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RESEARCH ARTICLE

An in vitro approach to understand contribution of kidney cells to human urinary extracellular vesicles

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Abstract

Extracellular vesicles (EV) are membranous particles secreted by all cells and found in body fluids. Established EV contents include a variety of RNA species, proteins, lipids and metabolites that are considered to reflect the physiological status of their parental cells. However, to date, little is known about cell-type enriched EV cargo in complex EV mixtures, especially in urine. To test whether EV secretion from distinct human kidney cells in culture differ and can recapitulate findings in normal urine, we comprehensively analysed EV components, (particularly miRNAs, long RNAs and protein) from conditionally immortalised human kidney cell lines (podocyte, glomerular endothelial, mesangial and proximal tubular cells) and compared to EV secreted in human urine. EV from cell culture media derived from immortalised kidney cells were isolated by hydrostatic filtration dialysis (HFD) and characterised by electron microscopy (EM), nanoparticle tracking analysis (NTA) and Western blotting (WB). RNA was isolated from EV and subjected to miRNA and RNA sequencing and proteins were profiled by tandem mass tag proteomics. Representative sets of EV miRNAs, RNAs and proteins were detected in each cell type and compared to human urinary EV isolates (uEV), EV cargo database, kidney biopsy bulk RNA sequencing and proteomics, and single-cell transcriptomics. This revealed that a high proportion of the in vitro EV signatures were also found in in vivo datasets. Thus, highlighting the robustness of our in vitro model and showing that this approach enables the dissection of cell type specific EV cargo in biofluids and the potential identification of cell-type specific EV biomarkers of kidney disease.

KEYWORDS
exosomes, extracellular vesicles, glomerular endothelial cells, kidney, mesangial cells, miRNA, podocytes, proteomics, proximal tubule cells, RNA

1 | INTRODUCTION

Extracellular Vesicles (EV) are cell-derived small membranous particles involved in cell-to-cell communication (Yáñez-Mó et al., 2015). As EV often retain protein, lipids and nucleic acids from their cell types of origin, they can reflect the underlying pathophysiological state of those cells (Barreiro & Holthofer, 2017; Iraci et al., 2016; Yuana et al., 2013) and since they are present in

body fluids, they are considered a promising source of non-invasive biomarkers for numerous diseases (Dickhout & Koenen, 2018; Liang et al., 2021; Pang et al., 2020; Xu et al., 2020).

EV in the urine has gained particular interest as non-invasive biomarkers of kidney disease. They are secreted by (and contain molecules from) all cell types constituting the nephron (Erdbrügger & Le, 2016). Numerous studies have begun to characterise uEV in kidney health and in disease (Braun et al., 2020; Ghai et al., 2018; Sonoda et al., 2019), although the methodologies to isolate and measure EV have been limited (Barreiro & Holthofer, 2017; Merchant et al., 2017).

Notably, due to the complexity and heterogeneity of uEV, it can be difficult to define the origin of the parental cells. As such, the profiles of uEV secreted by individual kidney cell types, and how their content directly reflects cellular protein/nucleic acid content, are not yet understood. Such knowledge would not only help to understand injury sites and target the cell for biological intervention but also design useful in vitro model systems. Some attempts have been made to identify cell (Connor et al., 2020; Müller-Deile et al., 2019) and tissue (Baker et al., 2017; Bazzell et al., 2018; Habuka et al., 2014) enriched miRNAs, mRNA or proteins and their contributions to biofluids composition (Cui & Cui, 2020; Hulstaert et al., 2020). Only recently, the contribution of cell and tissue derived EV to biofluids (plasma) was explored by gene expression signatures and deconvolution (Li et al., 2020). However, to date little is known about cell-type enriched EV cargo in urine.

Conditionally-immortalised kidney cell culture models have provided useful tools in studying molecular responses of individual kidney cell types (Audzeyenka et al., 2021; Grigorieva et al., 2019; Lau et al., 2012; Lay et al., 2017; Mutsaers et al., 2013; Ramnath & Satchell, 2020; Ramnath et al., 2014; Saleem et al., 2002; Wright et al., 2019) . Furthermore, the ease in which cell culture models can be 'up-scaled' to provide sufficient starting material when isolating EV and studying their cargo has obvious benefits.

Using our recently characterised EV isolation workflows (Barreiro et al., 2020), we here characterised the EV cargo from conditionally-immortalised human kidney cell lines to provide a clearer understanding of the composition and biology of EV secreted by distinct kidney cell types. We further cross-compare our findings from human cell lines with those from human uEV and kidney biopsies to further establish the utility of these models in studying human uEV signatures.

2 | MATERIALS AND METHODS

The experimental workflow is shown in Figure 1.

2.1 | Cell culture

Conditionally-immortalised human cell lines were grown to 70% confluence at 33°C with 5% CO_2 before thermo-switching to 37°C in 5% CO_2, allowing them to differentiate for 9–12 days. Conditionally immortalised human podocytes (POD) (Saleem et al., 2002) and mesangial cells (MC) (Sarrab et al., 2011) were maintained in RPMI-1640 containing L-glutamine and NaHCO3, supplemented with 10% or 20% FBS, respectively (Gibco). Conditionally immortalised human glomerular endothelial cells (GEC), (Satchell et al., 2006) were maintained in Endothelial Cell Growth Medium-2, containing microvascular SingleQuots Supplement Pack in 5% FBS (Lonza). Conditionally immortalised human proximal tubular cells (PTC) (Wilmer et al., 2010) were maintained in DMEM-HAM F-12 (Lonza) containing 36 ng/ml hydrocortisone (Sigma), 10 ng/ml EGF (Sigma) and 40 pg/ml Tri-iodothyronine (Sigma) and 10% FBS. Prior to study all cells were washed and incubated with 50 ml exosome-depleted FBS (Gibco) for 24 h. Conditioned media was centrifuged for 10 min at 2000 *g* to remove cell debris and stored at –80 before EV harvest.

2.2 | Extracellular vesicles isolation

Cell free culture media (CCM) samples were incubated at 37°C in a water bath and vortexed for 30 s. EV isolation by hydrostatic filtration dialysis was performed as described previously with some modifications (Barreiro et al., 2020; Musante et al., 2014). Briefly, 50 ml of CCM was poured into a cellulose ester (CE) dialysis membrane made with molecular weight cut-off (MWCO) 1000 kDa (Repligen Corp., Waltham, MA). The sample was first concentrated to 5–6 ml. Thereafter, the membrane was refilled with 100 ml of deionised water to wash remaining analytes below the MWCO. When the sample inside the membrane reached about 1 ml, it was collected in protein or DNA LowBind tubes (Eppendorf, Hamburg, Germany) and stored at –80°C. The HFD device was connected to a vacuum pump at a constant pressure of –20 kPa.

2.3 | Nanoparticle tracking analysis (NTA)

NTA was used to assess the concentration and size distribution of the isolated EV (Gardiner et al., 2013). EV samples were analysed using Nanosight model LM14 (Malvern Instruments Ltd, Malvern, UK) equipped with blue (404 nm, 70 mW) laser

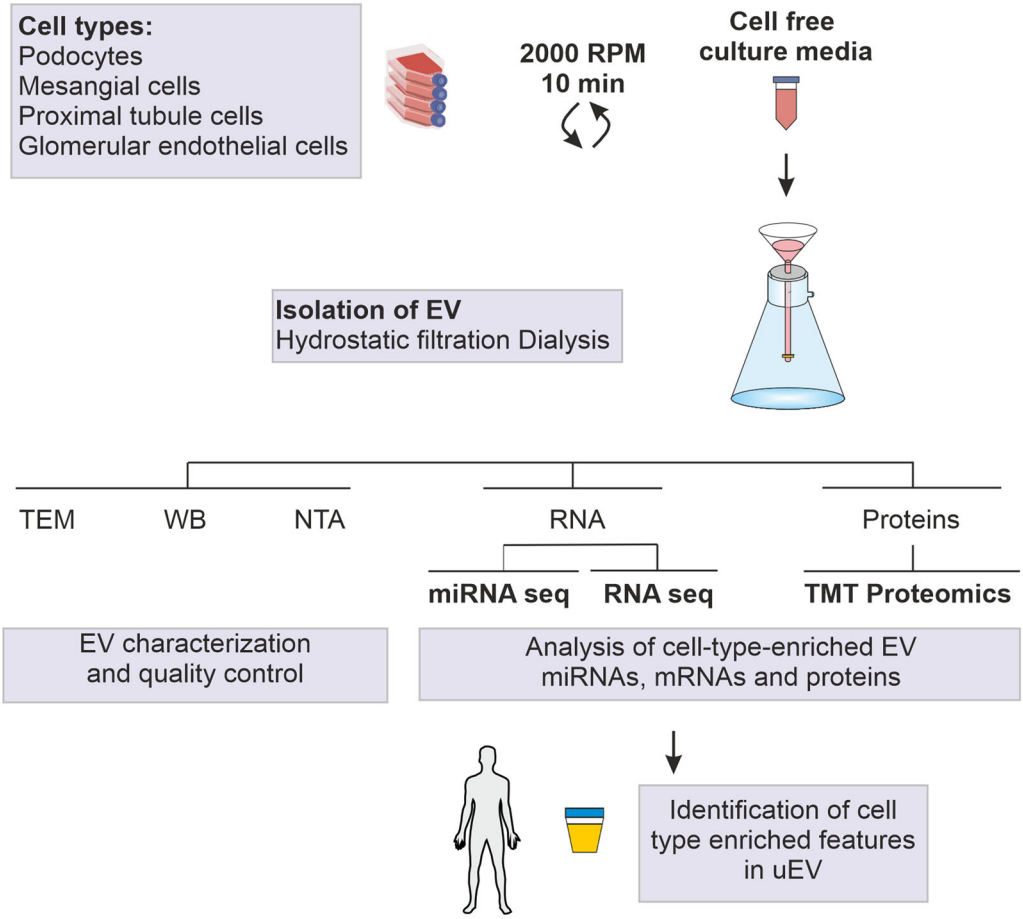

FIGURE 1 Experimental workflow: EV were isolated from 50 ml of cell free culture media by hydrostatic filtration dialysis. Vesicles were characterised by TEM, WB and NTA. Isolated total RNA was subjected to miRNA- and RNA-sequencing ($n = 5$ per cell type). Isolated proteins were subjected to quantitative proteomics (TMT approach). Omics dataset were used to define cell-type-enriched EV features. Lists of enriched features were tested in human uEV datasets. EV, extracellular vesicles; miRNA-seq, miRNA sequencing; NTA, nanoparticle tracking analysis; RNA-seq, RNA sequencing; TMT, tandem mass tag; TEM, transmission electron microscopy; uEV, urinary extracellular vesicles; WB, Western blotting

and SCMOS camera (Hamamatsu photonics K.K., Hamamatsu, Japan). To obtain 40–100 particles/view samples were diluted in 0.1 μm filtered (Millex VV, Millipore) DPBS. Five 30-s videos were recorded using camera level 14 and analysed with NTA software 3.0 (Malvern Instruments Ltd) with the detection threshold 5 and screen gain at 10.

