D-Serine, an emerging biomarker of kidney diseases, is a hidden substrate of sodium-coupled monocarboxylate transporters

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Abstract

Delay in diagnosis of renal injury has profound impacts on morbidity and mortality. Damage of proximal tubules by the injury impairs not only individual membrane transport proteins but also coordinated transport systems. In this study, we analyzed the proteome of apical membranes of proximal tubular epithelium from the mouse kidney with early ischemia-reperfusion injury (IRI), a well-known acute kidney injury (AKI) model. The proteome showed drastic elevations of the injury-responsive proteins and depressions of abundant transporters, leading to the prediction of biomarkers for early IRI. As the benchmark of our in-depth analysis from the proteome, we characterized transporters for D-serine, a promising renal diseases' biomarker. Using cell-based and cell-free methods, we identified sodium-coupled monocarboxylate transporters (SMCTs) as the D-serine transporters. This finding enlightens the role of non-canonical substrate transport and clarifies the D-serine transport systems in the kidney. Our approaches can be applied for investigation of other membrane transport systems.

Impact statement

Proteomics and biochemical analysis reveal D-serine as a non-canonical substrate of sodium-coupled monocarboxylate transporter SMCTs

Introduction

The renal system, where membrane transport proteins are the most diverse and abundant, plays crucial roles in body homeostasis, thereby often being the most impacted from critical illness and vulnerability to the subtle changes in molecular substances. Acute kidney injury (AKI) and chronic kidney diseases (CKD), which are common diseases worldwide, cause severe health problems but encounter the limitations on standard diagnosis and classification (Zhang and Parikh, 2019). AKI and CKD are considered as interconnected syndromes. Although AKI happens suddenly and is reversible, the extension of the injury and incomplete repairment of renal tubular cells consecutively lead to CKD progression (Devarajan and Jefferies, 2016). Hence, the gold-standard diagnosis at the early stages of AKI is an important key factor needed in order to maximize the therapeutic success and minimize CKD development. Conventional markers for AKI diagnosis include measuring serum creatinine levels and estimated Glomerular filtration rate (eGFR). Still, both methods are limited in their specificity, lack of standardized quantification, variation in individuals, and limited usage in patients with comorbidities (Ostermann and Joannidis, 2016). Lately, systemic analysis using innovative technologies, such as Integrative Omics and Next-Generation Sequencing, have opened new possibilities for detecting biomarkers of AKI and CKD. Yet, the new biomarkers have restrictions on their application and efficacy due to the specificity and the unclear characterization of the underlying molecular mechanism (Eddy et al., 2020; Kirita et al., 2020; Liu et al., 2020; Marx et al., 2018; Thongboonkerd, 2020; Zhang and Parikh, 2019).

Proximal tubule, the main part for reabsorption and secretion of metabolites and organic compounds in the kidney, is the primary target of AKI and CKD progression (Chevalier, 2016). Thus, apical (luminal) membrane proteins from proximal tubular epithelium would represent significant pathological membrane transport proteins that are the underlying mechanisms of metabolite biomarkers for AKI and CKD. D-Serine, a dextro isomer of serine, is discovered as

one of the promising biomarkers for AKI and CKD detections using the 2D-HPLC method (Hesaka et al., 2019; Kimura et al., 2020, 2016; Sasabe et al., 2014; Sasabe and Suzuki, 2018). In the normal condition, D-serine is in high ratio to urine but low in plasma. Contrarily, the plasma D-serine is elevated in patients and animal models with CKD and AKI, while the luminal D-serine is decreased. The amount of D-serine is strongly associated with kidney function and progression of these diseases. Transport of D-serine takes place at the proximal tubule (Kragh-Hansen and Sheikh, 1984; Sasabe and Suzuki, 2018; Shimomura et al., 1988; Silbernagl et al., 1999). However, such a corresponding transporter in both physiological and pathological conditions has not been clearly explained.

