with SDH has been found in certain tissues, first of all in the organs that are primary targets for diabetic lesions. It seems likely that PP may participate in podocyte lesions in diabetes. The present study characterizes PP expression and activity in podocytes.

Methods: Immortalized mouse podocytes (line SVI) were incubated in normal- and high glucose (NG 5.6 mM, HG 30 mM) and in normal-and high osmolar (Nosm 290 mOsm, Hosm 390 mOsm) media for 6 hours up to 5 days. AR expression and activities were determined using RT-PCR, Flow cytometry, immunofluorescence and spectrophotometry.

Results: Both AR and SDH are expressed in podocytes. Expression of AR increased by 50% after 6-hour incubation in NG-Hosm and HG-Nosm, while 1.8-fold rise of activity occurred after 6 hours incubation in NG-Hosm. After 6 hours incubation in the Nosm media, SDH activity significantly increased in response to high glucose (0.34±0.01 vs 0.26±0.01, HG vs NG, $P < 0.005$). At the same time, expression of the enzyme remained unchanged. After 5 days stimulatory effect of high glucose disappeared in the Nosm cells, while in the Hosm group, activity of SDH was markedly elevated by HG (0.40±0.02 vs 0.28±0.02, HG vs NG, $P < 0.001$). Simultaneously, SDH expression of the decreased by 29% in HG cells.

Conclusions: Podocytes possess a complete polyol pathway system which is regulated by glucose and osmolality. The PP enzymes are upregulated within a short time after increasing ambient glucose and osmolality Observed changes in the enzyme activity are not paralleled by respective changes in the protein expression. Active polyol pathway in podocytes may contribute to their impairment in diabetes.

I42

Podocyte-Specific Loss of the Negatively Charged Sugar Sialic Acid Leads to Kidney Failure in Mice

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Introduction: The role of sialylation in kidney biology is not fully understood. The synthesis of sialoglycoconjugates, which form the outermost structures of animal cells, requires CMP-sialic acid (Sia), which is a product of the nuclear enzyme CMAS. We could recently show in mice that a mutation in the Cmas gene, leading to reduced global expression of the enzyme, results in kidney failure within three days after birth. With the aim to analyze the importance of sialylation for the function of the podocytes as highly sialylated outermost layer of the glomerular filtration barrier, we now established a mouse model with a deletion of Cmas exclusively in podocytes (P-Cmas$^{-/-}$).

Methods: Urine analysis was performed with SDS-PAGE and Coomassie staining. The sialylation pattern of target proteins was investigated by Western Blotting and lectin staining. Histological aberrations were analyzed by H&E as well as immunohistological staining of paraffin embedded tissue sections of wildtype and P-Cmas$^{-/-}$ mice. Ultrastructural analysis was performed by transmission electron microscopy.

Results: P-Cmas$^{-/-}$ mice show growth retardation, develop massive proteinuria around postnatal day (P) 24, and die within 6 weeks after birth. Increasing loss of Sia, e.g. on podocalyxin and nephrin, was accompanied by a proceeding reduction of target protein levels. An enlargement of the glomerular urinary space together with expanded lumina of proximal and distal tubules filled with protein casts became visible around P28. At ultra-structural level a dramatic effacement of foot processes with loss of slit diaphragms was observed.

Conclusions: Loss of Sia on exclusively on podocytes entails symptoms that resemble the human 'Congenital Nephrotic Syndrome of the Finnish Type' (CNF). We aim to address the mechanisms involved in CNF disease with a detailed histological, ultrastructural and biochemical analysis of P-Cmas$^{-/-}$ mice and to identify the impact of Sia, e.g. on nephrin functions.

I43

High Throughput siRNA Screening Identifies Potential Novel Regulators of mTORC1 Signaling in Podocytes

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Introduction: The mTOR complex 1 (mTORC1) is a central regulator of cell metabolism and growth. Human and animal studies implicate excess mTORC1 activity in podocytes in the development of diabetic nephropathy (DN), while rapamycin, an mTORC1 inhibitor, induces proteinuria in some patients. Identifying regulators of the mTORC1 pathway in podocytes has the potential to further our understanding of the pathogenesis of DN and may identify targets for the development of more specific therapies.

Methods: To this end, we optimized a high throughput siRNA knockdown and in-cell western screening protocol using human immortalized podocytes cultured in hyperglycemic conditions, and monitoring ribosomal protein S6 phosphorylation (P-rpS6) as a surrogate of mTORC1 activity.

Results: To date, we have screened siRNAs targeting 7,317 genes in search of novel modulators of the mTORC1 complex activity under hyperglycemic culture conditions. Using robust selection criteria, we have identified 251 siRNAs that reduced P-rpS6 levels below 3 MADs (median absolute deviation) without affecting cell viability, including 28 protein kinases and 24 G-protein couple receptors. We also have identified 55 siRNAs that increased P-rpS6 levels over 3 MADs under the same conditions. Preliminary hits will be further validated by deconvolution of the target siRNA pools, followed by examination of their effect on mTORC1 signaling under normoglycemic conditions, and in the absence or presence of insulin. Validated hits will be further investigated to ascertain their ability to modulate mTORC2 signaling. We are also currently completing our screen to include the remaining 14,000 genes targeted in the genome-wide siRNA library.

Conclusions: These results demonstrate that conditionally immortalized podocyte cell lines are amenable to use in high-throughput siRNA screening and identify new genes potentially involved in the regulation of mTORC1 activity in podocytes during hyperglycemia.

I44

The Autophagy-Lysosome Pathway Is Altered in Alpha-Galactosidase A-Deficient Mouse Brain

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Introduction: Fabry disease is a rare, X-linked lysosomal storage disorder caused by mutations in alpha-Galactosidase A (α-Gal A). The resultant loss of α-Gal A enzymatic activity and accumulation of glycosphingolipid substrates leads to a widespread vasculopathy with particular detriment to the kidneys, heart and nervous system. Disruption in the autophagy-lysosome pathway has been documented previously in Fabry disease but its relative contribution to nervous system pathology in Fabry disease is unknown.

Methods: We examined brain pathology in α-Gal A hemizygous (deficient) and wild-type male mice with particular emphasis on the autophagy-lysosome pathway.

Results: Hemizygous mouse brains exhibited a relative lack of α-Gal A enzymatic activity compared to wild-type controls. Immunohistochemical analysis of brains of α-Gal A hemizygous mice revealed enhanced punctate perinuclear immunoreactivity for the autophagy marker microtubule-associated protein light-chain 3 (LC3) in the parenchyma of several brain regions, as well as enhanced parenchymal and vascular immunoreactivity for lysosome-associated membrane protein-1 (LAMP-1). Ultrastructural analysis revealed endothelial cell inclusions with electron densities and a pronounced accumulation of electron-dense lipopigment. Interestingly, ultrastructural analysis also revealed a relative absence of autophagosomes in α-Gal A hemizygous mouse brain. Previous studies have indicated enhanced LC3 immunoreactivity in the absence of autophagosome accumulation in mice genetically engineered to inhibit autophagy induction (Jaber et al 2012 PNAS 109:2003), as well as the association of LC3 specific to lipid droplets (Shibata et al 2009 Biochem. Biophys. Res Commun. 382:419). Results of these previous reports may explain the seemingly discrepant findings in our study indicating the aberrant enhancement of LC3 in the absence of autophagosome accumulation.

Conclusions: Future studies in our laboratory will use models of α-Gal A deficiency to further dissect the manner by which the autophagy-lysosome pathway is altered and to determine the role of autophagy-associated machinery in lipid metabolism.

I45

Tissue Transglutaminase Deficiency Augments Podocyte Autophagy and Increases the NRF2 Anti-Oxidant Pathway. A Major Modulation of Podocytes Phenotype That Protects from Crescentic Glomerulonephritis

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Introduction: We found podocyte expression of tissue transglutaminase (TG2) in mice and human kidneys in crescentic rapidly progressive glomerulonephritis (RPGN) with new roles for the intracellular enzyme.

Methods: We investigated the role of the TG2 in experimental RPGN. Global and podocyte specific *Tgm2* gene invalidation do not alter ultrastructural features of the filtration barrier.

Results: In the anti-glomerular basement membrane model of RPGN, *Tgm2* invalidation was associated with a marked attenuated glomerular injury. Albuminuria was 25 fold lower in Tgm2 KO mice at day 11 ($p < 0.001$) that displayed a complete absence of crescent formation (0±0% of the glomeruli vs. 45±6% at day 30, $p < 0.001$). Accordingly, TG2 deficiency prevented renal failure, as reflected by the preservation of BUN within normal ranges at day 30 ($p < 0.001$). Likewise, TEM demonstrated unaffected podocyte ultrastructure in *Tgm2* KO at day 4, compared to *Tgm2* WT mice which displayed severe foot process effacement. We next assessed the function of TG2 specifically in podocytes. Nphs2-Cre.*Tgm2* lox/lox mice also displayed significant protection against the development of the RPGN with a strong inhibition of podocyte migration and increase in constitutive autophagy. Furthermore, TG2 activity was required for routing of p62/SQSTM1 to the autophagosome. TG2 deficiency lead to increased p62 cytosolic abundance and activation of the Nuclear factor-erythroid 2 related factor 2 (Nrf2) pathway, a master transcription factor that regulates expression of antioxidant enzymes and cytoprotective proteins. Likewise, both *Nrf2* deficiency and podocyte-specific (Atg5) autophagy deficiency markedly accentuated RPGN. At last, pharmacological TG2 inhibition limited glomerular demolition and renal failure.

Conclusions: TG2 is required to severe experimental RPGN, through promotion of migration of podocytes, alteration of autophagy and inhibition of antioxidant defense. This study unravels a novel enzymatic pathophysiological pathway in podocytes and suggests that targeting TG2 may be clinically beneficial for treatment of severe RPGNs.

I46

mTORC1 Activation Links Insulin Signaling to Prohibitin-2 Function in Glomerular Podocytes

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Introduction: Mitochondrial dysfunctions have been implicated in a variety of human diseases and aging phenotypes. Fusion and fission of mitochondria maintain mitochondrial integrity and require prohibitin (PHB) ring complexes in the inner membrane of mitochondria. Recently, the function of prohibitins has been linked to the insulin signaling pathway in yeast and worms. Here, we studied the contribution of the mitochondrially localized protein, prohibitin-2 (PHB2), on podocyte function in general and its connection to insulin signaling and glomerular injury in particular.

Results/Methods: The impact of PHB2 on podocyte metabolism was analyzed in a podocyte-specific Phb2-knockout mouse. Loss of PHB2 resulted in the development of progressive proteinuria, glomerulosclerosis and endstage renal failure subsequently causing premature death of the animals. Podocytes deficient in Phb2 displayed severe structural abnormalities and alterations in mitochondrial morphology accompanied by increased phosphorylation of S6 ribosomal protein (S6RP), a known mediator of the mechanistic target of rapamycin (mTOR) signaling pathway, in vitro and in vivo. Interfering with insulin signaling by additional gene knockouts of both, the insulin and the IGF-1 receptors, prevented hyperphosphorylation of S6RP, delayed the onset of kidney failure and prolonged survival of podocyte-specific Phb2-knockout mice by several weeks despite progressive proteinuria. As proof of concept, treatment with the mTORC1-inhibitor rapamycin also enhanced survival of podocyte-specific Phb2-knockout mice.

Conclusions: In conclusion, we show for the first time that PHB2 is coupled to metabolic signaling in mammals. These data provide an important link between mitochondrial dysfunction and alterations in insulin/IGF-1/mTOR signaling and suggest that mitochondria-mediated diseases may be amenable to therapeutic intervention through modulation of insulin/IGF-1/mTOR signaling.

I47

Loss of Prohibitin-2 Leads to Progressive Proteinuria and Renal Failure in Mice

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Introduction: Loss of the mitochondrial scaffolding protein prohibitin-2 (PHB2) in podocytes results in progressive proteinuria, glomerulosclerosis and endstage renal failure within 4–5 weeks. By interfering with insulin and mTORC1 activation we were able to extend animal survival by 20–35%. However, the mice still developed renal failure and showed an impaired glomerular function. As PHB-domain containing proteins support the formation of functional lipid microdomains within cellular membranes we speculate on an additional extramitochondrial function of PHB2 in glomerular podocytes.

Results/Methods: In contrast to previous observations in proliferating fibroblasts and other cell types we did not observe an increased rate of apoptotic cell death of the non-dividing podocytes. Immunogold labeling and immunofluorescence stainings did not only localize PHB2 to mitochondria, but also to the slit diaphragm. Additional in vitro experiments revealed that PHB1 and PHB2 co-immunoprecipitated with podocin. Given the strong similarity of the slit diaphragm complex in mammals and a mechanosensory protein complex in C. elegans we also studied the localization and function of the PHB2 ortholog in touch neurons of the nematode (PHB-2). Again, immunofluorescence stainings showed co-localization of PHB-2 and MEC-2, the podocin ortholog in C. elegans. Moreover, knockdown of phb-2 led to a severe functional impairment of the mechanosensory complex. These data indicated that PHB2 may have a dual role, a function at the mitochondria as well as an additional function at mechanosensory protein complexes.

Conclusions: In conclusion, we provide several lines of evidence that PHB2 is not only found at the inner mitochondrial membrane but also localizes to the slit diaphragm in glomerular podocytes. Loss of PHB2 leads to renal failure and premature death resembling phenotypes of other slit diaphragm protein deficiencies.

I48

GIV/Girdin Links VEGF Signaling to Akt Survival Signaling in Podocytes

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Introduction: Podocytes are critically involved in the maintenance of the glomerular filtration barrier. It is essential to maintain podocyte survival and avoid apoptosis after acute glomerular injury. In normal glomeruli, podocyte survival is mediated via nephrin-dependent Akt signaling. In several glomerular diseases nephrin decreases, and podocyte survival correlates with increased VEGF signaling. How VEGF signaling contributes to podocyte survival and prevents apoptosis has remained unknown.

Methods: Glomeruli were isolated from normal and PAN rats. Expression and phospho-modification of GIV, VEGFR2 and Akt, and expression of VEGF were assessed by quantitative immunoblotting; localization of GIV was determined by immunofluorescence; interaction between endogenous VEGFR2 and GIV by co-immunoprecipitation; and in vitro interaction between GIV and Gαi3 by GST-pulldown assay. To mimic early PAN injury, in vitro differentiated mouse podocytes were treated with PA. GIV was depleted by siRNA and rescued by adenovirus, actin remodeling was assessed by rhodamine-phalloidin staining, cell migration by wound healing assay, and apoptosis by caspase 3 cleavage and quantitative PCR.

Results: We show here that GIV/girdin mediates VEGF receptor 2 (VEGFR2) signaling and compensates for nephrin loss: In puromycin aminonucleoside nephrosis (PAN) GIV expression increases, GIV is phosphorylated by VEGFR2, and p-GIV binds and activates Gαi3 and enhances downstream Akt2/mTOR signaling. In GIV-depleted podocytes, VEGF-induced Akt and mTOR activation is abolished, apoptosis is triggered, and cell migration is impaired. These effects are reversed by introducing GIV-WT but not a GIV mutant that cannot activate Gαi3.

Conclusion: Our data indicate that after PAN injury, VEGF promotes podocyte survival by triggering assembly of an activated VEGFR2-GIV-Gαi3 signaling complex and enhancing downstream PI3-kinase/Akt survival signaling which is mediated by both mTORC1 and 2. Due to its important role in promoting podocyte survival, GIV represents a novel target for therapeutic intervention in the nephrotic syndrome and other proteinuric diseases.

Subtheme II: Slit Diaphragm & Cytoskeleton

II1

Knockdown of Podocyte Foot Process Protein Klhl35 Results in Proteinuria in Zebrafish Pronephros

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Introduction: In our previous microarray study, a transcript encoding for Kelch-like family member 35 (Klhl35) was enriched in the glomerulus. As the protein is very poorly characterized, we aimed in this study to analyze the expression and function of Klhl35 in the kidney.

Methods: Expression was studied in adult and developing kidneys, as well as in cultured podocytes using RT-PCR, immunofluorescence and immune-electron microscopy. Functional studies were performed in zebrafish by inactivating the gene expression using morpholinos.

Results: Klhl35 was expressed by podocytes and it localized to foot processes. During glomerulogenesis Klhl35 was detected first at S-shaped stage glomeruli in where its expression seemed to preceed nephrin expression. In cultured podocytes, no significant expression was detected in undifferentiated cells. In differentiated podocytes the expression was induced and Klhl35 was detected in stress fibers. In zebrafish, the knockdown of Klhl35 orthologue resulted in pronephros abnormalities including foot process effacement. Functionally, pronephros was leaking as detected by the presence of of 500kD fluorescent dye in the tubular compartment.

Conclusions: Klhl35 is a novel podocyte foot process protein. It is important for the integrity of zebrafish pronephros. Based on its sequence, we speculate that Klhl35 is an actin-associated protein that regulates the organization of foot process cytoskeleton.

II2

The Molecular Architecture of Podocyte Foot Processes and Slit Diaphragm

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Introduction: The resolution of traditional microscopes, about 200 nm, is theoretically limited by the wave-length of light. Imaging structures smaller than 200 nm, like the foot processes of the podocyte and the slit diaphragm, has required electron microscopy. Super resolution imaging techniques are new methods of imaging that allow the diffraction barrier of the light microscope to be broken facilitating the traditional light microscope to image at 10X higher resolutions than previously thought to be possible. Recently, we used stochastic optical reconstruction microscope (STORM), a super resolution microscopic technique, to document the molecular ultrastructure of the glomerular basement membrane (GBM) of both mouse and human kidney tissue. Here we apply the method to study the architecture of the podocyte in normal and diseased tissue.

Methods: We applied STORM imaging to study the spatial distribution of key molecules of the podocyte foot process and slit diaphragm in the healthy mouse glomerulus. We used three color STORM imaging with the known reference molecules such as the GBM marker, agrin, to map the relative position of foot process and slit diaphragm molecules. Podocyte foot process components such as synaptopodin and cortactin, as well as slit diaphragm proteins such as nephrin, Neph1, podocin, Cd2ap and ZO-1 were examined.

Results: In the healthy glomeruli, synaptopodin labels the center of each foot process while nephrin is positioned between individual foot processes in the position of the slit diaphragm. As foot processes start to efface, nephrin redistributed away from the base of foot processes towards the apical surface in two models of podocyte injury models, Cd2ap knockout and adriamycin-injury mouse models. This change in nephrin distribution was accompanied with a change in synaptopodin staining. STORM-EM correlation shows that the change in the nephrin and synaptopodin distribution is an early event in the foot process effacement process.

Conclusions: Here, we establish the use of super resolution microscopy technique to image podocyte foot processes and slit diaphragm on a nanoscale. This method allows for single molecule imaging of the healthy as well as diseased kidney glomeruli. This technique will be very useful in identifying the early molecular changes governing the podocyte foot process effacement that lead to compromise glomerular filtration barrier and proteinuria.

II3

The Role of the FERM Domain Protein Epb41l5 for Glomerular Development and Maintenance

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Background: The FERM domain is approximately 150 amino acids in length and is found in a number of cytoskeletal-associated proteins that are localized to the plasma membrane and the cytoskeleton interface. The FERM domain is responsible for the PIP_2 regulated binding to the membrane, which associates the cytoskeleton with the membrane by linking actin filaments to adhesion proteins. Recently we identified a brain and podocyte-specific FERM domain protein Epb41L5 as a novel component of the slit diaphragm (SD).

Methods: To elucidate the precise function of Epb41l5 we utilized complementary transgenic mouse models, drosophila models, conventional biochemistry and high-resolution quantitative mass spectrometry-based proteomics.

Results: Ablation of *Epb41l5* in podocytes led to severe proteinuria, growth retardation and early lethality. At the same time the glomerular ultrastructure was completely abrogated. Interestingly, immunogold-labelling demonstrated that Epb41l5 located basolaterally in the foot processes at focal adhesion sites. Massive loss of KO podocytes into the urine suggests that Epb41l5 plays a central role in linking podocytes to the GBM. Unexpectedly, an interaction proteomics approach suggested that Epb41L5 links integrins, the SD, and focal adhesions.

Conclusion: We hypothesize that the Epb41L5 protein network could be the missing link coordinating the interplay of podocyte filter formation, adhesions properties and cortical actin dynamics.

II4

MAP-Kinase p38 – Pivotal Mediator of Slit Diaphragm Integrity

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Introduction: Albuminuria is an early symptom of diabetic damage of the glomerular filter. Diabetic albuminuria has been shown to be attenuated by inhibition of MAPK p38 but the underlying mechanism has not been elucidated yet. We could recently demonstrate that diabetic mice with albuminuria show increased nephrin endocytosis. Several other groups now confirmed that the

endocytosis of slit diaphragm proteins is paramount for podocyte function. Now we found that pharmacologic inhibition of p38 significantly attenuates nephrin endocytosis and albuminuria. In our project we further unravel the molecular mechanism of nephrin endocytosis and identify p38 as pivotal mediator of proteinuric signaling and slit diaphragm integrity.

Methods: Diabetes was induced in C57BL/6 mice by intraperitoneal injection of streptozotocin. For inhibition of p38, the mice were treated with SB202190. Albuminuria was quantified as albumin/creatinin ratio. Nephrin endocytosis was analyzed by immunofluorescence staining of glomeruli and biotin endocytosis assays. To quantify endocytosis in murine kidneys in vivo a special biotin-based endocytosis assay was established. Western blotting and kinase assays were performed to analyze the phosphorylation of nephrin mediated by p38 and PKCα.

Results: Hyperglycemic mice developed a significant albuminuria already four days after induction of diabetes application. In vivo analysis showed a significant increase in podocytic p38 activity and nephrin endocytosis only in albuminuric animals. Pharmacologic inhibition of p38 nearly completely prevented nephrin endocytosis and albuminuria. P38 mediated phosphorylation of the nephrin c-terminus facilitates the coupling of nephrin to the endocytotic machinery via PKCα – β-arrestin2 – signaling.

Conclusions: Here we show that p38 is a pivotal mediator of hyperglycemia-induced nephrin endocytosis and albuminuria. Our in vivo endocytosis assay allowed for the first time a quantification of nephrin endocytosis in murine kidneys under diabetic conditions. Inhibition of p38 decreases nephrin phosphorylation and endocytosis, supporting the hypothesis that stabilization of the slit diaphragm prevents albuminuria. These findings suggest p38 as pivotal mediator of early diabetic damage and thus as a promising therapeutic target.

II5

Paraoxonase PON2 Affects Lipid Peroxidation at the Slit Diaphragm

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Introduction: The mammalian slit diaphragm complex and the mechanosensory complex of C.elegans share highly similar and conserved proteins. The Podocin homologue MEC-2 orchestrates protein-lipid supercomplexes in the plasma membrane of C.elegans' touch neurons to allow for proper signalling via the degenerin cation channel MEC-4/-10. Part of the mechanosensory complex is also the lipid modifying enzyme MEC-6, which interacts with MEC-4/-10 and enhances its channel activity. The paraoxonase PON2 is homologue to MEC-6 of C.elegans. It is ubiquitously expressed and refers arylesterase, as well as lactonase activity and shares anti-oxidative properties which have impact on the progression of atherosclerosis and inflammation.

