Functionally Essential Tubular Proteins are Lost to Urine-Excreted, Large Extracellular Vesicles During Chronic Renal Insufficiency

Ryan J. Adam\textsuperscript{1}, Mark R. Paterson\textsuperscript{1}, Lukus Wardecke\textsuperscript{1}, Brian R. Hoffmann\textsuperscript{1,2,3,5}, and Alison J. Kriegel\textsuperscript{1,3,4}

\textsuperscript{1}Department of Physiology, \textsuperscript{2}Department of Biomedical Engineering, \textsuperscript{3}Cardiovascular Center, \textsuperscript{4}Center of Systems Molecular Medicine, \textsuperscript{5}Max McGee National Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin

Correspondence:
Alison J. Kriegel, Ph.D.
Department of Physiology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226
Phone: 414-955-4835
Fax: 414-955-6456
e-mail: akriegel@mcw.edu
ABSTRACT

**Background.** The 5/6 nephrectomy (5/6Nx) rat model recapitulates many elements of human chronic kidney disease (CKD). Within weeks of surgery 5/6Nx rats spontaneously exhibit proximal tubular damage including the production of very large extracellular vesicles and brush border shedding. We hypothesized that production and elimination of these structures, termed large renal tubule extracellular vesicles (LRT-EVs), into the urine represents a pathological mechanism by which essential tubule proteins are lost.

**Methods.** LRT-EVs were isolated from 5/6Nx rat urine 10 weeks after surgery. LRT-EV diameters were measured. LRT-EV proteomic analysis was performed by tandem mass spectrometry analysis. Data are available via ProteomeXchange with identifier PXD019207. Kidney tissue pathology was evaluated by trichrome staining, TUNEL staining, and immunohistochemistry.

**Results.** LRT-EV size and a lack of TUNEL staining in 5/6Nx rats, suggests LRT-EVs to be distinct from exosomes, microvesicles, and apoptotic bodies. LRT-EVs contained many proximal tubule proteins that, upon disruption, are known to contribute to CKD pathological hallmarks. Select proteins included aquaporin 1, 16 members of the solute carrier family, basolateral Na⁺/K⁺-ATPase subunit ATP1A1, megalin, cubilin, and sodium-glucose co-transporters (SLC5A1 and SLC5A2). Histological analysis confirmed the presence of apical membrane proteins in LRT-EVs and brush border loss in 5/6Nx rats.

**Conclusions.** This study provides comprehensive proteomic analysis of a previously unreported category of extracellular vesicles associated with chronic renal stress. Because LRT-EVs contain proteins responsible for essential renal functions known to be compromised in CKD, their formation and excretion may represent an underappreciated pathogenic mechanism.
INTRODUCTION

The kidney’s proximal tubules (PTs), when healthy, reabsorb the majority of total glomerular filtrate including large quantities of water, electrolytes, and nearly 100% of filtrate protein. Bulk reabsorption is enabled by the extensive surface area of apical microvilli, the so-called brush border, and their extensive reabsorption apparatus. Loss of the PT brush border has been reported in both acute and chronic kidney disease (CKD),¹,² and the pathological effects of progressive brush border loss on renal function has been established in a mouse model of acute-to-chronic kidney disease transition.³ The rat 5/6 nephrectomy (5/6Nx) remnant kidney model of CKD recapitulates many elements of human CKD including brush border loss, proteinuria, albuminuria, polyuria, and more.¹,³,⁴ We have observed the timing of brush border loss in these rats to coincide with renal functional decline, and the presence of large extracellular vesicles originating from PTs.¹ This process is distinct from acute kidney injury associated brush border necrosis and sloughing. We term these vesicles large renal tubular extracellular vesicles (LRT-EVs). Despite our identification of what could be LRT-EVs published as histological representations by other groups (earliest found dated 1914), we were unable to find explicit, in-text, literature reference to them, rendering their identification and functional consequence uncertain. LRT-EVs are too large to be exosomes (40-100 nm) or microvesicles (100-1000 nm).⁵ While the LRT-EV size range is compatible with apoptotic bodies (800-5000 nm),⁵ there is no evidence of PT epithelial apoptosis via TUNEL staining, and other methods, in our 5/6Nx model during the period of LRT-EV production.

