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Quantitative deep mapping of the cultured podocyte proteome uncovers shifts in proteostatic mechanisms during differentiation

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1Department II of Internal Medicine, University Hospital Cologne, Cologne, Germany; 2Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany; 3Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, University of Cologne, Cologne, Germany; and 4Systems Biology of Ageing Cologne, SybaCol, Cologne, Germany

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Rinschen MM, Schroeter CB, Koehler S, Ising C, Schermer B, Kann M, Benzing T, Brinkkoetter PT. Quantitative deep mapping of the cultured podocyte proteome uncovers shifts in proteostatic mechanisms during differentiation. Am J Physiol Cell Physiol 311: C404–C417, 2016. First published June 29, 2016; doi:10.1152/ajpcell.00121.2016.—The renal filtration barrier is maintained by the renal podocyte, an epithelial postmitotic cell. Immortalized mouse podocyte cell lines—both in the differentiated and undifferentiated state—are widely utilized tools to estimate podocyte injury and cytoskeletal rearrangement processes in vitro. Here, we mapped the cultured podocyte proteome at a depth of more than 8,800 proteins and quantified 7,240 proteins. Copy numbers of proteins mutated in forms of hereditary nephrotic syndrome or focal segmental glomerulosclerosis (FSGS) were assessed. We found that cultured podocytes express abundant copy numbers of endogenous receptors, such as tyrosine kinase membrane receptors, the G protein-coupled receptor (GPCR), NPR3 (ANP receptor), and several poorly characterized GPCRs. The data set was correlated with deep mapping mRNA sequencing (“mRNAseq”) data from the native mouse podocyte, the native mouse podocyte proteome and staining intensities from the human protein atlas. The generated data set was similar to these previously published resources, but several native and high-abundant podocyte-specific proteins were not identified in the data set. Notably, this data set detected general perturbations in proteostatic mechanisms as a dominant alteration during podocyte differentiation, with high proteasome activity in the undifferentiated state and markedly increased expression of lysosomal proteins in the differentiated state. Phosphoproteomics analysis of mouse podocytes at a resolution of more than 3,000 sites suggested a preference of phosphorylation of actin filament-associated proteins in the differentiated state. The data set obtained here provides a resource and provides the means for deep mapping of the native podocyte proteome and phosphoproteome in a similar manner.

glomerular disorders are an important cause for chronic kidney disease and renal failure. Glomerular disorders often rise from disorders of the renal filtration barrier. The renal filtration barrier consists of three components, endothelia, basement membrane, and glomerular podocytes. Podocytes are postmitotic cells and express gene products, which are mutated in hereditary nephrotic syndrome. These gene products localize to different compartments of the podocytes, among these are the specialized cell-cell contact, the slit diaphragm, the podocyte nucleus, the glomerular basement membrane, and the actin cytoskeleton. Examples for slit diaphragm-associated proteins include podocin, nephrin, and the cation channel TRPC6. Examples for cytoskeleton-associated proteins essential for podocyte homeostasis include actinin-4 and Rho GTPase-activating protein 24.

The advent of the era of systems biology, initiated by the human genome project, has rapidly pushed the limits of mass spectrometry-based identification and quantification of proteins. This is driven by three key developments. First, sample preparation methods are getting more sophisticated, second, mass spectrometers have rapidly evolved in both fragmentation speed and mass accuracy, and third, bioinformatics processes have been streamlined to achieve an accurate quantification and identification of proteins within a sample. This development has recently culminated in the near-comprehensive proteomics-based mapping of the human proteome. In cell physiological research, this versatile technology can be implemented as a more accurate "multiplex immunoblot", to discover unanticipated cellular mechanisms perturbed in health and disease, in vivo and in vitro, both for systems biology and hypothesis-driven research. For discovering cellular mechanisms, a deep coverage of the proteome is beneficial to delineate the quantitatively most important changes and to guide mathematical models.

In this paper, we performed an in-depth analysis of the cultured podocyte proteome. We and others have previously used proteomics and phosphoproteomics to elucidate signaling processes in podocytes in vivo and vitro. Cultured podocyte cell lines have been a valuable tool to translate some of these processes in vitro. The aim of this study was to generate a near-comprehensive map of the cultured podocyte proteome. By achieving this, we demonstrate unique features of the cultured podocyte proteome, including the presence of unanticipated signaling mechanisms, similarities and obvious differences to the native podocyte proteome.
and transcriptome, and alteration of global proteostatic mechanisms during podocyte differentiation.