D-Serine was firstly found highly abundant in the brain. Extensive studies have revealed that D-serine plays important roles in neurotransmitter modulation by acting as an obligatory physiological co-agonist of *N*-methyl-D-aspartate receptors (NMDARs) (Wolosker, 2018). Until now, three amino acid transporters, SLC1A4/ASCT1, SLC1A5/ASCT2, and SLC7A10/Asc-1 are recognized to mediate D-serine transport in the brain (Foster et al., 2016; Rosenberg et al., 2013). Despite the role and transport system of D-serine in brain, little is known about D-serine in peripheral tissues. Mammals acquire D-serine by biosynthesis via serine racemase function and absorption from dietary and gut microbiota via intestinal transport system(s) (Sasabe et al., 2016; Sasabe and Suzuki, 2018). The D-serine homeostasis in the plasma is controlled by luminal-membrane transporters and DAAO activities in the intestine and kidney. In the kidney, D-serine from blood, which passed through the renal glomeruli, is reabsorbed at proximal tubules. Subsequently, intracellular D-serine is degraded by DAAO, which is located in the proximal tubular cells, thereby maintaining the plasma D-serine at low levels (Sasabe et al., 2014). It is suggested by the serum and urinary D/L serine profile of the CKD patients and AKI animal models that the renal D-serine transport properties in

physiological conditions and in pathological conditions are different and have distinct stereoselectivity (Sasabe and Suzuki, 2018).

The reabsorption and excretion of a substance in the kidney is co-operated by multiple plasma membrane transport proteins (Chu et al., 2016; Ghezzi et al., 2018; Kandasamy et al., 2018). Malfunction of a single type of membrane transport protein causes a fluctuation in the chemical gradient of the substrates and affects other transport systems that also transport the same substance. Therefore, it is necessary to consider that renal dysfunction is caused by not only the breaking down of several transport systems themselves but also the disruption of the coordination between each transport system. Here, we applied proteomics of apical membranes from renal proximal tubules of the mice with ischemia-reperfusion injury (IRI), a well-known model for AKI and AKI-CKD transition (Fu et al., 2018; Sasabe et al., 2014), to reveal the membrane transport proteins that are responsible for the transport of AKI metabolite biomarkers, especially the D-serine transport system as a paradigm. By further screening and kinetic analysis, we identified two D-serine transport systems at the apical membranes of proximal tubules—sodium-coupled monocarboxylate transporters (SMCTs) and neutral amino acid transporter ASCT2, which explain the mechanism of chiral selection and elevation of D-serine transport in AKI and CKD.

Results

Membrane proteome of renal proximal tubules of the IRI model

To understand the molecular pathophysiology of AKI, we characterized proteomes of renal brush border membrane vesicles (BBMVs), the membrane fraction representing the apical membranes of proximal tubular epithelium, from the IRI mouse model. We analyzed the samples from 4 hours and 8 hours after ischemia-reperfusion (4h IRI and 8h IRI) because these time points are considered as early stages of AKI, where serum creatinine levels are slightly increased, and urine KIM-1 remains unchanged (Sasabe et al., 2014). The proteome of IRI samples was calculated as ratios of the negative control group (sham), yielding two groups of comparative proteome data: 4h IRI/sham (4h) and 8h IRI/sham (8h). In total, 4,426 proteins were identified in which 1,186 proteins were categorized as plasma membrane proteins and extracellular matrix proteins (Table supplement 1, Figure supplement 1A). Among them, 1,110 proteins were predicted to possess more than one transmembrane domain (Table supplement 1). We observed a great increase of two well-known early AKI biomarkers—CCN1 (CYR61: matrix-associated heparin-binding protein; the ratio of 4h/sham = 41.4; the ratio of 8h/sham = 29.9) and NGAL (LCN2: Neutrophil gelatinase-associated lipocalin; the ratio of 4h/sham = 1.8; the ratio of 8h/sham = 9.5), which confirmed the reliability of our proteomics on the IRI model for early AKI (Table supplement 1) (Marx et al., 2018). To characterize pathological changes in IRI, we selected 318 proteins which passed a cut-off value of 1.5 fold change ($\log_2$ fold of IRI/sham = 0.58: both increased and decreased) with $p\text{-value} \leq 0.05$ ($-\log_{10} p\text{-value} \geq 1.3$) and further analyzed the biological functions and toxicity functions by using IPA. Top-10 toxicity functions verified the damages of the kidney due to the injury and inflammation (Figure supplement 1B). Hierarchical heatmaps of the organ pathologies prognosticate various defects in the kidney which include tubule dilation, nephritis, kidney injury and damage, and

renal failure (Figure supplement 1C). Besides the renal pathologies, IPA also indicated the injury and damage in the liver and heart as several corresponding proteins are shared among the organs (Figure supplement 1C).