CKD now afflicts >15% of the U.S. adult population.⁶ New diagnostic and treatment strategies for CKD are urgently needed. Assessment of urine-excreted biomarkers has long been a diagnostic and prognostic cornerstone of kidney disease. Rapid technological advances are
changing this field. Old standbys such as urine albumin are now being supplemented by new-age “omics-based” (e.g. transcriptomics) urinary excreted biomarker analysis. Many such studies focused upon extracellular vesicles, especially exosomes and microvesicles, and provided novel insight into renal pathophysiology. We know the presence of our CKD rat LRT-EVs to temporally coincide with marked exacerbation of renal pathology. Based upon these observations we hypothesized that LRT-EVs are urine-excreted and contain proteins essential to healthy renal function. Toward this, and while mindful of insightful “omics-based” extracellular vesicle analysis from others, we performed a tandem mass spectrometry (MS/MS) proteomic analysis of LRT-EVs we isolated from CKD rat urine. We found LRT-EVs to contain a wide array of tubule proteins. Many of these were PT epithelial proteins that upon disruption are known to contribute to hallmarks of renal disease. This study provides needed information upon, hitherto, poorly characterized, renal pathology associated, LRT-EVs. Because LRT-EVs contain proteins essential to healthy renal function, their formation and excretion represents a potential newly described mechanism of renal pathology.
MATERIALS AND METHODS

Animal Model. All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee and performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Envigo, Madison, WI) were maintained on 0.4%NaCl chow (AIN-76A Purified Rodent Diet, Dyets, Inc., Bethlehem, PA) and water ad libitum under a 12hr light cycle. At 10 weeks of age animals underwent a sham or 5/6Nx operation as described previously.\textsuperscript{1,4} In brief, rats were anesthetized (ketamine, 50 mg/kg; xylazine, 8 mg/kg; and acepromazine, 5 mg/kg), and the entire right kidney and left kidney poles removed by surgical excision. Gelfoam coagulant was applied to resected surfaces. All subsequent rat data collection was performed 10 weeks following the 5/6Nx or sham surgery.

Tissue Collection and Histology. Tissues were excised from anesthetized animals and immediately immersion-fixed in 10% formalin. Kidneys were processed, paraffin-embedded, sectioned (4 µm), and stained with Masson’s Trichrome by the CRI Histology Core at MCW. Light microscopy was performed using an Eclipse E-400 microscope (Nikon). TUNEL staining was performed following manufacturer instructions (Click-iT™ Plus TUNEL Assay) with DNAase I pre-treatment as a positive control (Thermofisher). Mounting medium with DAPI (VECTOR Labs) was used to label nuclei. Imaging was performed on Nikon A1-R laser scanning confocal microscope. Immunohistochemistry for megalin was performed as previously described,\textsuperscript{7} with 1:50 megalin antibody (Abcam; AB184676) and 1:100 Texas Red-labeled anti-mouse (1:100, Invitrogen #T-862). Immunohistochemistry for NHE3 (Slc9a3) was performed in the same manner except with 1:50 NHE3 antibody (Millipore Sigma; MABN1813). Imaging was performed with an Eclipse 80i microscope (Nikon) or Nikon A1-R confocal microscope. Histological sections of proximal tubules, stained with Masson’s trichrome, from sham and 5/6Nx
rats were assessed for the presence of LRT-EVs. This analysis was performed on histological sections acquired from separate cohorts of sham and 5/6Nx rats at 2, 4, 5, 7, and 10 weeks post-surgery.

**LRT-EV Isolation and Diameter Measurement.** Sham-operated and 5/6Nx rats (n=3/group) were housed in wire-bottom metabolic cages and urine was collected into ice-immersed conical tubes for 24 hours. All subsequent steps were performed at room temperature. Urine was filtered (70 µm mesh), then centrifuged at 300 x g for 15 min (Spin A). The resulting pellet was resuspended in 1 mL of PBS and centrifuged again (Spin B). Supernatant from both spins (A & B) was combined and centrifuged at 650 x g for 10 minutes (Spin C) to remove any larger material or shed cells. The supernatant from Spin C was centrifuged at 2000 x g for 10 minutes (Spin D), to pellet the LRT-EV’s. The pellet was then washed in 1 mL of PBS and centrifuged at 580 x g for 10 minutes (Spin E). An aliquot of this final isolate was inspected and imaged under light microscopy for the presence of LRT-EVs. No LRT-EVs were observe in samples fractionated from sham-operated rat urine. The LRT-EV isolation methodology is summarized in Supplemental Figure 1. The size 5/6Nx LRT-EVs was measured from the light microscopy images using the Analyze Particles feature of FIJI Software (free download: https://imagej.net/Fiji/Downloads).

**Proteomic Sample Preparation and Analysis.** The remaining LRT-EVs were transferred to a Dounce homogenizer and mechanically lysed on ice followed by 30 minutes of water bath sonication at room temperature as previously described. Homogenized LRT-EVs were then buffer swapped (5x) and concentrated into 250 µL of 25 mM ammonium bicarbonate, pH 8.0, using 3K MWCO Amicon Ultra Filtration Units (Millipore) as described by the manufacturer protocol. The concentrated LRT-EV protein fractions then had 0.1% of the MS compatible
RapiGest surfactant (Waters) added, followed by incubation on ice for 10 minutes. Samples were then reduced (10 mM DTT for 30 minutes at 37°C), alkylated (20 mM IAA for 30 minutes in the dark at room temperature), and Trypsin Gold (Promega) digested as previously described. Following protein digestion, all samples were desalted/concentrated using OMIX C18 zip-tips (VARIAN) according to manufacturer protocol and prepared for liquid chromatography MS/MS analysis as previously described. Peptides were separated on a NanoAccuity UPLC system (Waters) with a C18 (Phenomenex) column with a 240 minute gradient (2%-98% acetonitrile) and analyzed with a LTQ-Orbitrap Velos MS (Thermo) as previously described.