METHODS

Podocyte cell culture. Mouse podocytes were cultured in RPMI-1640 medium (R8758; Sigma, St. Louis, MO) supplemented with 10% FBS, sodium pyruvate, and 20 mM HEPES. Briefly, podocytes at 33°C were cultured on Collagen I-coated 10-cm primary cell culture dishes, as previously described (25) and were harvested at 60% confluence on day 3, whereas podocytes at 37°C were cultured on normal uncoated dishes and harvested at a slightly higher confluence on day 14. 1 x 10 cm dish was used for each replicate at 33°C, while 3 x 10 cm dishes were used for each replicate at 37°C. Independent triplicates were collected for both cell culture conditions. Cells were washed with PBS, quickly trypsinized, and washed with RPMI medium. After a centrifugation with 1,000 rpm for 5 min at 4°C, the cell pellet was resuspended in 1 ml of sterile PBS and again centrifuged at 1,000 rpm for 5 min at 4°C. The final pellet was snap frozen in liquid nitrogen until further processing. Human podocytes were cultured as previously described (59). All cell lines were regularly tested for mycoplasma contamination by a regular kit. Cross-contamination with other cell lines was not observed and is currently frozen in liquid nitrogen until further processing. Human podocytes harvested on 10 cm dishes were cultured on Collagen I-coated 10-cm primary cell culture dishes. All cell lines were regularly tested for mycoplasma contamination by a regular kit. Cross-contamination with other cell lines was not observed and is currently frozen in liquid nitrogen until further processing.

Lyseate generation. Proteins were dissolved in urea buffer containing urea (8M) and amonium bicarbonate (100 mM) supplemented with 1X PPI (Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail). Protein lysates were sonicated for 30 s at 10% power. After centrifugation at 1,400 rpm for 30 min at 4°C, the supernatant was transferred to a new tube. Protein concentrations were measured using a commercial BCA kit (Thermo Scientific, Waltham, MA). The samples were incubated with DTT (10 mM) and iodoacetamide (40 mM) for 1 h at room temperature for the reduction and alklylation of disulfide bonds. 100 μg of protein from each sample were digested with trypsin using a 1:100 ratio (1 μg trypsin per 100 μg protein). Digestion was stopped the next day by adding 0.5% formic acid.

In-tip strong cation exchange fractionation for proteomic deep mapping. In-tip strong cation exchange fractionation (SCX) for proteomic analysis was performed as previously described (36) to fractionate minimal amounts of proteins. Samples were centrifuged at 1,400 rpm for 20 min. Six-layered tips with SCX resin (polystyrene-divinylbenzene copolymer modified with sulfonic acid) stage tips were conditioned with acetonitrile (ACN) and washed with 0.2% formic acid. Then, the supernatant was loaded on the in-tip columns and centrifuged until all of the peptide suspension passed the membrane. After washing the membrane of the stage tips with 0.2% formic acid, 6 different cationic buffers with increasing concentrations of ammonium acetate were used subsequently for elution: SCX 1: 50 mM ammonium acetate (AA), 20% (vol/vol) ACN, 0.5% (vol/vol) formic acid (FA); SCX 2: 75 mM AA, 20% (vol/vol) ACN, 0.5% (vol/vol) FA; SCX 3: 125 mM AA, 20% (vol/vol) ACN, 0.5% (vol/vol) FA; SCX 4: 200 mM AA, 20% (vol/vol) ACN, 0.5% (vol/vol) FA; SCX 5: 300 mM AA, 20% (vol/vol) ACN, 0.5% (vol/vol) FA; buffer X: 5% (vol/vol) ammonium hydroxide, 80% (vol/vol) ACN. The flow through was collected for each of the six buffers separately during centrifugation. In the end, all the collected flow-through was dried in the vacuum centrifuge for 30 min at 30°C.

Offline strong cation exchange fractionation for phosphoproteomic analysis. Analysis was performed as previously described with modifications (58). Briefly, ~2 mg of input material (harvested from the dish) of differentiated and undifferentiated podocytes was desalted using HLB columns (Oasics) and subjected to offline SCX fractionation using a LC system (Agilent). Gradients were as previously described (58). Eight fractions were generated by pooling subsequent peaks. Then IMAC (immobilized metal affinity chromatography, commercial kit by Thermo Scientific) phosphopeptide enrichment was performed as previously described (58). Finally, peptides were subjected to stage tip fractionation (51), as described by Rappsilber et al. (51) before injection into the mass spectrometer.

nLC-MS/MS. For nLC-MS/MS analysis of proteomic data, we followed the analysis previously described (4). nLC-MS/MS analysis of phosphoproteomic data was performed on an LTQ-Orbitrap XL machine coupled to a nLC with a 90-min gradient and machine settings as previously described (35). nLC-MS/MS analysis of proteomic samples were analyzed using a Q Exactive Plus (Thermo) machine coupled to a nLC as previously described, with a 2.5-h gradient (4).