Analysis of biological functions showed the proteome was highly involved in cellular compromise, cell survival, molecular transport, and system development (Figure 1A-B). The hierarchical heatmap exhibited that most of the biological functions are increased (with a decrease of cell death), which indicated the mechanisms related to the injury responses rather than the pathophysiological functions (Figure 1C). We categorized the injury responses into three groups: *rapid responses*, *prolonged responses*, and *slow responses*. The first group, *rapid responses*, shows biological functions are highly increased at 4h IRI but gradually reduced at 8h IRI. The responses related to pro-inflammation and innate immunity, which include the complementary system, free radical scavenging, metal ion elevation, lipid synthesis, lipid transport, and lipid metabolism (Figure 1C). The second group is involved in the biological functions that are rapidly increased at 4h IRI and maintained to 8h IRI, named *prolonged responses*. Cell viability and homeostasis, cell-cell interaction, epithelial cell movement, and vascularization are the biological functions in the *prolonged responses* in order to sustain an effective immune response (Figure 1C). The third group called *slow responses* are those increased functions at 8h IRI, including the adaptive immunity—leukocyte/lymphocyte migration and phagocytosis, immune cell and connective tissue adhesion, and endothelial tissue development (Figure 1C). At 8h, we observed the significant increase of cellular compromise and tissue development, implying the initiation of the repairment systems (Figure 1A-B). Analysis of diseases relevant to biological functions showed the remarkable elevation of the inflammatory response at both 4h and 8h IRI, confirming the significant contribution of the *rapid*, *prolonged*, and *slow responses* (Figure 1D). Although the immune-related responses and tissue repairment were progressive, we still observed the tissue abnormality, tissue damage,

and inflammation (Figure 1D). From the results, we concluded the biological functions in early AKI (both 4h and 8h IRI) as follows: 1) the immune system rapidly and extensively responded to the injury since 4h IRI, 2) the processes of tissue development and repair were initiated at 8h IRI, and 3) the pathological features of the injured kidney were prolonged observed since 4h IRI.

Next, we predicted a series of protein biomarkers to detect early stages of the kidney injury and found 34 proteins significant altered at both 4h and 8h IRI (Figure 1E). Table 1 summarizes the physiological function and previous applications of these proteins. The protein biomarkers previously proposed for AKI, CKD, and related kidney diseases are CCN1/CYR61, LTF, FPR2, CYBB, SERPINF1, and C5AR1. In this study, we predicted 28 protein biomarkers (Figure 1E, Table 1). The protein biomarkers are categorized into four types according to their physiological functions. BTF3, ZFP36L1, TPT1, CDA, RPSA, EIF3D, and RPS27A, which were increased in IRI, are molecules that play a role in protein synthesis. DCTN4, ARL3, PAFAH1B2, OCLN, PCOLCE, GPC4, and CMKLR1 are molecules involved in cell structure, cell-cell interaction, and tissue development. LDLR, ESYT1, and APOA2 are correlated with lipid transport and homeostasis. The last group of candidates which were mostly found decreased in IRI are membrane transport proteins: SLC15A2/PEPT2, SLC19A3/THTR2, SLC5A8/SMCT1, PLXDC2, SLC22A7/OAT2, CYB5B, SLCO4C1/OATP-M1, AQP7, SLC2A5/GLUT5, PIEZO1, and KCNAB2.

We analyzed the biological networks and found two top-scoring networks were related to the *rapid responses* (Figure 2A-B), while another network related to the *prolonged responses* (Figure 2C). Networks at the *slow responses* (8h IRI) have less scoring than the *rapid* and *prolonged* responses (Figure supplement 2). Although we did not examine the total proteome, we could predict the master regulators of the networks from our membrane proteome. In the networks of the *rapid responses*, NF- κ B, PI3K, and Myc are likely the key regulators (Figure

2A-B). HDL complex was found to be a central regulator for the *prolonged responses* and Akt was predicted to be involved (Figure 2C). BCL2, ANXA1, and integrin complex are important regulators for tissue development and repair in the *slow responses* (Figure supplement 2).