2.4 | Electron microscopy

Negative staining was performed as previously described (Barreiro et al., 2020; Puhka et al., 2017). Briefly, 5 μl of sample (equivalent to 0.5–1 ml of CCM) was loaded on Formvar/Carbon 200 mesh TH, Copper grids (Ted Pella Inc., Redding, CA), fixed with 2% PFA (Electron Microscopy Sciences, Hatfield, PA), negatively stained with 2% neutral uranyl acetate and embedded in methyl cellulose uranyl acetate mixture (1.8/0.4%). Images were viewed using Jeol JEM-1400 (Jeol Ltd, Tokyo, Japan) operating at 80 kV.

2.5 | Sample concentration and protein quantification

EV samples to be used for negative staining and Western blotting were 4X concentrated using Amicon Ultra-0.5 ml Centrifugal Filters 10k (Merck Millipore) following manufacturer's instructions.

Protein concentration was measured as previously described (Barreiro et al., 2020) using Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2.6 | Western blotting

Western blotting was performed as described previously (Barreiro et al., 2020). Briefly, 20 ug of EV samples were mixed with 5X Laemmli buffer, denatured, and loaded to 4%–20% Mini-PROTEAN® TGX Stain-Free Protein Gels (Bio-Rad laboratories Inc., Hercules, CA). After transference, membranes were blocked and incubated with primary antibodies (TSG-101, Santa Cruz Biotechnology SC-7964, dilution 1:200; CD63 MEM, ExBio 11-343-C100, dilution 1:500; CD81, Santa Cruz Biotechnology SC-166029, dilution 1:200) overnight at +4°C. Afterwards, membranes were incubated with secondary antibody. Secondary antibody (Rabbit anti-mouse, HRP, Jackson Immuno Research 315-035-045, dilution 1:6000) was detected using Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) and ChemiDoc Imager system (Bio-Rad laboratories Inc.).

2.7 | RNA isolation

Total RNA from 400 ul of EV samples (equivalent to 20 ml of CCM) was isolated using TRIzol LS (Life Technologies Corp., Carlsbad, CA) following manufacturer's instructions. After phase separation, we used miRNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol for Purification of Total RNA, Including Small RNAs, from Animal Cells. RNA was eluted in 30 μl of RNase free water. RNA concentration and profile were analyzed using Agilent RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA).

2.8 | RNA sequencing

The RNA cDNA libraries were prepared from 2 ng of total RNA using SMART-Seq® stranded kit (Takara BIo Inc., Mountain View, CA).

miRNA libraries were prepared using the QIAseq miRNA Library Kit (Qiagen) following the manufacturer's protocol, starting with ~1 ng of input RNA for each sample. Quality control and concentrations of individual libraries were assessed with Bioanalyzer 2100 Instrument and High Sensitivity DNA Kit (Agilent Technologies).

miRNA and RNA sequencing was performed on the Illumina HiSeq 4000 platform (Illumina Inc.) as 85 bp cycle single end read.

2.9 | RNAseq data analysis

Illumina reads from mRNA were converted to the industry standard FASTQ format and aligned to the human reference genome from Ensembl 84 using the STAR v2.5.2a aligner on default settings (Dobin et al., 2013). For increased alignment accuracy, the STAR genome index was generated to include splice junction annotations with the options '–sjdbGTFfile' and '–sjdbOverhang 49'. The quality of the RNA-Seq samples was verified with FASTQC version 0.11.2 and RNASeQC version 1.18 (DeLuca et al., 2012). The featureCounts tool of the subRead package version 1.5.0 (Liao et al, 2014) was used to quantify the per-gene read counts.

For the miRNA, adapter sequences were trimmed via CutAdapt v.1.16 and UMI reads were collapsed with a customised script. The trimmed reads were mapped against the human reference genome hg19 using STAR version 2.5.2a. Reads 16–26 nucleotides in length were filtered (samtools v0.1.18) to quantify mature miRNAs and reads mapping to the miRNAs annotated in miRBase release 20 were counted applying featureCounts v.1.6.2.

Filtering and normalisation: Protein-coding genes from the RNA-seq data were studied. For noise filtering, we kept only genes with log2 cpm (counts per million) $\geq$5 from at least one library in the analysis. The RNA-seq read count data were normalised with the TMM (trimmed mean of M-values) method from the edgeR R package (v3.22.5) (Robinson & Oshlack, 2010) and transformed to log2 cpm with the voom method from the limma R package (v3.38.3) (Law et al., 2014). For miRNA, we considered those with non-empty mapping in at least one library. The miRNA-seq log2 count data were normalised with the cyclic loess method also from limma.

2.10 | Tandem mass tag proteomics

EV secreted by a matched number of cells (95,000) was evaporated to dryness for each sample. As cell type and culture conditions can affect, for example, cell growth and number of EV secreted and currently no standardised guidelines on EV proteomics normalisation are in place, we considered that the best approach to answer our research question was to isolate proteins from

EV secreted by same number of cells (instead of doing the isolation from same amount of proteins or same amount of EV). Each pellet was resuspended in 50 μl RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) plus proteinase inhibitors (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich). The samples were centrifuged at 14,000 *g* for 15 min and the supernatant removed. The protein concentration was measured with Bradfords reagent (Bio-Rad, UK) using 10 μl of sample as per the manufacturer's instructions.

Isolated proteins were denatured, reduced, alkylated and tryptic digested following manufacturer's instructions.

For peptide labelling, 41 μl anhydrous acetonitrile was added to room temp TMT reagents, they were left to dissolve for 5 min with occasional vortexing, the tube was centrifuged to collect the label then 41 μl added to each sample. Samples were incubated for 1 h at room temperature. A total of 8 μl of 5% hydroxylamine was added to quench the labelling reaction and incubated for 15 min at room temperature. For each experiment all samples were combined and evaporated to dryness, resuspended in 5% formic acid and then desalted using a SepPak cartridge according to the manufacturer's instructions (Waters, Milford, MA). Eluate from the SepPak cartridge was again evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide, pH 10) prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 liquid chromatography system (Thermo Scientific). In brief, the sample was loaded onto an XBridge BEH C18 Column (130Å, 3.5 μm, 2.1 mm × 150 mm, Waters, UK) in buffer A and peptides eluted with an increasing gradient of buffer B (20 mM Ammonium Hydroxide in acetonitrile, pH 10) from 0%–95% over 60 min. The resulting fractions (15) were evaporated to dryness and resuspended in 1% formic acid prior to analysis by nano-LC MSMS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

2.11 | Proteomics data analysis

The raw data files were processed and quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt Human database (downloaded September 2018: 152,927 entries) using the SEQUEST HT algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.9949) as a variable modification and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT mass tag (+229.163) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 2 missed cleavages were allowed. The reverse database search option was enabled and all protein data was filtered to satisfy false discovery rate (FDR) of 5%.

The proteins with missing measurement in each EV preparation were ignored. The obtained proteomics scaled abundance data in log2 were normalised with the quantile method from the limma R package.

2.12 | Characterisation of cell-type-enriched EV features

A variant of the one-way analysis of variance (ANOVA) method was used to characterise miRNAs, mRNAs and proteins enriched in the four EV preparations. We exploited the oneway.test function in the stats R package (v3.5.1) (Welch, 1951), which tested whether multiple samples from normal distributions had the same mean value without assumption of equal variances between samples. Each sample contains measurements (normalised counts or abundances) of each mRNA, miRNA or protein in the corresponding quintuplicates for RNA-seq and miRNA-seq, or triplicates for proteomics from each EV preparation. It is noteworthy that only less than 5% of the features violated the assumption of normality with the Shapiro–Wilk test (Royston, 1982) despite the limited sample sizes. Pairwise *t*-tests were also performed to test for significant difference between all the pairs of samples, yielding *p*-values that are subsequently corrected for multiple testing (FDR) (Benjamini & Hochberg, 1995). Each sample is associated to the maximum FDR against the remaining samples. A log2 fold-change of each feature in one EV is also computed as the log2 ratio of average measurement in the corresponding EV against the average over all the others. A feature is considered as enriched in one EV preparation if it satisfies (i) log2 fold-change is positive; (ii) oneway.test *p*-value is less than 0.05 and (iii) only the corresponding cell type has FDR <0.05.

2.13 | Comparison of cell-type-enriched EV features to previous studies and uEV datasets

Datasets used to compare the cell-type-enriched EV features to human kidney expression data and to assess their presence in human uEV are detailed in Table S1. Gene/protein functions in Tables 2 and 3 were retrieved from Uniprot (UniProtConsortium, 2021) (https://www.uniprot.org/) and Humanmine (Kalderimis et al., 2014; Smith et al., 2012) (https://www.humanmine.org/humanmine) (both accessed on 20.05.21). Opentargets (Koscielny et al., 2017; Ochoa et al., 2021) (https://www.opentargets.org/) (accession date: 25.05.21), and Ingenuity pathway analysis (IPA) knowledge base (Qiagen) (accession date: 17.05.21) were used to retrieve genes associated with kidney disease. Vesiclepedia (Kalra et al., 2012) (http://microvesicles.org/) was used to retrieve miRNAs, mRNAs and proteins that had been reported as part of EV preparations (accession date 23.04.21). Bulk miRNA sequencing

data from kidney was obtained from miRNATissueAtlas2 (Keller et al., 2022) (https://ccb-web.cs.uni-saarland.de/tissueatlas2) (accession date: 06.05.22).

2.14 | Data availability

RNA and miRNA sequencing raw data as well as TMT proteomics data are deposited on the NCBI BioProject PRJNA905899.

3 | RESULTS

3.1 | Characterisation and quality control of kidney cell line EV preparations

EV isolated by hydrostatic filtration dialysis were characterised by EM, NTA and WB.

EM negative staining revealed typical round and collapsed membrane bounded vesicles of various sizes and with diverse electron density content in all kidney cell types (Figure 2a). NTA profiles demonstrated enrichment of EV between 100 and 300 nm (Figure 2b). Particle mean size and concentration did not differ significantly between cell types (Figure 2c,d). Notably, EV protein markers (TSG101, CD63 and CD81) were present in all isolated EV preparations (Figure 2e). Podocyte (POD), proximal tubule cell (PTC) and MC isolates had an RNA peak between 25 and 200 nt while GEC isolates presented a peak between 25 and 1000 nt (Figure 2f). EV RNA biotype distribution showed variability between cell types, that is, POD isolates had high content of miscellaneous RNA, PTC isolates contained more miRNAs compared to the other cell types, MC isolates had more mitochondrial and protein coding RNA while GEC isolates had higher content of sense intronic, processed transcript and long intergenic non-coding RNA (Figure S1).

3.2 | Kidney cell enriched EV miRNAs, RNAs and proteins

We next investigated the EV features which were enriched in each kidney cell type. Our Principal component analysis (PCA) from each of the three omics data sets in the different cell types revealed striking variation between EV cargo derived from GEC vs the other three cell types (Figure 3). This variation caused a bias towards GEC derived EV when studying enriched features in each EV isolate. Therefore, parallel comparative analysis, without GEC derived EV, to identify enriched features in each of the three other cell type derived EV, were performed and cell-type enriched EV feature lists were generated for each analysis (i.e., including and excluding GEC-EV (Figure S1).