Bioinformatics analysis. Analysis of raw spectral data (RAW files) were performed using MaxQuant v. 1.3.0.5 (phosphoproteomic data) or v. 1.5.1 (proteomic data) (18) with default settings. Phosphorylation was set as a variable modification for S,T,Y in the phosphoproteomic data. The data were searched against a reference mouse uniprot database (without isoforms) from March 2015. LFQ and match between run function was enabled (17). iBAQ values were calculated as previously described (61). Downstream bioinformatics analysis was performed using Perseus v 1.5 as previously described (35). Potential contaminants were removed. Both LFQ and phosphorylation site intensity were logarithmized (log2). Hierarchical clustering using Euclidean distance was utilized to cluster samples, protein LFQs, or phosphosite intensity sites. The summarized MS1 intensity of phosphorylation sites was normalized by subtraction of the mean in each column and row. Phosphorylation motifs were annotated using the phosphosite.org repository (26) generated with default settings by the Icelogo software using the Mus musculus proteome composition as a background (14). Quantitative proteomic analysis of LFQ was performed using an approach previously described as SAM by Tusher et al. (65) [false discovery rate (FDR) = 0.01, s0 = 1]. Statistical calculations and visualizations of cumulative histograms were performed with GraphPad (San Diego, CA). Box plots were visualized with boxplot (http://boxplot.tyerslab.com/). MS data were additionally searched against the mouse uniprot database by the SEQUEST algorithm embedded in the Proteome Discoverer 1.4 (Thermo) environment. Maximum cleavage sites were 2, minimal peptide length was 6, Delta Cn was 0.05, precursor mass tolerance was 10 ppm, and fragment mass tolerance was 0.8 Da. b and y ions were searched, max 3 equal and 4 dynamic modifications were allowed, and dynamic modification was methionine oxidation (M+15.995) and carbamidomethyl (+57.021). Protein identification FDR (stringent) was 0.01. Raw data, including staining intensities from the human protein atlas, were obtained from www.proteinatlas.org (accessed April 2016). Uniprot IDs of the study by Boerries et al. (7) (Supplemental Data Set S1/Table S1) were mapped against the current Uniprot DB (accessed June 26, 2016) to retrieve the gene name. Mapping of the generated data set was performed based on the gene symbol.

Bioinformatics analysis of mRNA sequencing data. mRNA sequencing data obtained from FACS-sorted podocytes (27) were reanalyzed for this study. Raw data from GEO (GSE64063) (accessible at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64063) were analyzed and FPKM values corresponding to mRNA copy numbers were generated using bowtie and cufflinks with default settings (for details, see Ref. 27).

Deposition of raw data. Spectral raw data were deposited in the Pride/Proteome exchange repository (67, 68). This can be accessed at https://www.ebi.ac.uk/pride/archive/ using the following accession numbers: Phosphoproteomic data—Project accession: PXD000413; Proteomic data—Project accession: PXD004040.

Proteasome assay. For each replicate of the experiment, 4 x 10 cm dishes (60% confluence) were used. Independent biological triplicates (mouse podocytes) or quintuplicates (human podocytes) were collected for both cell culture conditions. Mouse podocytes at 33°C were harvested on day 3, while cells at 37°C were harvested on day 14.
using trypsin. The harvested cells were pooled in RPMI-1640 medium and centrifuged at 1,000 rpm for 5 min at 4°C. The pellet was resuspended in 1 ml of sterile PBS and again centrifuged at 1,000 rpm for 5 min at 4°C. Pellets were immediately resuspended in a 50-μl proteasome buffer (containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 0.5 mM EDTA, 2 mM ATP, 1 mM DTT). The pellets were homogenized and centrifuged at 14,000 g for 10 min at 4°C. The protein concentration in the cytosolic extract was subsequently measured by a commercial Bradford Assay (Bio-Rad). Twenty-five micrograms of protein from each sample was loaded on a flat-bottom, black fluorescence 96-well microplate, together with 60 μl of proteasome buffer and 0.125 μg/μl of fluorogenic substrate (Z-Gly-Gly-Leu-AMC, bml-zw8505-0005, ENZO Life Science). As a fluorophore, we used AMC (7- amino-4-methylcoumarin). The temperature in the fluorescent plate reader, an EnSpire Multimode Plate Reader (PerkinElmer), was set to 37°C. The continuous recording of the fluorescence of the product (excitation wavelength of 380 nm and an emission wavelength of 460 nm) was done for 2 h at 37°C by measuring the fluorescence intensity every 5 min. Each of the samples was assayed in technical duplicates, with an additional control spiked with MG132 (10 μM, Calbiochem), a proteasome inhibitor. The measurement was stopped once saturation of the signal was achieved. We then quantified the relative slope of the data (as a fraction of 1) as a readout for proteasome activity.

**Immunoblot.** Cells were lysed in a buffer containing 10 mM Tris and 4% SDS and boiled at 95°C for 10–15 min. Then, debris was spun down by centrifugation at 16,000 g for 20 min. Immunoblots were performed on a 10% SDS gel and blotted semidry. The membrane was blocked with 1× RothiBlock (Roth) for 1 h and probed with 1:1,000 dilutions of a monoclonal Lamp1 antibody (cat. no. 9091; Cell Signaling, Danvers, MA) or a monoclonal Plk1 antibody (Cell signaling catalogue number no. 4513). Membranes were developed using an LI-COR Odyssey imaging system after incubation of membranes with an IRDye 680 (anti rabbit; LI-COR, Lincoln, NE) or IRDye 800 (anti mouse, dilution 1:25,000; LI-COR). Monoclonal antibody [β-tubulin, E7 (13), 1:2,000 dilution] was used for reprobe.