Alterations of membrane transport proteins in the kidney of the IRI model

To unveil the renal pathophysiological defect behind the injury responses, we analyzed the whole proteome without any cutoff to recognize slight changes of the silent molecules. The analysis depicted a high scoring of pathological functions related to kidney diseases and the circulatory system (Figure supplement 3). These data intimate that the proximal tubular membrane proteins involved in IRI pathological mechanisms altered less in the amounts than the injury-responsive proteins but were abundant in the membrane proteome. Among these membrane proteins, membrane transport proteins were one of the leading groups. Because the substrates of these membrane transport proteins are well-used metabolite biomarkers for AKI, CKD, and other diseases, we further investigated membrane transport proteins to unveil the pathogenic mechanisms. We selected the 319 membrane transport proteins comprising ATP-binding cassette (ABC) transporters, solute-carriers (SLCs), ATPase pumps, and channels to analyze their biological functions by IPA without any cutoff. The analysis unveiled two transport groups based on the types of substrates; 1) ions and 2) organic compounds (Figure 3A). The ions are composed of inorganic ions (Figure 3A: lower left) and charged organic compounds (Figure 3A: right). The organic compounds comprise both charged and uncharged compounds (Figure 3A: right). IPA analysis showed that the transport of ions remained unchanged at 4h IRI and slightly increased at 8h IRI. The sum of ions which indicated less change was actually made up from the combination of anions and cations in which both of them altered in some degrees at the opposite direction (Figure 3A: left). In particular, sum of ions which showed small alteration was caused by the dynamic alteration of various inorganic

ion transport proteins (both inorganic cations and inorganic anions; Figure 3B). Accordingly, the cells are suggested to have neural pH at 4h IRI and slight pH change at 8h. We suggest that the injured cells attempted to preserve cellular pH and ion homeostasis, which was a foundation for the function of organic compound transporters and substantial protein functions. In contrast to the ion transport group, the protein group that transports organic compounds was strongly decreased at 4h and gradually recovered at 8h (Figure 3A: right). Most proteins in this group were found to be decreased, indicating that their defect was from the damaged brush border membranes and delayed in recovery (Figure 3B). The levels of less alteration in membrane transport proteins also imply the low expression levels compared to the proteins in the injury responses.

Screening of candidate molecules for D-serine transporters

Membrane transport proteins derived from our proteome represent a good collection for identification of transport systems for the anticipated metabolite biomarkers of renal injury with unknown transporter(s) or novel metabolite biomarkers. Since D-serine has been proposed as an effective biomarker for AKI and CKD yet unknown transport mechanism, we aimed to characterize the D-serine transport system in the kidney. From the membrane proteome, we detected the increases of ASCT2 at both 4h and 8h IRI (Figure 4A, Table 2). Using HAP1 cells carrying CRISPR/CAS-mediated *ASCT2* knockout, we examined D-serine uptake and confirmed that ASCT2 is a D-serine transporter (Figure supplement 4A). ASCT2 is located at the apical membranes of all proximal tubular segments and transports serine with high affinity (K_m of 167 μ M for D-serine in oocyte system) but weak stereoselectivity (Foster et al., 2016). However, the previous studies demonstrated D-serine transport systems in S1-S2 and S3 segments are different and their kinetics are in mM range (Kragh-Hansen and Sheikh, 1984; Silbernagl et al., 1999). Silbernagl et al. also suggested ASCT2 is not (or not only) a D-serine

transporter at pars recta (Silbernagl et al., 1999). Notably, the serum and urinary D/L serine profile from CKD and AKI samples indicated the stereoselective transporter(s) (Sasabe and Suzuki, 2018). We thus postulated the existence of (an)other D-serine transporter(s). To screen and identify D-serine transporters, we choose ASCT2 as a pivotal molecule to make the cut-off line in our membrane proteomics data. We selected membrane transport proteins that altered more than ASCT2 at both 4h and 8h IRI (Figure 4A). To further focus on the reabsorption system at the lumen, we omitted transporters that are reported to be in basolateral membranes and organelles. Membrane transport proteins that recognize only inorganic ions were excluded. SLC36A1/PAT1 and SLC6A18/B⁰AT3, known small amino acid transporters, were included, although they only passed the cut-off value at one of the time points, either 4h or 8h. SLC5A12/SMCT2 was also included in the list with less significant alteration because SMCT1, another member of sodium-coupled monocarboxylate (SMCT) family, was selected. SMCT2 has a comparable role with SMCT1 in monocarboxylate reabsorption at apical membranes, but they localize at different segments of proximal tubules. Finally, ten candidates of D-serine transporters were listed (Table 2).