Results: Here we show that PON2 is expressed in podocytes, it localizes to the plasma membrane near the slit diaphragm in immunofluorescence and immunogold-labeling studies. In addition it not only co-fractionates with Podocin in cholesterol-rich detergent resistant membrane domains but also interacts directly with slit diaphragm proteins like Nephrin, Podocin and TRPC6 in pull-down experiments. We identify PON2 as an integral type II transmembrane protein that localizes to the plasma membrane with its enzymatically active domain facing extracellular. Trafficking of PON2 to the plasma membrane is increased upon elevated intracellular Ca2+ concentrations, induced by ionomycin and reversible after addition of EDTA to the cell culture medium. At the plasma membrane PON2 counteracts lipid peroxidation and affects the lipid composition of the membrane.

Conclusions: We report PON2 as a new type II transmembrane protein at the slit diaphragm that is shuttled to the membrane in a Ca^{2+}-dependent manner. The enzymatically active sites face the extracellular compartments and affect lipid peroxidation and lipid composition of the plasma membrane.

II6

Podocyte-Specific Deletion of the Planar Cell Polarity Gene Vangl2 Leads to Glomerular Abnormalities

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Introduction: The development and maintenance of podocyte architecture involves cytoskeletal re-organisation, but little is known about the pathways regulating this process. We hypothesised that the planar cell polarity pathway, which controls the uniform orientation and alignment of epithelial cells orthogonal to the apical-basal axis, may be critical in maintaining podocyte structure and the integrity of the glomerular filtration barrier. Our previous work demonstrated that Vangl2, a core PCP component, is expressed in podocytes and mice with a spontaneous homozygous mutation in this gene demonstrate impaired kidney branching morphogenesis and glomerular maturation. In this study we sought to establish the precise role of Vangl2 in podocyte morphogenesis and glomerular filtration.

Methods: Podocyte-specific Vangl2 knock-down mice ($Vangl2^{\Delta podocyte}$) were generated by combining floxed Vangl2 animals with Podocin-Cre mice (both on C57Bl/6 background); littermates without Cre were used as controls ($Vangl2^{flox/flox}$). Glomerular morphology, proliferation and overnight albumin excretion were assessed at 12 weeks of age. RNA was extracted from glomeruli isolated by Dynabead perfusion.

Results: $Vangl2^{\Delta podocyte}$ mice had a 40% reduction in Vangl2 glomerular mRNA levels compared with $Vangl2^{flox/flox}$. The expression of other PCP genes in isolated glomeruli (Vangl1, Celsr1, Scribble, MKP1&2) was unaltered. Abnormal glomerular mor-

phology, characterised by loss of capillary loops and tuft collapse, was seen in 16.6±4.8% of glomeruli of Vangl2$^{\Delta podocyte}$ mice compared with 3±1.5% in control littermates. This was accompanied by increased glomerular proliferation (0.17±0.03 vs 0.07±0.02 cells/glomerulus, $p < 0.05$) and nephrin phosphorylation. Expression of the extracellular matrix protein collagen (α_3) IV was also significantly reduced in Vangl2$^{\Delta podocyte}$ mice. Despite these findings, we did not observe any changes in albumin excretion at this time-point.

Conclusions: Our data indicates that loss of Vangl2 in the podocyte leads to subtle glomerular abnormalities including altered proliferation, matrix turnover and nephrin phosphorylation. However, this does not manifest as altered integrity of the filtration barrier and albuminuria.

II7

The Paraoxonase PON2 Regulates the Channel Activity of TRPC6 by Affecting the Lipid Composition of the Plasma Membrane

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Introduction: The mammalian slit diaphragm is a site of highly active intercellular signalling between adjacent podocytes. Signalling pathways e.g. via the non-selective cation channel TRPC6 depend on a distinct lipid composition of the plasma membrane. Hyperactivity of TRPC6 either through gain of function mutations, increased total protein levels or membrane abundance of the channel protein has been associated with glomerular disease. The slit diaphragm shares close homology with the mechanosensory complex of C. elegans. In the touch-sensitive neurons of the nematode the lipid modifying enzyme MEC-6 interacts with the stretch-activated cation channel MEC-4/10 to regulate its activity. In previous studies we have identified the anti-oxidative paraoxonase protein PON2 as the homologue of MEC-6 and delineated type II transmembrane topology and localization to the slit diaphragm. The present study aims to elucidate PON2-TRPC6 interaction biochemically and functionally and to provide evidence for mechanistic considerations.

Results: Pull-down experiments reveal that the N-terminal portion of PON2 interacts with TRPC6. Knockdown of PON2 in mouse podocytes affects TRPC6 activity and recovery both after chemical and after stretch activation of TRPC6. Additionally, mass spectrometry and atomic force microscopy disclose that PON2-deficiency alters the composition and the physical properties of the plasma membrane. To study the interaction of TRPC6 and PON2 in vivo PON2 knock-out mice, which are phenotypically normal at baseline, were challenged with adriamycin. In the adriamycin-induced nephropathy model, where TRPC6-signaling is enhanced, PON2-deficient mice show more pronounced renal failure, markedly increased proteinuria and aggravated glomerulosclerosis as compared to their wildtype and heterozygous littermates.

Conclusions: We provide evidence that the paraoxonase protein PON2 interacts with TRPC6 in the plasma membrane and affects signalling via TRPC6 by regulating the lipid environment of the channel protein. PON2 and PON2-regulating molecules may become therapeutic targets in the future.

II8

Ultrastructural Analysis of Drosophila Nephrocytes – Insights in Podocyte Development and Disease

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Introduction: Drosophila nephrocytes are podocyte-like mesoderm-derived cells with filtration slit diaphragms and a complex network of labyrinthine channels in the cell periphery. They are located inside the fly body cavity performing haemolymph filtration, thereby taking up toxins and wastes into the channel system in a size- and charge-selective manner, followed by endocytosis, life-long storage and thus inactivation. Drosophila homologs of NPHS1 and NEPH1 are required for slit diaphragm formation and function. During differentiation, apico-basal polarity of epithelia is determined by localization of specialized proteins. In case of the podocytes, the sub-apical adherens junction migrates to the basal aspect. Upon formation of the foot processes, it co-localizes with the slit diaphragms, suggesting that these cell polarity determinants (CPD) play a role in podocyte differentiation.

Methods: Expression levels of homologs to mammalian genes related to podocyte function or disease as well as cell polarity are specifically decreased or elevated in Drosophila nephrocytes using the UAS/GAL4-System. Shortly before puparation, larval garland nephrocytes are obtained and embedded in epoxy resin, followed by ultra-thin sectioning and transmission electron microscopy. Afterwards, the extent and complexity of the channel network and is quantified.

Results: A null-mutation (Δ1) of the myosin regulating factor and CPD downstream target PATJ severely impairs the development of both filtration slits and channel networks. The overall number of diaphragms is reduced and the distinct transition between the channel layer and the intracellular area is lost. Analyses of podocyte-related genes showed strong differences in channel complexity and depth.

Conclusions: Given the numerous similarities with podocytes, nephrocytes are well-suited to serve as an efficient model for fundamental podocyte research. Their morphological complexity can be used as a sensitive screen for genes, relevant for podocyte development and function. As an epithelial cell, they can also be employed to study cell polarity dynamics.

II9

Loss of Myo1c Protects Podocytes from Injury

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Introduction: Glomerular diseases including FSGS and nephrotic syndrome that induce podocyte effacement are leading causes of kidney failure. Podocyte proteins Nephrin and Neph1 are components of the slit diaphragm and it is now well documented that organization of Nephrin and Neph1 at the podocyte intercellular junction is altered in various glomerular disorders, which may contribute towards the development of disease pathology. Our recent preliminary observations suggest that Myo1c is directly involved in the turnover of these proteins at the podocyte intercellular junction. To further understand the function of Myo1c in podocytes, we genetically deleted Myo1c in mice in a podocyte-specific fashion.

Methods: Live FRAP (fluorescence recovery after photobleaching) analysis of cultured podocytes was performed to study the role of Myo1c in turnover of slit diaphragm proteins. Myo1c flox mice (Myo1c f/f) were created by homologous recombination and these mice were crossed with mice expressing podocyte-specific Cre recombinase.

Results: Myo1c variants on a variety of genetic backgrounds were born with normal Mendelian frequency and when aged to at least 6 months showed no evidence of proteinuria or structural abnormality. However, podocyte-specific Myo1c null mice bred to an adriamycin-sensitive (C57BL/6N) background showed remarkable resistance to adriamycin-induced glomerular injury; they did not develop proteinuria as compared to the control mice.

Conclusions: Nephrin and Neph1 were maintained at the podocyte intercellular junction following injury, indicating that loss of Myo1c prevented removal of these proteins in response to injury. Several published studies indicate that Myo1c is involved in cellular trafficking ranging from compensatory endocytosis to recycling. Our results suggest that Myo1c dependent trafficking mechanisms are required for intracellular movement of Nephrin and Neph1 that occur in response to podocyte injury and that is necessary for podocyte effacement.

II10

GLEPP1 Controls the Composition of the GBM

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Introduction: Proteinuria is one symptom of inherited and acquired glomerular kidney disease and evolves out of the altered glomerular filtration barrier. GLEPP1 is a receptor tyrosine phosphatase present in the podocyte foot processes. Recently, mutations in the GLEPP1 gene were shown to be associated with MCD and FSGS in childhood. The precise function of GLEPP1 is not fully understood.

Methods: GLEPP1 deficient and WT mice (129P3/J) were examined at the age of 4, 6 and 10 months. Urinary albumin/creatinine ratio was analyzed. RNA was obtained from isolated glomeruli and qPCR was performed for collagen IV alpha1-5 as well as laminin alpha 1, 5 and beta 1, 2. Kidneys were fixed in 4% paraformaldeyd. PAS staining was performed. For immunogold EM ultrathin sections of rat kidneys were labeled by an indirect immunogold protocol with a custom made GLEPP1 antibody.

Results: GLEPP1–/– mice display a significantly higher urinary albumin/creatinine ratio compared with +/+ controls at 6 and 10 months. However, at 4 months there was no difference in proteinuria. Histologically, this correlated with focal thickening of the GBM in GLEPP1–/– mice, resembling GBM humps. At the sights of humps foot process effacement was observed. qPCR from glomeruli revealed significant upregulation of collagen IV alpha1 and 2 chain expression while alpha3 expression was significantly down regulated. qPCR also showed significant upregulation of laminin alpha1 and beta1 chains. Immunogold EM detected GLEPP1 expression mainly at the apical part of the foot processes but interestingly also – in a smaller amount – in proximity to the glomerular slit.

Conclusions: Glepp1 deficiency mediates the formation of GBM humps. These humps seem to be formed by type IV collagen alpha 1 and 2 and laminin alpha 1 and beta 1. It can be speculated that GLEPP1 switches the GBM composition by expression of collagen IV and laminin from a mature to an immature composition. This switch can only be seen in aging mice and results functionally in proteinuria. These findings stress the protective and beneficial role of GLEPP1 for podocytes.

II11

Dynamin Oligomerization Reverses Podocyte Foot Process Effacement

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Introduction: Proteinuria, a common pathogenic feature of chronic kidney diseases, has been recognized as the consequence of podocytes dysfunction due to the dysregulation of the actin cytoskeleton. The large GTPase dynamin plays an essential role in the maintenance of podocyte structure and function. Dynamin has the propensity for self-assembly into higher order structures such as rings to regulate actin polymerization. We initiated this study to evaluate whether targeting the dynamin oligomerization into rings by a novel small molecule Bis-T-23, can restore global organization of actin cytoskeleton in both mouse and zebrafish proteinuria models.

Methods: PKCε knockout mice were used as a model of progressive glomerulosclerosis. The anti-proteinuric effects of Bis-T-23 were tested in PKCε knockout mice and in zebrafish with morpholino-mediated gene knockdown of PKCε, CD2AP, INF2 and nephrin. Bis-T-23 was delivered by intraperitoneal injection in mice and by cardinal vein injection in zebrafish. The impaired GFB function in zebrafish was determined by measuring the fluorescence intensity of EGFP-tagged vitamin D binding protein (EGFP-DBP) in the retinal vessel plexus of the fish eye.

Results: Loss of PKCε in mice leads to spontaneous foot process effacement and proteinuria. A single daily dose of Bis-T-23 over the course of 8 days into PKCε knockout mice ameliorated proteinuria, protected from development of glomerulosclerosis, and reversed foot process effacement. Knockdown of PKCε, CD2AP, INF2 and nephrin caused a generalized edema and proteinuria in zebrafish. Administration of Bis-T-23 significantly reduced proteinuria and increased the life span in zebrafish with knockdown of PKCε, CD2AP or INF2, but not in nephrin knockdown zebrafish. Additionally, foot process effacement was reversed to normal structure upon Bis-T-23 addition.

Conclusions: The dynamin oligomerization cycle is a potentially druggable pathway in chronic kidney diseases through affecting foot processes formation independent of defective upstream signaling mechanisms.

II12

Thymosin β4 Plays a Critical Role in the Progression of Glomerular Disease

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Introduction: Podocyte shape is essential to maintain the structure and function of the glomerular filtration barrier, hence molecules which alter this process may contribute to the progression of kidney disease. Based on this premise, we hypothesised that the actin-sequestering protein, thymosin-ß4, which regulates actin assembly and the cell cytoskeleton, may have a critical role in the glomerular filtration barrier.

Methods: Initially, we assessed thymosin-ß4 expression in developing and adult healthy kidneys by qRT-PCR, in-situ hybridisation and immunohistochemistry. To determine the role of thymosin-ß4 in healthy glomeruli we examined renal function and glomerular morphology in wild-type and total thymosin-ß4 knockout (Tß4ko) mice. Glomerular disease was also induced in wild-type and Tß4ko mice by intravenous administration of nephrotoxic serum. In-vitro, thymosin-ß4 was downregulated by siRNA in differentiated immortalised mouse podocytes. Cell migration (scratch-wound assay) and podocyte process number and length were assessed.

Results: Thymosin-ß4 mRNA and protein were highly expressed in developing and adult mouse glomeruli, both in podocytes and endothelial cells. The kidneys of Tß4ko mice had normal renal function and glomerular morphology. Renal thymosin-ß4 expression was upregulated in mice following glomerular injury. Lack of thymosin-ß4 accelerated glomerular disease in mice administered nephrotoxic serum compared with wild-type littermates as demonstrated by significantly increased albuminuria, plasma creatinine, blood urea nitrogen and decreased creatinine clearance. Tß4ko mice also had increased glomerular histological damage, inflammation and fibrosis. In vitro, thymosin-ß4 downregulation by siRNA increased podocyte migration and average process length, however, the number of cell processes per podocyte was not altered.

Conclusions: Our results suggest that although thymosin-ß4 is not essential for glomerular function in healthy mice, it may be critical in glomerular disease. Modulation of thymosin-ß4 may be a novel treatment strategy for renal disease in the future.

II13

The Glomerular Filter as An Electrical Powerhouse: How Much Voltage Is Required to Prevent Proteinuria?

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Introduction: It is still incompletely understood how the glomerular filter functions and why it never clogs. Recently, the electrokinetic model for glomerular filtration has been proposed: Streaming potentials generate an electrical field across the filtering capillaries, which in turn repell the negatively charged plasma proteins from entering the glomerular filter.

From mathematical considerations, it was argued against this concept was that an electrical field of approx. 1600 Volts/m (as measured by micropuncture in Necturus in vivo) may not be sufficient to prevent albumin from passing the across the glomerular filter. Therefore, we have determined the electrophoretic mobility of albumin experimentally in isolated perfused kidney (IPK) of a mammlian (rat) kidney in this study.

Methods: IPK were fixed with glutharaldehyde to block tubular or other cellular artifacts while preserving the electrical characteristics of the glomerular filter. An electrical field was applied externally to IPK via electrodes. The electrokinetic model requires that already relatively weak electrical fields (3.5 Volts, i.e. approximately 700 Volts/m) interfere with the endogenous electrical field of the glomerular filter resulting in significant increases of the albumin sieving coefficient. In contrast, mathematical modeling of the traditional pore model predicts that 100x more voltage is needed. As control, no current or 3.5 Volts high frequency alternating current (AC, 41 kHz) were used.

Results: Albumin sieving coefficient increased significantly when applying 3.5 Volts DC but not when applying AC or no current. No heat artifacts occurred.

Conclusions: The results show that already relatively weak extracellular streaming potentials influence the passage of albumin across the glomerular filter supporting the electrokinetic model.

II14

Nephrin Activation Induces Endosomal Signaling Necessary for Coordinated Focal Adhesion and Lamellipodial Dynamics

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Introduction: Nephrin ligation induces Nephrin tyrosine phosphorylation-dependent endocytosis. Nephrin endocytosis might be a mechanism by which podocyte junctions are disassembled and by which Nephrin protein and signaling is degraded in disease states. Alternatively, receptor-mediated endocytic trafficking can have signal-propagating functions, wherein 'signaling endosome' complexes signal to distinct subcellular compartments. We hypothesized that Nephrin signals from a signaling endosome.

Methods: We evaluated dynamics of Nephrin endosomal trafficking, focal adhesion (FA) turnover, and lamellipodial activity in cultured podocytes following chimeric CD16-nephrin cytoplasmic domain (CD16NCD) ligation focusing on the necessity of Nephrin-induced endosomal signaling in these integrated processes.

Results: Ligation of CD16NCD results in synchronized cellular events that culminate in lamellipodial induction peaking at 20 min. By 1 min, CD16NCD forms fine clusters on the cell surface, becomes tyrosine phosphorylated and is associated with Fyn, FAK, p130Cas, and caveolin. Soon thereafter, CD16NCD is dephosphorylated and undergoes endocytosis, disappearing from the cell surface and co-localizing with EEA1. By 5 min, FA proteins (e.g., FAK, p130Cas, paxillin, vinculin) disassemble and are transiently found associated with CD16NCD positive early endosomes between 5–10 min. By 20 min, FA proteins reassemble into linear FA when lamellipodial activity is first observed. At 40 min, CD16NCD endocytosis results in only limited CD16NCD degradation by endosomal trafficking via the late endosomal pathway. All aspects of this process require initiation by nephrin tyr phosphorylation, since Y-F mutation of all 10 CD16NCD tyr residues blocks endocytosis, FA turnover, and lamellipodial activity. Results using a mouse protamine sulfate model suggest a similar process in vivo.

Conclusions: Mechanisms governing lamellipodial activity in culture may be similar to those employed in vivo during foot process effacement. We suggest that podocyte injury-induced Nephrin tyr phosphorylation initiates Nephrin endosomal signaling that in turn is necessary for FA turnover, actin remodeling and foot process spreading.

II15

The Role of Cell Polarity Regulator Par3/Bazooka in Nephrocyte Development

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Introduction: The nephrocyte is a highly specialized cell type in Drosophila melanogaster which is responsible for the filtration of the haemolymph, thus removing harmful substances by endocytosis and life-long storage inside the cell. Resembling the mammalian podocyte in structure and function, the nephrocyte forms a size- and charge-selective filtration slit diaphragm and develops a network of channels in the cell periphery. A set of homologous proteins involved in forming the characteristic structures of these cells predestine the fly model for basic kidney research.

In a previous study, the cell polarity regulator Par3 has been shown to interact with Nephrin/Neph1 to cluster these proteins at the slit diaphragm. We have shown that RNAi knock-down of the Drosophila homologue of Par3 (Bazooka) has a strong effect on the correct development of the larval nephrocyte, manifesting in

reduced numbers of filtration slit diaphragms and channel networks. In addition, first experiments indicate an impaired interaction of a phosphorylation-mutant Bazooka with the Nephrin-complex. We furthermore apply an in vivo system to quantify the filtration efficiency upon cell-specific downregulation of Bazooka and Bazooka-associated proteins. Thus Drosophila nephrocytes provide a cheap and quick model to investigate the molecular mechanisms which are crucial for Bazooka/Par3-dependent establishment of the filtration barrier.

Methods: In this functional assay nephrocyte functionality can be quantified using confocal microscopy. Transgenic fly larvae secrete GFP into their haemolymph, which is taken up by the nephrocytes. A simultaneous RNAi knock-down of a gene of interest results in altering the ability of the nephrocyte for GFP-endocytosis and storage.

Results: The level of cell fluorescence differs depending on the importance of the knocked-down gene for nephrocyte development and functionality. Impaired ultrastructure reflects a decrease of endocytosed GFP.

Conclusions: In regard to the variety of genes playing a role in kidney diseases, we aim to further analyze the significance of clinically relevant genes during development and maintenance of the function of podocytes in a quick and easy-to-handle model system.

II16

Reactive Lipids Modulate Podocyte Physiology and Prosurvival Signaling – The Role of Redox Sensitive RhoA

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Introduction: Early podocyte loss is characteristic to chronic kidney disease in obesity, metabolic syndrome and diabetes. The role of redox imbalance has been implicated in podocyte dysfunction and loss in chronic kidney diseases but it is not known how metabolic syndrome related accumulation of toxic end products affects podocyte physiology and insulin sensitivity. Lipid radicals are produced in a lipid peroxidation process. Here, we propose that these species may not only be in excess in human metabolic syndrome and insulin resistance but they may also regulate redox sensitive processes in an adaptive to maladaptive manner.

Methods: In cultured mouse podocytes, we have generated lipid peroxyl radicals in a well controlled model system. In vivo, our models of metabolic syndrome were uninephrectomized DBA/2J mice on high fat diet and obese SHHF rats.

Results: Reactive lipids influenced motility of the cells in a steady-state concentration dependent manner. Interestingly, these radicals augmented basal Akt phosphorylation and WT-1 expression of the podocytes. Since the major motility regulator, RhoA is redox sensitive due to its Cys residues, we further looked into this redox regulatory mechanism upon lipid radical exposure. Lipid radicals seemed to influence the amount of active/total RhoA as a sensitive balance mechanism. Together with this, podocytes rearranged their F-actin filaments upon treatment. In vivo, our models showed impaired Akt or p42/44 MAPK phosphorylation in their podocytes upon insulin stimuli. Both models had increased lipid radical formation in the kidney detected by EPR spectroscopy. When animals were treated with a specific carbon centered radical scavenger POBN for 14 days, podocyte insulin sensitivity has significantly improved.

Conclusions: These results suggest that reactive lipids may be sensed through RhoA and could affect podocyte physiology, cytoskeletal rearrangements and pAkt signaling in a tightly regulated fashion on a scale from prosurvival mechanism to pathology.