**Immunofluorescence.** Podocytes seeded on cover slips were fixed by 4% PFA, washed with PBS, permeabilized, and blocked with 3% NDS in 0.1% Triton in PBS. Then coverslips were probed with phalloidin (1:100). Nuclei were counterstained with DAPI and mounted with Prolong Gold. Slides were imaged using a Zeiss epifluorescence microscope.

**RESULTS**

The podocyte cell line analyzed here has been generated by isolating primary podocytes from H-2Kb-tsA58 transgenic mice, which harbor a temperature-sensitive large T SV40
antigen under the control of an interferon-γ (IFNγ)-inducible (H-2Kb) promoter (25). At 33°C and in the presence of IFNγ, these cells usually proliferate (“undifferentiated”), and at 37°C in the absence of IFNγ, these cells stop cell cycle, thereby resembling the postmitotic nature of a podocyte (59). In this regard, the cell is similar, but not identical, to other available podocyte cell lines. In this study, we performed analysis of a cell culture model in both “differentiated” (cultured for 14 days at 37°C) and “undifferentiated” (at 33°C) podocytes. With differentiation, cell size increases and morphology is altered (Fig. 1A). Also, filamentous (F-) actin stress fiber formation is increased in the differentiated state (Fig. 1B).

To perform comprehensive analysis of podocyte proteome, we used an in-tip strong cation fractionation method to fractionate minimal protein amounts ([36], Fig. 1C). We were able to quantify 7,240 proteins in independent triplicates and found that there were distinct differences between both sample sets in hierarchical clustering analysis using Euclidean distance (Fig. 1D). The podocyte proteome quantification is compiled in a convenient way to use the database and is appended as Supplemental Data Set S1/Table S1 (also available at https://hpcwebapps.cit.nih.gov/ESBL/Database/PodocyteProteome/index.html). Additional proteins (including one peptide for the ion channel Trpc6) could be identified using another search algorithm, Sequest (Supplemental Table S2). In total, this list contains spectral evidence for 8,817 proteins.

We analyzed the data set by absolute quantification using the intensity-based absolute quantification (iBAQ) parameter (35, 61). The iBAQ parameter estimates protein copy numbers across the dynamic range of the proteome (Fig. 2). This parameter is calculated by summarizing the raw intensity of all identified peptides divided by the number of possible tryptic peptides (61). We found that the dynamic range of protein copy numbers within both undifferentiated (Fig. 2A) and differentiated (Fig. 2B) podocytes was ~7.4 orders of magnitude. The 10 most abundant proteins were mainly cytoskeletal proteins and histones. The proteins were β-actin (Actb), three histone proteins (H2afj, Hist2h2b, Hist1h4a), Gapdh, thymosin β-10 (Tmsb10), tubulin α-1B chain (Tubab1), α-enzolase (Eno1), Tubulin β-4A chain (Tubb4a), and peptidyl-prolyl cis-trans isomerase A, (Ppiu) in undifferentiated cells. The immense magnitude of the dynamic range can be illustrated by the fact that these 10 most abundant proteins comprise ~18% of the cellular protein copy numbers (Fig. 2C). Since stress fibers are used as a readout of podocyte injury particularly in the differentiated condition, we summarized iBAQ values of all proteins belonging to the stress fibers (indicated by GO: actin filaments), to other cytoskeletal proteins (GO: cytoskeleton or cytoskeletal part), Histones and “other proteins”. We observed no significant difference in expression of stress fiber proteins between the differentiated and undifferentiated podocytes (Fig. 2D).

We next used the iBAQ quantification to determine copy numbers across proteins essential for protein homeostasis. Cultured podocytes are used as a tool to study renal genetic disease. Thus, we checked for expression of proteins causing hereditary nephrotic syndrome or FSGS upon mutation [as determined by a recent review (6)]. The analysis revealed that cytoskeletal proteins (Fig. 2, E and F) are highly prevalent proteins within the data set, with Myh9 and Actn4 being very abundant. Eleven out of fifteen cytoskeletal proteins and eight out of eight basement membrane proteins were found. Only three out of six so-called slit membrane proteins [receptor-type tyrosine-protein phosphatase O (Ptpro), PLC-epsilon-1 (Plce), and Cdc2-associated protein (Cdc2ap)] were quantified, Trpc6 was detected with one peptide only, but not quantified (Supplemental Table S2). Nephrin (Nphs1) and podocin (Nphs2) and the podocyte-specific transcription factor WT1 were not identified. Only one nuclear protein associated with renal hereditary disease was quantified, WT1 was not identified. We then examined the data set for the presence of unanticipated signaling systems, which may be potential targets to modulate podocyte signaling (Fig. 2G). Among the expressed and annotated G protein-coupled receptors (GPCRs), we found that the atrial natriuretic peptide (ANP)-receptor type 3 (Gene symbol: Npr3) was most abundantly expressed (Fig. 2G), which is also abundantly expressed on transcript levels in vivo. We also found tyrosine-kinase membrane-coupled receptors, among these small abundances of the insulin receptor (Fig. 2H).