HEK293 cell line was used for the development of the screening method of D-serine transporters because the cells exhibited high transfection efficiency. First, we identified amino acid transporters from membrane proteome of HEK293 cells (Table supplement 2). Then, we examined which transporters are responsible for D-serine transport in the cells. From the results, D-serine transport in HEK293 cells required Na⁺ and was inhibited by ASCT2 substrates (L-Ser, L-Thr, and L-Met) (Figure supplement 4B-C). Inhibition by Ben-Cys and GPNA in HEK293 cells showed a similar pattern to that of ASCT2-expressed cells (Bröer et al., 2016), but MeAIB (system A inhibitor) had no effect (Figure supplement 4C). Using siRNA to knockdown ASCT2, we verified that ASCT2 is a main D-serine transporter in HEK293 cells (Figure 4B-C). A previous study showed that treatment with D-serine in a human proximal

tubular cell line impaired the growth (Okada et al., 2017). We found that this growth defect occurred in HEK293 cells and was due to the ASCT2 function. Although L-serine showed no effect, D-serine reduced cell growth in a concentration-dependent manner with IC₅₀ of 17.4 mM (Figure supplement 4D). In the ASCT2-knockdown cells, the growth inhibition by D-serine was attenuated (Figure 4D), indicating the D-serine taken up by ASCT2 contributes to the D-serine-suppressed cell growth.

Based on the findings above, we investigated the candidate transporters from the proteome data by the D-serine effect on cell growth. With D-serine treatment at both 15 mM (near the IC₅₀ concentration) and 25 mM (high concentration), Asc-1 transfected cells (positive control) showed significantly lower growth than the Mock cells, confirming this assay is effective to screen D-serine transporters. Among the ten candidates, PAT1 and B⁰AT3 attenuated growth suppression while SMCT1 and SMCT2 exaggerated growth suppression over Mock at both 15 and 25 mM D-serine treatment (Figure 5A-B), suggesting that both SMCTs transport D-serine into the cells. Accordingly, SMCT1 and SMCT2 are primarily selected for further analysis (Figure supplement 5).

Characterization of D-serine transport in SMCTs.

SMCTs are sodium-dependent monocarboxylate symporters. Their canonical substrates are short-chain fatty acids and monocarboxylates such as lactate, propionate, and nicotinate (Ganapathy et al., 2008). To characterize D-serine transport in both SMCT1 and SMCT2, we generated Flp-In T-REx 293 cells stably expressing human SMCT1 (FlpInTR-SMCT1) and SMCT2 (FlpInTR-SMCT2). Expression of SMCTs in FlpInTR-SMCT1 and FlpInTR-SMCT2 was verified by Western blot using anti-FLAG antibody to detect FLAG-tagged on SMCTs. SMCT1 and SMCT2 expression remain unchanged upon ASCT2 knockdown (Figure supplement 6A). The transport function of SMCTs was verified by

[¹⁴C]nicotinate uptake (Figure supplement 6B-C). Ibuprofen, an SMCT inhibitor, was used to verify the growth inhibition effect by SMCTs. Unlike Mock in which ibuprofen has no effect, the growth inhibition by D-serine treatment were gradually attenuated by ibuprofen in both FlpInTR-SMCT1 and FlpInTR-SMCT2 cells (Figure 5C).

Transport of D-[³H]serine was measured in the SMCT stable cell lines. Both SMCT1 and SMCT2 cells showed an increase of D-[³H]serine transport compared to Mock with or without ASCT2 knockdown (Figure 6A and supplement 6D). The D-[³H]serine transport in both SMCT1 and SMCT2 was inhibited by NSAIDs (ibuprofen and acetylsalicylate: SMCT inhibitors) (Figure 6B) (Gopal et al., 2007; Itagaki et al., 2006). D-Serine and nicotinate also inhibited D-[³H]serine uptake in the SMCT1 cells but much less effective in the SMCT2 cells suggesting the low affinity of D-serine in SMCT2 than SMCT1 (Figure 6B).

Transport properties and kinetics of D-serine in SMCT1

Kinetics of D-[³H]serine transport in SMCT1 was measured in FlpInTR-SMCT1 cells in the presence of ASCT2 knockdown. SMCT1 transported D-[³H]serine in a concentration-dependent manner, and the curve fitted to Michaelis–Menten kinetics with the apparent K_m of 5.0 mM (Figure 6C, supplement 6E).