A total of 16, 26, 21 and 70 enriched EV miRNAs (Tables S2 and S3; Figure S1), 80, 391, 218 and 132 enriched EV mRNAs (Tables S4 and S5; Figure S1) and 13, 8, 4 and 15 enriched EV proteins (Figure 6, Tables S6 and S7; Figure S2) were identified in POD, PTC, MC and GEC isolates, respectively. For the next sections, we refer to podocytes, proximal tubule cells, mesangial cell and glomerular endothelial cell EV-enriched features as POD-EV, PTC-EV and MC-EV and GEC-EV plus the feature we analysed, that is, podocyte EV enriched miRNAs are referred as POD-EV miRNAs. When we refer to cell-type-enriched features but without specifying the cell type, we use EV plus the feature we analyse, that is, cell-type-enriched EV miRNAs are referred as EV-miRNAs.

3.3 | Pathway enrichment analysis

To identify important biological pathways in the different cell type enriched EV-features, gene-set enrichment analysis (GSEA) was performed. Pathways were considered enriched if p-value <0.05 and q-value <0.1 in at least two omics analysis in any cell type.

Enrichment analysis of Gene Ontology (GO) biological processes showed that POD-EV miRNAs were enriched in cell growth and maintenance and a set of miRNAs and mRNAs were associated with cell adhesion (Figure 4). PTC-EV miRNAs and mRNAs were enriched in epithelial to mesenchymal transition, response to stress, signal transduction, cell growth and maintenance. A set of PTC-EV proteins were associated with cell growth and maintenance and cell adhesion (Figure 4). MC-EV miRNAs were enriched in biological processes related to epithelial to mesenchymal transition and responses to stress and signal transduction (also mRNAs) (Figure 4). GEC-EV miRNAs and proteins were enriched in response to stress while GEC-EV mRNAs were enriched in processes related to cell growth and maintenance and cell adhesion (Figure 4). A full list of enriched biological processes is shown in Figure S3. Analysis of GO molecular function enrichment (Figure 5) revealed that GEC-EV miRNAs, mRNAs and proteins were enriched in growth factor binding, collagen binding, structural extracellular matrix constituents and miRNAs and proteins in heparin binding. PTC-EV miRNAs and proteins were enriched in cadherin binding, cell adhesion mediator

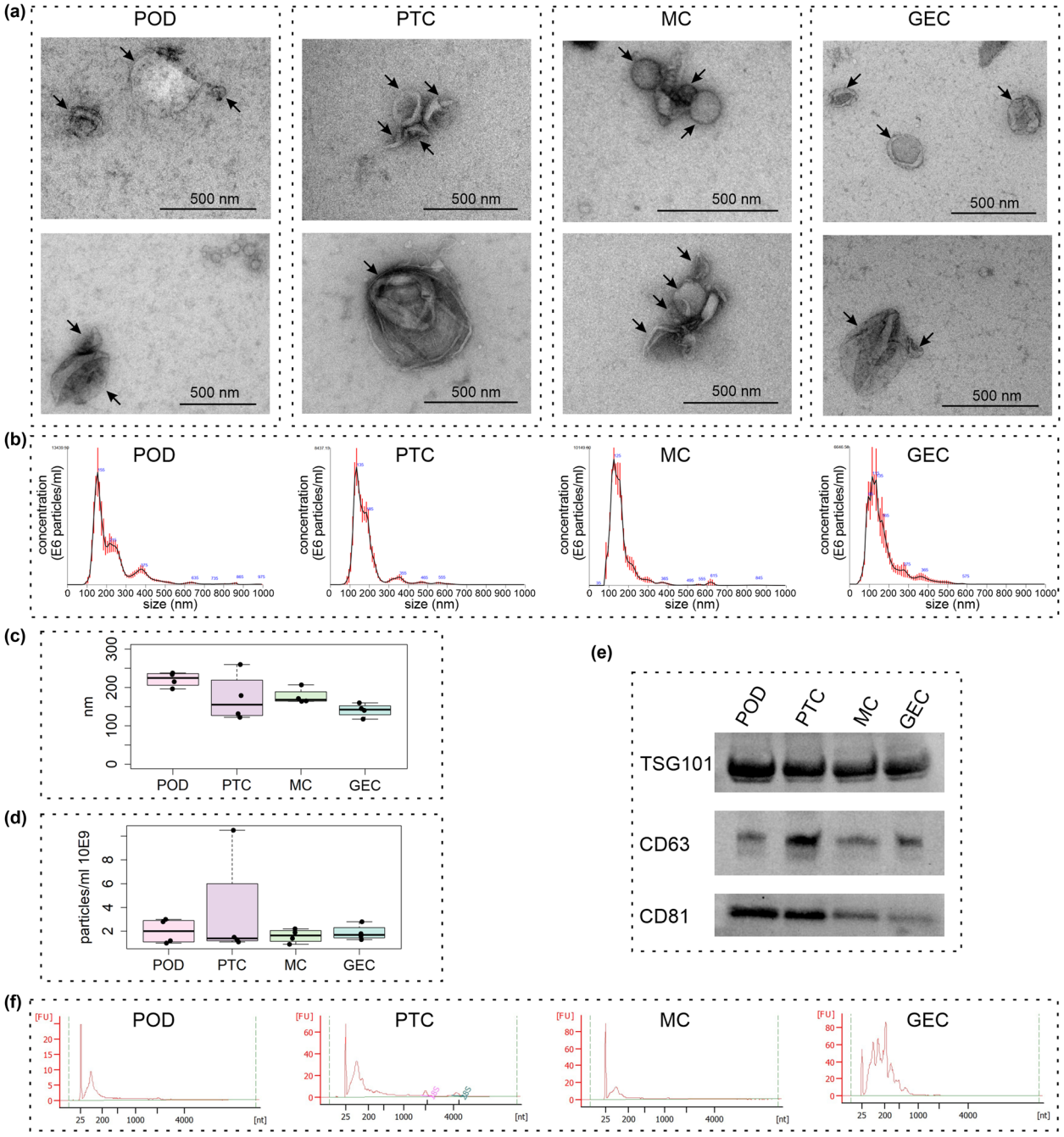

FIGURE 2 EV preparations characterisation and quality control. (a) Electron microscopy micrographs of EV samples isolated by HFD. (b) Representative NTA profiles of uEV total RNA. (c) Diameter mean per cell type EV evaluated by NTA. (d) Particle yield per ml of cell free culture media as measured by NTA. (e) Western blotting. Immunodetection of EV markers (TSG101, CD63 and CD81). (f) Representative RNA electropherograms obtained by Agilent RNA 6000 Pico Kit. Black arrows: extracellular vesicles. GEC, glomerular endothelial cells; EV, extracellular vesicles; MC, mesangial cells; NTA, nanoparticle tracking analysis; POD, podocytes; PTC, proximal tubule cells

activity while only miRNAs were enriched in collagen binding, heparin binding and structural extracellular matrix constituent. No significant molecular function terms were found for POD-EV and MC-EV miRNAs, mRNAs or proteins.

3.4 | Assessment of identified cell-type-enriched EV features in human datasets

Our identified EV features enriched in the four kidney cell types were benchmarked against different human datasets reported in the literature. The comparison was done separately for each type of feature (EV-miRNAs, EV-mRNAs and EV-proteins) for

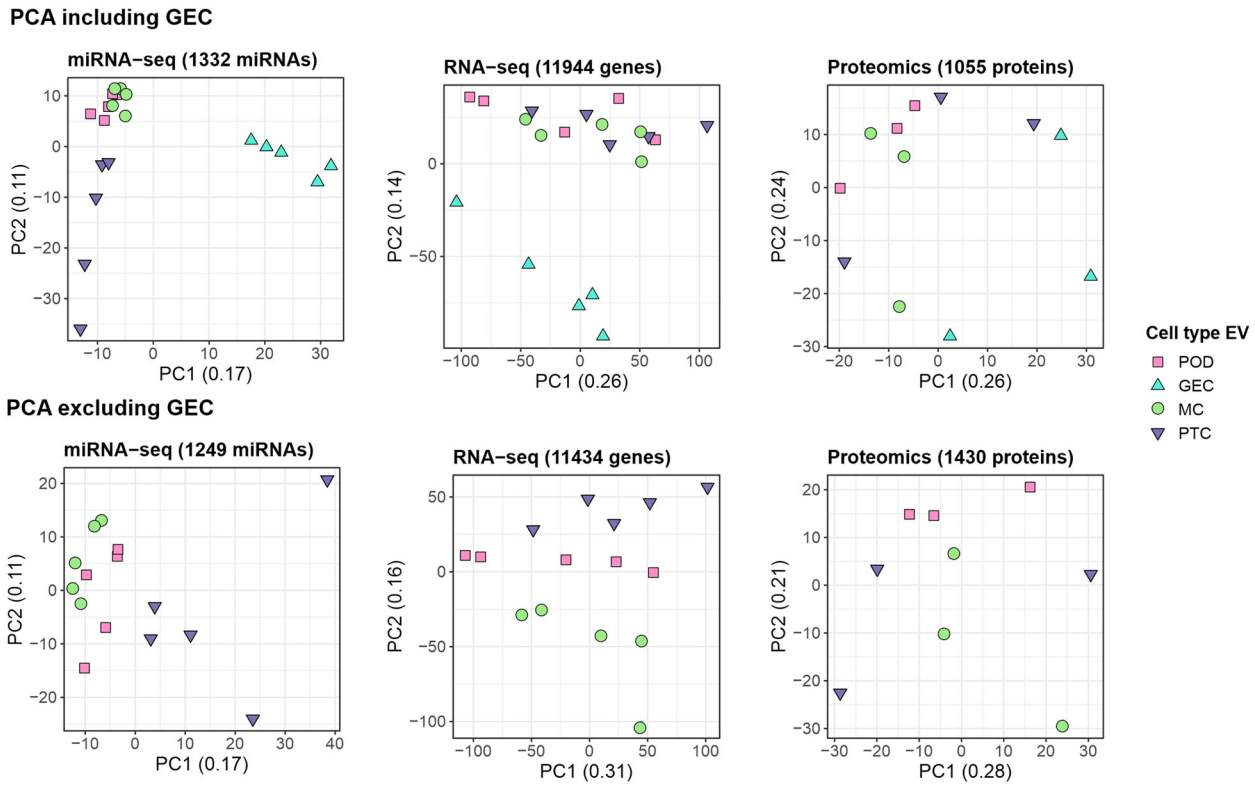

FIGURE 3 Principal component analysis (PCA) on miRNA-seq, RNA-seq and proteomics data from the four extracellular vesicles (EV) isolates. (a) PCA including GEC, (b) PCA excluding GEC in order to avoid GEC bias. GEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