The MS/MS spectral data was searched against the rodent UniprotKB protein databases in Mascot and SEQUEST algorithms, followed by comparative analysis using the Visualize proteomic software. Within the software, preference was given to rat, and the Mascot/SEQUEST matches were combined for each run using the combine search function that matches each scan to the best spectral match from either algorithm to ensure no redundancy. Filters were applied for each search, including a Visualize P>0.85 (FDR<5%). All biological replicates (N=3 with two technical replicates of each; 6 total runs) were then combined in Visualize where a peptide filter of ≥2 peptides and a scan count filter of 6 was applied. The unique proteins detected in the experiments were uploaded to Ingenuity Pathway Analysis using the Uniprot Accession number for subsequent categorization of proteins by known cellular location and function/type. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019207 and 10.6019/PXD019207.

**Statistical Analysis.** Histological assessment for the presence of LRT-EVs in proximal tubules (Figure 1D) are represented as average ± SEM. These data were not normally distributed as
assessed by the Shapiro-Wilk Test. Therefore, the non-parametric, Mann-Whitney Test was used to determine the presence of significant differences ($P<0.05$) between groups.
RESULTS

We observed vesicles budding from proximal tubules 10 weeks post 5/6Nx surgery (Figure 1A), a time when these rats exhibit substantial pathology including tubular damage, proteinuria, and cardiovascular dysfunction. Sham-operated rats had intact brush borders and lacked lumenal vesicles. In 5/6Nx rats, the PT epithelial cells in regions of large vesicle production had intact nuclei upon visual inspection, were not TUNEL positive, and no TUNEL or DAPI signal was detected within the vesicles (Figure 1B), suggesting that these vesicles are not apoptotic bodies.

We isolated LRT-EVs (Figure 1C) from 5/6Nx rat urine using a purpose-developed, filtration and differential centrifugation methodology (Supplemental Figure 1). We histologically determined the presence of LRT-EVs in PTs in sham and 5/6Nx rats at 2, 4, 5, 7, and 10 weeks post-surgery (Figure 1D). The presence of LRT-EVs in PTs was time-dependent. At two weeks post-surgery 1.9 ± 1.4% (Avg ± SEM) of 5/6Nx PTs had LRT-EVs; at four weeks, 8.0 ± 2.7%, at five weeks 7.0 ± 2.7%, at seven weeks 51.1 ± 6.4%, and at 10 weeks 59.2 ± 4.8. The PTs of sham operated rats had virtually no LRT-EVs at any of those time points. Microscopic image analysis of LRT-EVs isolated from urine at 10 weeks post-surgery revealed them to be circular in shape (in the 2D images) with an average diameter of 2.8 ± 1.5 µm (std. deviation), and median diameter of 2.5 µm. It is possible that LRT-EVs of larger diameter were eliminated as part of the isolation methodology. LRT-EV size and protein markers render them distinct from exosomes, microvesicles, and apoptotic bodies (Figure 1E).

Proteomic analysis of isolated LRT-EVs detected 447 proteins (Supplemental Table 1). These proteins were of diverse functionality and derive from numerous cellular locations (Figure 2A). Cytoplasmic proteins comprised 46.3% of the total, extracellular space proteins 23.7%, nuclear proteins 7.4%, and plasma membrane proteins 20.1%. Proteins within these respective
cellular domains were subdivided by functionality. Subgroups included: transporters, enzymes, ion channels, kinases, peptidases, transcriptional regulators, phosphatases, G-protein coupled receptors, cytokines, and transmembrane receptors. The LRT-EVs contained megalin (low density lipoprotein-related protein 2; LRP2) in relatively high quantities. Megalin is a PT microvilli reabsorption mediator of numerous ligands including vitamin carrier proteins, lipoproteins, hormones, enzymes, immune-related proteins, and select drugs and toxins.14 Consistent with the proteomics, immunohistochemical analysis indicated the presence of megalin within LRT-EVs (Figure 2B). We also observed that megalin was abundant throughout the PT in 5/6Nx rats, but its intracellular localization was abnormally distributed. Sodium-hydrogen antiporter 3 (NHE3, aka Slc9a3) is a PT protein that was not identified in LRT-EVs. Consistent with these data, immunohistochemical analysis indicated robust NHE3 expression in sham operated rat PT epithelial cells, reduced expression in PT epithelial cells of 5/6Nx rats, and no expression in LRT-EVs (Figure 2C).
DISCUSSION