II17

SUMOylation is a Novel Mechanism That Determines Turnover and Localization of Nephrin at the Plasma Membrane

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Introduction: Podocyte effacement and organization of foot processes and slit diaphragms can be induced experimentally within minutes. Therefore, it seems likely that the slit diaphragm proteins underlie orchestrated recycling mechanisms under the control of posttranslational modifiers. A disordered endocytosis of nephrin could cause a misplaced localization and could therefore lead to destabilization of the slit diaphragm. SUMO (small ubiquitin-like modifier) is a ubiquitin-like protein with 20% identity to ubiquitin. In vertebrates the SUMO-family has at least four members: SUMO-1, -2, -3 and -4. Modification of SUMO could block ubiquitination of the same site thus lead to stabilization. The aim of our studies was to analyze the role of Sumo-modification in regulating localization and stability of nephrin.

Methods: We mapped potential sites by a SUMOylation prediction algorithm. By immunofluorescence and immunoprecipitation we analyzed if nephrin is a substrate of SUMO. Furthermore we designed by site-directed mutagenesis lysine mutants of the predicted sites. Treatment of mice with the SUMOylation inhibitor ginkgolic acid supported the mechanistic importance of nephrin SUMOylation in vivo.

Results: We can demonstrate in vitro and in vivo that nephrin is a substrate modified by SUMO-proteins thereby increasing its steady state level and its expression at the plasma membrane. A conversion of lysines on predicted SUMO-sites at the intracellular tail of murine and human nephrin leads to a decreased protein stability of nephrin, a decreased expression at the plasma membrane as well as decreased PI3K/AKT signaling. Furthermore treatment of podocytes with ginkgolic acid showed a reduced plasma membrane expression of nephrin after 10 hours. Treatment with ginkgolic acid led to proteinuria, reduced SUMOylation and diminished expression of nephrin in mice after 24 hours.

Conclusions: Since SUMOylation is a reversible process, our results suggest that SUMOylation participates in the orchestration of nephrin turnover at the slit diaphragm.

II18

mTORC2 Signaling Pathway Regulates TRPC6 in Podocytes

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Introduction: Transient receptor potential cation channel protein 6 (TRPC6) is a member of nonselective cation channels. Abnormal expression and gain of function of TRPC6 were involved in the pathogenesis of hereditary and nonhereditary forms of renal diseases. Although underlying molecular mechanisms of this remain poorly understood, recent research found that there were many cell signaling pathways involved in regulating the expression and function of TRPC6. We examined the effect of mTOR signaling pathway on TRPC6 in podocytes.

Methods: Mouse podocytes were exposed to rapamycin and ku0063794. By using real-time reverse transcription PCR and western blotting, TRPC6 and downstream targets of mTOR complexes were examined. Fluorescence calcium imaging was used to study the function of TRPC6 in podocytes.

Results: Rapamycin, an inhibitor of mTORC1 signaling pathway, with different time points and concentrations, have no effect on both mRNA and protein expression levels of TRPC6. Ku0063794, a dual inhibitor of mTORC1 and mTORC2, could down-regulate mRNA and protein of TRPC6 in podocytes. In addition, ku0063794, but not rapamycin suppressed TRPC6-dependent calcium influx in intracellular.

Conclusions: These findings indicate that mTORC1 signaling pathway has no effect on TRPC6, while mTORC2 signaling pathway regulates TRPC6 in podocytes.

II19

Synaptic Vesicle Protein 2B is Essential for the Maintenance of the Integrity of the Slit Diaphragm

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Introduction: We have previously reported synaptic vesicle protein 2B (SV2B) was expressed in podocyte, and the cultured podocyte of which SV2B was knock-downed with siRNA failed to arrange the proper localization of CD2AP (JASN 17, 2006). SV2B is expressed on a vesicle surface in neurons and plays a role in the vesicle trafficking. However, the trafficking system with a synaptic vesicle like vesicle in podocyte and the role of SV2B are not well understood.

Methods: To elucidate the function of SV2B in podocyte, we generated and analyzed SV2B knockout (KO) mice.

Results: In SV2B KO mice, an increase in the amount of proteinuria. In the electronmicroscopic analysis, the effacement of foot processes and the increased thickness of glomerular basement membrane were detected in SV2B KO mice. Immunohistochemical study showed that the alterations in the staining pattern of CD2AP, nephrin, NEPH1 and laminin were detected in KO mice. We observed that SV2B was colocalized with CD2AP and nephrin in the normal rat glomeruli by immunofluorescence and immunoprecipitate study. In the developmental study with the kidney section of E20.5 rat, the staining of SV2B was found on whole cell surface of presumptive podocyte of the early stage of the S-shaped glomeruli when nephrin expression was not detected yet. To identify the molecules related with SV2B in podocyte, the expression of other synaptic vesicle associated proteins was analyzed in kidney cortex of SV2B KO mice by real-time PCR. The mRNA expression and the staining of neurexin, a molecule expressed on the presynaptic membrane and podocyte, were clearly decreased in SV2B KO mice.

Conclusions: These results indicated that SV2B is involved in the intracellular trafficking for the slit diaphragm molecules probably via neurexin and that the trafficking system with a synaptic vesicle like vesicle is essential for the maintenance of the integrity of the silt diaphragm.

II20

Defining the Glomerular Matrisome

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Introduction: Efficient glomerular filtration requires a selectively permeable barrier composed of unique cellular and extracellular matrix (ECM) components. Specific ECM proteins are vital for barrier function, yet many more may contribute. We hypothesised that global analysis, coupled with structural investigation of glomerular ECM in health and disease, would identify novel components and build understanding about ECM regulation in the glomerulus.

Methods: We employed label-free mass spectrometry (MS) and transcriptomics for a global analysis of human and murine glomerular ECMs, in addition to ECMs derived from glomerular endothelial cells (GEnC) and podocytes. Furthermore, we interrogated ECM structure with transmission electron microscopy and serial block-face scanning electron microscopy, which enables 3D image reconstruction.

Results: In human glomeruli, we robustly identified a proteome of 144 ECM proteins and validated expression with colocalisation studies. Topological network analysis revealed a core of highly clustered and connected ECM proteins, which are likely

necessary for ECM assembly. We found that podocyte and GEnC ECMs were distinct, suggesting differential contributions of these cell types to glomerular ECM and interestingly coculture resulted in basement membrane (BM)-like ECM deposition between cells. Analysis of murine glomeruli from disease-susceptible FVB mice and disease-resistant B6 mice revealed structural ECM defects, most notable in 3D reconstructions, including thickened BMs with sub-podocyte expansions in FVB mice. Finally, whole glomerular microarray and proteomic analysis of glomerular ECM from these mice enabled the identification of ECM proteins and pathways that associated with reduced barrier function.

Discussion: We have identified a core set of ECM proteins that may be important for glomerular ECM assembly and are conserved from mouse to man. We also found distinct ECM signatures demonstrating the complex and dynamic nature of this extracellular niche. Changes in ECM composition and structure associated with barrier dysfunction and systems-level-analysis revealed potential regulatory pathways.

II21

The Integrity of the Glomerular Filtration Unit Is Dependent on the E3 Ubiquitin-Ligase HUWE1

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Introduction: As a terminally differentiated cell featuring an intricate cytoskeletal architecture, podocytes rely heavily on a tight cell cycle control and the regulation of cell differentiation and protein turn-over at the slit diaphragm. In a protein interaction screen, we recently identified the E3 ubiquitin ligase HUWE1, a known modulator of these signaling pathways, as an interactor of slit diaphragm proteins. In the present work, we examined the function of HUWE1 on the homeostasis of podocyte and its influence on the integrity and function of the glomerular filtration unit.

Methods: We generated mice with a podocyte-specific knockout (Huwe1-flox/pod.cre) and employed qualitative and quantitative immunofluorescence- and Coomassie-stainings, Western blot, ELISA, as well as biochemical assays of blood sera to analyze the renal phenotype.

Results: Huwe1-flox/pod.cre mice show a significantly reduced life expectancy and develop signs of progressive kidney damage at the age of 6–8 weeks. These include glomerular crescent formation, sclerosis, mesangiolysis, and glomerulo-parietal synechia and hint at primary damage of the filtration apparatus. The degree of these glomerular lesions varies greatly intra- and interindividually. Markers of podocyte differentiation are downregulated. Concurrent with the histological alterations, animals display a sharp decline in kidney function. To further elucidate the role of HUWE1 in podocytes, we looked for a differential regulation of apoptosis/proliferation. The proliferation marker Ki67 is strongly upregulated in HUWE1-deficient glomeruli suggesting an effect on cell cycle control. Moreover, the coexpression of HUWE1 and the slit diaphragm protein podocin causes a marked increase in ubiquitination of podocin. Thus, HUWE1 might regulate slit diaphragm turn-over by ubiquitin-dependent protein degradation.

Conclusion: We could show that the E3 ubiquitin ligase HUWE1 is involved in the maintenance of the integrity of the glomerular filtration unit possibly by controlling podocyte proliferation, differentiation, and slit diaphragm protein turn-over.

II22

Misexpression of Mouse Nephrin in the Drosophila Developing Eye Leads to Irregular Cell Sorting Resulting in a 'Rough Eye' Phenotype

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Introduction: Mutations in the murine NPHS1 gene which encodes the slit diaphragm protein Nephrin result in incomplete formation of podocyte cell processes and the development of proteinuria. Nephrin binds to the cell adhesion molecule Neph1 at the slit diaphragm and upon activation mediates signals to the actin cytoskeleton in a phosphotyrosine-dependent fashion. In the D. melanogaster developing eye Nephrin orthologs hibris and sns are expressed by ommatidial cells while Neph1 orthologs kirre and roughest are expressed by interommatidial cells and mediate cell sorting. This work aims at analyzing molecular mechanisms of Nephrin family signaling to the actin cytoskeleton.

Methods: An in vivo model was established which allows analyzing the signal transduction of murine Nephrin in the Drosophila developing eye. Wild-type as well as several mutant murine UAS Nephrin transgenes were manufactured and integrated into the genome of Drosophila by ÖC31-based transformation to assure equal expression strength.

Results: We expressed wild-type or one of several different mutant murine UAS Nephrin transgenes in cells of the developing eye using the GMR-Gal4 driver. Flies that express wild-type murine Nephrin in the developing eye show a characteristic rough eye phenotype which arises from defective cell sorting during the development of the compound eye. To analyze the functional role of tyrosine residues within the cytoplasmic domain of murine Nephrin, we generated a murine Nephrin mutant in which all tyrosine residues within the cytoplasmic domain were mutated to phenylalanine. Expression of this mutant Nephrin protein in the developing eye results in a weaker rough eye phenotype, compared to flies expressing the wild-type murine Nephrin.

Conclusions: SH2 domain protein-dependent Nephrin signaling as well as phosphotyrosine-independent Nephrin signaling appears to play a role in this in vivo model. The Drosophila model is a valid system to decipher Nephrin signaling.

II23

Contribution of the Functional Domains of Myosin 1e, A Component of the Slit Diaphragm Complex, to Its Localization and Functions

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Introduction: Myosin 1e (myo1e) is an actin-dependent molecular motor expressed in podocytes. Loss of myo1e activity in mice and humans leads to proteinuria. We have shown that myo1e is a component of the slit diaphragm complex that regulates junction assembly in podocytes. Genetic studies have identified mutations in the MYO1E gene associated with FSGS. This study was aimed at testing the role of the functional domains of myo1e in its localization and activity. We hypothesized that mutations in the motor domain of myo1e may have particularly dramatic effects on its localization and functions.

Methods: Motor domain mutations expected to disrupt or enhance myo1e interactions with actin, as well as deletions of tail domains, were introduced into the GFP-myo1e construct. The localization and protein-protein interactions of these constructs were analyzed using live cell imaging and protein binding assays.

Results: Myo1e construct containing a novel FSGS-associated motor domain mutation was severely mislocalized in podocytes. A motor domain mutant predicted to have strong actin binding properties showed enhanced localization to the leading edge and promoted formation of protrusions at the cell edge. In addition to the motor domain, we have identified a proline-rich region in the tail domain of myo1e as one of the key determinants of its intracellular localization and tested its role in myo1e-actin interactions.

Conclusion: This study highlights the key roles of the myo1e motor domain and proline-rich region in its intracellular localization and functions.

II24

Expression of Podocyte Associated Proteins Nephrin and CD2AP in Patients with Hypertensive Kidney Injury

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Introduction: We found that nephrin and CD2AP played important roles in the maintenance of glomerular structural integrity in mice, and this study focused on the expression changes of nephrin and CD2AP induced by hypertensive kidney injury in patients with proteinuria.

Methods: The involved patients were divided into two groups as follows. Collecting twenty hypertensive patients with urinary trace protein as a study group (except patients with ventricular dysfunction, diabetes, urinary tract infections, tumor, rheumatic diseases) and sixteen healthy persons as a control group. Hypertensive group: patients with hypertension and proteinuria who were diagnosed as hypertensive kidney injury via kidney biopsy. Control group: patients with kidney trauma but without hypertension or proteinuria. Using the immersion-fixation method, we fixed the kidney biopsy section taken from hypertensive group and the normal kidney tissues taken from control group via urologic surgical procedures. Then immunohistochemistry staining was performed with HE, DAB, immunofluorescence, and uranyl acetate, while observed by light microscopy, confocal laser scanning microscopy and immunoelectron microscopy.

Results: In the control group, the capillary loops were smooth and plump. Nephrin and CD2AP were observed staining along the glomerular capillary loops (GCLs) continually and evenly. However, in the hypertensive group the capillary loops became wizened, and the Bowman's space and luminal spaces of the proximal or distal tubules became more widely opened in the renal cortices. Additionally, nephrin and CD2AP decreased and stained intermittently. Through the immunoelectron microscopy, different degrees of foot processes effacement were observed in the hypertensive group. Nephrin and CD2AP decreased and stained weakly along the podocyte basal membrane, while in the control group, they distributed evenly in podocytes.

Conclusions: Hypertension induced dysregulation of podocyte cytoskeletal proteins, which may be an important cause that leads to the development of proteinuria and decline of renal function in hypertensive kidney injury patients.

II25

SIK1 Localizes with Nephrin in Glomerular Podocytes and Its Polymorphism Predicts Kidney Injury

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Introduction: Mutant α-adducin and endogenous ouabain (EO) levels exert a causal role in hypertension by affecting renal Na-K ATPase. In addition, mutant β-adducin is involved in glomerular damage through nephrin down-regulation. Recently, the salt-inducible kinase 1 (SIK1) has been shown to exert a permissive role on mutant α-adducin effects on renal Na-K ATPase activity involved in blood pressure (BP) regulation and a SIK1 rs3746951 polymorphism has been associated to changes in vascular Na-K ATPase activity and BP.

Methods: Here, we addressed the role of SIK1 on nephrin and glomerular functional modifications induced by mutant β-adducin and ouabain, by using congenic substrains of the Milan rats expressing either mutant α- or β-adducin, alone or in combination, ouabain hypertensive rats (OHR) and hypertensive patients.

Results: We showed that SIK1 co-localized and co-immunoprecipitated with nephrin from glomerular podocytes and associated with caveolar nephrin-signaling. In cultured podocytes, nephrin-gene silencing decreased SIK1 expression. In mutant β-adducin congenic rats and in OHR, the podocyte damage was associated with decreased nephrin and SIK1 expression. Conversely, when the effects of β-adducin on podocytes were blocked by the presence of mutant α-adducin, nephrin and SIK1 expressions were restored. Ouabain effects were also reproduced in cultured podocytes. In hypertensive patients, nephrinuria, but not albuminuria, was higher in carriers of mutant SIK1 rs3746951 than in wild type, implying a more direct effect of SIK1 on glomerular damage.

Conclusions: These results demonstrate that, through nephrin, SIK1 is involved in the glomerular effects of mutant adducin and ouabain and a direct effect of SIK1 is also likely to occur in humans.

II26

Interleukin-13-Induced Vav1-Rac1 Pathway Associated with B7-1 Activation and Podocyte Foot Process Effacement

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Introduction: In an established rat model of minimal change nephrotic syndrome (MCNS), we have shown that overexpression of IL-13 gene resulted in podocyte foot process (FP) effacement and up-regulation of B7-1 in the glomeruli. We have also demonstrated increased glomerular IL-4Rα and IL-13Rα2 gene expression, and increased expression of IL-4Rα in podocytes of nephrotic rats, suggesting that IL-13 may act on podocytes in the glomeruli. We hypothesized that IL-13 acts through activation of B7-1, causing podocyte FP effacement and proteinuria. This study aimed to delineate glomerular 'gene signature' related to our IL-13 rat model of MCNS, with subsequent validation in cultured podocytes in order to investigate the mechanism of IL-13-induced podocyte injury.

Methods: Glomerular RNA from six control and six IL-13-overexpressed rats with MCNS were analysed in Sentrix® BeadChip Array RatRef-12v1 platform. Vav1 protein expression on glomeruli and podocytes were validated using immunohistochemical staining on rat kidney as well as immunofluorescence staining and Western blotting on podocytes. Podocyte actin cytoskeleton was examined using phalloidin staining and RhoA/Rac1 activity was measured using ELISA. The role of vav1 in IL-13 induced podocyte injury was studied using vav1 knock-down podocytes.

Results: Glomerular transcriptional profile of IL-13-overexpressed rats showed characteristic features of podocyte injury, with more than 87% of genes related to podocytes significantly down-regulated. Gene expression of vav1 was highly up-regulated in the glomeruli of IL-13-overexpressed rats and MetaCore™ pathway analysis of the differentially expressed genes suggested a possible role of vav1 in podocyte cytoskeleton remodeling. Moreover, immunohistological staining demonstrated the presence of vav1 in glomeruli and podocytes. In-vitro IL-13 stimulation of human podocytes resulted in significant increased expression of IL-13Rα2, B7-1 and phosphorylated vav1. This was associated with actin cytoskeleton rearrangement and Rac1 activation, which was abrogated in vav1 knock-down podocytes.

Conclusions: IL-13 induced podocyte FP effacement was mediated through activation of B7-1-vav1-Rac1-induced actin cytoskeleton rearrangement.

II27

Comparative Phosphoproteomic Analysis of Mammalian Glomeruli Reveals Podocin C-Terminal Phosphorylation as Determinant of Slit Diaphragm Complex Architecture

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Introduction: Signaling in podocytes in health and disease largely depends on phosphorylation, but technologies to understand its signaling have not been available. Recently, we performed in-depth analysis of the murine glomerular phosphoproteome by tandem mass spectrometry and confidently identified more than 4000 phosphorylation sites (Rinschen et al. JASN 2014). Comparisons of phosphoproteomic evidence across species is a powerful mean to functionally prioritize phosphorylation sites (Beltrao et al. Cell 2012).

Methods: We performed an extensive phosphoproteomic analysis of glomerular fractions of bovine and rat kidneys and performed a bioinformatics comparison of the dataset.

Results: These dataset significantly expands the number of known phosphorylation sites in glomeruli. In the rat dataset, we discovered several phosphorylation sites with potentially high biological relevance, among these tyrosine phosphorylation of synaptopodin and neph-1 (Kirrel) at non-characterized sites. Comparison of the dataset partly confirms significant cross-species conservation of signaling events in mouse, rat and cow. Phosphorylation sites were found on disease-relevant proteins whose mutation is known to lead to proteinuria in human disease. We determine that across mammalian species, nephrin serine/threonine phosphorylation is predominantly found at an acidic cluster at the intracellular terminus of the protein, and podocin phosphorylation is predominantly found at the C-terminus of the protein. We studied a highly conserved podocin phosphorylation site, S382 (murine/rat sequence), which was found to be conserved and phosphorylated in rat, mouse and cow. A phosphoablating mutant of podocin, S382A was not associated with altered membrane abundance in cell culture systems, but had stronger nephrin interaction as compared with the phosphomimicking (S382D) mutant.

Conclusions: The results clearly demonstrate that our understanding of the architecture of the slit-diaphragm complex itself is far from being complete. Interaction between the respective proteins is regulated not only via tyrosin phosphorylation, but also via serine/threonine phosphorylation.

II28

ARP3 Controls Podocyte-ECM Interaction via Modulation of the Actomyosin Contractile Machinery

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Introduction: Podocyte foot processes (FP) are essentially involved in the formation of the glomerular filtration barrier and provide at the same time tight adherence of the podocyte to the underlying GBM. We've previously demonstrated that N-WASP mediated actin branching is involved in the stabilization and maintenance of podocyte FPs. However, it remained so far unaddressed, whether and how actin branching might influence podocyte-GBM interaction.

Methods: We generated a set of complementary conditional mouse models allowing for deletion of *N-Wasp* and *Arp3* at decisive developmental stages of podocytes. Furthermore, a novel primary podocyte culture system with proven genetic origin was employed to investigate cellular functions of either N-WASP or ARP3.

Results: Deletion of N-Wasp at different developmental stages demonstrated only minor alterations in podocyte FP morphology at first. Interestingly, also deletion of *Arp3* as an ultimate effector of N-WASP resulted in comparable phenotypes. Analysis of *Arp3* deficient primary podocytes showed a decreased spreading capacity, migrational speed and altered focal adhesion morphology. Employing traction force microscopy revealed that *Arp3* deficient podocytes exert higher force levels compared to control cells and display an activated actomyosin machinery. Inhibition of the actomyosin machinery reversed the inefficient protrusion formation of *Arp3* deficient cells in 3D culture conditions.

Conclusion: Our observations demonstrate that the mechanism of actin-branching is not essentially required for intial podocyte FP assembly, but rather for the stabilization of FPs under mechanical stress. Furthermore, we provide evidence for a novel link between ARP2/3 dependent actin networks, the actomyosin machinery and podocyte-GBM interactions. These findings further deepen our global understanding of actin dependent podocyte FP function and plasticity.

II29

Podocyte Protein Turnover Analysis by Combining High Throughput Podocyte Purification Protocols with SILAC Mouse Models

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Background: Analysis of podocyte cellular signaling cascades, protein degradation machineries or protein turnover rates *in vivo* have been hampered due to limited accessibility of podocytes.

Methods: Based on a transgenic fluorescent mouse model we have pioneered a high throughput podocyte purification method. For detailed analysis of protein stability and turn-over rates metabolic labeling of mice with stable isotope labeling by amino acids (SILAC) was used. Sub-saturating SILAC labeling was achieved by feeding mice for 1,2 or 3 weeks with a diet containing $^{13}C_6$-labeled lysine. Subsequently, high-resolution mass spectrometry was employed from freshly isolated podocytes to quantify protein dynamics. The ratio of externally supplied (for 1,2 or 3 weeks) versus endogenous amino acids to de novo protein synthesis allowed to determine the accurate turnover rates of various podocyte proteins including the SD protein machinery.

Results: We gathered robust data of about 1700 podocyte proteins. We were able to draw conclusions about heavily consumed podocyte proteins, stability of the slit diaphragm complex and metabolic circuits as well as lifetime of different podocyte intracellular organelles.

Conclusion: Our proteomics approach to analyze *in vivo* protein dynamics is able to fundamentally enhance our knowledge about podocytes cellular physiology.

Subtheme III: Genetics, Epigenetics & Regeneration

III1

Five Children with Fabry Disease and Significant Tissue Damage in Renal Biopsies Despite Normal Clinical Renal Parameters

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Introduction: In Fabry disease globotriaocylceramid (GL3) starts to accumulate in kidney cells in utero, and continues to accumulate throughout childhood and adulthood.