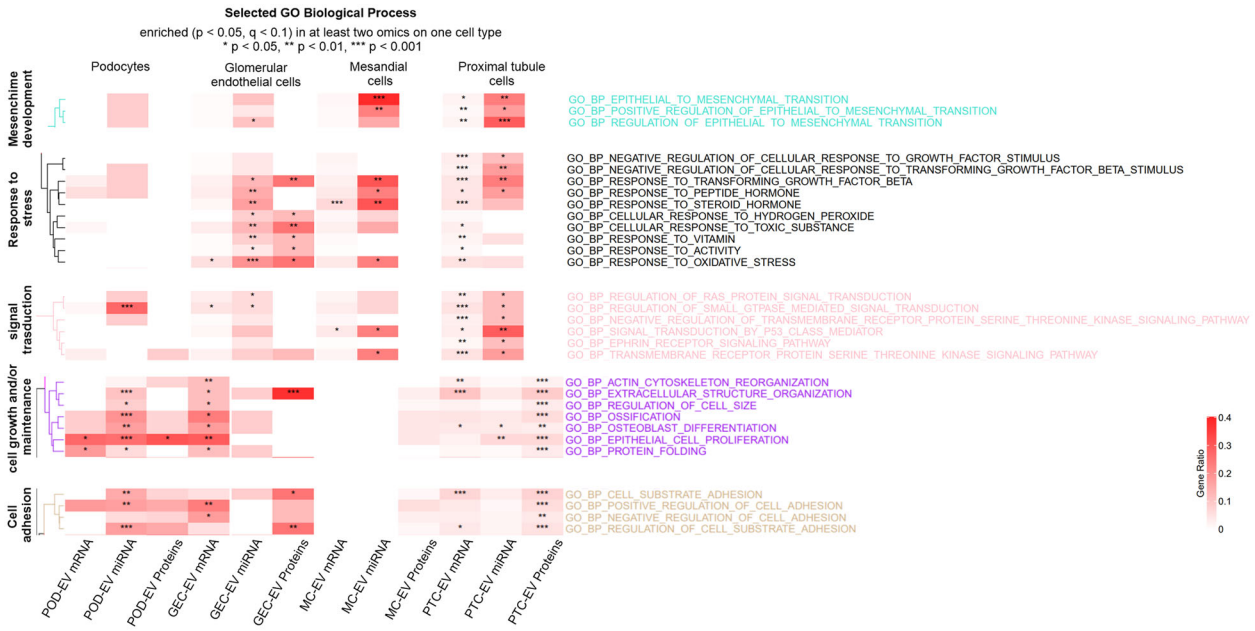

FIGURE 4 Gene ontology biological process enrichment analysis with multi-omics data. Heatmap depicts selected enriched biological processes per cell and molecule type. Analysis was done using cell-type-enriched EV features (POD-EV, PTC-EV, MC-EV and GEC-EV). GGEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

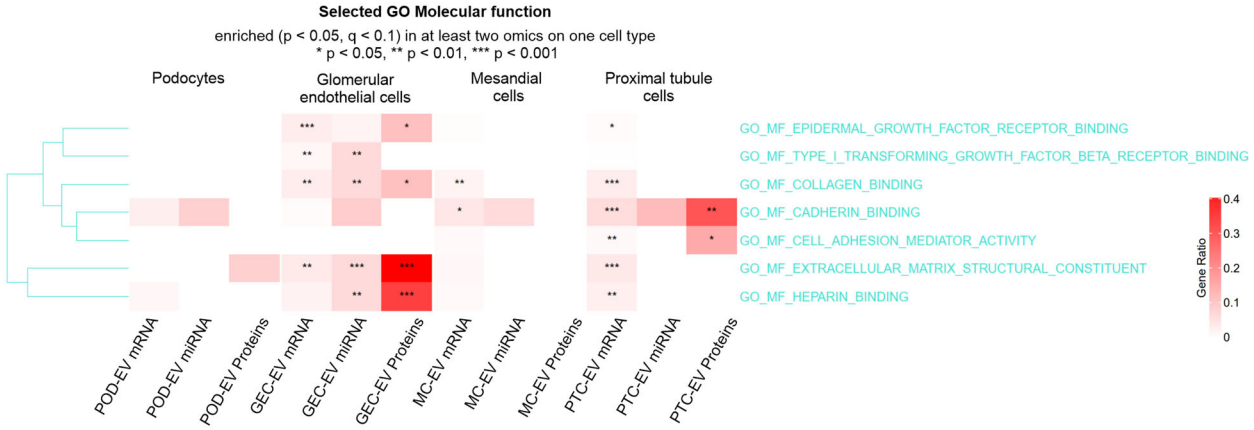

FIGURE 5 Gene ontology molecular function enrichment analysis with multi-omics data. Heatmap depicts selected enriched molecular functions per cell and molecule type. Analysis was done using cell-type-enriched EV features (POD-EV, PTC-EV, MC-EV and GEC-EV). GEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

each cell type (POD, MC, GEC and PTC). The number of cell-type-enriched EV features detected in a dataset is presented as percentage range, that is, the values correspond to cell types with the lowest and highest percentage of EV-features overlapping with a particular dataset. To see the results for each cell type separately please refer to the respective figures and/or tables.

We initially cross-referenced the 'vesiclepedia' (Kalra et al., 2012) database to assess evidence that EV-features had previously been reported as EV cargo. We found that 69%–81% of EV-miRNAs, 71%–85% of EV-mRNAs and 100% of EV-proteins had been reported previously as present in EV from diverse biofluids (Tables S8–S10).

We next compared our cell-type-enriched EV transcriptomic profiles with those derived from human urine samples. For miRNA and mRNA, we used our previously published data (Barreiro et al., 2020) comprising uEV mi- and mRNA sequencing datasets of healthy controls and individuals with type-1 diabetes and macroalbuminuria ($n = 10$). Of note, from Barreiro et al. (2020) we retrieved only the datasets from uEV isolated by hydrostatic filtration dialysis. To work with RNAs consistently found in uEV, we selected miRNAs and mRNAs with raw counts ≥5 in at least 80% of the samples. We found that 31%–63% of the EV-miRNAs and 61%–85% of the EV-mRNAs were also detected in human uEV (Figures 6 and 7, Tables S8 and S9).

To compare EV-proteins to those from uEV, several published proteomics datasets were analysed (Braun et al., 2020; Gonzales et al., 2009; Oeyen et al., 2018; Xu et al., 2019) (see Table S1 for details). As the majority of the published datasets reported proteins by gene name without including unique identifiers, for example, UNIPROT ID, we could only compare EV-proteins with associated gene name from our lists (see Table S10 for UNIPROT IDs without associated gene name in our cell-type-enriched EV protein lists). We found that 58%–67% of the EV-proteins have previously been detected in human uEV (Figure 8).

With the aim of verifying kidney expression of the cell-type-enriched EV features, we compared them to miRNA, mRNA and proteins found in human kidney biopsies. Studies selected included bulk small RNA sequencing (miRNATissueAtlas2), bulk RNA sequencing (Levin et al., 2020), proteomics data (Miyamoto et al., 2007; Yoshida et al., 2012) and single cell sequencing (Wu et al., 2018). We found that 57%–69% of the EV-miRNAs were expressed in human kidneys (Table S8). Regarding mRNAs we found that 91%–94% of the EV-mRNA were found in human kidneys (Table S9). For proteins, between 0% and 84% of the EV-proteins have been described in human glomeruli. Protein results showed high variability between datasets and between cell types (Table S10). To ascertain whether EV-enriched genes were expressed by the same cell types in human kidneys, we explored scRNA-seq data (see Table S1). We found that 9%–29% of the EV-mRNAs expression pattern was concordant with human cell type expression pattern.

Finally, we verified if any of our identified EV-uEV (EV features that were also found in human uEV) molecules had previously been described in pathways associated to kidney disease and/or other diseases. Interestingly, we found several of them were, for example, miR-29a-3p (Wang et al., 2012), thioredoxin interacting protein (Huang et al., 2016), and complement component 3 (Kelly et al., 2015) as detailed in Tables 1–3, which reveals a potential to qualify our findings functionally from cells in vitro to in vivo.

4 | DISCUSSION

The potential of EV as carriers of biomarkers for diverse diseases is high, which reflects in the large number of studies that are currently searching for EV biomarkers, especially in biofluids. Urinary EV are an attractive approach to search for non-invasive biomarkers of urinary tract diseases, as urine is easy to collect. Most of the approaches are based on bulk isolation

TABLE 1 Cell-type-enriched EV miRNA also detected in human urinary extracellular vesicles

miRNA	Functions associated to disease	Association with kidney disease	References
Podocytes			
miR-204-5p	highly expressed in the kidney; miR-204-5p plays a prominent role in safeguarding the kidneys against common causes of chronic renal injury; urinary exosomal level as biomarker for Xp11.2 translocation renal cell carcinoma	yes	(Cheng et al., 2020; Kurahashi et al., 2019)
miR-99b-5p	miR-99b-5p is associated with response to tyrosine kinase inhibitor treatment in clear cell renal cell carcinoma patients	yes	(Lukamowicz-Rajska et al., 2016)
miR-194-5p	miR-194-5p was significantly downregulated in patient urine exosomes, in murine Pkd1 cystic kidneys and in human PKD1 cystic kidney tissue	yes	(Magayr et al., 2020)
miR-99a-5p	miR-99a-5p was upregulated in macro-albuminuric patients compared with normo-albuminuric and micro-albuminuric patients. Transfection of miR-99a-5p in cultured human podocytes downregulated mTOR protein expression and downregulated the podocyte injury marker vimentin	yes	(Uil et al., 2021)
miR-425-5p	miR-425-5p is a potential predictor of extreme response to tyrosine kinase inhibitors in renal cell cancer	yes	(Garrigós et al., 2020)
miR-99b-3p	miR-99b-3p promotes angiotensin II-induced cardiac fibrosis in mice by targeting GSK-3β	no	(Yu et al., 2021)
miR-625-3p	miR-625-3p promotes migration and invasion and reduces apoptosis of clear cell renal cell carcinoma	yes	(Zhao et al., 2019)
miR-625-5p	involved in regulation of Wnt/β-catenin and NF-kappa-B activation	no	(Tang et al., 2019)
miR-503-5p	miR-503-5p functions as an oncogene in various cancer diseases, such as oral squamous cell carcinoma	no	(Fei et al., 2020)
miR-15a-5p	decreased urinary exosomal level in incipient T2D kidney disease	yes	(Xie et al., 2017)
Mesangial cells			
let-7b-5p	TGF-β1-regulated miRNA which is associated with an increased risk of rapid progression to end-stage renal disease	yes	(Pezzolesi et al., 2015)
miR-200a-3p	MicroRNA-200a-3p suppresses tumor proliferation and induces apoptosis by targeting SPAG9 in renal cell carcinoma	yes	(Wang et al., 2016)
miR-429	increased urinary exosomal level upon acute kidney injury	yes	(Sonoda et al., 2019)
miR-101-3p	potential earyl biomarker for acute kidney injury	yes	(Aguado-Fraile et al., 2015)
miR-664a-5p	decreased serum exosomal level in patients with pancreatic ductal adenocarcinoma	no	(Wang et al., 2021)
miR-181a-5p	miR-181a-5p regulates the proliferation and apoptosis of glomerular mesangial cells by targeting KLF6	yes	(X. Liang & Xu, 2020)
let-7b-3p	involved in the pathogenesis of chronic thromboembolic pulmonary hypertension	no	(Gong et al., 2021)
miR-542-3p	MiR-542-3p drives renal fibrosis by targeting AGO1 in vivo and in vitro	yes	(Li et al., 2020)
miR-200a-5p	increased urinary exosomal level upon acute kidney injury; miR-200a Prevents renal fibrogenesis through repression of TGF-β2 expression	yes	(Sonoda et al., 2019; Wang et al., 2011)
miR-3605-3p	miscellaneous	no	

(Continues)