We demonstrate there to be innumerable protein-laden, LRT-EVs in the PTs and urine of 5/6Nx rats 10 weeks post-surgery. LRT-EVs house proteins essential to PT reabsorption. The importance of PT brush border reabsorption is made clinically evident by the severity of genetic diseases in which it is comprised (e.g. Dent disease, Fanconi syndrome, etc.). The loss of numerous functionally important proteins through LRT-EV formation in a CKD-like model of chronic renal stress/insufficiency has not been characterized. These shed proteins are varied in function, and therefore their loss likely contributes to a spectrum of CKD related phenotypes. The effect size of these lost proteins upon cellular function is likely dependent upon a complex relationship between the magnitude of protein loss, cellular demand for that protein’s function, and the cell’s ability to compensate through translational replacement and/or compensatory mechanisms. Loss of proximal tubule proteins has been described in other models of renal injury.\textsuperscript{3,15} It is possible this protein loss is caused, in part, by LRT-EV formation and excretion, although additional studies would be required to confirm this.

LRT-EV size, lack of protein markers specific to other extracellular vesicles, and presence only in specific pathological settings, render them distinct from relatively well characterized urine extracellular vesicles such as exosomes, microvesicles, and apoptotic bodies. To the best of our knowledge, this is the first explicit description of LRT-EVs. While much work remains to more fully characterize LRT-EVs, this study enables us to draw several inferences about them. First, LRT-EVs are likely derived, at least in part, from the PT epithelium. This is suggested by their absence in Bowman’s capsule, presence in the PT, their temporal correspondence to PT brush border loss, and their containment of many PT specific proteins including aquaporin 1, cubilin, megalin, and others. Second, LRT-EV presence in CKD
rats but not sham 5/6Nx rats may suggest LRT-EVs lack membership within the “healthy” tubule extracellular vesicle milieu, but rather, exist consequent to pathology. In this respect, LRT-EVs bear resemblance to oncosomes and exophers. Oncosomes are very large EVs (1-10 µm), carry oncogenic proteins, and have only been observed in association with cancer.\textsuperscript{16,17} Exophers, also large (4 µm), are EVs produced by \textit{C. Elegans} neurons only under specific conditions of neuronal stress. Exophers appear to be a means by which these neurons package and jettison neurotoxic components.\textsuperscript{18} Not only are LRT-EVs similar to oncosomes and exophers in size, but also in their derivation from a particular tissue type only under specific pathological conditions. In contrast, exosomes and microvesicles are found in urine from healthy individuals. Third, urine exosomes, microvesicles, and apoptotic bodies are each associated with specific protein markers. These markers often reflect the mechanism of their respective vesicle’s formation. For example, exosomes are endosomal in origin and contain protein markers (e.g. TSG101, Alix, etc.) associated with the endosomal system.\textsuperscript{5,19,20} Similarly, microvesicles form through outward budding of the plasma membrane; some proteins involved in this process, such as ARF6, are microvesicle protein markers.\textsuperscript{19} Apoptotic bodies are associated with markers of apoptosis such as caspase 3.\textsuperscript{19,20} The protein markers of exosomes, microvesicles, and apoptotic bodies were largely absent from LRT-EVs, perhaps suggesting LRT-EVs have a formation mechanism distinct from those extracellular vesicles.

Many LRT-EV proteins are important for maintenance of cellular homeostasis (Figure 3A). For example, regucalcin (RGN) is an important nuclear and cytoplasmic Ca\textsuperscript{2+} regulator. Superoxide dismutase 1 & 3 (SOD1 & SOD3) are critical to the cellular response to reactive oxygen species. VDAC1, VDAC2 and ATP Synthase subunits (ATP5F1A, ATP5F1B, ATP5PB) play key roles in mitochondrial energetics. Loss of such proteins may reduce the PT’s capacity
for reabsorption, albeit indirectly. LRT-EVs also contained many proteins directly involved in reabsorption (Figure 3B). Their collective loss likely contributes to a spectrum of CKD related phenotypes. Our proteomic analysis identified 16 proteins of the solute carrier family; plasma membrane transporters that facilitate reabsorption of many solutes. Among them were SLC3A1 and SLC34A1, functional loss of which is associated with cystinuria and phosphateuria, respectively.\textsuperscript{21,22} Proteomics identified sodium-glucose co-transporters (SLC5A1 and SLC5A2) in LRT-EVs. Their functional inhibition is associated with glucosuria, which is reported to occur with progressive brush border loss.\textsuperscript{3} Megalin and cubilin, both found within LRT-EVs, are receptors that facilitate reabsorption of a wide variety of ligands including proteins. LRT-EVs contained 5 subunits of vacuolar-ATPase (ATP6V1A, ATP6V1B2, ATP6V0D1, ATP6V0C, ATP6V1E1), a proton pump that is in PT epithelia as well as distal tubule intercalated cells.\textsuperscript{23} It regulates organelle pH and endocytic reabsorption of filtrate proteins, among other functions.\textsuperscript{24,25} Also lost in urine-excreted LRT-EVs was G-protein coupled receptor family C group 5 member C (GPRC5C). Gprc5c KO mice have reduced blood pH and elevated urine pH.\textsuperscript{26} LRT-EVs contained Aquaporin 1 (AQP1), an important mediator of water balance. AQP1 loss impairs the kidney’s ability to concentrate urine.\textsuperscript{27} LRT-EVs contained Na\textsuperscript{+}/K\textsuperscript{+}-ATPase subunit alpha 1 (ATP1A1), a primary component of the basolateral Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. The transmembrane Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is responsible for generating the electro-chemical gradient that facilitates virtually all energy-dependent tubular reabsorption in the PT.