Methods: We here present a case series of a 14 year old girl and four boys (median age 12.5 years, range 11–16 years) with classical Fabry phenotype that were started on enzyme replacement therapy (ERT) due to severe acroparesthesia. All patients had normal GFR and normal albumin creatinine ratio (< 30 mg/g). Before start of ERT a kidney biopsy was performed. In all patients significant GL3-accumulation was found in several types of kidney cells with high amounts of GL3 in the podocytes. Despite a normal albumin creatinine ratio, podocyte effacement was present on the electron microscopy (EM) sections in all patients.

Results: The four male patients were treated with agalsidase alfa or beta every other week (EOW) for a median time of 4.5 years, range 2–7 years. All patients had good responses to ERT with less neuropathic pain and better quality of life; GFR and albuminuria remained normal during follow-up. The female patient was initially treated with pain killers and angiotensin II receptor antagonist, and was then started on ERT at 21 years because of increased albuminuria and increased pain. Two patients (12 and 16 years at baseline) had a repeat kidney biopsy after five years on ERT (agalsidase alfa 0.2 mg/kg/EOW), one also had a biopsy after 3 years of ERT. Glomerular endothelial and mesangial cells were cleared after three and five years, whereas no significant change of podocyte GL3-accumulation was found. Two male patients (11 and 13 years old) have been started on agalsidase beta 1.0 mg/kg/EOW, with repeat kidney biopsy planned after three years of ERT.

Conclusions: Podocyte effacement was found in five young classical Fabry patients with neuropathic pain without any clinical evidence of Fabry nephropathy. Kidney biopsies are valuable in the early diagnosis of nephropathy and in the evaluation of response to ERT.

III2

FAT1 Mutations Cause Combined Renal Glomerular and Tubular Disease with Neurologic Defects

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Introduction: Steroid-resistant nephrotic syndrome (SRNS) causes 15% of chronic kidney disease (CKD) in the first two decades of life with the main features of gross proteinuria, hypoalbuminemia, and edema. Nephronophthisis-related ciliopathies (NPHP-RC) cause 5% of CKD and are distinct from SRNS by absence of proteinuria.

Methods: To identify monogenic causes of NS or NPHP-RC we combined homozygosity mapping with whole exome sequencing followed by high-throughput screening. The function of FAT1 was studied in IMCD3 cells.

Results: We identify recessive mutations in FAT1 in 9 different families, causing a previously undescribed renal disease with the combined features of NS, NPHP-RC, neurodevelopmental delay (4/9) and hematuria (7/9). We demonstrate that knockdown of Fat1 in IMCD3 causes an decreased migratory phenotype and decreases active GTP-bound RAC1 and CDC42, but not RHOA, indicating that RAC1 and CDC42 are more relevant to the pathogenesis of the kidney disease caused by FAT1 mutations. In addition we also show that knockdown of Fat1 impairs lumen formation in IMCD3.

Conclusions: We here identify mutations in FAT1 as causing a novel syndrome with an overlapping phenotype of NPHP-RC and NS, and reveal that RHO GTPase signaling is a pathogenic mediator of this disease.

III3

Studying the Function of APOL1 in the Zebrafish Pronephros

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Introduction: APOL1 which encodes for a secreted high density lipoprotein (HDL) is expressed in a number of human tissues, including the kidney. Recently it was shown that genetic variants of APOL1 are associated with non-diabetic kidney diseases, kidney diseases attributed to hypertension and focal segmental glomerulosclerosis (FSGS) in African Americans. In human, APOL1 is expressed mainly in podocytes, in extraglomerular endothelial cells and tubules. Since APOL1 is not expressed in mice, the zebrafish is an ideal model to study the function of this protein.

Methods: To knockdown (KD) zApol1 specific morpholinos were injected into fertilized eggs. For immunhistochemistry zebrafish larvae were fixed and sections were cut. To determine whether the KD of zApol1 influences also the glomerular filtration barrier we injected two different fluorescence-labeled dextran molecules (10 and 500 kDa) into the vein of zebrafish larvae and measured the fluorescence intensity in vessels and in the pronephric tubules.

Results: We have found that the expression of zApol1 in zebrafish larvae is similar to the expression in human tissue. After the KD of zApol1 in zebrafish larvae by the use of morpholinos, the larvae developed severe pericardial edema accompanied by a lower number of glomerular capillaries. Furthermore, we have observed that the expression of nephrin was significantly affected after zApol1 KD. Moreover, we have found that a KD of zApol1 affects the glomerular filtration barrier.

Conclusion: Since APOL1 plays an essential role in the development of kidney diseases and APOL1 is not expressed in the mouse, we have used the zebrafish model to study the function of APOL1. We have found that the expression of zApol1 in podocytes is important for the proper formation of the glomerular tuft. Furthermore, we show that zApol1 KD significantly reduces the expression of nephrin accompanied by a compromised filtraton barrier measured in zebrafish larvae in vivo.

III4

Epigenetic Modifications in Renal Aging

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Introduction: Aging is a known driving force for chronic glomerular kidney disease and the kidney represents a prime target of age-associated organ damage, which is reflected by the clear association of renal functional decline with age. Due to its complexity and its postmitotic nature, the epithelial podocyte represents the most fragile component of the glomerular filtration barrier. A decrease in the number of glomerular podocytes is the best predictor for kidney aging and the progression of renal diseases. The variability of podocyte response to environmental factors and the increased disease susceptibility of postmitotic podocytes with age indicate that epigenetic modifications might be involved.

Methods: A detailed molecular and epigenetic analysis of podocytes requires the isolation of these cells from their complex three-dimensional glomerular association. We have generated a double labelled transgenic mouse model, where green fluorescent

podocytes are contrasted by red fluorescence of all other kidney cells. Using this mouse model, we have pioneered a cell sorter based purification of glomerular podocytes from kidney single cell suspensions allowing genome-wide methylations screens and expression arrays comparing glomeruli and podocytes of young (4 weeks), middle aged (12 months) and old (18 months) male mice.

Results: We generated a comprehensive molecular regulatory network analysis defining kidney aging. Our data indicate that kidney aging is associated with changes in gene expression profiles and modulations in DNA methylation (hyper- and hypomethylation). Comparing our results to a proteomics screen of isolated podocytes, we could identify several highly podocyte specific regulatory proteins that were methylated in aged samples and non-methylated in young controls in the respective promoter regions of the gene.

Conclusions: In summary, these data ascertain DNA hyper- and hypomethylation of CpG rich promoters important regulating elements in podocyte aging.

III5

WT1, A Key Regulator of Podocyte Identity

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Introduction: The Wilms tumor suppressor gene, WT1, is essential for kidney development, podocyte maturation and homeostasis. Alternative splicing of WT1 produces several isoforms that appear to serve distinct molecular functions: While WT1(-KTS) variants act as transcriptional regulators, the precise role of WT1(+KTS) variants remains to be identified. Mutations interfering with the production of this latter variant lead to Frasier syndrome, a condition characterized by XY sex reversal and focal segmental glomerulosclerosis. We have previously generated genetically modified mice carrying mutations identical to those found in Frasier patients. While heterozygous mutants develop glomerulosclerosis and thus represent a model for Frasier syndrome, mice homozygous for this mutation fail to develop mature podocytes indicating an absolute requirement for WT1+KTS in foot process formation.

Methods: Here we addressed the role of WT1 isoforms using a combination of microarray, ChIP-seq and functional analysis.

Results: In vivo ChIP-seq analysis determined the exact binding matrix of WT1 during kidney development and identified a large number of podocyte specific target genes. Exon-array profiling in Frasier mutant mice defined a set of podocyte specific genes that appear to be under control of WT1(+KTS). One of these genes, the membrane associated guanylate cyclase 2 (MAGI2) localizes to the cytoplasmic side of the slit diaphragm. Interestingly, inactivation of Magi2 in vivo causes severe defects in foot processes formation, a mislocalisation of Nphs1 and an almost complete lack of the filtration barrier. Thus, these malformations recapitulate the phenotype observed in WT1(+KTS)$^{-/-}$ mice.

Conclusions: In conclusion, our approach led to a better knowledge of the molecular events acting downstream of WT1 and identified MAGI2 as a key gene in podocyte maturation.

III6

Co-Inheritance of Functional Podocin Variants with Heterozygous Collagen IV Mutations Is a Potential Cause of Renal Failure

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Introduction: Recent studies have shown that a subset of thin basement membrane nephropathy (TBMN) patients develops proteinuria and focal segmental glomerulosclerosis due to the inheritance of heterozygous mutations in *COL4A3/COL4A4* genes. In patients over the age of 50 years, end-stage kidney disease (ESKD) is observed in ~20% of the carriers. Two different studies have shown that *NPHS2*-p.R229Q, a podocin variant, may act as a modifier of phenotype in TBMN patients, increasing the risk for proteinuria and chronic renal failure (CRF).

Methods and Results: In order to identify more candidate modifier variants in *NPHS2* gene, we screened 35 TBMN patients. The only non-synonymous variants found in these patients were the *NPHS2*-p.R229Q and *NPHS2*-p.E237Q. Further evaluation of these in the TBMN cohort illustrated that for *NPHS2*-p.R229Q or *NPHS2*-p.E237Q, eight carriers were patients of the 'Severe' category while none were found for the 'Mild' one ($p = 0.053$). The eight carriers belong in two distinct TBMN families segregating the same mutation, *COL4A3*-p.G1334E. Segregation analysis in all family members revealed that both *NPHS2* variants perfectly correlated with the severe *COL4A3* p.G1334E phenotypes. Moreover we performed immunoprecipitation (IP) and immunofluorescence (IF) experiments in order to investigate any possible effect of these variants on the normal function of podocin. Immunoprecipitation experiments showed a stronger binding of podocin to nephrin at the presence of 229Q or 237Q amino-acid residue, contradicting previous published results that showed the opposite effect regarding p.R229Q. Immunofluorescence experiments in undifferentiated podocytes after double transient transfection with *NPHS1 and NPHS2* cDNA in the presence of 229Q or 237Q, showed that when wild-type podocin was expressed with wild-type

nephrin, both proteins were properly localized to the plasma membrane. This was not observed for p.R229Q or p.E237Q, where both variants showed to interfere with the normal trafficking of both proteins, demonstrating a perinuclear staining.

Conclusions: Our findings indicate that certain variants of podocin may act as modifiers for TBMN, resulting in worse prognosis.

III7

APOL1 Disease Mechanisms: Explaining Recessive Inheritance with a Model Involving Gain of Toxic Function Prevented by the Non-Risk Allele

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Introduction: APOL1 G1 and G2 coding variants (compared to ancestral G0) are associated with kidney disease in African-Americans, with largely recessive inheritance. Recessive inheritance patterns are usually due to loss of function mutations, but the recent appearance of APOL1 in primate evolution and an apparently healthy man with two null alleles argues against a critical renal function. We sought to test the hypothesis that G1 and G2 variant proteins increase toxicity while the G0 protein rescues the phenotype.

Methods: Recombinant GST-APOL1 was expressed in insect cells. HeLa and HEK-293 cells were transiently transfected. Cytotoxicity was measured using SyTox Blue. Endoplasmic reticulum (ER) stress and autophagy were measured using standard markers.

Results: Overexpression of APOL1 G0, G1, and G2 revealed similar RNA expression but altered steady-state protein levels. In HeLa cells, G1 steady state levels were decreased and G2 steady state levels were increased with regard to G0, while in HEK-293 cells the reverse was true. Cycloheximide chase, protease degradation inhibition, and secretion experiments suggested distinct mechanisms. In vitro, recombinant APOL1 showed multimerization. In cells co-transfected with two APOL1 constructs, one His-tagged and the other GFP-tagged, immunoprecipitation demonstrated that APOL1 proteins forms monomers, homodimers and homotrimers. In HeLa cells, the risk variants increased ER stress but not autophagy. In HEK-293 cells both risk variants decreased autophagy and did not affect ER stress. Cytotoxicity was increased by both risk variants in both cell types. On-going experiments are addressing whether co-expression of the G0 protein reduces the greater toxicity seen with the G1 and G2 proteins.

Conclusions: The present work suggests that APOL1 risk variants may cause a gain of toxic function, with APOL1 G0 functioning in a dominant-negative fashion in mixed multimers to prevent or limit toxicity. This molecular mechanism would reconcile the evolutionary data with recessive inheritance.

III8

How Missense Mutations in the COL4A5 Gene Cause X-Linked Alport Syndrome and the Effect of Chemical Chaperones

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Introduction: Forty percent of mutations in X-linked Alport syndrome are caused by missense variants in the COL4A5 gene. These result in an abnormal collagen IV α5 chain that is retained intracellularly increasing ER stress. This study examined the effects of COL4A5 missense mutations on the cell, and the consequences of treatment with a chemical chaperone.

Methods: Cell lines were produced from skin fibroblasts from two male and four female subjects with X-linked Alport syndrome caused by missense mutations, and from 4 non-hematuric controls. Levels of intracellular collagen IV α3, α4 and α5 chains were measured using western blots, and the corresponding mRNA quantitated using RT-PCR (Applied Biosystems 7500). Growth curves were examined over five days. Levels of mRNA corresponding to ER stress (BiP, CHOP and ATF6), autophagy (ATG5, BECN1 and ATG7) and the pro- and antiapoptotic pathways (caspase 3, BAD and Bcl2) were also quantitated. ER size in electron micrographs, and mRNA corresponding to ER stress, autophagy and apoptosis markers were measured at baseline and after treatment with 10 mM 4-PBA.

Results: Cell growth rates were reduced in the affected male but not female cell lines. Affected male and female cell lines had larger ER than normals, and increased mRNA corresponding to ER stress, autophagy and apoptosis markers. Treatment with PBA reduced the size of the ER but also increased apoptosis markers.

Conclusions: Missense COL4A5 mutations have an adverse effect on cell growth and ER stress. Treatment with chemical chaperones reduces the amount of stored collagen IV α5 chain but increases the adverse cellular effects.

III9

ChIPseq of WT1 Coupled with mRNA Expression Analysis in Podocytes in Vivo Identifies the Focal Adhesion Pathway as a Novel WT1 Target

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Introduction: The Wilms' Tumor Suppressor protein Wt1 is an abundant transcription factor in podocytes whose mutations cause focal and segmental glomerulosclerosis (FSGS). Only a few target genes of Wt1 have been identified in podocytes. A comprehensive analysis of Wt1-directed transcription and its relevance to FSGS is lacking. Therefore, we characterized Wt1 binding sites in podocytes in vivo by ChIPseq and analyzed mRNA expression by RNAseq in healthy and diseased podocytes.

Methods: Chromatin immunoprecipitation for Wt1 followed by next generation sequencing was carried out on wildtype kidneys. For RNA expression analysis, GFP was conditionally expressed in podocytes. RNAseq was carried out on RNA obtained from FAC-sorted podocytes of mice injected with adriamycin and controls. Bioinformatic analysis was carried out according to ENCODE guidelines.

Results: ChIPseq identified more than 11.000 Wt1 bound sites, located at promoters and enhancers in the cis-regulatory regions (CSR) of 5.900 genes. Genes with Wt1 binding in their CSR were significantly more likely to be expressed in podocytes and included most genes responsible for hereditary podocyte disease. Gene ontology (GO) analysis of Wt1 bound regions identified significant enrichment of terms in key podocyte components such as the actin cytoskeleton, slit diaphragm, and cell adhesions, indicating that Wt1 has a major function in maintaining physiologic gene expression levels in podocytes. Coupling GO analysis of Wt1 ChIPseq data and of mRNAs differentially regulated between healthy and damaged podocytes, revealed significant enrichment of focal adhesion terms, suggesting that Wt1 transcriptionally regulates the focal adhesion pathway, a key pathway in FSGS. Indeed, several integrins and laminins were both, bound by Wt1 and differentially expressed between different podocyte conditions.

Conclusions: In summary, ChIPseq suggests WT1 as a master transcription factor in podocytes. Focal adhesions are revealed as novel WT1 targets by linking Wt1 ChIPseq results to RNA expression analysis.

III10

Assessment of the Functional Impact of New Steroid Resistant Nephrotic Syndrome (SRNS) Candidate Genes Using a Combined Approach of in Vitro Experiments and the Drosophila Model

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Introduction: Inherited forms of SRNS are genetically heterogeneous and more than 25 genes have been implicated in isolated or syndromic SRNS. However, the disease-causing mutation(s) remain(s) unknown in approximately half of familial cases and 87% of sporadic cases. We sought to identify new genes implicated in SRNS.

Methods: Exome sequencing was performed on 3 affected members of a consanguineous family, presenting SRNS, cardiomyopathy and neurologic impairment. After candidate gene knock-down (KD) in an immortalized human podocyte cell line, cellular processes (adhesion, migration, proliferation) were assessed in real-time using xCELLigence technology. Invalidation experiments, using the GAL4-UAS system, were performed in Drosophila nephrocytes to explore defects on cytoskeleton structure, expression of slit diaphragm proteins or the nephrocyte filtration and endocytic functions.

Results: We identified two homozygous missense variants, predicted to be deleterious, in two candidate genes, ADD3 and KAT2B, both expressed in podocytes. Segregation analysis in non-affected family members did not exclude any mutation. The first gene encodes adducinγ, an important regulator of both the spectrin membrane skeleton and actin cytoskeleton, and the second gene encodes the lysine acetyl transferase2B (KAT2B), which is involved in acetylation of histones and modulation of several transcription factors. Adducinγ KD in podocytes led to decreased adhesion and migration, whereas KAT2B KD led to decreased adhesion only. Additionally, KD of the ADD3 orthologue in garland nephrocytes disrupted the cytoskeleton architecture and decreased the expression of the NEPH1 orthologue kirre. Neither ADD3 nor KAT2B orthologue KD decreased filtration or endocytosis in larval nephrocytes.

Conclusions: Our results point to a major effect of ADD3 in podocyte biology. Further experiments will be necessary to confirm or exclude the role of KAT2B. Simultaneous invalidation of both candidate genes and transgenic fly lines carrying the human mutations will help to define the precise role of each mutation.

III11

A Homozygous Missense Mutation in the Ciliary Gene TTC21B Causes Familial FSGS

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Introduction: Several genes, mainly involved in podocyte cytoskeleton regulation, have been incriminated in familial forms of primary focal segmental glomerulosclerosis (FSGS). Nevertheless, in ~50% of familial cases, the causal gene mutation(s) remain(s) unknown.

Methods: Whole exome sequencing combined to homozygosity mapping was performed in consanguineous families with late-onset FSGS. Functional studies included immunostainings on human kidney biopsies and podocyte cell lines, gene knock-down and nocodazole treatment on cultured podocytes.

Results: We found a homozygous missense mutation (p.P209L) in the TTC21B gene in 7 families with FSGS. Mutations in this ciliary gene were previously reported to cause nephronophthisis, a chronic tubulo-interstitial nephropathy. Interestingly, analysis of biopsies of FSGS patients carrying the p.P209L mutation revealed tubular basement membrane thickening reminiscent, of that observed in nephronophthisis. Conversely, 3 families initially diagnosed with nephronophthisis bearing the p.P209L homozygous mutation also had FSGS lesions. Consistent with the rat podocytes, we showed that fetal and undifferentiated human podocytes display a primary cilium that disappears at mature and differentiated stages. TTC21B gene product IFT139, an intraflagellar transport-A component, mainly localized at the base of the primary cilium in developing podocytes from human fetal tissue and in undifferentiated cultured podocytes. In contrast, in non-ciliated adult podocytes and differentiated cultured cells, IFT139 relocalized along the extended microtubule network. We further showed that knock-down of IFT139 in podocytes led to primary cilia anomalies (shortening and retrograde transport defects), abnormal cell migration and cytoskeleton alterations which could be partially rescued by p.P209L over-expression, indicating its hypomorphic effect.

Conclusions: Our results unexpectedly demonstrate the involvement of a ciliary gene in a glomerular disorder and point to a critical function of IFT139 in podocytes. Altogether, these data suggest that this homozygous TTC21B p.P209L mutation leads to a novel hereditary kidney disorder associating a spectrum of glomerular and tubulo-interstitial damages.

III12

Genetic Variants in NPHS1, NPHS2 and INF2 in Patients with Primary Focal Segmental Glomerulosclerosis in Denmark

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Introduction: Variants in genes encoding several glomerular proteins have been identified to be causative in a subset of patients with focal segmental glomerulosclerosis (FSGS) and steroid resistant nephrotic syndrome (SRNS). We aimed at examining the incidence and spectrum of disease causing variants in NPHS1, NPHS2 and INF2 in patients with FSGS and/or SRNS in Denmark.

Methods: Genotyping of NPHS1, NPHS2 and INF2 was performed in DNA samples from 36 patients with biopsy proven sporadic FSGS. These patients were identified from the Danish FSGS registry. Three patients with SRNS were directly recruited from local Pediatric Clinics.

Results: The previously described variant in INF2 c.550G>A (p.Glu184Lys) was identified in heterozygous form in one patient. Another patient was shown to be hemizygous for the NPHS2 c.855_856del (p.Arg286Thrfs*17) variant in one allele and had a whole gene deletion of the other allele. Co-existence of variants in NPHS1 and NPHS2 was observed in 3 patients. Compound heterozygous variants of unknown significance in NPHS1 were observed in 9 patients.

Conclusions: The presence of gene variants could explain the disease phenotype in 13% (5/39) of patients. The type of gene variants did not differ significantly in Danish FSGS and/or SRNS patients compared to published reports. We report the first gross

deletion of the NPHS2 gene. There is a need for functional studies of glomerular proteins to reduce the uncertainty about genotype-phenotype correlations in NS genes. Comprehensive screening strategies including known NS genes can help obtain a high yield of molecular genetic diagnosis.

III13

Efficacy and Safety of the Renin-Angiotensin System Inhibitor in Chinese Children with Alport Syndrome

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Introduction: Alport syndrome (AS) is a hereditary renal disease, clinically characterised by persistent hematuria, proteinuria, progressive renal failure. It has been proven that the podocyte injury contributes a crital step in the pathogenesis of glomerular proteinuria in AS. Experimental studies and few clinical studies demonstrate that angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) reduce proteinuria and retard progression of AS. Most of the patients with AS in the reports came from European. The lack of multi-center, prospective, randomized controlled study limited the assessment of efficacy and safety of ACEI and ARB used for AS treatment. More clinical observation studies on the treatment of AS are necessary to provide data for further study.

Methods: Eighty three Chinese patients with AS under ACEI and ARB therapy were investigated. All the patients were followed up more than 6 months. Clinical data, 24 hour urinary protein and renal function were collected and updated via outpatient and telephone follow-up.

Results: Thirty nine patients were treated with ACEI, and 44 patients were treated with ACEI plus ARB. 95.2% (79/83) of the patients were <18 years old. The average age at onset of therapy was 9.43±5.76 ys (1.5 ys to 39.5 ys). Proteinuria decreased significantly in the1st and 2nd year follow-up and kept stable in six year follow-up after treatment. 11 patients were at very early stage of AS (proteinuria <0.3 g/d) at the beginning of the treatment and proteinuria decreased or kept stable in 82% of them after one year of therapy. There is no statistically difference of the proteinuria controlling effect for ACEI and ARB in patients with missense or truncating mutations and patients with or without extrarenal menifestations after one year of therapy.