TABLE 1 (Continued)

miRNA	Functions associated to disease	Association with kidney disease	References
Proximal tubule cells			
miR-532-5p	miR-532-5p suppresses renal cancer cell proliferation by disrupting the ETS1-mediated positive feedback loop with the KRAS-NAP1L1/P-ERK axis; increased urinary exosomal miR-532-5p level in intermediate-risk prostate cancer	yes	(Kim et al., 2021; Zhai et al., 2018)
miR-95-3p	invoved in angiogenesis; increased in ectosomes obtained from patients with T2D	no	(Stępień et al., 2018)
miR-31-5p	miR-31-5p acts as a tumor suppressor in renal cell carcinoma by targeting cyclin-dependent kinase 1	yes	(Li et al., 2019)
miR-187-3p	Mesenchymal Stem/Stromal Cells Increase Cardiac MIR-187-3P Expression in Polymicrobial Animal Model of Sepsis	no	(Ektesabi et al., 2021)
miR-218-5p	miR-218-5p is expressed in endothelial progenitor cells and contributes to the development and repair of the kidney microvasculature	yes	(Wang et al., 2020)
miR-181a-2-3p	miR-181a-2-3p alleviates the apoptosis of renal tubular epithelial cell via targeting GJB2 in sepsis-induced acute kidney injury	yes	(Yi et al., 2021)
miR-185-5p	miR-185-5p ameliorates endoplasmic reticulum stress and renal fibrosis by downregulation of ATF6	yes	(Yuan et al., 2020)
miR-708-5p	considered as a therapeutic agent against metastatic lung cancer	no	(Wu et al., 2016)
miR-130a-3p	miR-130a-3p inhibition protects against renal fibrosis in vitro via the TGF-β1/Smad pathway by targeting SnoN	yes	(Ai et al., 2020)
miR-335-3p	aldosterone regulates miR-335-3p in the cortical collecting duct to alter sodium transport	yes	(Edinger et al., 2014)
miR-500a-5p	involved in epithelial-mesenchymal transition	no	(Tang et al., 2020)
miR-500b-5p	involved in phenotypic switching in vascular smooth muscle cells and is involved in the pathogenesis of aortic dissection.	no	(Wang et al., 2021)
Glomerular endothelial cells			
miR-125b-5p	decreased urinary exosomal miRNA level in individuals with diabetic kidney disease	Yes	(Zang et al., 2019)
miR-10b-5p	miR-10a-5p has been associated with disease progression, proliferation and invasion of renal cell carcinoma	yes	(Kowalik et al., 2017)
miR-29a-3p	acts anti-fibrotic, mainly by targeting different types of collagen via a Smad3-dependent mechanism, thus leading to decreased ECM accumulation; Linagliptin, a DPP-4 inhibitor used as an anti-diabetic drug, can also confer renal protection and decrease fibrosis in a mouse model of DN by inducing miR-29a, which targets DPP-4	yes	(Assmann et al., 2018; Kanasaki et al., 2014)
miR-29c-3p	acts anti-fibrotic, mainly by targeting different types of collagen via a Smad3-dependent mechanism, thus leading to decreased ECM accumulation; linagliptin and telmisartan-induced restorative effects on miR-29c expression were reflected in urinary exosomes	yes	(Assmann et al., 2018; Delić et al., 2020)
miR-29b-3p	acts anti-fibrotic, mainly by targeting different types of collagen via a Smad3-dependent mechanism, thus leading to decreased ECM accumulation	yes	(Assmann et al., 2018)
miR-199a-3p	acts as a tumor suppressor in clear cell renal cell carcinoma and acts in a pro-fibrotic manner in chronic diabetic kidney disease; telmisartan and linagliptin suppressed the induction of miR-199a-3p	yes	(Delić et al., 2020; Liu et al., 2018)

(Continues)

TABLE 1 (Continued)

miRNA	Functions associated to disease	Association with kidney disease	References
miR-199b-3p	miscellaneous	no	
miR-361-5p	involved in regulation of epithelial-mesenchymal transition	no	(Yin et al., 2020)
miR-107	increased urinary exosomal miRNA level in clinical responders (lupus nephritis patients)	yes	(Garcia-Vives et al., 2020)
miR-221-3p	increased urinary level after drug-induced kidney injury	yes	(Chorley et al., 2021)
miR-660-5p	miR-660-5p is associated with cell migration, invasion, proliferation and apoptosis in renal cell carcinoma	yes	(He et al., 2018)
miR-532-3p	decreased expression in kidneys obtained from patients with progressive chronic kidney disease	yes	(Rudnicki et al., 2016)
miR-501-3p	high expression levels of exosome miR-501-3p contribute to arteriosclerotic changes	no	(Toyama et al., 2021)
miR-500a-3p	miR-500a-3P alleviates kidney injury by targeting MLKL-mediated necroptosis in renal epithelial cells	yes	(L. Jiang et al., 2019)
miR-10b-3p	miscellaneous	no	
miR-126-3p	relevant for DKD pathogenesis and endothelial to mesenchymal transition	yes	(Wang et al., 2019)
miR-19b-3p	exosomal miRNA-19b-3p of tubular epithelial cells promotes M1 macrophage activation in kidney injury	yes	(Lv et al. 2020)
miR-20a-5p	miR-20a-5p is enriched in hypoxia-derived tubular exosomes and protects against acute tubular injury	yes	(Yu et al., 2020)
miR-185-5p	reduces endoplasmic reticulum stress and fibrosis in vitro and in a mouse model of kidney fibrosis.	yes	(Yuan et al., 2020)
miR-760	increased urinary exosomal microRNA signatures in nephrotic, biopsy-proven DN	yes	(Lee et al., 2020)
miR-324-5p	miR-324-5p inhibits lipopolysaccharide-induced proliferation of rat glomerular mesangial cells by regulating the Syk/Ras/c-fos pathway	yes	(Wang et al., 2020)
miR-195-5p	miR-195-5p alleviates acute kidney injury through repression of inflammation and oxidative stress by targeting vascular endothelial growth factor A	yes	(Chen et al., 2017)

Note: Functions associated with disease (reported in bibliography).
Abbreviations: AGO1, argonaute RISC component 1; ATF6, activating transcription factor 6; COL I/III, collagen I/III; DKD, diabetic kidney disease; DPP-4, dipeptidyl peptidase-4; ECM, extracellular matrix; ETS1, ETS proto-oncogene 1; GJB2, gap junction protein beta 2; GSK-3β, glycogen synthase kinase 3 beta; KLF6, Kruppel Like factor 6; KRAS, KRAS proto-oncogene; MLKL, mixed lineage kinase domain like pseudokinase; mTOR, mammalian target of rapamycin; NAP1L1, nucleosome assembly protein 1 like 1; NF-kappa-B, nuclear factor kappa beta; P-ERK, phospho-ERK; Pkd1, polycystic kidney disease 1; SnoN, nuclear Smad-interacting protein; SPAG9, sperm associated antigen 9; Syk, spleen associated tyrosine kinase; T2D, Type 2 diabetes; TGF-β1, transforming growth factor beta 1; TGF-β2, transforming growth factor beta 2.

and processing of vesicles and hence, it is not possible to identify which cell types are affected. Understanding which cell types contribute to disturbances in EV profiles in complex biofluids is highly relevant to identify pharmacological targets but also to decide and follow up pharmacological interventions. Moreover, as proposed for miRNAs (Chorley et al., 2021; Koturbash et al., 2015), cell type EV signatures could be used in, for example, clinical trials to define which region of the kidney are drug effects most likely derived from and safety and efficacy. Some attempts have been made to shed light to this topic. One approach which has shown promise involves single cell EV isolation (Nikoloff et al., 2021), but this is still in its infancy. Moreover, it is not clear if the RNA and protein yield would be sufficient for profiling applications. Another approach used earlier for plasma derived EV is deconvolution (Li et al., 2020), which consists of using cell sequencing datasets (or tissue bulk RNA sequencing datasets) to derive cell/tissue signature matrices and using deconvolution algorithms to predict, for example, the cell/tissue origin of EV mixtures. However, at present it is not yet clear how expression of EV contents correlates with the expression in their cells of origin, that is, correlation or anti-correlation. A recent study that compared the transcriptome of prostate cancer tissue to paired uEV samples, showed that while the complexity for mRNA between sample types was comparable, a set of mRNAs were significantly enriched in uEV (Almeida et al., 2022). Moreover, the same study showed that uEV were enriched in mature cytoplasmic mRNA, which was confirmed also in vitro (Almeida et al., 2022). Hence, the applicability of tissue/single cell signatures for deconvolution are not straightforward.

TABLE 2 Top 10 cell-type-enriched EV mRNA also detected in human urinary extracellular vesicles

Gene name	description	Function	Association with diseases	References	Open targets	IPA
Podocytes						
PLEKHB2	pleckstrin homology domain containing B2/evectin-2	Involved in retrograde transport of recycling endosomes.	Role in proliferation of metastatic cells by activating Yes-associated protein in cardiomyocytes.	(Matsudaira et al., 2017)	yes	
COBLL1	cordon-bleu WH2 repeat protein like 1		Associated with prostate cancer progression. It stimulates androgen receptor signalling pathway and modulates the cell morphology. Also, its upregulation is associated with chronic lymphocytic leukaemia reduced survival. Associated with apoptosis and fibrosis in cardiomyoblasts.	(Plešingerová et al., 2018; Takayama et al., 2018; Xiong et al., 2021)	no	
MRPL57	mitochondrial ribosomal protein L57	Component of the mitochondrial large ribosomal subunit.			no	
LCP1	lymphocyte cytosolic protein 1	Actin-binding protein.	Downregulation reduces motility and proliferation of breast cancer cells in vitro and in a mouse model. Involved in Hypereosinophilia by activating mTORC2/Akt. Candidate circulating biomarker for kidney cancer.	(Ma et al., 2020; Pillar et al., 2019; Su Kim et al., 2013),	yes	Renal cancer
GNG4	G protein subunit gamma 4	Subunit required for the guanine nucleotide-binding proteins GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction.	Upregulated in liver cancer, promotes cell proliferation and adhesion. Downregulation in colon cancer cells reduced proliferation and migration and invasion. Association with cognitive decline as a consequence of aging.	(Bonham et al., 2018; Liang et al., 2021; Tanaka et al., 2021)	yes	
WSB1	WD repeat and SOCS box containing 1	Probable substrate-recognition component of a SCF-like ECS (Elongin-Cullin-SOCS-box protein) E3 ubiquitin ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins.	In cancer, WSB1 upregulates hypoxia inducible factors by pVHL ubiquitination and proteosomal degradation. It is also associated with thyroid hormone homeostasis and immune regulation.	(Haque et al., 2016; Kim et al., 2015)	yes	
ALDH6A1	aldehyde dehydrogenase 6 family member A1	Plays a role in the valine and pyrimidine catabolic pathways.	Downregulation in renal cell carcinoma which was associated with poor survival. Also survival predictive value for metastatic prostate cancer.	(Cho et al., 2018; Lu et al., 2020)	yes	
ORC4	origin recognition complex subunit 4	Subunit of the ORC, which is essential for the initiation of the DNA replication in eukaryotic cells.	Postulated as a gene which could be involved in eGFR for kidney disease of diverse origin.	(Morris et al., 2019)	yes	

(Continues)

TABLE 2 (Continued)