Some PT proteins abundantly expressed in health were identified in LRT-EVs (e.g. SLC2A1 and SLC34A1), while others were not, such as SLC9A3 (NHE3). This could indicate that inclusion of specific proteins into LRT-EVs is not based strictly upon abundance. It may also reflect intrinsic protein expression changes consequent of pathology. For example, NHE3
expression, highly expressed in health, is significantly reduced only two weeks following 5/6Nx in rats. Our immunohistochemistry analysis of NHE3 expression (Figure 2C) is consistent with these previous studies. Thus, LRT-EVs may lacked NHE3, and other PT proteins abundantly expressed in health, due to a pathology-related reduction in expression. Interestingly, NHE3-regulator 1 (NHE3R1) was identified in LRT-EVs. NHE3R1 is involved in NHE3 intracellular trafficking. Thus, loss of NE3R1 to LRT-EVs may have contributed to the differences in NHE3 expression between sham and 5/6Nx rat PT epithelia. From this study we are unable to determine how and why specific proteins were included in LRT-EVs while others not. Inclusion likely depends upon a complex amalgam of regulated and stochastic factors.

Several studies have made important contributions to our knowledge of the PT and urinary proteome. The “shotgun” proteomics-based analysis of 5/6Nx LRT-EVs provided here adds to our understanding of renal tubular pathophysiology. Hypothesis driven investigations of LRT-EV production and characteristics in different disease states and model systems may provide deeper understanding of changes that occur during CKD progression. This study had a number of limitations. First, the marked increase in LRT-EV formation and excretion that occurred between week 5 and 7 post-surgery coincided with the timing of a sharp decline in renal function in this model including elevations in proteinuria, and an abrupt increase in histological evidence of PCT pathology including hypertrophy and dilation. Despite this, we are unable from this study to determine conclusively that LRT-EVs are a pathogenic mechanism of CKD. However, that they house functionally important tubule proteins suggests they are, at a minimum, formed during a period of rapid worsening of renal function in the 5/6Nx model of CKD. Second, many LRT-EV proteins were specific to the proximal tubules, but others were not. For example, LRT-EVs contained FABP3, which, in health, is expressed in greater abundance in distal than proximal
tubules, and is considered a urinary biomarker of distal tubule damage. While PTs appear to be the predominate site of LRT-EV formation, LRT-EVs may be generated at additional sites. Third, we performed our proteomic analysis on LRT-EVs collected from 5/6Nx rats 10 weeks post-surgery. The LRT-EV proteome likely varies with renal injury type and with advances (or retreats) in disease progression.

In conclusion, the 5/6Nx rat model of CKD spontaneously produces LRT-EVs that are urine-excreted. Our proteomic analysis indicates LRT-EVs to contain a wide assortment of proteins, including those specific to the PT epithelium such as transporters, enzymes, transmembrane receptors, and endocytic receptors (Figure 4). This study adds to the rapidly growing field of “omics-based” renal disease extracellular vesicle analysis. Therapies designed to limit LRT-EV formation may improve renal function. Loss of important tubule proteins via excretion of LRT-EVs may represent an underappreciated pathogenic mechanism of renal disease.
DISCLOSURES

All authors have nothing to disclose.

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AUTHOR CONTRIBUTIONS

R Adam: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft; Writing – review and editing

M Paterson: Methodology; Writing - review and editing

L Wardecke: Investigation; Methodology; Writing - review and editing

B Hoffmann: Formal analysis; Validation; Writing - original draft; Writing - review and editing

A Kriegel: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Supervision; Validation; Visualization; Writing - original draft; Writing -
review and editing

SUPPLEMENTAL MATERIAL TABLE OF CONTENTS

- **Supplemental Table 1, Page 1 (legend) and attached Microsoft Excel spreadsheet.**
  List of LRT-EV proteins detected by our proteomic analysis and their IPA-identified cellular location and function.

- **Supplemental Figure 1, Page 1.** LRT-EV isolation methodology.