Conlusion: Our findings indicate that long-term use of ACEI and ARB can efficiently decrease or stabilize proteinuria in AS and can be tolerated well, even in patients at very early stage of AS. And the antiproteinuria effect of ACEI and ARB in AS is not significantly associated with genotype and extrarenal manifestations.

III14

Monogenic Cause of Steroid-Resistant Nephrotic Syndrome Can Be Revealed in 28% of Cases Worldwide

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Introduction: In steroid-resistant nephrotic syndrome (SRNS) >26 single-gene causes are known. To determine the frequency of monogenic cause of SRNS we examined a large worldwide cohort with SRNS using a high-throughput PCR-based microfluidic approach for these 26 single-gene causes of SRNS.

Methods: We performed mutation analysis in 1,783 families (2,016 individuals) with SRNS for the 3 most frequent nephrotic syndrome genes (NPHS2, NPHS1, WT1). A subset of 1,036 families (1,152 individuals) was included in our high-throughput PCR-based mutation analysis to screen for 26 (21 recessive and 5 dominant genes) known genes mutated in SRNS.

Results: We identified the disease causing mutation in 506/1,783 families (28%) in 20/26 genes examined. 392/1,783 (22%) mutations were identified by Sanger sequencing for the 3 most frequent causative genes and 114/1,036 (11%) mutations were detected using the high-throughput PCR-based microfluidic approach. We found the causative mutations for the different genes in the following percentages: NPHS2 (10%), NPHS1 (7%) and WT1 (5%), PLCE1 (2%), LAMB2 (1%), SMARCAL1 (1%), INF2 (0.5%), COQ6 (0.5%) and TRPC6 (0.5%). In another 2.6% the causative mutation was detected for 11 additional nephrotic syndrome genes. The frequency of disease-causing mutations depended on age of onset and showed the following distribution: First 3 months of life (72%), 4 to 12 months (51%), first year combined (63%), children 1–6 years old (27%), children 7–12 years old (19%) and adolescents 13–18 years old (11%).

Conclusions: When studying the 26 genes known to be mutated in SRNS, our high-throughput mutation analysis detects the molecular cause in 28% of families with nephrotic syndrome from a worldwide cohort. For the management of SRNS, our study will strongly facilitate molecular genetic diagnostics by different regions and will allow identification of individuals in whom a treatment directed toward the cause is available.

III15

Whole Exome Sequencing Identifies Mutations of ARHGAP4 as a Novel Single-Gene Cause of Nephrotic Syndrome

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Introduction: Identification of single-gene causes of the steroid resistant nephrotic syndrome (SRNS) has furthered the understanding of its pathogenesis. However, additional genes and disease mechanisms remain unknown.

Methods: We performed whole exome sequencing (WES) combined with homozygosity mapping in 43 sib pairs with SRNS. To identify additional families we screened our worldwide cohort of ~800 individuals with severe SRNS by an array-based multiplex PCR (Fluidigm Access Array™) and next generation sequencing (NGS).

Results: By WES in two affected male sibs and their affected maternal grandfather, we detected a hemizygous missense mutation (p.A425V) in the gene ARHGAP4 (Rho GTPase activating protein 4), which resides on the X chromosome. After screening our worldwide cohort of 800 children with SRNS by multiplex PCR and NGS, we identified two additional families (affected males) with hemizygous missense mutations (p.G810A and p.G830D) in this gene. All mutations segregated with the affected status in their families and were absent from control individuals in the Exome Variant Server. Knockdown of ARHGAP4 in cultured human podocytes showed an increased migration phenotype, which was rescued by overexpressing wild type mouse Arhgap4, but not mutant Arhgap4, that is equivalent to the human p.A425V mutation.

Conclusions: We have identified mutations of ARHGAP4 as a new cause of NS, implicating another component of Rho GTPase signaling in the pathogenesis of NS.

III16

NPHS1 and NPHS2 Mutations in Brazilian Children with Nephrotic Syndrome

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Introduction: Inherited impairments of proteins in the glomerular filtration barrier (GFB) have been identified as important causes of Nephrotic Syndrome (NS). Two of these proteins are nephrin and podocin, encoded by NPHS1 and NPHS2, respectively. NPHS1 is the major gene involved in Congenital NS (CNS) while mutations in NPHS2 are a frequent cause of early onset autosomal recessive (AR) steroid resistant nephrotic syndrome (SRNS). The aim of this study was to evaluate the frequency of mutations in these two genes in a Brazilian cohort of 150 children, 3 with CNS and 147 with SRNS or steroid sensitive NS (SSNS), all sporadic cases.

Methods: 5'UTR and coding sequences of NPHS1 and NPHS2 genes were PCR and DNA sequenced.

Results: We identified compound heterozygous mutations in NPHS1 gene in two of the three CNS patients. The third patient presented two mutations in cis. After NPHS2 analysis we identified compound heterozygous mutations in four SRNS patients, corresponding to 2.7% (4/147), one of them had already received a living donor kidney transplant. Seven patients (4.8%), one responsive to the steroid treatment and 6 with frequent relapses, presented heterozygous missense variants, corresponding to a mild and late onset form of NS. In 4 patients, 2 with SRNS, one SSNS and one with frequent relapses, corresponding to 2.7% (4/147), we identified four novel heterozygous variants in the promoter region; they were absent in 278 control individuals. In silico analyses evaluated the possible deleterious role for novel variants.

Conclusions: This study, which analyzed a Brazilian cohort for the first time, emphasizes the relevance of the molecular characterization of NS in childhood to guide further treatment. Additionally, living donor transplantation might be considered since SRNS patients with homozygous or compound heterozygous mutations in NPHS2 have reduced risks for recurrence of focal segmental glomerular sclerosis after renal transplant compared with children without mutations.

III17

Podocyte Loss in Nephropathic Cystinosis Is Associated with Increased Cell Motility

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Introduction: Cystinosis is an autosomal recessive disorder caused by mutations in the CTNS gene that encodes a lysosomal cystine transporter and results in lysosomal cystine accumulation. Cystinosis causes proximal tubular dysfunction progressing towards end-stage renal failure. Glomerular damage is also present in cystinosis, with high molecular weight proteinuria and morphological changes of podocytes. We tested the hypothesis whether urinary podocyte loss described in other glomerular diseases is present in cystinosis. We next studied the mechanisms of urinary podocyte loss.

Methods: We analyzed mRNA isolated from urine samples from healthy children (n = 9) and cystinosis patients (n = 14). Conditionally immortalized podocyte cell lines were generated from living exfoliated cells present in urine. The stable cell lines were characterized for expression of specific markers (CD2AP, synaptopodin, nephrin, podocin) and used for studying cell motility. The results were confirmed using conditionally immortalized podocyte cell line with CTNS down-regulated by means of specific siRNA.

Results: We demonstrated pronounced podocyturia in cystinosis using qPCR test to quantify the amount of exfoliated podocytes in urine (normalized by creatinine for each sample, $p < 0.05$ for CD2AP and synaptopodin). Urine samples from cystinosis patients provided more viable podocytes suitable for further culturing in comparison with healthy donors. We established 11 conditionally immortalized podocyte cell lines that accumulated cystine (mean cysteine content was 15.6 nm/mg protein vs 0.47 in controls, $p < 0.05$). Cystinosis podocytes demonstrated increased motility in wound-healing assay associated with enhanced phosphorylation of Akt kinase (p-Ser473) at the leading edge of the migrating cell. Actin cytoskeleton was altered, including partial dissociation of alpha-actinin-4 from actin filaments. Cellular adhesion sites were perturbed in cystinosis cells, indicative of impaired ability to adhere to the substrate.

Conclusions: We demonstrate podocyte dysfunction in cystinosis that can be associated with progression of this disease and can be a target of therapeutic interventions.

III18

LMX1B is Essential for the Maintenance of Differentiated Podocytes in the Kidneys of the Adult Organism

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Introduction: Nail-patella syndrome (NPS) is a rare autosomal-dominant hereditary disorder. The prognosis of NPS patients is determined by chronic kidney failure. In 1998 the first mutations in LMX1B, a gene encoding the LIM-homeodomain transcription factor 1 beta, were reported to be responsible for NPS. Ultrastructural analyses of affected kidneys revealed pronounced alterations of the glomerular filtration barrier, in particular podocyte effacement. This prompted us to develop and characterize inducible podocyte-specific Lmx1b knock-out mice in order to identify the underlying pathogenetic events.

Methods: Putative Lmx1b target genes were identified by microarray analysis of isolated glomeruli from induced and non-induced Lmx1b knock-out mice. Candidate genes encode actin cytoskeleton-associated proteins and were validated by chromatin immunoprecipitation. Studies on actin dynamics and cell adhesion were carried out on primary podocytes gained from glomeruli isolated from inducible podocyte-specific Lmx1b knock-out mice by magnetic bead perfusion. Ultrastructural studies were performed with transmission electron microscopy.

Results: The knock-out of Lmx1b leads to an upregulation of genes encoding actin cytoskeleton-associated proteins. Biophysical studies on podocytes from Lmx1b knock-out animals support these data. Lmx1b knock-out podocytes exhibit significantly more F-actin and show reduced cytoskeletal turnover rates and F-actin regeneration dynamics. In addition, podocytes from Lmx1b knock-out animals exhibit an increased adhesiveness to different substrates. Knockdown studies in zebrafish confirm the connection between Lmx1b and actin modulating proteins. These findings are in line with the onset of marked proteinuria one week after the inactivation of Lmx1b in adult mice and drastic ultrastructural changes of podocytes.

Conclusions: Our report describes an essential role of LMX1B for the maintenance of an appropriately structured actin cytoskeleton in podocytes. The podocyte cytoskeleton represents an often affected target in glomerular diseases. Similar to hereditary forms of focal-segmental glomerulosclerosis caused by mutations in ACTN4 or INF2 the inactivation of Lmx1b leads to a stiffer actin cytoskeleton.

III19

Rostafuroxin Protects from Podocyte Injury and Proteinuria Induced by Adducin Genetic Variants and Ouabain

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Introduction: Glomerulopathies are important causes of morbidity and mortality. Selective therapies addressed to the underlying mechanisms are still lacking. Recently, our group identified two mechanisms, mutant β-adducin and ouabain, involved in glomerular podocytopathies and proteinuria through nephrin downregulation. Here, we proposed that rostafuroxin, a novel antihypertensive agent developed as a selective inhibitor of Src-SH2 interaction with mutant adducin and ouabain-activated Na-K ATPase, may protect podocytes from adducin and ouabain effects, thus representing a novel pharmacological approach for the therapy of podocytopathies and proteinuria caused by the above mentioned mechanisms.

Methods: To study rostafuroxin effect on podocyte protein changes and proteinuria, mice carrying mutant β-adducin and ouabain hypertensive rats, OHR, were orally treated with 100 μg/kg/day rostafuroxin. Primary podocytes from congenic rats carrying mutant α- (NA) or β-adducin (NB) from the Milan hypertensive strain, MHS, and normal rat podocytes incubated with 10^{-9} M ouabain, were cultured with 10^{-9} M rostafuroxin.

Results: We showed that mutant β-adducin and ouabain caused podocyte nephrin loss and proteinuria in these animal models. Such alterations were reproduced in primary podocyte cultures from NB rats and normal rat incubated with ouabain. Treatment of animals, or incubation of cultured podocytes with rostafuroxin, reverted mutant β-adducin and ouabain-induced effects on nephrin protein expression and proteinuria.

Conclusions: These findings indicate that rostafuroxin prevented podocyte lesions and proteinuria due to mutant adducin and ouabain in animal models. This suggests a potential therapeutic effect of rostafuroxin also in patients with glomerular disease progression associated with these two mechanisms.

III20

APOL1 Protein in Podocytes: A Role for Endogenous Synthesis and Cellular Uptake

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Introduction: Apolipoprotein L1 gene (APOL1) variants are strongly associated with a spectrum of progressive non-diabetic nephropathies in individuals with recent African ancestry. APOL1 protein synthesis, uptake, and localization in kidney cells were evaluated since mechanisms of kidney disease remain unknown.

Methods: APOL1 protein and mRNA localization were examined in kidney tissue from African Americans and European Americans and in human kidney-derived cell lines.

Results: Indirect immunofluorescence microscopy performed on non-diseased nephrectomy cryo-sections from African Americans and European Americans with normal kidney function revealed that APOL1 protein was markedly enriched in podocytes (co-localizing with synaptopodin and Wilm's tumor suppressor), and present in far lower abundance in renal tubule cells. Fluorescence in situ hybridization detected APOL1 mRNA in glomeruli (podocytes and endothelial cells) and tubules, consistent with endogenous synthesis in these cell types. Quantitative RT-PCR in renal-derived cell lines did not detect APOL1 mRNA in human mesangial cells; however, abundant levels of APOL1 mRNA were observed in proximal tubule cells (PTCs) and glomerular endothelial cells (GECs), with lower expression in podocytes. Corresponding levels of APOL1 protein were observed in these cell lines by Western blot analysis. To explain the discrepancy between marked abundance of APOL1 protein in kidney podocytes observed in cryo-section versus lesser abundance in podocyte cell lines, APOL1 cellular uptake was examined. APOL1 protein was readily taken up by human podocytes in vitro, but not efficiently by mesangial cells, GECs, or PTCs.

Conclusions: We hypothesize that the higher levels of APOL1 protein in human cryo-sectioned podocytes reflects both endogenous protein synthesis and APOL1 uptake from the circulation or glomerular filtrate.

III21

Combining Genetic Fate Mapping, Podocyte Isolation Protocols and FACS Analysis to Precisely Quantify Podocyte Turnover

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Introduction: Although recent studies have shown that a subset of parietal epithelial cells can serve as podocyte progenitors, the role of podocyte regeneration in aging and nephron loss remains unclear.

Methods: Here we combined genetic fate mapping with highly efficient podocyte isolation protocols to precisely quantify podocyte turnover.

Results: We demonstrate that parietal epithelial cells can give rise to fully differentiated visceral epithelial cells indistinguishable from resident podocytes, and found that limited podocyte renewal occurs during glomerular development as well as in a diphtheria toxin-model of acute podocyte ablation. In contrast, the compensatory programs in response to nephron loss mainly evoke podocyte hypertrophy, but not podocyte regeneration. Also under physiological conditions no turnover of podocytes could be detected in aging mice. In addition, Adriamycin nephropathy and nephrotoxic serum model of rapid progressive glomerulonephritis did not result in podocyte replacement from the parietal epithelial cell compartment.

Conclusions: In summary, quantitative investigation of podocyte regeneration *in vivo* provides novel insights into the mechanism and capacity of podocyte regeneration in mice. Our data reveal that podocyte generation is mainly confined to glomerular development and may occur after acute glomerular injury, but fails to regenerate podocytes in aging kidneys or in response to nephron loss, Adriamycin nephropathy and nephrotoxic serum nephritis.

Subtheme IV: Various Aspects of Podocyte Function & Translational Medicine

IV1

Isolation and Characterization of Podocyte-Derived Microparticles in Hypoxia

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Introduction: Microparticles (MPs) shed from cell membrane in various stimuli such as oxidative stress or hypoxia. Ischemic injury might result in multitude of cellular processes, including cell survival. Podocytes are highly differentiated glomerular cells with the actin cytoskeleton. We performed this study to investigate the isolation and characteristics of podocyte-derived MPs in acute hypoxia condition.

Methods: We isolated and characterized MP from podocyte. Podocytes were cultured in serum deprived RPMI for 24 hours and treated without or with hydrogen peroxide (H_2O_2, 500 uM) stimulation for 2 hrs to mimic hypoxia condition. MPs were isolated from supernatants by differential ultrafiltration. The presence of MP was confirmed by flow cytometric analysis.

Results: By FACS analysis, podocyte-derived MPs were mainly detected at a region below the forward scatter signal corresponding to 1 μm beads, which were used as internal size standards. Podocyte-derived MPs expressed nephrin, podocin, podocalyxin, which were also expressed on podocyte plasma membranes. FACS analysis also showed that the expressions of podocyte specific markers and annexin V were more in MPs from podocyte treated with H_2O_2 (control vs. H_2O_2: nephrin 11.3% vs. 67.6%, podocalyxin 15.8 % vs. 65.9%, podocin 12.8 vs. 30.8%, annexin V 9.4% vs. 55.8%).

Conclusions: The results might show that ischemic injury promotes more release of podocyte-derived microparticles.

IV2

Urine Podocyte mRNAs as Useful Biomarker in Human Glomerular Diseases

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Introduction: Proteinuria is widely used for noninvasive assessment of kidney diseases. However, proteinuria is a nonspecific marker of diverse forms of kidney injury, therefore, more glomerular disease specific biomarkers are required. Podocyte depletion is a major mechanism driving glomerulosclerosis, and that persis-

tent podocyte loss is the likely driver for most forms of glomerular disease progression. Podocyte cell lineage specific mRNAs can be recovered from urine pellets of model systems and man. We have previously reported that urine podocyte mRNAs could serve as useful glomerular disease biomarker in model system (Fukuda A et al. KI 2012). The purpose of this study was to test whether the urine podocyte mRNAs could be useful biomarker in human glomerular diseases.

Methods: From January 2008 to December 2013, early morning voided urine samples were obtained from 125 patients with histology-proven glomerular diseases (minimal change nephrotic syndrome (n = 11), crescentic glomerulonephritis (n = 16), membranous nephropathy (n = 22), IgA nephropathy (n = 67) and lupus nephritis (n = 9)). A total of 30 urine samples were collected from healthy volunteer who had no known kidney disease or hypertension. We examined urine podocyte mRNAs excretion and urine protein/creatinine ratio (U-PCR), also examined the relationships between urine podocyte mRNAs and U-PCR, or renal histological findings.

Results: Compared with controls, urine podocyte mRNAs significantly increased in patients with glomerular diseases. Compared with no proteinuria (U-PCR <0.3), urine podocyte mRNAs were significantly increased in with proteinuria (U-PCR ≥0.3), however, urine podocyte mRNAs did not reveal linear correlation with U-PCR in any glomerular diseases. Furthermore, urine podocyte mRNAs were significantly increased in patients with histological findings of acute extracapillary proliferative lesions (n = 47) compared with without these lesions (n = 78), however, proteinuria was not.

Conclusions: Urine podocyte mRNAs not only may indicate podocyte loss in potentially progressive glomerular diseases but also reflect acute extracapillary proliferative lesions.

IV3

Quantification of Messenger RNA Levels of Podocyte Associated Molecules in Patients with Active or Remission Lupus Nephritis

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Introduction: Podocyte loss plays an important role in the pathogenesis of lupus nephritis (LN). We hypothesize that quantification of messenger RNA of podocyte associated genes in urine sediment could be related with LN severity.

Methods: We studied 15 urine samples of patients with systemic lupus erythematosus (SLE): 5 SLE without LN, 5 remission LN (RLN) (1.8±0.58 g/day) and 5 active LN (0.43±0.21 g/day); and 5 healthy controls. Messenger RNA levels of podocalyxin, podocin, Wilms' tumor-1 (WT1) and synaptopodin in urinary sediment were quantified by real-time polymerase chain reaction and compared to other clinical parameters.

Results: The highest mRNA values of podocalyxin, podocin and synaptopodin were shown in patients with active LN (3.3-fold change, 2.6-fold change, and 1.5-fold change, respectively) compared to controls. Patients with remission LN showed the highest values of mRNA WT1 (2.6-fold change) compared to controls. Similar mRNA values were found between active or remission LN mRNA levels and between patients with SLE without LN and control group for all podocyte associated molecules. In addition, the highest significant correlations between mRNA molecules were obtained either in active or remission LN groups (<0.01 for all molecules). Urinary podocalyxin, podocin, synaptopodin and WT1 mRNA were correlated with proteinuria (r = 0.791, r = 0.794, r = 0.632 and r = 0.640, respectively) only in patients with remission LN, and with SLEDAI (r = 0.738, r = 0.740, r = 0.720 and r = 0.724, respectively) in active LN group.

Conclusions: Urinary podocalyxin, podocin and WT1 are higher in the urinary sediment and are correlated with lupus activity (proteinuria and SLEDAI) either in active or remission LN groups. Urinary mRNA levels of podocyte associated molecules could be a valuable method for studying LN and its evolution in these patients.

IV4

Podocytes Modulate the Inflammatory Environment Through NEMO-Dependent Signaling in Nephrotoxic Nephritis

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Introduction: Inflammatory diseases lead to acute and chronic glomerular injury and are a major cause of end stage renal disease. NF-kappaB signaling is among the most important regulators of pro-inflammatory signaling. However the role of podocytes in the modulation of inflammatory kidney diseases through NF-kappa B is only poorly understood. In this study we investigated the inflammatory role of podocytes by analyzing a podocyte-specific knockout of the NF-kappaB essential modulator NEMO.

Methods: We crossed a NEMO-flox-mouse with the Podocin:Cre mouse and analysed proteinuria, histological structure and EM ultrastructure of NEMO-flox/Podocin:Cre and wild-type mice after injection of a nephrotoxic antibody at several timepoints. We analysed the glomerular expression of chemokines by qPCR and quantified the amount of different immune cell populations by FACS. Using an mRNA-Array approach we aimed to define the pro-inflammatory transcriptome that underlies the phenotype.

Results: After induction of nephrotoxic nephritis (NTN) both NEMO-podocyte KO (PKO) and wild-type mice developed nephrotic-range proteinuria within 3 days after disease induction. At

day 7, podocyte NEMO-PKO mice recovered significantly faster and showed decreased levels of proteinuria compared to wild-type littermates. Morphological analysis as well as immunohistochemistry and electron-microscopy of the glomeruli revealed restoration of the slit diaphragm morphology in NEMO-PKO while wild-type showed significant foot-process effacement. We were able to show a significant reduction of proinflammatory chemokines in glomeruli. In a FACS analysis of infiltrating cells in kidneys we were able to show a significant reduction of CD8-positive T-lymphocytes at day 3. By using mRNA arrays of untreated and NTN-treated mice, we analysed the pro-inflammatory transcriptome to reveal potential therapeutic targets.

Conclusions: In this study we show that podocytes are able to influence the proinflammatory environment through NF-kappaB signaling by modulation of chemokine expression and immune cell infiltration. Inhibition of this intrinsic proinflammatory signaling is beneficial in nephrotoxic nephritis in mice.

IV5

Profiling of Urinary Proteases and Protease Inhibitors Associated with Extracellular Vesicles in Patients with Diabetic Nephropathy

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Introduction: Urinary extracellular vesicles (UEVs) have attracted increasing research attention as found to be a precious source of diagnostic and prognostic disease biomarkers. We have recently deviced a new and simple method to enrich vesicles from urine and used it to detect the variety of proteases and protease inhibitors associated with UEVs in patients with type 2 diabetic nephropathy.