Because there were the high backgrounds of D-serine transport in cells, even in the ASCT2-knockdown condition, we assumed that endogenous transporters, including uncharacterized molecules, and metabolites impede the examination of the SMCT1 properties precisely. We then characterized the D-serine transport properties of the purified SMCT1 in SMCT1-reconstituted proteoliposomes. Liposomes are spherical-shaped vesicle made from lipid mixtures to mimic the membrane lipid bilayer. The proteoliposomes are the liposomes with a protein incorporated. With the proteoliposome system, both internal and external substances can be squarely defined. 3xFLAG-tagged SMCT1 was purified from Expi293F cells

transfected with pCMV14-SMCT1 by anti-FLAG affinity column. Purified SMCT1 showed a single band on SDS-PAGE corresponding to the expected size (Figure 7A), and the protein was confirmed by Western blot using anti-FLAG antibody (Figure supplement 7A). SDS-PAGE showed the successful incorporation of the purified SMCT1 in the proteoliposomes (SMCT1-PL) (Figure 7A). We measured the uptake of L- and D-[³H]serine in SMCT1-PL. In the presence of external Na⁺, SMCT1 transported both L- and D-[³H]serine as well as the canonical substrates ([³H]lactate and [³H]propionate) (Figure 7B, supplement 7B). The transport was dramatically inhibited by ibuprofen, confirming the function of SMCT1 (Figure 7B, supplement 7B). The time courses of D-[³H]serine uptake were measured in both Na⁺ and Na⁺-free buffers. The results showed that SMCT1 transported D-[³H]serine in Na⁺-dependent condition (Figure 7C and supplement 7C). The uptake value in Na⁺-free buffer is a result of non-specific accumulation in liposomes/proteoliposomes, which is slightly linear upon the incubation time (Figure supplement 7C). SMCT1 recognized both L- and D-serine over other small amino acids (L- and D-alanine), acidic amino acids (L- and D-glutamate), or large neutral amino acids (L- and D-tyrosine) (Figure 7D). Uptake of L- and D-serine was approximately 30% and 60% of lactate, respectively (Figure 7D). These results indicated that SMCT1 transported serine with lower affinity than its canonical substrates, and the recognition is more stereoselective to D- than L- isomer.

D-Serine reabsorption in renal proximal tubular cells

To investigate the function of transporters at the apical membranes of proximal tubules under controlled conditions of chemical gradients, we used mouse brush border membrane vesicles (BBMVs), which enriched the apical membrane transporters. D-Serine transport in mouse BBMVs was examined in different conditions to distinguish the contribution of ASCT2 and SMCTs. Because ASCT2 functions as an antiporter that influxes one amino acid with an

efflux of L-glutamine while SMCTs are symporters (Ganapathy et al., 2008; Scalise et al., 2018), we performed D-serine transport assay in BBMVs with or without L-glutamine preloading. In L-glutamine-preloaded BBMVs, transport of D-[³H]serine arose quickly and reached the saturated point at 20 sec (Figure 8A, supplement 8A). The uptake was ibuprofen-insensitive at 10 – 30 sec and became partially ibuprofen-sensitive at 1 min (Figure 8A). In contrast, transport of D-[³H]serine in BBMVs without the preloading increased slowly (Figure 8B). D-[³H]Serine transport was initiated after 30 sec and reached the optimum at 2 min (Figure 8B, supplement 8B). Notably, the transport was largely inhibited by ibuprofen which is a SMCT inhibitor, but not by L-threonine which is an ASCT2 substrate (Figure 8B-C). These results suggested that, in the L-glutamine preloading condition, positive D-[³H]serine transport at 10 - 30 sec was derived from the antiport mode of Asct2, which occurred rapidly. Meanwhile, SMCTs function started slowly at 60 sec as seen in the non-preloading condition. As a result, D-[³H]serine transport at 1 min in L-glutamine preloading condition was from the combinational functions of both ASCT2 and SMCTs (Figure 8A).