Gene name	description	Function	Association with diseases	References	Open targets	IPA
STYXL1	serine/threonine/tyrosine interacting like 1	STYXL1 is an inactive phosphatase. It binds to G3BP1 and inhibits the formation of G3BP1-induced stress granules.	Upregulated in glioma and hepatocellular carcinoma. Upregulation is associated with poor prognosis	(Tomar et al., 2019; J. Z. Wu, Jiang, Lin & Liu, 2020)	yes	Renal clear cell adenocarcinoma
MESANGIAL CELLS						
KRT19	keratin 19	Intermediate filament Involved in the organisation of myofibers.	Associated with increased risk of developing tumours in kidney failure. Upregulated in diverse models of tubular injury. High expression in breast and thyroid cancer.	(Djudjaj et al., 2016; Martínez-Camberos et al., 2022; Sarlos et al., 2018)	yes	Autosomal dominant polycystic kidney disease
TXNIP	thioredoxin interacting protein	Protects cells from oxidative stress by regulating of cellular redux status.	Role in kidney fibrosis related to aging. Upregulated by hyperglycaemia in kidney. Knock-out attenuates DKD histopathological changes in a streptozotocin model of diabetes.	(De Marinis et al., 2016; Shah et al., 2015)	yes	Oxidative stress response, damage of renal glomerulus, failure of kidney, injury of podocytes
MT-ND2	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I).	Associates with higher risk to develop nephropathy in T2D. Mutations associated with maternal transmitted T2D.	(Jiang et al., 2021; X. Yang, Zhang, Ma, Zhao & Lyu, 2015)	yes	
MT-ND1	mitochondrially encoded NADH:ubiquinoı oxidoreductase core subunit 1	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I)	Mutations associated with maternal transmitted T2D. In colorectal cancer, circulating levels are associated with progression and response to chemotherapy.	(Jiang et al., 2021)	yes	Chromophobe renal cell carcinoma
PABPC1	poly(A) binding protein cytoplasmic 1	PABPC1 binding to poly(A) promotes ribosome recruitment and translation initiation. It is also involved in poly(A) shortening.	Upregulated in oesophageal squamous cell carcinoma, which promotes angiogenesis and malignant progression. Upregulated in gastric cancer and hepatocellular carcinoma where can act as a prognostic biomarker. Mutations of these genes were found in renal cancers.	(An et al., 2021; Yamamoto et al., 2021; YuFeng & Ming, 2020; Y. Zhang et al., 2022)	yes	Renal cancer
S100A11	S100 calcium binding protein A11	It may play a role in cell motility, invasion and tubulin polymerisation	level of expression in primary clear cell renal cell carcinoma can predict disease free survival. Regulates cell proliferation, invasion and migration in renal cell carcinoma through EGFR/Akt.	(Gabril et al., 2016; Liu et al., 2017)	yes	Renal cell carcinoma
RPS27A	ribosomal protein S27a	Part od the 40s ribosomal subunit	Associated with cervical cancer prognosis. In in lung adenocarcinoma cells regulates apoptosis, proliferation and cell cycle. It may control microglia activation in neurodegenerative diseases.	(Khayer et al., 2020; Li et al., 2022; Wang et al., 2021)	yes	

(Continues)

TABLE 2 (Continued)

Gene name	description	Function	Association with diseases	References	Open targets	IPA
ATP5F1A	ATP synthase F1 subunit alpha, mitochondrial	Subunit of mitochondrial ATP synthase	In HeLa cells, overexpression of ATP5F1A regulated the expression of genes related to innate immune response, angiogenesis and collagen catabolic processed. It also regulated the alternative splicing of genes involved in glucose homeostasis. Associated with clear cell renal cell carcinoma prognosis.	(Song et al., 2021; Yuan et al., 2018)	yes	
CNDP2	carnosine dipeptidase 2	It is a nonspecific dipeptidase, hydrolyses a variety of dipeptides including L-carnosine	Variants of these genes are associated with risk of kidney disease in T2D. Upregulated in substantia nigra pars compacta during Parkinson disease. Role in cancer growth and metastasis of ovarian cancer.	(Ahluwalia et al., 2011; Licker et al., 2012; Zhang et al., 2019)	yes	
SKP1	S-phase kinase associated protein 1	Component of the SCF complexes, participates in ubiquitination of specific protein substrates for their degradation at the proteosome.	Role in colon cancer cell proliferation. Role in the progression of hepatocellular carcinoma.	(Zhai et al., 2017; Zhu et al., 2021)	yes	
Proximal tubule cells						
GPX3	glutathione peroxidase 3	It protects cells against oxidative damage by catalysing the reduction of organic hydroperoxides and hydrogen peroxide by glutathione.	Downregulated in a mouse model of DN. Associated with cardiac disease. Acts as a tumour suppressor in kidney cell lines	(An et al., 2018; Morito et al., 2014; Pang et al., 2018)	yes	Chromophobe renal cell carcinoma, papillary renal cell carcinoma
ASCC2	activating signal cointegrator 1 complex subunit 2	As part of the ASCC complex, it is involved in DNA damage repair. As part of the ASC-1 complex plays a role enhancing NF-kappa-B, SRF and AP1 transactivation.	Associated with coronary artery disease. In cancer, somatic mutations interferes with ASCC2-ASCC3 interaction, which disturbs DNA repair.	(Jia et al., 2020; Y. Yang & Xu, 2021)	yes	
B2M	beta-2-microglobulin	Component of the class I major histocompatibility complex.	Associated with acute kidney injury severity. Indicator of tubular injury and glomerular filtration.	(Barton et al., 2018; Grubb et al., 1985)	yes	Acute kidney injury, acute renal allograft rejection, DN, ostertag type, injury of renal glomerulus
DAP	death associated protein	Dap is a positive regulator of apoptosis induced by interferon-gamma and a negative regulator of autophagy.	In breast cancer, role in cell migration, proliferation and adhesion.	(Wazir et al., 2015)	yes	
MARCKSL1	Myristoylated alanine-rich C kinase substrate-like 1	It plays a role in the formation of adherens function. It controls cell movement through the regulation of cytoskeleton homeostasis.	Downregulated in DN. Anti-angiogenic function preventing the VEGF phosphorylation mediated by Akt/PDK-1/mTOR pathway.	(Kim et al., 2016; Tang et al., 2020)	yes	

(Continues)

TABLE 2 (Continued)

Gene name	description	Function	Association with diseases	References	Open targets	IPA
BASP1	brain abundant membrane attached signal protein 1	Membrane bound protein with several transient phosphorylation sites and PEST motifs. PEST motif is associated with proteins that have a short intracellular half-life.	Mediates albumin induced apoptosis in tubular cells. In podocytes, pro-apoptotic in DN.	(Sanchez-Niño et al., 2010; Zhang et al., 2021)	yes	Renal cell carcinoma
OSBP	oxysterol binding protein	It is a lipid transporter which delivers sterol to the Golgi in exchange for PI4P to the endoplasmic reticulum. It is also believed to transport sterols from lysosomes to the nucleus.	Plays a role in insulin granule formation in rat insulinoma cell line. Role in regulating the proatherogenic protein profilin-1 under diabetic like conditions.	(Hussain et al., 2018; Romeo & Kazlauskas, 2008)	yes	
LMNA	lamin A/C	Component of the nuclear lamina.	Some variants are associated to cardiac disease. Mutation of these gene is associated with proteinuric renal disease and membranous glomerulonephritis.	(Fountas et al., 2017; Fujita & Hatta, 2018; Lazarte et al., 2022)	yes	Renal clear cell adenocarcinoma
PDLIM5	PDZ and LIM domain 5	It binds protein kinases to the Z-disk in striated muscles. It may have a role in cardiomyocyte expansion and in preventing postsynaptic growth of excitatory synapses.	Recruited to integrin adhesion complexes in Podocyte. Polymorphisms of this gene are associated with T2D and hypertension.	(Maier et al., 2021; Owusu et al., 2017)	yes	
STARD10	StAR related lipid transfer domain containing 10	The protein encoded by this gene transfer phospholipids. It can transfer phosphatidylcholine and phosphatidylethanolamine between membranes.	Deletion of STARD10 impairs insulin secretion. Upregulated in breast cancer.	(Carrat et al., 2017; Olayioye et al., 2004)	yes	
Glomerular endothelial cells						
TMSB4X	thymosin beta 4 X-linked	Actin sequestering protein that inhibits actin polymerisation. It is also involved in cell proliferation, migration and differentiation.	Reduces lung inflammation and fibrosis induced by LPS. Administration of TMSB4X reduced the kidney damage in a model od DN.	(Tian et al., 2022; Zhu et al., 2015)	yes	
FDFT1	farnesyl-diphosphate farnesyltrans-ferase 1	Role in cholesterol biosynthesis. Enzyme that catalyses the dimerisation of 2 farnesyl pyrophosphate groups to form squalene.	Antitumoral effect by inhibiting AKT/mTOR/HIF1α pathway during fasting in colorectal cancer. Associated with fibrosis progression in chronic hepatitis C.	(Stättermayer et al., 2014; Weng et al., 2020)	yes	Cell death of kidney cell lines, metastatic renal cancer, renal cell carcinoma, renal osteodystro-phy
SELENOW	selenoprotein W	It plays a role as a glutathione-dependent antioxidant and may have a redox function	During nervous system development, it Protects neurons against oxidative stress.	(Chung et al., 2009)	yes	Adhesion of multiple myeloma endothelial cells

(Continues)

TABLE 2 (Continued)

Gene name	description	Function	Association with diseases	References	Open targets	IPA
SNCG	synuclein gamma	It plays a role in neurofilament network integrity and may modulate the keratin network in skin	Protection against ER stress by regulating autophagy in colon cancer cells. Promotes cancer metastasis. Downregulation in endometrial cancer cells prevented proliferation, migration and invasion, and promoted apoptosis.	(Liu et al., 2022; Ni et al., 2021; Ye et al., 2021)	yes	
GLUL	glutamate-ammonia ligase	It is involved in ammonia and glutamate detoxification, acid-base homeostasis, cell signalling and cell proliferation.	In early DN, GLUL is downregulated in podocytes. Associated with cardiovascular disease in T2D.	(Look AHEAD Research Group, 2016; Wei et al., 2021)	yes	Early stage DN, migration of endothelial cells, migration of vascular endothelial cells, sprouting of vascular endothelial cells
ARHGDIB	Rho GDP dissociation inhibitor beta	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them.	Development of antibodies against ARHGDIB protein are associated with kidney graft loss. Upregulated in breast cancer and associated with prognosis.	(Kamburova et al., 2019; Wang et al., 2020)	yes	
GTF3A	general transcription factor IIIA	It is involved in ribosomal large subunit biogenesis.	Dysregulated in secondary hyperparathyroidism.	(Santamarea et al., 2005)	yes	
CALCOCO2	calcium binding and coiled-coil domain 2	It acts as a receptor for ubiquitin-coated bacteria and plays and a role in innate immunity by mediating macroautophagy.	Dysregulated in dilated cardiomyopathy with increased autophagy. Role in mitophagy.	(Gil-Cayuela et al., 2019; Yamano & Youle, 2020)	no	
RBM3	RNA binding motif protein 3	It enhances global protein synthesis. It is Induced by cold shock and low oxygen tension.	Role in inducing the proliferation of hepatocellular carcinoma. In hypoxic conditions, promotes neural stem cells proliferation.	(Miao & Zhang, 2020; Yan et al., 2019)	yes	Renal cancer
TCEAL4	transcription elongation factor A like 4	Involved in transcription modulation in a promoter context-dependent manner.	Downregulated in gestational diabetes. Down regulated in anaplastic thyroid cancer.	(Akaishi et al., 2006; Alur et al., 2021)	yes	Renal clear cell adenocarcinoma

Note: Top 10 mRNAs were selected based on their level of expression in urinary extracellular vesicles dataset. Functions (as reported in uniprot, humanmine and bibliography) and examples of association to kidney disease and/or other diseases (evaluated using open targets kidney disease entries, ingenuity pathway analysis (IPA) and bibliography).
Abbreviations: AP1, activator protein 1; ASC1, activating signal cointegrator 1 complex; BAX, Bcl-2-associated X; BCL-2, B-cell lymphoma 2; DN, diabetic nephropathy; EGFR, epidermal growth facrtor receptor; eGFR, estimated glomerular filtration rate; G3BP1, Ras GTPase-activating protein-binding protein 1; GDP, guanosine diphosphate; GTP, guanosine triphosphate; JAK, Janus kinases; mTOR, mammalian target of rapamycin; mTORC2, mammalian target of rapamycin complex 2; NADH, reduced nicotinamide adenine dinucleotide; NF-kappa-B, nuclear factor kappa beta; ORC, origin recognition complex; PDK-1, 3-phosphoinositide dependent protein kinase 1; PEST motif, Proline, glutamic acid, serine and threonine; PI3K, phosphoinositide 3-kinase; PI4P, phosphatidylinositol-4-phosphate; PTEN, phosphatase and tensin homolog; SCF complex, Skp, Cullin, F-box containing complex; SRF, serum response factor; STAT3, signal transducer and activator of transcription protein 3; T2D, type 2 diabetes; VEGF, vascular endothelial growth factor.