Next, we mined the data set for similarities to the native podocyte proteome (Fig. 3A). Boerries et al. (7) performed a first label-free quantitative proteomic fingerprint of the podocyte. In this study, 116 podocyte-specific proteins were determined by label-free quantification. Eighty-nine of these proteins could be mapped to the current uniprot database (April 2016). Of these 89 proteins, 15 proteins could not be found in the data set obtained here (Table 1). Because deeper native podocyte proteomic analyses are not currently available, we mapped copy numbers (FPKM values) of a published deep transcriptomic map of the native podocyte against the data set of protein copy numbers (27). This comprehensive analysis of the podocyte transcriptome has been generated by deep mapping of the messenger RNA (mRNA) using RNAseq technology and is made publically available under https://hpcwebapps.cit.nih.gov/ESBL/Database/Podocyte_Transcriptome/index.htm (accessed December 2015) (27). We found that 5,877 (80.1%) proteins were mapped against the database, 1,436 did not match any transcript (based on gene symbol, Fig. 3A). We asked whether transcript copy numbers determined by RNAseq would correlate with copy numbers determined by iBAQ in the cell culture model. To this end, copy numbers on protein level (iBAQ) and on transcript level (FPKM) were plotted against each other (Fig. 3, B and C). In fact, a significant correlation between FPKM and iBAQ values was observable [Pearson’s correlation; r = 0.46 for undifferentiated and 0.45 for differentiated podocytes; −log (P) > 14].

To delineate which factors were relatively increased in the transcriptome and which were relatively increased in the proteome, we performed two-dimensional-enrichment analysis (19). The algorithm annotates proteins and genes with their keywords, combines proteins with the same term and places the terms—FDR controlled—in a two-dimensional space to visualize their preferential overrepresentation in either data set. Proximity to a diagonal line (x=y, dashed line) means that the respective term has similar expression both on transcriptome and proteome level. When performing this analysis for the proteome vs. the transcriptome, we found that basement membrane proteins were more highly expressed in the transcriptome compared with the proteome. This can be visualized in the initial scatterplot (Fig. 3, D and E). Threonine proteases (violet) had, for example, high copy numbers in both data sets, and telomere proteins (cyan) had low copy numbers in both data
sets (Fig. 3E). The results of the 2D GO enrichment are depicted in Supplemental Data Set S3 and S4/Table S3 and S4 and can be mined by each reader for the difference in the protein class of interest. In conclusion, comparison of the proteomic data and transcriptomic data set demonstrated agreement between functional protein classes in vivo and in vitro.

We further asked whether high intensity in podocytes can also predict potential novel markers in podocytes. To this end,
we mapped iBAQ values against the data set of glomerular staining in the human protein atlas (22). In fact, we found proteins with a high glomerular staining had, in fact, higher iBAQ values compared with proteins detected with lower staining intensities ($P < 0.01$; two-way ANOVA). When merging both data sets, proteins with high staining intensity in glomeruli and high iBAQ values can be extracted, e.g., galecin-1 (LGALS) and the ion channel anoctamin 10 (ANO10). In conclusion, similarities between native podocyte proteome and cultured podocyte proteome do clearly exist, but the cell culture line lacks the expression of several proteins and genes found in vivo.

To find the most abundant changes between podocytes in the differentiatied and undifferentiated state, we performed label-free quantitative proteomic analysis (Fig. 4) and found that 116 proteins were highly significantly increased in differentiated cells compared with undifferentiated cells, and 225 proteins were decreased in differentiated cells compared with undifferentiated cells ($FDR = 0.01$, $s0 = 1$, Fig. 4A). We performed GO enrichment analysis of the increased or decreased protein population compared with the unchanged protein population (Fishers exact test, $FDR < 0.02$, Fig. 4, B and C). The analysis revealed that undifferentiated cells had a strong enrichment for cell cycle proteins (as demonstrated by the GO terms mitotic cell cycle, cell division) (Fig. 4B). The proteins increased in differentiated cells, however, exhibited a strong and significant enrichment for lysosome-related proteins (as demonstrated by the GO terms lysosome, catabolic processes, and hydrolase activity), but also for adhesion proteins (Fig. 4C).