Methods: Urine samples from 16 healthy volunteers were collected among the laboratory staff and 36 representative patient samples from the Finnish Diabetic Nephropathy (FinnDiane) Study Group and divided into three groups based on the level of albuminuria.

Isolated UEVs were screened on a nitrocellulose membrane blot array to detect simultaneously the relative changes of 34 different proteases and 32 protease inhibitors, respectively. Protease and protease inhibitor profiles and quantitation were established from the pixel average of fluorescent density changes of spots using an infrared Odyssey scanner and plotted with its image analysis software. Quantitations with more than 1.5-fold difference were considered.

Results: Arrays showed a progressive increase of cathepsin-C, -D, and -X/Z/P in the samples from patients with macroalbuminuria while no appreciable changes were observed in the array for kallikreins. Further, the array showed a moderate altered expression of metalloproteases with a progressive decrease of MMP-2 and bimodal trend for MMP-9 which increased in the normoalbuminuric cohort while a decrease in the micro- and macroalbuminuric groups were observed. No major variations were observed for the set of tissue inhibitors for metalloproteases (TIMPs). On the other hand, substantial variation was found for the cystatins.

Conclusions: This study shows for the first time characteristic alterations in protease and protease inhibitor profiles associated with UEVs in DN. These results suggest that the underlying mechanisms may reveal important mechanistic, prognostic and diagnostic features in advancing kidney damage.

IV6

Combined Treatment with Pioglitazone and Glucocorticoids Provides Enhanced Protection Against Nephrotic Syndrome

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Introduction: Oral glucocorticoids (GCs) are the primary therapy for nephrotic syndrome (NS), although GCs have serious side effects and are clinically ineffective in ~20% of patients. We previously reported that like GCs, PPARγ agonists also act directly on podocytes to protect against PAN-induced actin cytoskeletal disruption and cell death, and to modulate the GC receptor pathway. We thus hypothesized that the PPARγ agonist, Pioglitazone (Pio), could enhance the reduction in proteinuria seen with GCs during NS.

Methods: Proteinuria was induced in Wistar rats (N = 13/group) by single PAN injections (50 mg/kg), while control rats received saline. Treatment groups received PAN+low-dose GCs (5 mg/kg), PAN+high-dose GCs (15 mg/kg), PAN+Pio (10 mg/kg), PAN+Pio+low-dose GCs, and PAN+Pio+high-dose GCs. Analyses included proteinuria and glomerular gene expression 11 days after PAN injection. Translation to a human case of refractory NS included the addition of Pio to GCs and other immunosuppressive drugs at 15 mg/day for 4 weeks, followed by 30 mg/day for 12 weeks. Subsequent analyses included 1st AM proteinuria and drug dosing details.

Results: PAN induced severe proteinuria, which was significantly reduced by high-dose GCs (79%; P = 0.005) but not low-dose (25%; P = NS). Pio alone reduced proteinuria moderately (61%; P = NS), but low-dose GCs+Pio reduced proteinuria significantly (63%; P = 0.025), similar to high-dose GC alone. High-dose GCs+Pio reduced proteinuria to almost control levels (97%; P = 0.001). PAN increased glomerular cycloxygenase-2 and α-actinin-4 expression, and decreased podocyte synaptopodin expression, while Pio+GCs treatment restored levels to control values. Analogous treatment of refractory human NS with Pio correlated with a 60–75% decrease in proteinuria.

Discussion: Repurposing Pio as a combination therapy with GCs has the potential to notably increase the clinical efficacy of GCs in reducing proteinuria during NS, and potentially enable reduced GCs dosing and toxicity. This efficacy likely results in part from the restoration of podocyte/glomerular expression of synaptopodin, cycloxygenase-2 and α-actinin-4.

IV7

Detection of Glomerular CD80 (B7-1) mRNA by qRT-PCR and on Podocytes by Immunstains on Paraffin Embedded Biopsies with FSGS

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Introduction: CD80 (B7-1) on podocytes has been described as a promising target for therapy with abatacept in focal and segmental glomerulosclerosis (FSGS). In the original manuscript, CD80 positivity is described in all cases responding to abatacept therapy, suggesting CD80 positivity as predictive for therapy response. As a first step towards a predictive test for abatacept responsiveness we tried to establish quantitative RT-PCR from microdissected glomeruli and immunohistochemical stains for CD80 on paraffin tissue.

Methods: CD80 immunostains with two primary antibodies (abcam and R&D) were established on paraffin embedded lymphatic tissue. 13 paraffin embedded biopsies from 12 patients with primary FSGS and one biopsy with lupus glomerulonephritis class IV-G (SLE-GN) were subjected to qRT-PCR after preamplification and immunostained for CD80. PLA2R1 served as control for qRT-PCR.

Results: CD80 immunostains with both primary antibodies delivered a valid and strong signal on lymphatic tissue and on nodular lymphoid aggregates as internal controls on the examined biopsies with FSGS and lupus glomerulonephritis. CD80 immunostaining of podocytes was negative in all 13 FSGS biopsies and positive in the SLE-GN biopsy. Glomerular CD80 mRNA was detectable by qRT-PCR in 2/13 FSGS biopsies and undetectable in SLE-GN. Relative CD80 mRNA expression levels in the two FSGS cases was 0.005 and 0.010. Glomerular PLA2R1 mRNA was detectable by qRT-PCR in all biopsies, the median of the relative expression was 5.54 (minimum 0.346, maximum 12.80).

Discussions & **Conclusions:** Immunohistochemical detection of CD80 on podocytes is technically difficult on paraffin tissue, most likely due to very low mRNA expression. Further studies incorporating immunostains on frozen sections, qRT-PCR and improved staining of paraffin tissue with novel anti-CD80 antibodies are needed to establish podocyte CD80 positivity as a valid predictive marker for abatacept responsiveness in FSGS.

IV8

miRNA Expression Profiling of Microdissected Human Parietal Epithelial Cells by qRT-PCR Low Density Arrays

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Introduction: miRNAs are known to regulate mRNA levels and mRNA translation. Parietal epithelial cells (PECs) are key players in focal and segmental glomerulosclerosis (FSGS) and crescent formation. As a first step towards deeper analysis of miRNA function in PECs and as a proof of principle study, we determined their miRNA expression profile.

Methods: Human PECs were microdissected from 5 paraffin embedded biopsies with minimal tubulointerstitial nephritis. Patients were under no medication at the time of biopsy.PECs were microdissected, mRNA transcript levels (PAX2, WT1, CD44) were determined by qRT-PCR after preamplification. miRNA expression profiles were obtained by low density qRT-PCR arrays after preamplification.

Results: In the tissue compartments microdissected as PECs, relative expression levels of PAX2 (1.6±0.6), WT1 (2.9±1.5) and CD44 (0.9±0.4) mRNA suggested high purity of PECs. Out of 758 miRNA species 194 were detectable by qRT-PCR. The three most most abundant miRNAs were miR-1274B (99.0±89.0), miR-720 (44.9±38.4) and miR-30c (3.7±2.1).

Conclusions: It appears possible to obtain valid miRNA expression profiles from PECs obtained by microdissection from paraffin embedded biopsies. Our results have to be confirmed by single qRT-PCR and in situ hybridisation. Comparison with PECs from diseased kidneys will show shifts in miRNA expression that could explain PEC dysregulation in FSGS and crescent formation.

IV9

Vac14 – A Powerful Mediator of Membrane Identity Interacts with Rab7 GAP TBC1D15 and Rab9

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Introduction: It is assumed that in 50–80% of all glomerular diseases podocytes are directly or indirectly damaged. Apart from foot process effacement vacuolization is a most common feature of injured podocytes in vivo. However, the mechanisms leading to

vacuolization and its significance in the pathogenesis of glomerular disease are unknown. Vacuolization indicates defects in membrane sorting and membrane transport processes (membrane identity). Here, we investigate the role of Vac14, which regulates the interconversion of the phosphoinositide phosphate PI3P into PI(3,5)P2, thereby controlling endo-lysosomal or autophagy-associated intracellular trafficking.

Methods: We established a doxycycline-dependent expression system of Vac14 to generate stable cell lines (including podocytes) by retro- and lentiviral based transductions. To address the vacuolization phenotype in more detail we used live cell imaging in combination with different fluorescence-labeled constructs. By using a FLAG-based pulldown assay in combination with MudPIT, we could identify several interaction partners of Vac14, which were confirmed by Co-IPs or imaging afterwards.

Results: High levels of Vac14 resulted in vacuolization of cells and an accumulation of endo-lysosomal as well as autophagy markers. Endolysosomal markers were found on the limiting vacuolar membranes, while GFP-LC3 as a marker of autophagosomal membranes was not. Thus, the alterations affect predominantly endolysosomal maturation processes than autophagy-linked events. Hence, the accumulation of LC3-II could be an indirect consequence of dysfunctional endolysosomal maturation or could arise due to a delay in autophagic processes. Moreover, we identified TBC1D15 and Rab9 as Vac14 interacting proteins, which are powerful regulators of intracellular trafficking pathways.

Conclusions: Taken together, our findings give evidence that Vac14 is crucial for Rab7 dependent endolysosomal maturation processes and Rab9 dependent transport of lysosomal membranes to the trans-Golgi network. Hence, our data support a hypothesis that regulators of membrane identity might also be crucial factors in onset and progression of renal diseases.

IV10

Transcriptome Analysis of Glomerular Disease Using PodNet, a Protein-Protein Interaction Network of the Podocyte

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Introduction: Recently, we have built PodNet, a literature-based mouse network of protein-protein interactions (PPIs) in the podocyte (Warsow et al., Kidney Int. 2013). Mapping expression data of podocytes on PodNet, we gained valuable insight into podocyte differentiation in vivo and the loss of differentiation in culture. The aim of the present study was to analyze the glomerular transcriptome in various glomerular diseases using PodNet2, an updated and improved version of PodNet.

Methods: Recently published findings (2011–2013) were integrated into PodNet2. A mouse and a human version of PodNet2 were built and expanded to XPodNet2 by incorporation of PPIs from STRING database. The most differentially regulated interactions were identified with the Cytoscape plugin ExprEssence. In addition, we isolated podocytes from mouse kidneys by fluorescence sorting and determined the transcriptome using microarrays.

Results: After mapping glomerular transcriptomes of healthy and diseased kidney from the European Renal cDNA Bank on XPodNet2, the most differentially regulated interactions in FSGS, MCD, MN and DN were identified. For example, small networks around collagen I and lipoprotein lipase were found to be up- and downregulated, respectively, in FSGS. Weighted gene correlation network analysis demonstrated that several modules with correlated gene expression changed in glomerular disease. As an example, FSGS modules with correlated gene expression contained frequently the gene ontology terms 'cell cycle' or 'mitosis'. To assess the likelihood that a gene is expressed in podocytes and/or other glomerular cells, we developed a measure ('podocyticity index') from podocyte and non-podocyte transcriptomes. Surprisingly, podocyte specific essential interactions (e.g. those of the slit diaphragm) were hardly affected in glomerular disease.

Conclusions: PodNet2 is a valuable tool for the comprehensive analysis of mouse and human expression data. Our analysis of human glomerular transcriptomes provides novel insight into gene expression in glomerular disease.

IV11

Kidney Culture as a Screening Tool for Epigenetic Regulation of Kidney Development

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Introduction: Nephrons are the filtering units of the kidney. Their numbers in humans range between approximately 250,000 – 2 million with an average of 900,000 nephrons per kidney. Individuals with a low nephron endowment have a much higher risk of developing chronic kidney disease. Nephron number is determined during embryonic development and influenced, among others, by genes and nutrition. However, many factors determining nephron number are still unclear.

Methods: Here, we established an *in vitro* kidney culture system to analyze the role of epigenetic regulation on nephron induction.

Results: Culturing of embryonic kidneys on transwell filters is a well-established *in vitro* system and results in the induction and 2D-growth of the metanephric mesenchyme. Using transgenic Six2.Cre;Tomato/EGFP reporter mice and whole mount stainings, we established a kidney culture setup to screen epigenetic inhibitors for their potential to regulate ureteric bud branching, cap mesenchyme and nephron induction. We identified several inhibitors leading to severe, dose-dependent growth retardation of the embryonic kidneys with diverse developmental phenotypes.

Conclusions: In the future, further investigation of these epigenetic mechanisms will contribute to our knowledge on nephron induction during kidney development and might help identify risk factors leading to decreased nephron endowment.

IV12

Medical Informatics and Machine Learning Solutions for Personalized Renal Care

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Introduction: Chronic Kidney Disease (CKD) is recognized by the WHO as major non-communicable disease that affects more than ten percent of adults in industrialized nations. While the lifetime risk for CKD is over 50 percent, the lifetime risk for end stage renal disease (ESRD) ranges from two to seven percent depending on population. The longterm aim of this work is to develop and validate informatics tools that incorporate available clinical, genomic, and biomarker data to estimate precisely the risk for major CKD outcomes ESRD and cardiovascular disease (CVD) in each individual in order to focus personalized preventive renal care to patients likely to experience these major outcomes in the future.

Methods: We developed multiple technologies using data available in electronic health records (EHR): 1) automated case definition algorithms (CDA) to identify CKD cases, 2) Bayesian machine learning models to define individual CKD progression trajectories, and 3) predictive modeling to estimate likelihood of future rapid decline of kidney function.

Results: 1) An automated CDA identifies diabetic and/or hypertensive patients with CKD stage three or higher with 98% positive and 99% negative predictive value, respectively. 2) A validated alignment and clustering model accurately classifies patient records with non-progressor, progressor, and rapid progressor kidney function trajectories (slope) at different stages of kidney disease sampling four or more years of longitudinal estimated glomerular filtration rate (eGFR) lab results. 3) A weighted and segmented Ridge regression model uses all available patient data, including clinical measurements and lab results, diagnostic codes, and medication use to predict a 20% or more future decline of eGFR over a two year period with good precision and recall and C-statistic over 0.8.

Conclusions: These tools and methods can be bundled as software applications to enable accurate early identification and targeted preventive intervention for advanced clinical trials and precision renal care.

IV13

Anti-PLA2R Antibody Could Not Differentiate Idiopathic and HBV-Associated Membranous Nephropathy in Chinese Patients

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Introduction: Membranous nephropathy (MN) is a well-known podocyte disease, which affected more patients in developing countries recently. Serum anti-phospholipase A2 receptor (PLA2R) autoantibody plays an important role in idiopathic MN. The aim of this study was to investigate the prevalence of anti-PLA2R autoantibody, and analyze their associations with clinical and pathological features and renal outcomes of Chinese idiopathic MN and hepatitis B virus (HBV) associated MN patients in a large cohort.

Methods: Clinical and renal histopathological data of 226 patients with biopsy-proven MN were retrospectively analyzed, including 23 patients with HBV-associated MN. Serum anti-PLA2R antibody at the time of biopsy was detected by indirect immunofluorescence (EUROIMMUN, Germany).

Results: In the 226 patients with MN, 203 patients were diagnosed as idiopathic MN (89.8%), and 23 with HBV-associated MN (10.2%). The average age was 49.7±15.5 years (14 to 78) at presentation. 127 (56.2%) patients were male and 99 (43.8%) were female, with a male to female ratio of 1.28:1. The ratio of anti-PLA2R antibody positive in idiopathic MN group was 66.5% (135/203), which was 69.6% (16/23) in HBV-associated MN group, and there was no significant difference between the two groups (P = 0.768). Patients with anti-PLA2R antibodies presented with significantly lower level of serum albumin than those without it (27.0 vs. 29.2 mg/dl, P = 0.014). During the follow-up time of average 39 (7.5~115.6) months, 85 (37.6%) patients achieved complete remission, 89 (39.4%) with partial remission and 30 (13.3%) with treatment failure. Four patients were dead and 3 were with ESRD, all of them were anti-PLA2R antibody positive. Positive of anti-PLA2R antibody was a risk factor for treatment failure (P = 0.017).

Conclusions: PLA2R antibody could be detected in both idiopathic and HBV-associated MN. The presentation of PLA2R at initiation may associate with poor prognosis and indicate treatment failure.

IV14

Detection of Activated Parietal Epithelial Cells on the Glomerular Tuft Distinguishes Early FSGS from Minimal Change Disease

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Introduction: Parietal epithelial cells (PECs) migrating onto the glomerular tuft participate in the formation of FSGS lesions. We investigated whether immuno-histological detection of PECs in the initial biopsies of patients with first manifestation of idiopathic nephrotic syndrome can improve the sensitivity to detect sclerotic lesions compared to standard methods.

Methods: Ninetyfive out of 168 renal biopsies of adult patients from 5 different pathology centers containing 8 or more glomeruli were stained for claudin-1 (PEC marker), CD44 (activated PECs) and LKIV69 (PEC matrix). Of these, 38 had been diagnosed by a pathologist as 'early' primary FSGS and 57 patients as minimal change disease (MCD).

Results: Immuno-hiostological detection of PEC markers on the glomerular tuft resulted in a diagnosis of FSGS, or else of MCD. PECs were detected on the tuft in 87% of the biopsies of patients diagnosed as primary FSGS. PECs were detected in FSGS lesions from the earliest stages of disease. In MCD, no PEC activation was observed by immunohistology. However, in 25% of biopsies originally diagnosed as MCD the presence of small FSGS lesions was detected, by the detection of PEC markers on the glomerular tuft. Staining for the PEC matrix marker LKIV69 detected lesions with the highest sensitivity. Two novel PEC markers AKAP12 and ANXA3 exhibited similar sensitivity.

Conclusions: Detection of PECs on the glomerular tuft by immunostaining improves the differentiation between MCD and primary FSGS and may serve to guide clinical decision-making.

IV15

Effect of Lupus Nephritis Severity on Urinary Protein Levels of Podocyte Associated Molecules

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Introduction: Decrease in podocyte number is a critical determinant contributing to the development of lupus nephritis (LN). The aim of the study is analyze the effect of LN degree on urinary sediment podocyte associated proteins.

Methods: We studied 15 urine samples of patients with systemic lupus erythematosus (SLE): 5 active LN (0.43±0.21 g/day), 5 remission LN (RLN) (1.8±0.58 g/day) and 5 SLE without LN; and 5 healthy controls. The protein quantity of podocalyxin, podocin, Wilms' tumor-1 (WT1) and synaptopodin in urinary sediment were measured by western-blot and compared to clinical variables. Furthermore, cells obtained from the urine were grown under cell culture conditions.

Results: The highest values of podocalyxin, podocin, WT1 and synaptopodin were shown in patients with active LN compared to controls (<0.05 for all comparisons) and patients with SLE without LN (<0.05, except to synaptopodin). However, no significant differences were found between active and remission LN. In addition, significant correlations between proteins were obtained in SLE and controls (<0.05 for all molecules), but there was no significant correlation in LN groups. Urinary podocalyxin was significantly correlated with proteinuria ($r = 0.603$, $p = 0.048$); synaptopodin and WT1 were significantly correlated with SLEDAI ($r = 0.706$, $p = 0.020$, and $r = 0.616$, $p = 0.008$, respectively). On the other hand, we show that cells from urine of patients with RLN and SLE that grew ex vivo stained strongly positive for podocyte specific markers (podocalyxin, podocin and synaptopodin).

Conclusions: Protein levels of podocyte associated molecules are higher in urinary sediment of patients either with active or remission LN, indicating podocyte damage in both situations. Urinary podocalyxin, synaptopodin and WT1 are correlated with clinical parameters such as proteinuria and SLEDAI even in remission LN. Finally, podocytes that are detached from patients with RLN and SLE into the urine space grew, indicating they are viable, but not from active LN samples.

IV16

Podocytes Derived from Undifferentiated Kidney Cells Isolated from Amniotic Fluid and Urine

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Introduction: Podocytes are terminally differentiated and highly specialized epithelial cells. Loss of podocytes is the main cause of many glomerular diseases. Subpopulations of cells found in amniotic fluid (AF) and urine have been shown to express progenitor cell features and may have the potential to differentiate into several cell lineages. We aimed to obtain podocytes derived from kidney stem/progenitor cells (KSPC) isolated from (AF), principally composed of fetal urine, and freshly voided urine from neonates and adult donors.

Methods: Fetal progenitor cells (fPC) were isolated from AF of the second trimester of pregnancy and urine progenitor cells were isolated from neonates (nPC) at day 1 and healthy adult donors (29±7 y.o). Clonal cell lines were characterized as KSPC and KSPC-derived podocytes by gene expression analyses using quantitative PCR and protein expression by flow cytometry and immunofluorescence. Podocytes differentiation was induced by incubation of progenitor cells in culture medium supplemented with retinoic acid and vitamin D.

Results: KSPCs expressed the mesenchymal stem cell markers CD24, CD90, CD105, CD73, CD166, CD13 and were negative for hematopoietic markers CD34, CD45 and CD14. While fPCs and nPCs were positive for the fetal renal markers SIX2, PAX2 and CITED1, aPCs expressed the adult renal epithelial markers CD133 and CD24. KSPC-derived podocytes presented MTE transition and many of the cells became bi- or multi-nucleated with arborized cytoplasm comparable to conditionally immortalized podocytes. Cells presented up-regulation of podocyte-specific genes such as Lmx1b, podocalyxin, synaptopodin, CD2AP and nephrin.

Conclusions: Amniotic fluid contains cell subpopulations committed to renal cell fates. Stem/progenitor cells can be isolated from neonatal and adult urine and are committed to the renal lineage. These cells presenting renal phenotype can be differentiated into podocytes and may provide a novel and non-invasive source of cells for regenerative medicine aiming kidney repair.

IV17

A Recipe for an 'All Purpose' Perfusion Fixation of Rodent Kidneys for Morphology and Cytochemistry

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Introduction: Current advances in rodent transgenic technology have increased the need for high resolution morphology and histochemistry protocols including suitable fixation recipes. Adequate preservation of the kidney requires particular care owing to the complexity of its parenchyma. Immersion fixation results in poor morphology and inadequate cell biological analysis. Conventional in vivo vascular perfusion protocols may require separate approaches for ultrastructural and histochemical investigation, resulting in large animal group sizes and resulting conflicts with the '3R' directive for animal welfare. We aimed to establish a simple fixation protocol which permits global structural evaluation at high quality standards within a single experimental animal.

Methods: Retrograde perfusion of the abdominal aorta was done under anesthesia using flushing buffer and aldehyde fixative. Parameters varied were the perfusion pressure, composition of the carrier and fixative solutions, post-fixation protocols, and sample processing techniques including cryopreparation. Paraformaldehyde (PFA) fixatives were compared to standard glutaraldehyde fixation. Protocols were optimized for podocyte structural preservation and cytochemical evaluation.

Results: The best quality was obtained using perfusion at 180 cm H_2O, modified phosphate buffer as carrier solution, 4% PFA as fixative, followed by various post-fixation treatments adapted for cryo- and paraffin sectioning, conventional EM, freeze substitution or Tokuyasu technique, and immuno-EM. Variations in carrier osmolalities were adapted to the conditions required for the individual parenchymal zones with particular focus on podocyte preservation. State-of-the-art confocal LM and high-end EM techniques including tomography and STEM led to excellent results.