Here, we present an in vitro approach to identify cell-type enriched EV signatures with applicability to research on human uEV. In our approach, we isolated EV from cell culture media from four kidney cell types and profiled miRNA, mRNA and proteins. Thereafter, we defined cell-type-enriched EV RNAs and proteins and compared them to human datasets.

Vesicles of typical morphology and variable sizes were identified in all EV preparations. We did not find differences in EV size and concentration between cell types. In contrast, we did find that GEC Bioanalyzer profile differ from the other cell types. Thus, we explored if differences in the RNA biotype distribution could explain the wider RNA peak in the GEC Bioanalyzer profiles.

TABLE 3 Cell-type-enriched EV proteins also detected in human urinary extracellular vesicles

Gene name	Description	Function (Uniprot and/or Humanmine)	Disease association	Association with kidney disease	References
Podocytes					
PSMB2	Proteasome subunit beta type-2	Component of the 20S core proteasome complex which is involved in the proteolytic degradation of most intracellular proteins.	knock-down suppresses hepatocellular carcinoma cell proliferation and invasion. Up-regulated in muscle tissue from burnt patients where correlated with cellular respiration rates.	no	(Ogunbileje et al., 2016; Tan et al., 2018)
CUL2	Cullin-2	Component of Cul2-RING ubiquitin ligase complex, which mediate the ubiquitination of target proteins.	Required for the activity of hypoxia-inducible factor and vasculogenesis.	no	(Maeda et al., 2008)
C3	Complement C3	Plays a central role in the activation of the complement system.	Highly expressed in the kidneys of T2D rats with DN; deposits in glomeruli from individuals with C3 Glomerulonephritis. Protects pancreatic β-cells against pro-inflammatory cytokines induced apoptosis.	yes	(Dos Santos et al., 2017; Huang et al., 2019; Kelly et al., 2015; Sethi et al., 2012)
CSTA	Cystatin-A	Intracellular thiol proteinase inhibitor.	Up-regulated in hypoxic cells, anti-apoptotic effect.Role in maintaining cell-cell adhesion. Upregulated in several epithelial-derived malignancies, including squamous cell carcinoma.	no	(Gupta et al., 2015; Shou et al., 2020)
KRT14	Keratin, type I cytoskeletal 14	Involved in promoting KRT5-KRT14 filaments to self-organise into large bundles and enhances the mechanical properties involved in resilience of keratin intermediate filaments in vitro.	Expressed in progenitor cells of salivary glands, trachea, prostate, bladder and lung. Regulates epidermal homeostasis and barrier function.	no	(Abashev et al., 2017; Guo et al., 2020)
PLBD2	Putative phospholipase B-like 2		Presence in autolysosomes in human aged brains (accumulate undegraded proteins and lipids) may be associated with Parkinson disease.	no	(Zucca et al., 2018)
ERP44	Endoplasmic reticulum resident protein 44	Acts as a chaperone in the secretory pathway. It is activated by pH.	ER resident chaperone protein, has been implicated in the modulation of ER stress; its depletion worsen ER stress and aggravates DN in db/db mice. Elevated in urine from individuals with T1D	yes	(Pang et al., 2018; Van et al., 2017)
MESANGIAL CELLS					
CKM	Creatine kinase	Cytoplasmic enzymewith a role in energy homeostasis.	Hyperoxia-responsive, Nrf2-dependent in lung.	no	(Zervou et al., 2017)

(Continues)

TABLE 3 (Continued)

Gene name	Description	Function (Uniprot and/or Humanmine)	Disease association	Association with kidney disease	References
CLSTN2	Calsyntenin-2	May modulate calcium-mediated postsynaptic signals.	Involved in homophilic cell adhesion via plasma membrane adhesion molecules. Positively correlated with Immune Infiltration level and immune markers in colon adenocarcinomas. Hyperoxia-responsive, Nrf2-dependent in lung.	no	(Cho et al., 2012; Wu et al., 2020)
CRIM1	Cysteine-rich motor neuron 1 protein	May have a functionin tissue development by interacting with members of the transforming growth factor beta family.	In a gene-trap mouse line, abnormal glomerular development (enlarged capillary loops, podocyte effacement and mesangiolysis) was observed. Involved in endothelial maintenance and integrity. CRIM1 loss contributes also to defects in the extraglomerular vasculature	yes	(Wilkinson et al., 2007; Wilkinson et al., 2009)
Proximal tubule cells					
HEXB	Beta-hexosaminidase subunit beta	Hydrolyses glycoconjugates.	Mutations cause Sandhoff disease (lysosomal storage disorder) and accumulation of ganglioside GM2 in brain kidney and liver. Knockout associated with apoptosis in the central nervous system	yes	(J. Q. Huang et al., 1997; Merscher & Fornoni, 2014)
EEF1G	Elongation factor 1-gamma	Member of the elongation factor-1 complex (responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome).	Upregulated in kidney clear cell carcinoma. Positive regulator of autophagy. Required for autophagy signalling upstream of mTORC1	yes	(Dengjel et al., 2012; Hassan et al., 2018)
PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	Subunit from protein phosphatase 1, which dephosphorylate several biological targets.	Associated with renal control of ion homeostasis in distal convoluted tubule. May have a role in the regulation of ciliary structure and function.	no	(Luo et al., 2019; Penton et al., 2019)
PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	Subunit from protein phosphatase 1, which dephosphorylate several biological targets.	Catalytic subunit of protein phosphatase 1. Its deletion causes remodelling of the heart, interstitial fibrosis and contractile dysregulation.	no	(Liu et al., 2015; Penton et al., 2019)
BASP1	Brain acid soluble protein 1	Membrane protein that has several transient phosphorylation sites and PEST sequences. PEST are associated with proteins that have a short intracellular half-life.	Mediates glucose-induced and albumin-induced apoptosis in tubular cells. proapoptotic factor in DN and possibly also in hypertensive nephropathy	yes	(Sanchez-Niño et al., 2015; Sanchez-Niño et al., 2010)

(Continues)

TABLE 3 (Continued)

Gene name	Description	Function (Uniprot and/or Humanmine)	Disease association	Association with kidney disease	References
HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2			no	
Glomerular endothelial cells					
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1 isoform 1	Extracellular matrix glycoprotein member (member of the fibulin family).	Pro-angiogenic. Overexpression in HUVECs increased Tube formation and proliferation. Overexpressed in IgA nephropathy. Reduces phosphate-induced vascular smooth muscle cell calcification by Inhibition of oxidative stress. Activates MAPK and Akt pathways in pancreatic carcinoma cells by binding to EGFR.	no	(Camaj et al., 2009; Cheng et al., 2020; Luong et al., 2018; Paunas et al., 2019)
PSAP	Prosaposin	involved in the catabolism of glycosphingolipids with short oligosaccharide groups	Increased in urine from children with urinary tract infection. dysregulated in urine from individuals with Fabry nephropathy. Elevated in the urine of T1D children.	yes	(Forster et al., 2019; Manwaring et al., 2013; Riccio et al., 2019)
PTX3	Pentraxin-related protein PTX3	Plays a role in the regulating inflammatory reactions, angiogenesis and tissue remodeling.	PTX3 increased the respiratory capacity in human endothelial cells. Induced vascular dysfunction and changes in endothelial layer in vitro. PTX3 inhibits acute renal injury-induced interstitial fibrosis by suppressing IL-6/Stat3 pathway in mice. Increased circulating levels associated with lower GFR in the elderly.	yes	(Carrizzo et al., 2015; Carrizzo et al., 2019; Sjöberg et al., 2016; Xiao et al., 2014)
LTBP2	Latent-transforming growth factor beta-binding protein 2	Extracellular matrix protein with multiple domains involved in diverse functions such as member of the TGF-beta latent complex, as a structural component of microfibrils, and a role in cell adhesion.	Predictor for acute kidney injury and major adverse kidney events after open heart surgery. LTBP2 can upregulate TGF-β1 expression and secretion in fibroblasts which may be significant in connective tissue disorders.	yes	(Haase et al., 2014; Sideek et al., 2017)
TXNRD1	Thioredoxin reductase 1, cytoplasmic	Member of the thioredoxin (Trx) system. TrxRs reduce thioredoxins and other substrates. Plays a role in redox homoeostasis.	TXNRD1 levels were high in a model of gestational diabetes in rats. Upregulated in high glucose treated HMVEC. Alleviates tubular damage and interstitial fibrosis by inducing the expression of genes that protects against oxidative stress.	yes	(Fu et al., 2021; Nezu & Suzuki, 2020; Patel et al., 2013)

(Continues)

TABLE 3 (Continued)

Gene name	Description	Function (Uniprot and/or Humanmine)	Disease association	Association with kidney disease	References
CSPG4	Chondroitin sulphate proteoglycan 4	Proteoglycan involved in cell proliferation and migration. It is a cell surface receptor for collagen alpha 2(VI), which may facilitate the mobility in this substrate.	In human gliomas induces cell proliferation and migration. In gliomas it is highly expressed in pericytes where has a role in neoangiogenesis. Involved in fibrogenic/adipogenic differentiation in skeletal muscle tissues	no	(Schiffer et al., 2018; Takeuchi et al., 2016)

Note: Functions (as reported in uniprot, humanmine and bibliography) and examples of association to kidney disease and/or other diseases.
Abbreviations: DN, diabetic nephropathy; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GFR, glomerular filtration rate; HMVEC, human microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; T1D, Type 1 diabetes; T2D, Type 2 diabetes; TGF-β1, transforming growth factor beta 1.