We then followed up on this data functionally and checked for the expression of proteins belonging to different mechanisms involved in protein homeostasis (proteostasis) (Fig. 5). Proteins can be either degraded by a lysosome or the proteasome, both of which were covered with a significant protein number. Mapping proteins related to lysosome (magenta) and proteasome (cyan) on the volcano plot quantification suggested a substantial shift in expression of both proteasomes and lysosomes during differentiation (Fig. 5A). Cumulative histograms of proteasomal and lysosomal proteins compared with all other proteins are depicted in Fig. 5, B and C, and the distribution of proteins of both classes were significantly different to the other proteins (two-way Kolmogorov-Smirnov test, $P < 0.01$; Fig. 5, B and C). To validate this functionally, we conducted a proteasome assay by measuring chymotrypsin-like activity of podocytes in both differentiated and undifferentiated cells. The assay is based on cleavage of fluorogenic peptide substrate (32). Proteasome activity as indicated by the slope of the increase of florescence was significantly lower in differentiated podocytes compared with undifferentiated podocytes (an exemplary depiction of the slope can be seen in Fig. 5D). In both cases, proteasome activity could be nearly completely blocked by the addition of MG132 (10 μM) to the lysate prior to the measurement (Fig. 5E). To validate key findings of the analysis, we performed immunoblot analysis of lysates of a second, human podocyte cell line generated by Saleem et al. (59). The results showed that the expression of lysosomal marker lysosome-associated membrane glycoprotein 1 (Lamp1) was increased in differentiated cells, and the cell cycle kinase Polo-like kinase (Pkl1) was markedly decreased, consistent with findings from the previous data set (Fig. 5F). Both findings are consistent with an increase of lysosome abundance and a decrease in cell cycle activation in the differentiated cell line. Proteasome activity assays confirmed that proteasome activity was decreased in the differentiated human podocytes (Fig. 5G).

To gain initial insights into podocyte posttranslational modifications, we performed an initial phosphoproteomic analysis of the mouse cell line. To this end, we profiled the phosphoproteome of differentiated and undifferentiated podocytes. Of the 4,152 identified phosphorylation sites, we confidently detected 3,534 with MS1 intensities, among these 2,251 in both samples; 280 of the 2,251 sites were previously undescribed in the phosphosite.org database (26). The data are compiled in a user-friendly format to a cPODOPHOS database. This is appended as Supplemental Data Set S5/Table S5. This database will be made online at https://hpcwebapps.cit.nih.gov/ESBL/Database/PodocytePhosphoproteome/index.htm. The raw data and quantification data are deposited also in the PRIDE/ProteomeExchange database (see METHODS for details). Phosphorylation sites on four proteins involved in hereditary nephrotic syndrome were found, among these phosphorylation sites on Cd2-associated protein (Cd2ap) (five residues; positions S188/T189, S193, S510, S514, S458; uniprot accession: Q9JLQ0; S188 or T189 cannot be distinguished), Integrin α-3 (Itga3) (3 residues positions S1044, T1050, Y1053; uniprot Q62470), Myosin-9 (Myh9) (2 residues, T1943, S1939, uniprot Q8VDD5) and synaptopodin (Synpo) (31 residues, uniprot E9Q3E2 and Q8CC35). Several of the sites confirmed recent phosphoproteomic findings in vivo, e.g., the high abundance of proline-directed synaptopodin phosphorylation (56, 57).

Phosphorylation sites can be bioinformatically organized according to their phosphorylation motif, which determines the kinase class involved in phosphorylating these residues (41). Proline-directed motifs (e.g., $S\times P$, with a $P$ at the +1 position) are typical substrates of MAP-kinases and cyclin-dependent kinases (41). Basophilic motifs (e.g., $R\times X\times S^*$, with a $R$ or $K$ at the −3 position) are typical substrates of AGC kinases, e.g.,

Fig. 2. A: absolute quantification of podocyte proteins. Rank of decadic logarithm of iBAQ values indicative of podocyte protein copy numbers in undifferentiated podocytes is depicted against the rank of each protein. The sigmoid plot demonstrates the dynamic range of the undifferentiated podocyte proteome (−7.4 orders of magnitude). B: similar analysis was performed for differentiated proteins. C: illustration of the order of magnitude. The bar graph represents absolute numbers of the 10 most abundant proteins (10/7,313 = 0.14%) and the representation of absolute copy numbers (−18% of the protein mass) within the cell. D: absolute quantification of histone proteins, actin stress fiber proteins, other cytoskeletal proteins, and other proteins and their contribution to total podocyte protein mass. There was no difference in the sums of each category between differentiated and undifferentiated podocytes. E: distribution of proteins causing podocyte disease (hereditary nephrotic syndrome and focal segmental glomerulosclerosis) within the dynamic range of the undifferentiated podocyte proteome. The proteins essential for podocyte homeostasis span the entire order of magnitude of the proteome. The colors indicate the compartments as represented by Ref. 6. F: analysis of the differentiated podocyte proteome provides a similar result. G: iBAQ values of proteins with Gene Ontology (GO) term “G-protein coupled receptor activity” (GO:0004930) in both differentiated and undifferentiated conditions. Small G proteins (e.g., the inhibitory G protein Gna1) were removed from the list. H: iBAQ values of proteins with GO term transmembrane tyrosine receptor signaling activity (GO:0004714).
PKA or G (41). Motif analysis of both data sets using a binary decision tree (66) determined no vastly different representation of phosphorylation motifs within both samples, although proline-directed motifs were more common in the undifferentiated sample at the expense of basophilic motifs (Fig. 6B). An initial comparison using unsupervised hierarchical clustering of normalized MS1 intensities between both profiles demonstrated three clusters with markedly different intensities between both data sets: two clusters of proteins demonstrated high intensity in differentiated cells (clusters 1 and 2), and one cluster of proteins showed low intensity in differentiated cells (cluster 3) (Fig. 6C). Analysis of the phosphorylation motifs of these sites revealed that phosphorylation sites with high intensity in differentiated cells (clusters 1 and 2) mainly consisted of both proline-directed sites and basophilic sites (R-X-X-S/T-P, marked with arrows) (Fig. 6D). In contrast, downregulated motifs (cluster 3) showed almost exclusively proline-directed sites [S-P, +1 and -3 positions marked with arrows (Fig. 6D)]. GO enrichment analysis of the clusters increased in phosphory-
Table 1. Fifteen podocyte-specific proteins only found in native podocyte, identified in the data set by Boerries et al.