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Table 1: Summary of protein diversity in LRT-EVs. This table summarizes the 3 most abundant proteins (by scan count and if present) within each functional category (type) of select cellular locations identified by IPA. The 10 most abundant transport proteins in the plasma membrane are indicated. Uniprot accession numbers, protein symbols and Entrez Gene name are also provided. See Supplemental Table 1 for a complete list of IPA-identified protein location/types.

| Location            | Category       | UniProt/Swiss-Prot/GenPept Accession | Symbol | Peptide Count | Scan Count | Entrez Gene Name                      |
|---------------------|----------------|-------------------------------------|--------|---------------|-----------|---------------------------------------|
| Cytoplasmic         | Enzymes        | P19468                              | GCLC   | 25            | 260       | glutamate-cysteine ligase catalytic subunit |
|                     |                | P04764                              | ENO1   | 17            | 139       | enolase 1                              |
|                     |                | P07632                              | SOD1   | 8             | 109       | superoxide dismutase 1                |
| Ion Channels        | P81155         | VDAC2                               | 10     | 44            | 74        | voltage dependent anion channel 2     |
|                     | Q9Z2L0         | VDAC2                               | 6      | 22            | 10        | voltage dependent anion channel 1     |
| Kinases             | Q4KL26         | TKFC                                | 16     | 74            | 36        | triokinase and PKM cyclase            |
|                     | P16617         | PGK1                                | 10     | 40            | 30        | phosphoglycerate kinase 1             |
|                     | P07379         | PCK1                                | 8      | 30            | 30        | phosphoenolpyruvate carboxykinase 1   |
| Peptidases          | P00758         | KLK1                                | 8      | 165           |           | kallikrein 1                          |
|                     | P00787         | CT5B                                | 12     | 147           |           | cathepsin B                           |
|                     | P16675         | CTSA                                | 10     | 125           |           | cathepsin A                           |
| Phosphatases        | P25113         | PGAM1                               | 8      | 36            | 36        | phosphoglycerate mutase 1             |
|                     | P20611         | ACP2                                | 8      | 27            | 27        | acid phosphatase 2, lysosomal         |
|                     | Q91YE9         | NTS1C1B                             | 8      | 18            | 18        | 5'-nucleotidase, cytosolic IB         |
| Transporters        | P55054         | FABP9                               | 10     | 60            | 60        | fatty acid binding protein 9          |
|                     | P19511         | ATP5PB                              | 3      | 59            | 59        | ATP synthase peripheral stalk-membrane subunit b |
|                     | P10719         | ATP5FB                              | 14     | 44            | 44        | ATP synthase F1 subunit beta          |
| Cytokines           | P08721         | SPP1                                | 7      | 86            | 86        | secreted phosphoprotein 1             |
|                     | Q09CP74        | MYDGF                               | 3      | 14            | 14        | myeloid derived growth factor         |
|                     | P06684         | CS                                  | 4      | 12            | 12        | complement C5                         |
| Growth Factors      | P07522         | EGF                                 | 44     | 590           | 590       | epidermal growth factor               |
|                     | P01015         | AGT                                 | 15     | 189           | 189       | angiogenin                        |
|                     | P23785         | GRN                                 | 7      | 98            | 98        | granulin precursor                   |
| Extracellular       | Other Proteins | P17475                              | SERPINA1 | 40  | 2659       | serpin family A member 1              |
|                     |                | P05545                              | Serpina3c/Serpina3m | 32  | 2252       | serine (or cysteine) proteinase inhibitor, clade A, member 3C |
|                     |                | P27590                              | UMOD    | 24  | 1724       | uromodulin                             |
| Peptidases          | Q01177         | PLG                                 | 58     | 1172          | 1172      | plasminogen                             |
|                     | P01026         | C3                                  | 78     | 949           | 949       | complement C3                         |
|                     | P06866         | HP                                  | 24     | 401           | 401       | haptoglobin                             |
| Transporters        | P02770         | ALB                                 | 9      | 16638         | 16638     | albumin                                 |
|                     | P12346         | TF                                  | 65     | 3405          | 3405      | transferrin                             |
|                     | P14046         | Tmgl1                               | 74     | 1583          | 1583      | Muringoglobin 1                        |
| Plasma Membrane     | Enzymes        | P07314                              | GGT1   | 21            | 324       | gamma-glutamyltransferase 1           |
|                     | P97675         | ENPP3                               | 10     | 23            | 23        | ectonucleotide pyrophosphatase/phosphodiesterase 3 |
| Category            | Accession | Code | Position | Description                                           |
|---------------------|-----------|------|----------|-------------------------------------------------------|
| G-Protein Coupled Receptors | Q8CFN2    | Cdc42| 3        | cell division cycle 42                               |
|                     | Q3XRC4    | GPRC5C| 2        | G protein-coupled receptor class C group 5 member C   |
| Ion Channels        | Q9Z0W7    | CLIC4| 10       | chloride intracellular channel 4                      |
|                     | Q9Z0Y8    | CACNA1I| 2       | calcium voltage-gated channel subunit alpha1 I        |
|                     | Q91YD4    | TRPM2| 2        | transient receptor potential cation channel subfamily M member 2 |
| Kinases             | Q01279    | EGFR | 4        | epidermal growth factor receptor                      |
| Other Proteins      | P11977    | EZR  | 28       | ezrin                                                |
|                     | Q9R0T4    | CDH1 | 13       | cadherin 1                                            |
|                     | Q9U19     | SLC9A3R1| 25     | SLC9A3 regulator 1                                    |
| Peptidases          | P15684    | ANPEP| 30       | alanyl aminopeptidase, membrane                       |
|                     | P07861    | MME  | 34       | membrane metalloendopeptidase                         |
|                     | P14740    | DPP4 | 31       | dipeptidyl peptidase 4                               |
| Transmembrane Proteins | P07151   | B2M  | 8        | beta-2-microglobulin                                  |
|                     | Q07244    | CUBN | 17       | cubulin                                               |
|                     | Q63257    | IL4R | 4        | interleukin 4 receptor                                |
| Transporters        | P98158    | LRP2 | 106      | LDL receptor related protein 2/megalin               |
|                     | Q64319    | SLC3A1| 31      | solute carrier family 3 member 1                     |
|                     | Q0J40     | PDZK1| 20       | PDZ domain containing 1                               |
|                     | P29975    | AQP1 | 8        | aquaporin 1                                           |
|                     | P15083    | PIGR | 21       | polymeric immunoglobulin receptor                     |
|                     | Q62687    | SLC6A18| 12      | solute carrier family 6 member 18                     |
|                     | Q63424    | SLC15A2| 16     | solute carrier family 15 member 2                     |
|                     | Q91WY7    | SLC23A1| 11     | solute carrier family 23 member 1                     |
|                     | Q80W57    | ABCG2| 9        | ATP binding cassette subfamily G member 2             |
|                     | P50516    | ATP6V1A| 9      | ATPase H+ transporting V1 subunit A                   |
Figure 1