FIGURE 6 Identification of cell-type-enriched extracellular vesicles (EV) miRNAs in urinary extracellular vesicles (uEV). Venn diagrams depict overlapping between cell-type-enriched EV miRNAs (POD-EV, PTC-EV, MC-EV and GEC-EV miRNAs) and human uEV miRNAs. GEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

GEC-EV had a higher percentage of long intergenic non-coding RNA and sense intronic RNAs compared to other cell types. For both RNA types, size exceeds 200 nt, which could explain at least partly the differences in Bioanalyzer RNA profiles. In addition, previous reports have mentioned differences in Bioanalyzer RNA profiles between cell types and EV types (Crescitelli et al., 2013).

We successfully derived lists of miRNA, mRNA and proteins enriched in EV isolated from different cell types. Firstly, we characterised the list of features by enrichment analyses and we found that most of the cell type EV shared differentially represented gene ontology terms related to cell adhesion, cell growth and maintenance as well as response to stress. As expected, not all the pathways were shared, for example, PTC-EV and MC-EV were enriched in epithelial to mesenchymal transition and signal

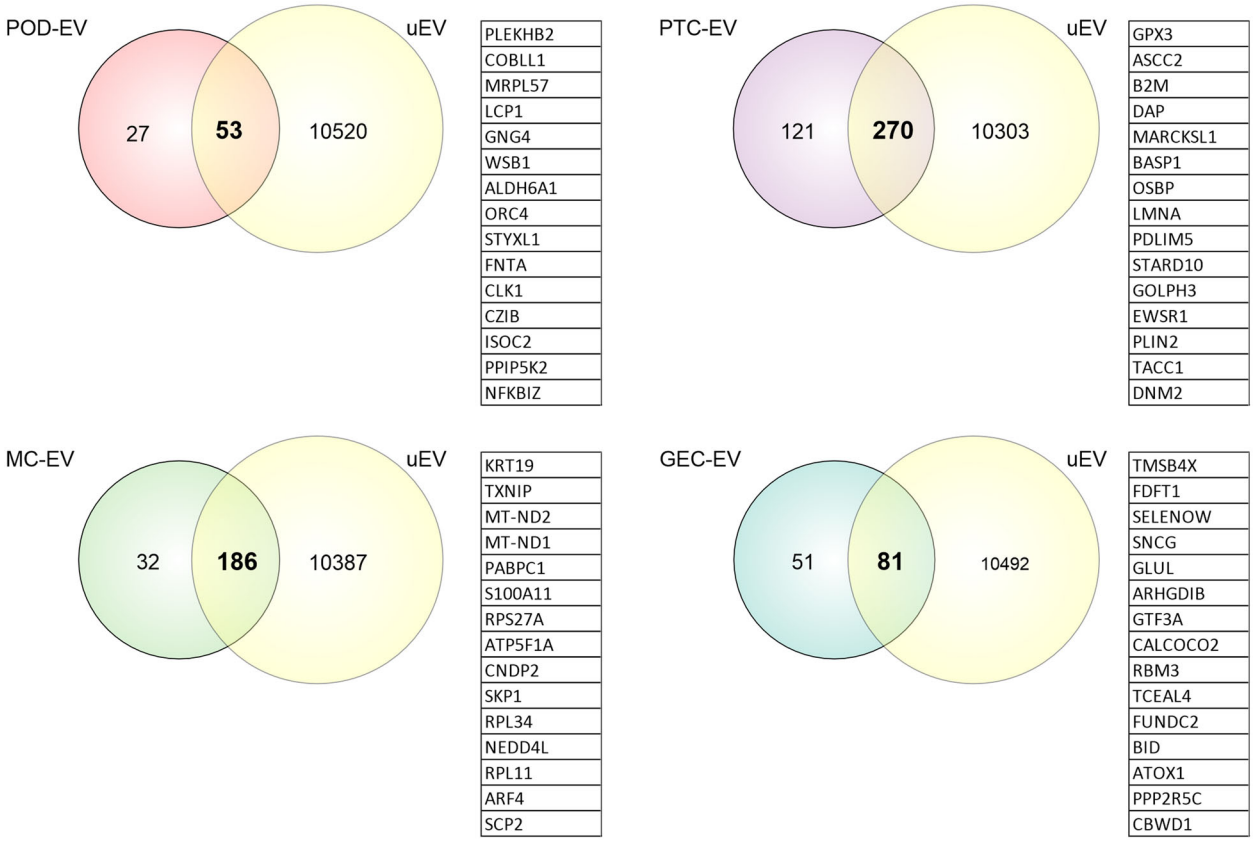

FIGURE 7 Identification of cell-type-enriched extracellular vesicles (EV) genes in urinary extracellular vesicles (uEV). Venn diagrams depict overlapping between cell-type-enriched EV mRNAs (POD-EV, PTC-EV, MC-EV and GEC-EV mRNAs) and human uEV mRNAs. Top 15 mRNA (by expression in uEV) are listed for each cell type. GEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

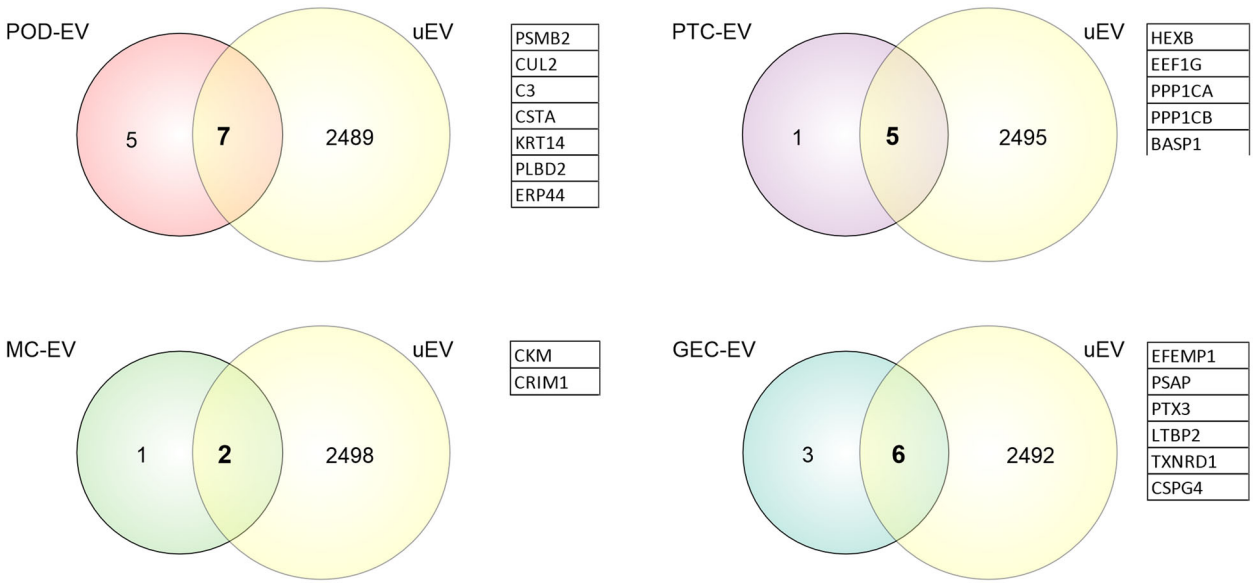

FIGURE 8 Identification of cell-type-enriched extracellular vesicles (EV) proteins in urinary extracellular vesicles (uEV). Venn diagrams depict overlapping between cell-type-enriched EV proteins (POD-EV, PTC-EV, MC-EV and GEC-EV proteins) and human uEV proteins. GEC, glomerular endothelial cells; MC, mesangial cells; POD, podocytes; PTC, proximal tubule cells

transduction and PTC-EV and GEC-EV shared structural extracellular matrix constituent and heparin and collagen binding. We then compared our lists to in-vivo datasets, that is, human uEV transcriptomics and kidney biopsy data (bulk transcriptomics and proteomics and single cell sequencing). Between 31%–85% and 44%–94% of the EV RNAs (miRNA and mRNA) and proteins were found in human uEV datasets and bulk (bulk RNA sequencing or proteomics) biopsy data, respectively. On the contrary, none of the three MC-enriched EV proteins were found in proteomics datasets from kidney biopsies. In addition, the comparison of our lists to single cell sequencing data showed that less than 30% of EV enriched mRNAs had previously been detected using this technology. The variability on the number of features occurring in human datasets could arise from factors such as low translatability between in vitro models and in vivo data or specific cell sorting of some of the features to EV (Carnino et al., 2020; Garcia-Martin et al., 2022; O'grady et al., 2022). In addition, more abundant features from other cell types (including in EV derived from non-kidney cell types) could mask the expression of some of the cell-type-enriched features. This possibility could be assessed by targeted detection methods such as qPCR. Moreover, the missing cell-type-enriched mRNAs in the single cell sequencing data could be partly due to technical limitations, for example, high level of drop-outs (lack of detection of transcripts due to low efficiency in mRNA capture or amplification) (Haque et al., 2017). Taken together, since many of the features were found in human datasets and importantly in human uEV, our in vitro model showed to be robust. Moreover, our approach appears to be more specific than deconvolution because our datasets consist of cell type specific EV instead of single cell sequencing. Furthermore, our approach can be easily scaled up to yield enough EV isolates and perform different omics from the same sample.

The limitations of our study include a low samples number, especially for proteomics profiling, which could be one of the reasons for the low number of enriched EV proteins per cell type. In addition, we included only a subset of kidney cells that contribute to uEV heterogeneity (POD, PTC, MC, and GEC) and we did not include urothelial cells. However, our line of research is focused on diabetic nephropathy and POD, PTC, MC and GEC are key targets of this disease (reviewed in (Jefferson et al., 2008)). Thus, major disturbances in uEV feature profiles will most likely occur in these cells. Finally, EV isolates can contain contaminants, that is, non-vesicular RNAs or proteins (Théry et al., 2018). We found that most of the features in our EV signatures had been described previously as part of EV isolates in urine or other biofluids, but we cannot discard that not all the features are enclosed in vesicles.

In summary, we propose here an in vitro approach to study cell type enriched EV cargo that could be translated to in vivo findings. Our profiles of EV enriched miRNAs, mRNAs and proteins by cell type, specifically the ones that were also found in uEV, could be applied to understand cell type contribution to complex EV mixtures such as urine and could translate to improve diagnostics. Moreover, as our in vitro model showed to be robust, the approach presented here could be further developed to find cell type specific biomarker for kidney disease such as diabetic kidney disease.

AUTHOR CONTRIBUTIONS

Karina Barreiro: Conceptualization; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft. **Abigail C. Lay**: Conceptualization; Investigation; Methodology; Validation; Writing – original draft. **German Leparc**: Formal analysis; Software; Writing – review & editing. **Van Du T. Tran**: Formal analysis; Software; Visualization; Writing – original draft. **Marcel Rosler**: Formal analysis; Software; Visualization; Investigation; Writing – review & editing. **Lusyan Dayalan**: Investigation; Writing – review & editing. **Frederic Burdet**: Supervision; Writing – review & editing. **Mark Ibberson**: Resources; Supervision; Writing – review & editing. **Richard J. M. Coward**: Conceptualization; Funding acquisition; Resources; Supervision; Writing – original draft. **Tobias B. Huber**: Funding acquisition; Writing – review & editing. **Bernhard K. Krämer**: Supervision; Writing – review & editing. **Denis Delic**: Conceptualization; Resources; Supervision; Writing – original draft. **Harry Holthofer**: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing – original draft.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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