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Entry</th>
<th>Protein Names</th>
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<td>Clic3</td>
<td>Q9DJP7</td>
<td>Chloride intracellular channel protein 3</td>
</tr>
<tr>
<td>Coro2b</td>
<td>Q8BH44</td>
<td>Corinon-2B</td>
</tr>
<tr>
<td>Npl</td>
<td>Q9DCJ9</td>
<td>N-acetylneuraminate lyase</td>
</tr>
<tr>
<td>Marc2</td>
<td>Q922Q1</td>
<td>Mitochondrial amidoxime reducing component 2</td>
</tr>
</tbody>
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Fifteen podocyte-specific proteins only found in native podocyte, but not in the current data set. Boerries et al. (7) defined a set of 116 podocyte-specific proteins by proteomic label-free quantification. Of these, 89 could be mapped to a current uniprot ID. The data set by Boerries et al. (7) did not contain the bonafide podocyte markers, nephrin, and WT1.

DISCUSSION

Podocytes, like any other cell type, are best studied in vivo. Upon genetic or pharmacological injury, however, podocytes are oftentimes first reacting with a morphological change, effacement, which involves simplification of their complicated architecture and initiates proteinuria (10). Then, podocytes detach from their basement membrane and get washed away by the urine, a process that is leading to further increase of proteinuria (53). In many cases, it is indiscernible whether proteinuria observed in the glomerulus also demonstrated a significantly increased iBAQ value compared with “medium,” “low,” or “nondetected” proteins. Center lines show the medians; box limits indicate the 25th and 75th percentiles, as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots.

A variety of podocyte cell lines have been generated in the past (25, 42, 46, 59, 60, 62). They still serve as model for some aspects of podocyte function due to their expression of podocyte-specific proteins. For example, high-throughput screens have analyzed cytoskeletal, drug-induced rearrangements that helped to identify potential drug targets in podocyte disease (37). Analysis of leading-edge dynamics revealed significant insights into foot process dynamics (23). Cell culture studies can also be used to investigate signal transduction (21). Immortalized podocyte cell cultures, however, also do have obvious significant disadvantages compared with the native podocyte. They do not form a functional filtration slit. This may be related to the fact that they express only very low numbers of nephrin and podocin (in fact, none of these “hallmark podocyte proteins” were identified in the data set). These proteins, however, can be easily detected in very high abundances, also by mass spectrometry, from native podocytes (reviewed in Ref. 57). Immortalized podocytes grown in cell culture may have additional limitations, but these only can be elucidated by an equally resolved native podocyte proteome (the current native podocyte database is somewhat limited and only comprises a bit more than 100 podocyte-specific proteins (Fig. 3)).

Here, we performed a deep quantitative proteomic analysis to dissolve specificities of each of the differentiated and undifferentiated state. First, the dynamic range of the podocyte proteome is similar to other described cell culture lines (24, 44). However, we demonstrate that the podocyte cell line analyzed maintains some of the unique features of the podocytes. For instance, it expresses a majority of those proteins that are causing genetic podocyte disease upon mutation, with good coverage of cytoskeletal proteins and complete coverage of proteins involved in maintaining the basement membrane. This is consistent with the finding that composition of basement membrane can be efficiently and comprehensively studied in vitro (11). On a global level, a surprisingly good correlation of 5,877 proteins with its respective mRNA levels could be observed. With an R of 0.46 (undifferentiated) or 0.45 (differentiated), the correlation is within the range of mRNA and protein copy number correlation in large-scale data sets, even within one single biological sample (69). Two-dimensional GO-enrichment analyses revealed that the proteome has rather low levels of protein copies related to “basement mem-
brane”, an artifact probably caused by harvesting the cells and removing them from their matrix. Further strong differences can be found in metabolic pathways (Fig. 3, C and D). Interestingly, proteomic and transcriptomic data agreed on high copy numbers of “threonine proteases”, a protein class which is important for podocyte stress response (72), but the intracellular protease network is not entirely characterized (33). By taking advantage of a repository containing immunostainings for more than 80% of human genes (22), we could show that proteins with a “high” glomerular staining have, in
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...ing of native glomeruli (56, 57). This study reveals the mechanism at the renal filtration barrier using phosphoproteomic (Fig. 6).