A Renal Cortex, Masson’s Trichrome

B Renal Cortex, TUNEL (Apoptosis Marker)

C Isolation and Brightfield Microscopy of Urinary LRT-EVs

D Percentage of Proximal Tubules with Intraluminal LRT-EVs on Histological Examination

*P<0.05 vs. Sham of same week post Nx
#P<0.05 vs. 10 Wks Post Nx, 5/6Nx Group

E Size and Protein Markers of LRT-EVs vs. Other Extracellular Vesicles

- LRT-EVs: Diameter: 1-20 μm
  (Individual sizes shown)
  Markers: Unknown

- Apoptotic Bodies: Diameter: 0.8-5 μm
  Markers: Prohibin, histones, caspase 3

- Microvesicles: Diameter: 0.1-1 μm
  Markers: VCAMP3, ARF6, CD40 ligand

- Exosomes: Diameter: 40-100 nm
  Markers: CD9, CD53, CD81, AUX, Tsg101, HSP70, HSP90
**Figure 1.** LRT-EV isolation and evaluation. A) Examination of renal cortical tissue collected from 5/6Nx and sham-operated controls 10 weeks post-surgery. 5/6Nx rats exhibited extensive brush border loss and the presence of large extracellular vesicles (LRT-EVs) in PT lumens (arrows). B) TUNEL indicates apoptosis by labeling DNA double strand breaks. Kidney sections from sham-operated (sham; left) and 5/6Nx (center) rats were TUNEL negative (488 nm autofluorescence, green; DAPI, blue). Importantly, the large vesicles within the lumen and the nuclei of the cells producing the vesicles were both TUNEL negative. A section treated with DNase (right) serves as a positive control for the assay (DAPI in blue co-localized with TUNEL in bright green). C) These vesicles were isolated from 5/6Nx rat urine through a sequence of filtration and centrifugation (see Supplemental Figure 1 for detailed methodology). Sham rat urine lacked LRT-EVs. D) Proximal convoluted tubules were scored for the presence of LRT-EVs in separate cohorts of sham and 5/6Nx rats at 2, 4, 5, 7, and 10 weeks post-surgery. Each dot represents one animal. E) The size, as measured by microscopic analysis, and lack of apoptotic markers suggest these vesicles (LRT-EVs) represent a unique category of urine-excreted, extracellular vesicle, prompting proteomic analysis to characterize their composition. A subset of published diameters and protein markers of exosomes, microvesicles, and apoptotic bodies are represented here. \(^5,^{19,20}\) Individual LRT-EV diameter measurements are shown; horizontal bar equals average.
Figure 2

A. LRT-EV Proteins by Cellular Compartment

| Extracellular Matrix (24%) | Nucleus (7%) | Cytoplasm (46%) | Other (3%) | Plasma Membrane (20%) |
|---------------------------|-------------|------------------|-----------|----------------------|