Conclusions: A universal fixation protocol for rat or mouse kidney tissues has been adapted for a wide spectrum of structural and cytochemical techniques within a single experimental animal. Our methodological approach is easy to perform and sets high standards for adequate, state-of-the-art glomerular, tubular and interstitial evaluation techniques.

IV18

Loss of AATF in Murine Developing Podocytes Leads to FSGS – Diverging Need of the DNA Damage Response in Postmitotic vs. Proliferating Cells

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Introduction: Genomic integrity is continously being challenged by DNA damage of endogenous or exogenous sources. AATF is a key regulator of the tumor suppressor p53 in the DNA-damage response signaling cascade. AATF inhibits the ability of p53 to transactivate pro-apoptotic target genes. The conventional knockout of AATF in mice is pre-implanational lethal. The contribution of DNA-damage signaling in postmitotic cells, such as podocytes, has not been studied in detail. Here we show that deletion of AATF in podocytes leads to FSGS in adult animals contrasting the demand of AATF in postmitotic and proliferative cells.

Methods: Animal handling, coomassie blue and western blot SDS-PAGE, electron microscopy, IHC, IF, PCR.

Results: AATF was knocked out specifically in developing podocytes, postmitotic cells of the renal filtration barrier. The mice show progressive proteinura, weight loss and ESRD at the average age of 8–12 weeks. Histologically and ultrastructurally, the kidneys demonstrate the classical signs of FSGS. Furthermore, positive cleaved caspase 3 and TUNEL-stainings point towards a programmed cell death in podocytes, presumably due to their inability to inhibit p53-driven apoptosis in the absence of AATF. In contrast, AATF knockout in developing keratinocytes, a highly proliferating cell type, leads to a severe developmental phenotype with early postnatal death due to thin skin with a strong barrier defect. Keratinocytes are lost by a massive wave of programmed cell death just when the embryonic skin of both wildtype and knockout animals show highest levels of endogenous DNA-damage. The p53-driven initiation of apoptosis in response to DNA-damage needs to be strongly antagonized by AATF for proper epidermal development.

Conclusions: Podocytes tolerate loss of AATF significantly longer presumably due to their low level of DNA-damage that is still being high enough to rely on AATF to antagonize the activity of p53 to initiate apoptosis.

IV19

Deletion of Ebf1 Abrogates Cox-2 Expression Within Podocytes

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Introduction: We have recently demonstrated that the transcription factor Early B Cell Factor 1 (Ebf1) is spatially and temporally regulated during postnatal renal development, and its deletion has significant consequences including a thinned cortex and accumulation of immature peripheral glomeruli. Functional defects in podocytes arise at postnatal day (P14) coincident with the expression of Ebf1 in podocytes. The transcriptional targets of Ebf1 in these cells are, however, unknown.

Methods: RNA-Seq was performed on the Illumina HiSeq 2000 system using isolated podocytes from either WT or Ebf1$^{-/-}$ mice. Three separate ages were analyzed: P2 for cells that do not yet express Ebf1, P14 to identify early changes once Ebf1 is upregulated, and P21 for fully mature cells that include secondary damage-induced changes. The sequence reads were analyzed with the TopHat suite of analysis software.

Results: P2 cells from either genotype were quite similar. The same was true of the WT P14 and P21 samples. The Ebf1-deficient P14 and P21 samples were however, very different from either of the other two groups. Ebf1-deficient cells were found to have significantly reduced expression of the inducible cyclooxygenase Ptgs2 (Cox-2). Basal expression of Cox-2 was reduced by 5 fold at P14, and remained suppressed even once secondary damage was observable at P21.

Conclusions: There is a strong similarity between the renal phenotypes of Ebf1-deletion and inhibition of Cox-2 expression/function. While all of these changes are not intrinsic to a function of Cox-2 in the podocyte, Cox-2 is suppressed at the whole kidney level as well. Additionally, there is a conspicuous absence of fibrotic accumulation in the damaged Ebf1-deficinent podocytes, a process attributable to pathogenic Cox-2 induction. Taken together these data suggest that regulation of Cox-2 by Ebf1 is a major function of this transcription factor in kidney.

IV20

Live Intracellular Calcium Dynamics During Podocyte Development and Injury in Zebrafish

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Introduction: Nephrotic syndrome genes, including TrpC6 and PLCe1, affect podocyte calcium signaling. However, the role of calcium signaling during podocyte development remains unknown. We aim to understand podocyte calcium signaling during development using live imaging of zebrafish embryos.

Methods: Transgenic zebrafish Tg(podocin:Gal4) x Tg(uas:GCaMP3) expressing the calcium biosensor GCaMP3 under the podocin promoter were generated and imaged using live confocal microscopy. Larvae were treated with drugs to determine the source of calcium. Cellular events during glomerulus formation were captured by imaging Tg(wt1b:GFP) x Tg(flk:mCherry) and Tg(podocin:Gal4) x Tg(uas:YFP) embryos. The role of calcium signaling in podocyte differentiation, was assayed in zebrafish plce1 morphants.

Results: Immature podocytes are dynamic and interact with the dorsal aorta to form glomerular capillaries. By 4 dpf podocytes stabilize and the filtration barrier is mature. We observed spontaneous intracellular calcium transients in podocytes at 2–3 dpf which were silenced by 4 dpf suggesting a role for calcium signaling in podocyte maturation. Elimination of filtration pressure by blocking heartbeat with the drug amperozide or by Tnnt2 knockdown, resulted in glomerular collapse but did not block podocyte calcium transients. Calcium transients were blocked by cyclopiazonic acid, thapsigargin and 2APB but not by cilnidipine or nifedipine, indicating calcium release was from intracellular stores. plce1 knockdown resulted in podocyte defects, disorganized capillaries, and loss of podocin expression, further suggesting a requirement for calcium signaling in podocyte differentiation. Significantly, calcium transients were reinitiated in mature podocytes (6dpf) by puromycin injury.

Conclusions: The dynamic behavior of developing podocytes is associated with spontaneous calcium transients that occur independently of vascular stretch. Stable podocytes lack calcium transients, however, transients are reinitiated upon injury. Further studies on upstream regulators and downstream effectors of calcium signaling in podocyte are ongoing.

IV21

Podocyte Number in the Normal Human Kidney: Are All Glomeruli the Same?

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Introduction: Podocyte number and density are critical parameters used to define podocyte depletion. However, the variability of these parameters between and within subjects without kidney disease is unknown. The aim of this study was to assess the variation of podocyte number and density in normal human kidneys.

Methods: Kidneys were collected at autopsy in Mississippi, USA. We included tissue from 12 male Caucasian American adults without renal disease. 50 serial sections (14 μm) were cut from one paraffin block per subject. Every second section was used for podocyte counting. Podocyte identification was based on Wilms' Tumour-1 immunolabelling and nuclear location outside capillary loops. Individual glomerular volume (IGV) was estimated by the physical disector/Cavalieri principles – 30 glomeruli per subject. The 3 smallest and 3 largest glomeruli from each subject were selected for podocyte counting based on series of confocal optical images and the optical disector/fractionator principles. Podocyte density was calculated by dividing number of podocytes per glomerulus by IGV.

Results: Overall, glomeruli contained between 263 and 983 podocytes (3.7-fold range) and between 114 and 526 podocytes per 10^6 μm^3 of glomerular tuft (4.6-fold range). Within subjects, the difference in podocyte number between small and large glomeruli was as low as 141 podocytes (ranging from 354 to 495) and as high as 470 podocytes (ranging from 438 to 908). For podocyte density, the difference between small and large glomeruli within subjects was as low as 53 podocytes per 10^6 μm^3 (ranging from 206 to 259) and as high as 391 podocytes per 10^6 μm^3 (ranging from 135 to 526).

Conclusions: There is great variability in podocyte number and density within and between subjects without renal disease, raising questions about when and how this variability was established. These results also highlight the importance of a reliable method to assess podocyte number and density.

IV22

Podocyte Number in Children and Adults: Associations with Glomerular Hypertrophy

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Introduction: Podocytes have very limited capacity for replication and sources for podocyte gain are scarce. Numbers of podocytes present during early childhood should allow physiological adaptations associated with adult body growth (i.e. glomerular hypertrophy) without the development of sclerosis. This study aims to determine associations between glomerular hypertrophy and numbers of glomerular resident cells, with a focus on podocytes.

Methods: Kidneys from 16 Caucasian American males, including 4 children (≤3 years old) and 12 adults (≥18 years old) without renal disease, were collected at autopsy in Mississippi, USA. We used a combination of immunohistochemistry, confocal microscopy and design-based stereology to estimate individual glomerular volume (IGV), and numbers of podocytes, non-epithelial cells (NECs, tuft cells other than podocytes) and parietal epithelial cells (PECs). Podocyte density was calculated. Values are median and interquartile range (IQR).

Results: Glomeruli from children were small and contained 452 podocytes (IQR: 335–502), 389 NECs (IQR: 265–498), and 146 PECs (IQR: 111–206). Adult glomeruli contained significantly more cells, including 558 podocytes (IQR: 431–746; $P < 0.01$), 1383 NECs (IQR: 998–2,042; $P < 0.0001$) and 367 PECs (IQR: 309–673;

$P < 0.0001$) than glomeruli from children. Interestingly, large adult glomeruli showed markedly lower podocyte density (183 podocytes per $x10^6$ μm^3) than small glomeruli from adults and children (932 podocytes per $x10^6$ μm^3; $P < 0.0001$). IGV was strongly correlated with numbers of NECs ($R = 0.97$; $P < 0.0001$) and PECs ($R = 0.88$, $P < 0.0001$). While glomeruli from children contained more podocytes than NECs, large adult glomeruli had more than three NECs per podocyte.

Conclusions: Large adult glomeruli contained more podocytes than glomeruli from children and small adult glomeruli, raising questions about the origin of these podocytes. The increased number of podocytes in large glomeruli does not match the increase in glomerular volume observed in adults, resulting in relative podocyte depletion. This may render these large glomeruli susceptible to pathology.

IV23

Podocyte Depletion and Its Association with Age, Hypertension and Nephron Number in Subjects Without Renal Disease

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Introduction: Podocyte depletion can be absolute (reduced podocyte number) or relative (reduced podocyte density). Podocyte depletion has been linked to glomerulosclerosis in rodents. This study aims to determine associations between CKD risk factors, including older age, hypertension and low nephron number (N_{glom}), and podocyte depletion.

Methods: Kidneys from 14 adult male Caucasian Americans without renal disease were collected at autopsy in Mississippi, USA. Age and history of hypertension were obtained from medical records. N_{glom} was estimated using the disector/fractionator method. Age was dichotomized into younger and older (cut-off: 40 years), and N_{glom} as normal and low (cut-off: 0.6 million nephrons). We used a combination of immunohistochemistry, confocal microscopy and stereology to estimate podocyte number and density in six randomly sampled glomeruli per subject.

Results: Median age was 39 years (IQR: 21–50). 31% of subjects had a history of hypertension. N_{glom} ranged from 0.25 to 1.66 million (median: 0.95). Podocyte number in younger (median: 433; IQR: 386–512), normotensive (median: 424; IQR: 358–506) and normal N_{glom} subjects (median: 424; IQR: 356–493) was higher than in older (median: 357; IQR: 317–425; $P < 0.001$), hypertensive (median: 359; IQR: 315–433; $P < 0.05$) and low N_{glom} subjects (median: 358; IQR: 301–409; $P < 0.05$). Similarly, podocyte density (podocytes per 10^6 μm^3 of glomerular tuft) was lower in subjects who were older (median: 195; IQR: 139–241), hypertensive (median: 194; IQR: 94–241) and with low N_{glom} (median: 121; IQR: 71–266) compared to subjects who were younger (median: 275; IQR: 216–318; $P < 0.0001$), normotensive (median: 260; IQR: 194–295; $P < 0.001$) and with normal N_{glom} (median: 240; IQR: 194–289; $P < 0.01$).

Conclusions: This preliminary report suggests that older age, hypertension and low N_{glom} are associated with absolute and relative podocyte depletion in adults without kidney disease. Further studies are needed to establish the threshold at which podocyte depletion leads to glomerulosclerosis in humans.

IV24

Defining the Immunodominant Epitope in Phospholipase A2 Receptor (PLA2R)

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Introduction: PLA2R was identified as a major target antigen involved in idiopathic membranous nephropathy (IMN). 70% of patients have circulating autoantibodies, which recognise this receptor leading to immune complex deposition. This study aimed to define the epitope(s) recognised by anti-PLA2R autoantibodies.

Methods: The smallest reactive peptides constituting the epitope were identified using trypsin fragmentation of full length extracellular PLA2R (N-C8), followed by multi-dimensional separations. The peptides blotted with anti-PLA2R from patient sera were cut from the gel and analysed by mass spectrometry (MS). A peptide inhibition assay was used to validate the epitope and surface plasmon resonance was developed to assess binding kinetics of patient antibodies to PLA2R.

Results: We established the smallest fragment recognised by anti-PLA2R was from the N-terminus to CTLD3 (N-C3) and N-terminus to CTLD2 was not reactive. In an ELISA screen of 43 IMN patients we measured the ability of an N-C3 fragment to inhibit the binding of the autoantibodies to N-C8 and found inhibition in 90% of the patients suggesting the presence of more than one epitope with the dominant epitope in the N-C3 region. To further define this epitope we analysed immuno-reactive trypsin polypeptides by MS and assessed the potential of the MS-identified peptides to block the binding site between the autoantibody and PLA2R and found one peptide located in the Ricin domain with reproducible inhibition of 85%. We determined that the autoantibodies bound with strong and equal affinity (~0.1 nM) to N-C8 and N-C3 and the affinity was similar in all five patient sera tested.

Conclusions: We report the major epitope is in the Ricin domain of PLA2R and highlight the importance of correct conformation for epitope functionality. The interaction between anti-PLA2R and PLA2R is high and there is no difference in affinity between patients. This investigation has clinical implications for the therapy of patients with IMN.

IV25

Purinergic Signalling Induces Clathrin-Mediated Flux of Albumin into Podocytes

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Introduction: Increased amounts of albumin filtrated through the slit membrane between the foot process of podocytes may lead to albuminuria, strongly associated with progression of chronic kidney disease. Albumin may be taken up in the podocyte by clathrin-mediated endocytosis and their exposure to albumin initiates an injury response. Podocytes function may be modified by extracellular nucleotides acting through purinoceptors and podocytes express G protein-coupled purinoceptors. Thus we investigated the action of purinoceptors agonists (2-methythioATP, ATP-γ-S, 10^{-6} M) on albumin routed and involvement of clathrin in this action.

Methods: Endocytosis was studied in primary cultured rat podocytes using FITC-albumin by direct quantitative assay and their permeability was measured with a transmembrane albumin flux assay. Nucleofection with construct RFP-GFP-LC3 were used to detect the autophagy process.

Results: 2-MeSATP increased albumin endocytosis (52.042.59 vs. 40.10±1.33 μg/mg protein) and transmembrane albumin flux (89.59±7.26 vs. 36.28±3.60 μg/ml). Similar actions were observed for ATP-γ-S. Those effects were abolished by Pitstop2. Furthermore, Pitstop 2 decrease the basal level of albumin endocytosis (29.39±0.54 μg/mg protein) but not transmebrane albumin flux (32.72±2.33 μg/ml). Incubation of podocytes with albumin (1 mg/ml, 2 days) activates the autophagy.

Conclusions: Extracellular nucleotides lead to clathrin-mediated albumin endocystosis and transmembrane flux, these may result in podocyte injury.

IV26

Endothelial PAI-1-Mediated Beta 1 Integrin Endocytosis Promotes a Vicious Cycle of Podocyte Loss

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Introduction: Podocyte injury causes microangiopathy due to local reduction of VEGF, however it is not clear whether microangiopathy accelerates podocyte injury. We previously showed that plasminogen activator inhibitor-1 (PAI-1) from glomerular endothelial cells was increased prior to the reduction of podocytes by using NEP25/LMB2 mice. PAI-1/uPA complex binds uPAR on the cell membrane and promotes integrin endocytosis causing cells to detach from the matrix by loss of adhesive function. This study shows the glomerular intra-capillary response to primary podocyte injury and its role in glomerulosclerosis, using NEP/LMB2 mice and immortalized podocytes.

Methods: Podocyte injury was induced in two groups of NEP25/LMB2 mice (with or without PAI-1 inhibitor (PI)) by injecting LMB2 on Day 0. PI was administered from Day 0 to 12, and histological and clinical parameters were analyzed on Day 12. Next, we treated cultured podocytes with PAI-1/uPA complex (P/U), uPA (U), PAI-1 (P), or an antibody for blocking uPAR with P/U (B-P/U). After incubation, attached cells were counted, and localization of β1 integrin and uPAR was detected by immunofluorescence and immunolabeling electron microscopy (IEM). Cytoplasmic β1 integrin was analyzed by Western blot.

Results: PI blocked proteinuria and thrombosis, and preserved podocyte number ($P < 0.05$). Subsequent intracytoplasmic localization of β1 integrin in podocytes was not seen in NEP/LMB2 with PI mice but was observed in NEP25/LMB2 mice. In immortalized podocytes, the P/U group caused significant cell detachment ($P < 0.05$), while the U, P or B-P/U groups did not. Furthermore, confocal microscopy and Western blotting showed that β1 integrin on the cell surface translocated to the cytoplasm, and IEM revealed co-localization of β1 integrin and uPAR in podocyte vesicles in the P/U group.

Conclusions: Primary podocyte injury-caused in situ thrombotic microangiopathy promotes extra podocyte damage by PAI-1/uPA/uPAR complex mediated β1-integrin endocytosis.

IV27

Utilizing 'Fast FLIM' to Discriminate Multiple Fluorescent Labels in Glomerular in Vivo Imaging

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Introduction: The use of multiphoton microscopy for intravital glomerular imaging has opened new avenues for glomerular research. But the fixed filter sets of external detectors make it difficult to discriminate between dyes with similar emission spectra. In Fluorescence Lifetime Imaging (FLIM) time-correlated single photon counting (TCSPC) determines the fluorescence lifetime for each pixel recorded in the image. A curve is fitted on the fluorescence decay histogram and the average lifetime (τ_{av}) is calculated. But for optimal results slow scan speeds and long acquisition times are necessary for sufficient pixel counts, which is problematic in the in vivo imaging setting. We therefore used the 'Fast FLIM' image, where the color of each pixel encodes the lifetime and the brightness the fluorescence intensity. This can be obtained much quicker than regular FLIM and serves as a tool to discriminate signals of different labels in the same detector channel.

Methods: Pod:cre mTomato/mGFP mice, expressing a membrane-targeted GFP in podocytes and Cre negative litter mates expressing mTomato in podocytes were prepared for multiphoton microscopy. The imaging was performed on a Leica SP8 multiphoton microscope with external Hybrid detectors (NDD) connected to a PicoHarp300 TCSPC system. 'Fast FLIM' was acquired with the SymphoTime software.

Results: By adjusting the thresholds of a rainbow colored 'Fast FLIM' image we were able to discriminate dyes within seconds after the imaging acquisition in a resolution of 512x512 pixels. In the red channel we could separate autofluorescence, tdTomato, Texas red dextrane and Propidium iodide. In the green channel we could visualize autofluorescence, GFP, Fluorescein dextrane and Hoechst in different colors.

Conclusions: Separation of labels with 'Fast FLIM' extends the possibilities to monitor several structures or processes at the same time, helps to better visualize glomerular diseases and will increase our knowledge about podocyte biology in the intact kidney in vivo.

IV28

In Vitro Culture of Human Renal Progenitors

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Introduction: How renal cells, like podocytes or tubule cells, respond to injury may determine their survival under disease conditions. A better understanding of the developmental pathways active in these cells may shed light on their response to injury and provide insights into novel therapies. Currently, no culture system exists, without the use of genetic manipulation, that can stably propagate human nephron progenitors that can be differentiated into mature renal cells. We previously showed the presence of a renal progenitor population within human amniotic fluid (hAKPC-P) that can be induced to differentiate into functional podocytes. Most importantly, this population contains cells that express specific developmental renal genes, like Six2 and Cited1, suggesting their identity as nephrogenic precursors with self-renewal and nephron specification capabilities.

Methods: Subpopulations of nephron progenitors were isolated by FACS sorting using RNA fluorescent probes for Six2 and Cited1 and characterized by gene and protein expression by RT-PCR, microarrays, staining, western blot and secretome profiling.

Results: Nephron progenitor cells represent 0.3–0.5% of the amniotic population as assessed by FACS analysis for both protein and RNA. Cells exhibit a fast growth rate and can be easily expanded. This subpopulation isolated from amniotic fluid expressed nephrogenic markers, including HOXA11, HOXC11, Eya1, and Notch. Moreover, this nephrogenic population was successfully differentiated into various renal lineages, including podocytes.

Conclusion: Second trimester human amniotic fluid harbors a small population of renal progenitors that can be easily isolated without the use of genetic manipulation and expanded in vitro maintaining self-renewal and renal differentiation. This research could lead to important discoveries concerning renal developmental pathways in humans.

IV29

Increased PARsylation Activates Canonical Wnt Signalling in CD2AP Deficient Podocytes

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Introduction: CD2-associated protein (CD2AP) is essential for the structural and functional integrity of the glomerular filtration barrier as mice lacking CD2AP develop nephrotic syndrome with heavy proteinuria and effacement of podocyte foot processes. To study the role of CD2AP in podocytes, we performed a yeast two-hybrid screening on a glomerular library, and found that CD2AP bound tankyrase 2. Tankyrases are poly-ADPribose polymerases that catalyze PARsylation [poly-(ADP-ribosyl)ation], a post-translational modification of proteins involved in diverse cellular processes. PARsylation has been suggested to play a role in the pathological manifestations of diabetic nephropathy. The aim of this study was to characterize the significance of tankyrase-CD2AP interaction and the PARsylation activity of tankyrases in podocyte function and injury.

Methods: The CD2AP-tankyrase interaction was confirmed by co-immunoprecipitation and GST-pull down assays. Wild-type and CD2AP knockout mouse kidneys and cultured podocytes were analyzed by immunohistochemistry and colorimetric PARsylation assay. Western blotting and quantitative RT-PCR were used for mRNA and protein expression studies.

Results: Tankyrases are expressed in normal mouse and rat kidneys in vivo. CD2AP interacts with both tankyrase 2 and its close homolog tankyrase 1. Tankyrases are downregulated in CD2AP–/– podocytes, but in contrast, the total PARsylation activity is elevated in CD2AP–/– podocytes in culture, and in the kidneys of CD2AP–/– mice. Additionally, inhibition of tankyrases with a small molecule inhibitor lowers the total PARsylation of cultured CD2AP–/– podocytes. Accumulation of the active form of β-catenin was observed in CD2AP–/– podocytes indicating activation of the canonical Wnt signaling, that is known to induce podocyte injury. Inhibition of tankyrases could partially inactivate β-catenin in these cells.

Conclusions: CD2AP inhibits the PARsylation activity of tankyrases. The overactivity of tankyrases leads to the activation of the Wnt/β-catenin signaling pathway that may contribute to podocyte injury in the absence of CD2AP.