We previously discovered unanticipated signaling mechanism at the renal filtration barrier using phosphoproteomic analysis of native glomeruli (56, 57). This study reveals the presence of several signaling machineries, which are not clearly characterized, and suggests that cultured podocytes could be utilized as a model for these. It has to be stressed that the presence of these proteins does not prove a functional, physiological signaling cascade downstream of the receptor. For some proteins, however, a further elucidation would be of interest. Npr3, for example is an ANP receptor, which is abundantly expressed in podocytes (surprisingly, it is in fact, based on stoichiometry of the GPCR-subclass, the most abundantly expressed receptor in cultured podocytes, Fig. 3, G and H). The impact of ANP signaling on podocytes is studied in vitro (38), but not in vivo, but the receptor is expressed also in vivo on mRNA level and protein level as shown by this and previous studies (29). ANP is elevated in congestive heart failure (9, 12). Thus, it is tempting to speculate that signaling through ANP—through podocytes—may be a component of the physiological “heart-kidney-axis” and could contribute to the onset of albuminuria in CHF patients (9).

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Fig. 6. Comparative phosphoproteomic analysis of undifferentiated and differentiated mouse podocytes. A: comparison of phosphorylation sites identified in undifferentiated and differentiated podocytes. B: motif analysis of phosphorylation sites identified in undifferentiated and differentiated podocytes using a binary decision tree (66). The numbers indicate % of total sites. C: clustering analysis of intensities of 2,251 phosphorylation sites detected in both differentiated and undifferentiated podocytes. Five clusters were found with a difference in expression of more than seven log2 steps. Clustering was performed using Euclidean distance to cluster rows (proteins). D: motif analysis of 2,251 phosphorylation sites for each individual cluster was performed using motif logo software (icelogo). The position-weighted matrices demonstrate the occurrence and statistical overrepresentation of residues surrounding the phosphorylation site (in the center of the matrix). The phosphorylation clusters increased in differentiated podocytes (clusters 1 and 2) contained also basophilic phosphorylation sites and proline-directed sites (R at the -3 position, P at the +1 position, motif R-X-X-S-P, marked with arrows). The phosphorylation site cluster 3 (decreased in differentiated podocytes) contained a strong abundance of proline-directed phosphorylation sites (P at the +1 position, motif: SP, marked with an arrow and an asterisk). E: GO-term enrichment analysis of clusters 1, 2, and 3. GO term enrichment for each cluster was performed compared with all of the other clusters, and relative enrichment was performed. The fold enrichment compared with all other clusters is depicted. In clusters 1 and 2 (decreased in differentiated podocytes) phosphorylation sites on stress fiber and actin filament proteins are overrepresented. In cluster 3 (decreased in the differentiated podocyte) cell cycle proteins are overrepresented. All GO terms depicted in this panel are highly and significantly overrepresented compared with all other proteins in the remaining clusters [FDR controlled (FDR < 0.02), Fisher’s exact test].
kinase activity at the membrane were discovered. Some of them, such as the insulin and Igf receptor, are extensively studied in vivo and in vitro and are very important in the development of diabetic nephropathy (reviewed in Ref. 15, 16). The role of other tyrosin kinase receptors is currently unclear. For example, the druggable cancer-associated tyrosine receptor kinase Axl and Epha are highly expressed in cultured podocytes (2, 47). This is interesting given the prominent role for nephrin tyrosine signaling in podocytes (45).

On a general, cellular physiological level, this study analyzed the global effect of the expression of the temperature sensitive SV 40 large T antigen, a commonly used cell culture method. As expected, cell cycle-related protein expression (Fig. 4)—and presumably phosphorylation (Fig. 6)—was largely decreased once the cells stopped proliferation (in the differentiated condition). The most striking difference between these states was that cultured podocytes have a stronger expression of lysosomal proteins, compared with undifferentiated podocytes. Conversely, proliferating podocytes express increased abundance of proteasomal proteins and show a functional increase in proteasome activity and expression of proteasomes. This is consistent with the fact that cell cycle progression requires proteasome activity (48, 75). Presumably, there are thus differences in differentiated and undifferentiated cells regarding the degradation mechanisms of proteins. For only a few podocyte proteins, however, the precise degradation mechanism is known (55). Upon podocyte injury, the expression of ubiquitin and presumably the ubiquitin-proteasome activity increases (40)—this network needs further investigation.

In summary, the data presented here form a resource for the means and the rationale for large-scale proteomic and phosphoproteomic analysis of other cell lines and native mouse podocytes in a similar fashion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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human podocyte cell line demonstrating nephrin and podocin expression. 