LRT-EV Protein Groups Within Cellular Compartments

- Cytokine (4%)
- Other (49%)
- Peptidase (15%)
- Growth factor (4%)
- Transporter (15%)
- Enzyme (12%)
- Enzyme (24%)
- Ion channel (3%)
- Kinase (9%)
- Other (42%)
- Transcription regulator (18%)
- Transporter (3%)
- Kinase (3%)
- Peptidase (9%)
- Transporter (9%)
- Ion Channel (1%)
- Other (24%)
- Phosphatase (2%)
- Enzyme (51%)
- Transcription regulator (1%)
- Transporter (32%)
- Kinase (1%)
- Enzyme (11%)
- Peptidase (9%)
- G-protein coupled receptor (1%)
- Other (31%)
- Ion Channel (3%)

B. Rat Cortex Immunohistochemistry. Megalin (red), DAPI (blue), Autofluorescence (green).

Sham

5/6Nx

5/6Nx (inset)

C. Rat Cortex Immunohistochemistry. NHE3 (red), DAPI (blue), Autofluorescence (green).

Sham

5/6Nx

5/6Nx (inset)
Figure 2. Cellular location and type of LRT-EV proteins identified through proteomic analysis. A) Proteins identified through MS/MS data analysis (N=3 with two technical replicates of each; six runs total) and meeting a minimum scan count of 6 and peptide count of 2, were uploaded to Ingenuity Pathway Analysis to categorize these proteins based on cellular compartment and protein type/function. The percentage of identified LRT-EV proteins per cellular compartment (location) is depicted on the horizontal bar at the top. The proteins within each cellular compartment are then subcategorized by type, with percentage within each compartment indicated at the right of vertical bars. B) Proteomic analysis identified the plasma membrane transporter megalin in LRT-EVs. Representative immunohistochemistry of kidney tissues collected 10 weeks post-surgery show presence of megalin (red) localized at the base of the brush border on the apical membrane in sham-operated (sham) rats. In 5/6Nx rats megalin can be seen in LRT-EVs that are within the tubule lumen and LRT-EVs emerging from the proximal tubule cells. The distribution of megalin is diffuse (yellow) or absent in some tubular cells. Image on right is an inset from center image. DAPI, blue; autofluorescence at 455 nm (green); Cal. bar = 100 µm. C) Proteomic analysis failed to detect NHE3 in LRT-EVs. Immunohistochemistry of kidney tissue collected 10 weeks post-surgery show the presence of NHE3 (red) in sham and 5/6Nx rat proximal tubule epithelial, but not in LRT-EVs. DAPI, blue; autofluorescence at 455 nm (green); Cal. Bar = 50 µm.
Figure 3

A. Select Proteins Important For Cellular Homeostasis Lost To LRT-EVs

| Protein                                                        | Anticipated Consequent Impairment       |
|----------------------------------------------------------------|----------------------------------------|
| Regucalcin (Cytosolic, nuclear)                                  | Ca\(^{2+}\) handling                    |
| CLIC 1 & 4 (Membrane, cytosolic, nuclear)                       | Cl\(^{-}\) handling                     |
| ATP synthase                                                    | Mitochondrial energetics               |
| VDAC2                                                          | Mitochondrial oxidative metabolism     |
| Vitamin D binding protein (Serum)                               | Vitamin D handling                     |
| Malate dehydrogenase 1                                          | Many metabolic pathways                |
| FABP 3, 6, & 9                                                  | Fatty acid handling                    |
| SOD 1 & 3                                                      | ROS handling                           |

B. Select Proximal Convoluted Tubule Proteins Lost To LRT-EVs

| Basolateral            | Apical                                      | Anticipated Consequent Impairment       |
|-----------------------|---------------------------------------------|----------------------------------------|
| Aquaporin 1           | Ability to concentrate urine                |
| SLC34A1               | Phosphate handling                          |
| SLC3A1                | Cystinuria                                  |
| Na\(^{+}\)/K\(^{+}\)-ATPase | Transmembrane electrochemical gradient balance |
| Alanine Aminopeptidase | Na\(^{+}\), Ang III handling                |
| Vacuolar ATPase (Organelle & Apical membranes)                | Acid-base regulation                     |
| Megalin               | Proteinuria                                 |
| Cubilin               | Proteinuria                                 |
| GPRC5C                | Acid-base regulation                        |
| Neprilysin            | Peptide catabolism including ANP, and Ang II |
| SGLT1 & 2             | Glucosuria                                 |
Figure 3. Graphical summary of select LRT-EV proteins important for maintenance of cellular homeostasis (A), and directly related to PT reabsorption (B).

Figure 4. Schematic summary. Proteins with essential proximal tubule functions are lost to LRT-EVs and expelled in urine in the 5/6Nx rat model of CKD.