IV30

Insulin Activation of Orai1 Channel Contributes to Podocyte Dysfunction and Proteinuria

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Introduction: The Ca^{2+}-mediated signaling pathways including TRPC5 and TRPC6 have been implicated either directly or indirectly in podocyte dysfunction. Nevertheless, store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} influx pathway in non-excitable cells like podocyte. However, the expression and functional role of molecular components of SOCE have not been investigated in podocyte. Moreover, pathophysiology of insulin-mediated Ca^{2+} handling via SOCE also remains largely elusive. Here, we examined the molecular mechanism by which insulin regulates Orai1, a predominant component of SOCE, and contributes to Orai1-mediated podocyte dysfunction and proteinuria.

Methods: Orai channel activity was measured by patch-clamp techniques and live-cell Ca^{2+} imaging using Fura-2. Protein expression and morphological analysis were confirmed by immunohistochemistry, immunofluorescence, quantitative-PCR, western blot and EM. db/db mice was used as hyperinsulinemic type II diabetic animal model.

Results: Orai1 channel was a critical molecular component of SOCE in podocyte. Insulin stimulated cell surface abundance of Orai1 via activating its VAMP2-dependent secretory pathway in a PI3K-Akt-dependent manner. In contrast, insulin had no effect on intrinsic channel properties such as activation kinetics and current-voltage relationship. Exaggerated Ca^{2+} influx by Orai1 mediated cytoskeletal rearrangement of podocyte followed by causing increased albumin permeability. Overexpression of Orai1 caused proteinuria that was augmented by insulin treatment confirmed by in vivo gene delivery experiments in mice. Furthermore, in hyperinsulinemic db/db mice, disruption of podocyte slit-diaphragm and proteinuria were significantly reduced by blockade of Orai1 channel or its downstream effector calcineurin supporting the notion that insulin-mediated Orai1 activation may, at least partly, involve podocyte dysfunction followed by proteinuria.

Discussions & Conclusions: Orai1 is a crucial molecular component of SOCE and novel Ca^{2+} influx pathway in podocyte. Insulin stimulates membrane expression of Orai1 via PI3K-Akt-dependent its exocytic pathway that mediates remodeling of actin cytoskeleton causing podocyte injury and proteinuria in mice.

IV31

Podocyte MDM2 Prevents p53 Overactivation-Related Cell Death (Podoptosis), Proteinuria, and Glomerulosclerosis

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Introduction: Murine double minute-2 (MDM2), an E3 ligase regulating the cell cycle and inflammation and the main negative regulator of tumor suppressor gene p53, is highly expressed in podocytes. In podocyte injury, MDM2 drives podocyte loss via mitotic catastrophe but the function of MDM2 in resting podocytes is unknown. We speculated that resting podocytes need MDM2 to maintain homeostasis and sought to define in which manner.

Methods: To test our hypothesis that MDM2 may have a protective effect in vivo and in vitro we blocked the MDM2 or MDM2/p53 in cultured mouse podocytes by siRNA, in zebra fish by using morpholino oligonucleotides, and by generating podocyte-specific knockout mice.

Results: MDM2 knock down by siRNA caused podocyte death in vitro, which was completely rescued by co-knock down of p53. Apoptosis, pyroptosis, necroptosis, ferroptosis, and parthanatos were excluded as modes of this p53-overexpression-related cell death, here referred to as podoptosis. Podoptosis was associated with cytoplasmic vacuolization, ER stress, and dysregulated autophagy previously described as paraptosis. MDM2 knock down caused podocyte loss and proteinuria in a zebra fish model, which was consistent with the phenotype of podocyte-specific MDM2-knockout mice that also showed the aforementioned ultrastructual podocyte abnormalities before and during progressive glomerulosclerosis. The phenotype of both animal models was entirely rescued by co-deletion of p53.

Conclusion: We conclude that podocytes need MDM2 to maintain homeostasis and long-term survival. MDM2 prevents podoptosis, a p53 overexpression-related form of cell death, which in podocytes was not apoptosis or any of the known modes of regulated necrosis but showed unspecific features previously named paraptosis.

IV32

Gene Expression Profiling of Young and Aged Wild-Type Mice Reveals Pathways Involved in Kidney Aging

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Introduction: The worldwide demographic increase in the elderly population has made prevention and therapy of aging-related chronic kidney disease become a major health concern. Mild to severe impairment of kidney function is common amongst old patients, yet models to study renal aging are currently lacking. In order to identify molecular mechanisms involved in glomerular and kidney aging we analyzed gene expression profiles of young and aged wild-type mouse glomeruli and whole kidney tissue.

Methods: Wild-type B6 mice were sacrificed at 14 and 96 weeks of age. Glomeruli were isolated using magnetic beads. RNA was extracted, reverse transcribed and cDNA was hybridized to Affymetrix microarrays. Statistical analysis was conducted using R/Bioconductor. For GO enrichment and network analyses the DAVID server and NetBox software was used.

Results: GO term enrichment analysis revealed differentially expressed genes to be involved in immune and defense response in both glomerular and whole kidney samples. In a network analysis, these genes were part of inflammation signaling, complement response, growth receptor and chemokine receptor signaling. Several genes differentially expressed in our whole kidney screen were already known to be implicated in renal damage, such as Kim1, LFABP1 or N-gal. Histologically, a significant increase in glomerular tuft area and glomerular external diameter was present. In immunofluorescence we detected a strong myeloperoxidase staining predominantly localized to glomeruli of 96 week old mice compared to 14 week old mice.

Conclusions: Aged mouse kidney tissue exhibited glomerular hypertrophy and aging associated histologic alterations. Genes differentially expressed in aged glomerular and kidney samples were preferentially involved in immune response and inflammation. Our results will contribute to identifying novel pathways and genes involved in regulating the process of kidney aging.

IV33

TNFα Mediated Impairment of Cholesterol Efflux Affects Podocyte Function

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Introduction: Diabetic Kidney Disease (DKD) is the most common cause of end-stage renal disease worldwide and serum Tumor Necrosis Factor alpha (TNF levels correlate with the development and progression of DKD. In DKD, decreased podocyte number and glomerular cholesterol accumulation are associated with albuminuria. We tested the hypothesis that TNFα apoptosis in podocytes and that inhibition with Infliximab, a monoclonal antibody against TNF can protect from the development of albuminuria in experimental DKD.

Methods: Caspase 3 activity was measured to quantify apoptosis in TNF treated human podocytes. Lipid droplets were quantified in Bodipy 493/503 stained cells using the Opera High Content Screening system. Cholesterol efflux experiments were performed loading human podocytes with ^{3}H-cholesterol. Gene expression profiling was performed on human kidney biopsies from patients with DKD using U133plus2 Affymetrix and GeneChip arrays.

Results: TNF treatment of human podocytes induced apoptosis ($p < 0.05$) which was associated with a significant increase in the number of lipid droplets per cell ($p < 0.01$), decreased ApoA1-mediated cholesterol efflux ($p < 0.05$), and decreased ATP-binding cassette transporter A1 (ABCA1) mRNA ($p < 0.001$) and protein ($p < 0.01$) expression. Decreased ABCA1 expression was also observed in glomeruli of patients with type 2 diabetes and DKD ($N = 70$) when compared with healthy living donors ($N = 32$). TNF treatment of human podocytes increased the total cholesterol content ($p < 0.05$) whereas the esterified cholesterol pool was significantly decreased ($p < 0.05$). Cholesterol depletion with cyclodextrin partially prevented TNFα-induced podocyte apoptosis ($p < 0.05$). Recombinant TNF injection in mice led to significant albuminuria. TNF inhibition with Infliximab administered to BTBR ob/ob mice, a mouse model of DKD that is characterized by increased glomerular cholesterol accumulation, attenuated the degree of albuminuria.

Conclusions: TNF attenuates ABCA1-mediated reverse cholesterol transport in podocytes leading to increased cholesterol accumulation and apoptosis. Our data suggest Infliximab may protect podocytes from cholesterol-mediated injury in DKD.

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Podocyte High Content Screening Assays for the Identification of Novel Therapeutic Leads for Treating Proteinuric Kidney Diseases

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Introduction: Focal segmental glomerulosclerosis (FSGS) is a leading cause of idiopathic nephrotic syndrome with limited therapeutic options. Podocytes represent a crucial target for therapeutic interventions in FSGS and other proteinuric glomerular diseases. However, current methods are inadequate for podocyte targeted drug discovery. Here, we aim to develop novel, podocyte cell-based assays for use in a drug discovery environment and use these assays to identify new therapeutic leads.

Methods: Normal actin cytoskeletal machinery is essential for maintaining podocytes in a healthy state in vivo and in vitro. We developed a novel, high content screening (HCS) based methodology to quantitatively measure actin cytoskeleton and other cellular features in healthy podocytes. We further developed a computational methodology to quantitatively measure changes in podocytes after treatment with an increasing dose of podocyte damaging agent puromycin aminonucleoside (PAN). Cellular classification based on various phenotypic feature allowed a robust determination of PAN induced changes in cells to separate the damaged and healthy cell populations and the data was used to generate a percent healthy cell statistic per well. Treatment with known podocyte-protective agents, such as mizouribine, showed a quantitative, dose-dependent protection of podocytes from damage in our assay. Next, we applied a chemical library to this assay to identify compounds that could potentially protect podocytes PAN-induced damage.

Results: Our assay quantitatively defined dose-dependent changes in a variety of podocyte markers and phenotypes, including changes in cellular morphology, F-actin and focal adhesion markers. Our assay is robust and has a Z-prime factor >0.5, which makes it suitable for use in a high-throughput screening environment. Screening with a small chemical library identified a number of novel compounds that protected podocytes from PAN-induced damage (~1% hit rate).

Conclusions: We describe a novel, high-throughput assay for unbiased quantification of changes in podocyte health that has wide applicability. Using this newly developed assay, we have identified a few novel compounds that show podocyte protection in a dose-dependent fashion. Our current efforts are focused at validating the hits and understanding their mechanism of action so that the new agents could be developed as potential therapeutics for treating proteinuric kidney diseases.

IV35

Mechanotransducing Elements in Podocytes: Role of Purinergic P_2X Channels

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Introduction: Hypertension and the resulting increase in mechanical load to podocytes are believed to result in podocyte damage. However, the molecular identity of the proteins sensing the mechanical force is completely unknown. It has been speculated whether the classical transient receptor channel type 6 (TRPC6) in complex with the MEC-2 homologue podocin and possibly other slit-membrane protein could form a mechanosensitive channel complex in podocytes. We investigated mechanotransducing elements of podocytes with focus on the role of TRPC6.

Methods: We isolated primary podocytes from wild type mice and used electrophysiological whole cell recordings and fluorimetric calcium recordings to determine how podocytes respond to hypoosmotically induced membrane stretch.

Results: Podocytes indeed respond to mechanical stretch with increased inward cation current densities in electrophysiological whole-cell measurements that are not mediated by TRPC6 since podocytes isolated from TRPC6 knock-out mice responded to mechanical stretch in a similar way as control podocytes. By using the selective $G_{q/11}$-protein inhibitor YM-254890 in the bath solution, we could rule out an involvement of mechanosensitive $G_{q/11}$-protein coupled receptors. Instead, the observed currents could be reduced by using the specific purinergic P_2X_4 channel blocker 5-BDBD in electrophysiological measurements. Quantitative RT-PCR confirms expression of several purinergic receptor channels including P_2X_2 and P_2X_4. Extracellular perfusion of podocytes with ATP resulted in a similar inward current as observed with hypotonic solution. Moreover, we found out that hypoosmotically induced membrane stretch leads to release of ATP.

Conclusions: Primary podocytes respond to hypoosmotically induced membrane stretch with increased inward current densities similar to those observed by extracellular perfusion of podocytes with ATP. Mechanical activation results in the release of ATP from podocytes which subsequently activates P_2X channels thereby determining mechanosensitivity of podocytes.

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The Role of Survivin in Podocyte Injury Induced by Puromycin Aminonucleoside

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Introduction: Survivin (Birc5), is a member of inhibitior of apoptosis family, which is the strongest inhibitor of apoptosis factor by now. The expression of survivin is low or absent in the terminally differentiated normal tissues/cells. While various studies have been demonstrated that survivin is Over-Expressed in different cancers tissues/cells, and the high level of survivin expression play a siganificant role in the apoptosis inhibition. Recent studies have demonstrated that survivin is expressed in some normal adult cells. In the present study, we aimed to investigate the significance of suivivin expression in the terminally differentiated epithelial cells injury of the kidney, podocyte injury.

Methods: Survivin expression and location were detected by Real-Time PCR, western blot and immune fluorescence conforcal methods in mouse podocytes of normal and injury. Survivin cytopection function was also studied by analyzing PAN-Induced damage in cultured podocyte with knocking down and overexpression survivin expression.

Results: PAN stimulation podocytes could Up-Regulate expressions of survivin and apoptosis related molecules, the activation of caspase 3. Knockdown of survivin expression by siRNA increased the activation of caspase 3, increased podocyte apoptosis and induced a remarkable dearrangement of actin cytoskeleton in podocytes compared with the universal negative control siRNA group. Moreover, overexpression of survivin via the transfection decreased podocyte apoptosis and cytoskeleton rerangement induced by PAN.

Conclusions: Our data provides evidence that survivin played an important role in preventing podocytes from apoptosis in PAN-Induced injury. The potential mechanism of survivin anti-apoptotic might also involve the caspase. Survivin might be an essential mediator of cytoprotection in podocyte injury.

IV37

Circulating Zonulin Regulates Podocyte Morphology and Glomerular Permeability via Protease-Activated Receptor 2

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Introduction: Zonulin, a 47 kDa protein in the small intestine, activates protease-activated receptor (PAR) 2 on enterocytes in an autocrine manner, leading to the phosphorylation of zonula occludens (ZO)-1, the transient disassembly of tight junctions (TJ) and a reversible elevation in paracellular transport across the gut. In patients with Celiac Disease (CD) zonulin levels increase in response to dietary gluten, leading to abnormal gut permeability and diarrhea. Since zonulin is also elevated in the serum of patients with CD as well as other autoimmune diseases, zonulin potentially functions as a circulating modulator of TJs in other tissues. Podocytes express PAR2 and the slit diaphragm (SD) contains TJ

proteins. We hypothesize that zonulin can directly target podocytes, increase paracellular permeability in the glomerulus, and induce proteinuria.

Methods: Immortalized murine podocytes (wild type and PAR2 knockdown) were treated with AT1002, a zonulin synthetic peptide analogue. Albumin permeability was determined in isolated rat glomeruli treated with AT1002 or with sera from FSGS patients in the presence of a PAR2 inhibitor. Urine albumin levels were monitored in C57Bl/6 mice on a gluten-rich diet with high serum zonulin.

Results: AT1002 caused a loss of actin stress fibers and changes in RhoGTPase activities, induced tyrosine-phosphorylation and redistribution of ZO-1, and increased cell migration and paracellular transport. AT1002 and FSGS sera elevated albumin permeability in isolated rat glomeruli in a PAR2-dependent manner, and mice on a gluten-rich diet developed mild proteinuria.

Conclusion: Zonulin-mediated PAR2 activation in podocytes might function as a novel regulator of the SD and paracellular permeability in the glomerulus. Future studies aim to determine if aberrant zonulin elevation can induce podocyte injury and proteinuria in mice, if serum zonulin levels in patients with glomerular disorders are increased, and if albumin excretion rates in patients with CD are elevated.

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MicroRNA-30 Family Inhibits Calcium-Calcineurill Signaling in Podocytes

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Introduction: Calcium-calcineurin signaling has recently been implicated in the injury of podocytes. Several reagents, including TGF-beta, Lipopolysaccharides (LPS) and puromycin-aminonucleoside (PAN), can induce calcium-calcineurin signaling in podocytes, but the underlying mechanisms are still unknown. We have recently found that miR-30 members are abundantly expressed in podocytes, but all dowlll'egulated by TGF-beta, LPS or PAN, leading to podocyte injury. Bioinformatics analyses suggest miR-30s may protect podocytes by inhibiting calcium-calcinellrin signaling, and dowlll'egulation ofmiR-30s would enhance calcium-calcineurin signaling, leading to podocyte injury.

Methods: Conditionally-immortalized human podocyte cell line treated with TGF-beta, LPS or PAN, PAN-treated rats, and the biopsies ofFSGS patients were used for the study. miR-30 target validations were performed by lllciferase reporter assay and western blotting.

Results: We treated podocytes with TGF-beta, LPS or PAN, and found an increase of calcium influx and calcineurin activity, accompanied by dowlll'egulation of miR-30s and upregulations of calcium-calcineurin signaling components (TRPC6, PPP3ca, PPP3cb, PPP3rl and NFATc3, which are the predicted miR-30 targets) in the cells. However, mexogenous miR-30 expression that sustained the overall level of miR-30s in the podocytes prevented the increase of calcineurin activity and upregulation of TRPC6, PPP3ca, PPP3cb, PPP3rl and NFATc3 in the treatment of PAN. In PAN-treated rats, upreglllation of calcineurin and dowlll'egulation of miR-30s were also observed in the]lodocytes. However, transferring exogenous miR-30a to the]lodocytes of the rats prevented the upregulation of calcium-calcinel'llin components and ameliorated]lodocyte injury. Calcineurin upregulation was also found in the podocytes of FSGS patients, in which miR-30s were dowlll'egulated. Finally, luciferase reporter assays confirmed that TRPC6, PPP3ca, PPP3cb, PPP3r1 and NFATc3 are the direct targets ofmiR-30s in podocytes.

Conclusions: miR-30s inhibit calcium-calcineurin signaling in podocytes, both in vivo and in vitro, by directly targeting calcineurin signaling components. Dowlll'egulation of miR-30s and the consequent upregulation of calcium-calcineurin signaling may be an alternative mechanism involving in podocytes injury.

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Proteomic Analysis of Formalin-Fixed Paraffin-Embedded Glomerular Tissue Suggests Depletion of Glomerular Filtration Barrier Proteins in Two-Kidney One-Clip Hypertensive Rats

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Introduction: It is well known that hypertension may cause glomerular damage, but the molecular mechanisms involved are still incompletely understood.

Methods: In the present study we used formalin-fixed paraffin-embedded (FFPE) tissue to investigate changes in the glomerular proteome in the non-clipped kidney of 2-kidney-1-clip (2K1C) hypertensive rats, with special emphasis on the glomerular filtration barrier. 2K1C hypertension was induced in 6-weeks-old Wistar-Hannover rats (n = 6) that were sacrificed 23-weeks later and compared to age-matched sham operated controls (n = 6). Tissue was stored in FFPE tissue blocks and later prepared on tissue slides for laser microdissection. Glomeruli without severe morphological damage were isolated and the proteomes were analyzed using liquid chromatography tandem mass spectrometry.

Results: 2K1C glomeruli showed reduced abundance of proteins important for slit-diaphragm complex, such as nephrin, podocin and neph1. The podocyte foot process had a pattern of reduced abundance of transmembrane proteins but unchanged abundances of the podocyte cytoskeletal proteins synaptopodin and α-actinin-4. Lower abundance of important glomerular basement membrane proteins was seen. Possible glomerular markers of damage with increased abundance in 2K1C were transgelin, desmin and nestin.

Conclusions: Microdissection and tandem mass spectrometry could be used to investigate the proteome of isolated glomeruli from FFPE tissue. Glomerular filtration barrier proteins had reduced abundance in the non-clipped kidney of 2K1C hypertensive rats.

IV40

Patch Clamp and Calcium Measurements: New Old Tools to Study TRPC Function in the Podocytes of the Freshly Isolated Glomeruli

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Introduction: The key role for podocytes in the pathogenesis of glomeruli diseases has been previously established. The goal of this study was to develop techniques allowing to investigate TRPC channels in the podocytes of the freshly isolated decapsulated glomeruli and to apply these approaches to determine the effects of Angiotensin II on intracellular Ca^{2+} levels and endogenous channels in intact podocytes.

Methods: Single channel analysis of native ion channels in the podocytes of the intact freshly isolated glomeruli has been established here. In addition to patch clamp analysis, we employed high resolution confocal fluorescence ratiometric measurements to quantify calcium flux in the podocytes.

Results: We examined the effects of Angiotensin II on intracellular Ca^{2+} concentration levels and endogenous channels activity in intact podocytes of the murine glomeruli. An ion channel with distinct TRPC6 properties was identified in the WT animals, and was absent in $TRPC6^{-/-}$ mice. Single channel analysis demonstrated that Angiotensin II acutely activates native TRPC-like channels in the podocytes as well as TRPC6 channels transiently overexpressed in CHO cells, and this effect is mediated by changes in the channel open probability. Angiotensin II also evoked intracellular calcium transients in the wild type podocytes, which were blunted in $TRPC6^{-/-}$ glomeruli. Pan-TRPC inhibitors gadolinium and SKF96365 reduced the response in wild type glomerular epithelial cells, whereas the transient in $TRPC6^{-/-}$ animals was not affected.

Conclusions: Developed single channel analysis and calcium measurements allow real-time quantification of channels' activity and calcium flux in their native settings. These new tools could be instrumental in identifying the function of endogenous ion channels in podocytes, and elaborating how native signaling pathways regulate these channels. In addition to the technical advantages, our data provide direct evidence of Angiotensin II-dependent activation of TRPC6 channels in podocytes.

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Studying the Signaling Network of the NEPH-NEPHRIN Protein Complex in *Drosophila* Nephrocytes

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Introduction: The functional and molecular similarities between nephrocytes and podocytes are remarkable and allow combining the power of genetic *Drosophila* tools with *in situ* organ analysis.

Methods: Use of the GAL4/UAS-system enables the nephrocyte specific expression of mammalian Neph proteins. Various readouts like SEM and TEM were established to assess the precise nature of phenotypes resulting from these manipulations. The functional properties of toxin removal from the hemolymph by nephrocytes were monitored by quantifying the uptake efficiency of an endogenously secreted fluorescence labeled protein (MHC-ANF-RFP). By coexpression of RNAi constructs the NEPH protein misexpression phenotypes can be studied in detail.

Results: Loss of the NEPH ortholog Kirre in *Drosophila* nephrocytes resulted in a strong loss of function phenotype. Rescue experiments using the GAL4/UAS-system revealed that of the three mammalian Neph Proteins only Neph1 is able to reverse this phenotype. Furthermore, rescue experiments with cytoplasmic deletion constructs of Kirre indicate a role of a cytoplasmic domain present in Kirre and Neph1 but not in Neph2 and Neph3. Based on Neph1 overexpression phenotypes an RNAi suppressor screen is currently being set up to identify downstream components of the Neph1 induced signal transduction cascade.

Conclusions: Due to similarities with podocytes, nephrocytes are a powerful platform to study SD biology. Proper nephrocyte function requires an intact NEPH-NEPHRIN protein complex. Our rescue data indicate an involvement of NEPH signaling by a cytoplasmic domain. Based on NEPH overexpression phenotypes suppressor screen will be performed to identify components of the signal transduction cascade.

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