Previous studies described the localization of SMCT1 at S3 segment. SMCT2 is found in all segments and enriched in S1 of proximal tubules (Gopal et al., 2007; Kirita et al., 2020). Although ASCT2 has been intensively studied in cells or other organs, the localization and function in the kidney have not been well characterized. We generated affinity-purified Asct2 antibodies to recognize both N-terminus (NT) and C-terminus (CT) of Asct2 (Figure supplement 8C) and detected Asct2 in mouse kidney. Asct2 was co-immunostained with both Sglt2 (Slc5a2; sodium/glucose cotransporter 2) and Agt1 (slc7a13, aspartate/glutamate transporter 1), which are apical membrane markers for S1+S2 and S3 segments, respectively (Figure 8D-E) (Ghezzi et al., 2018; Nagamori et al., 2016a). In contrast, Asct2 did not co-localize with Na⁺/K⁺-ATPase, which is a basolateral membrane marker (Figure 8F). The results demonstrated that Asct2 is localized at the apical side in all segments of proximal tubules.

Discussion

Our membrane proteome revealed two major groups of the proteins classified by biological functions. The first group is involved in injury/inflammatory responses and tissue repairs. Most proteins in this group exhibited high degrees of elevation and various subcellular localization. Many of them are often found in the omics research and the list of protein biomarkers in kidney diseases (Eddy et al., 2020; Marx et al., 2018; Thongboonkerd, 2020; Zhang and Parikh, 2019). On the other hand, the second group is rather specific to the pathogenic proteins in IRI. The dominant protein types in the second group are membrane transport proteins. Many of the membrane transport proteins showed low degrees of alteration in IRI, adding difficulty to their identification from the whole-cell/tissue proteome and transcriptome. In this study, we succeeded in identifying multiple membrane proteins, in particular transporters located at the apical membranes of proximal tubules. The membrane proteome allows us to examine how transporters play roles in the normal condition or in the injured condition.

Based on the proteome data, we utilized the intensive biochemical assays including “*in vitro*” cultured cells, “cell-free” proteoliposomes, and “*ex vivo*” BBMVs methods to identify SMCT1, SMCT2, and ASCT2 as D-serine transporters at the apical membranes of renal proximal tubules. SMCT1 has preference on D-serine over L-serine and other amino acids (Figure 7D). The apparent K_m of D-serine transport in SMCT1 is 5.0 mM which is considered to be low affinity (Figure 6C). We attempted to examine the kinetics of D-serine transport in SMCT2. However, the uptake values at high D-serine concentration were low and concealed by the high background, indicating a lower affinity of SMCT2 than that of SMCT1. These results are in agreement with the physiological properties of SMCT1 and SMCT2 as the low-affinity transporters for their canonical substrates although the affinity of SMCT1 is higher than that of SMCT2 (Ganapathy et al., 2008). We conclude that SMCT1 and SMCT2 transport

D-serine with low affinity and high stereoselectivity toward the D- isomer. On the contrary, ASCT2 transports D-serine with high affinity (μM range) although approximately 8 folds less than L-serine (Foster et al., 2016).

It is surprising, but reasonable once we found that SMCTs transport D-serine. Several studies reported the transport of non-canonical substrates by membrane transport proteins especially the recognition between amino acids, carboxylates and amines among SLC transporters (de Carvalho and Quick, 2011; Matsuo et al., 2008; Metzner et al., 2005; Schweikhard and Ziegler, 2012; Wei et al., 2016). D-Serine structure consists of a hydroxypropionic acid with an amino group at the α -carbon. It is likely that the hydroxypropionic acid residue on D-serine is the main part to interact with SMCTs because hydroxypropionic acid shares similar moiety to lactic acid and propionic acid of SMCT canonical substrates.

Previous studies indicated D-serine transport takes place at the proximal tubules by the distinct transport systems between S1-S2 and S3 segments. Although the transport properties of each system were not clearly distinguished, both systems exhibited the characteristics of Na^+ dependency, electrogenicity, low affinity (mM range), and partial stereoselectivity (Kragh-Hansen and Sheikh, 1984; Shimomura et al., 1988; Silbernagl et al., 1999). These D-serine transport properties suit well to SMCTs and convince the contribution of SMCTs to renal D-serine transport in addition to ASCT2. It was reported that a fair amount of D-serine from gut microbiota mitigates AKI (Nakade et al., 2018). However, a high dose of D-serine administration induces nephrotoxicity by targeting proximal tubules and confining at the S3 segment (Hasegawa et al., 2019; Morehead et al., 1945; Silbernagl et al., 1999). It is most likely that the D-serine-induced proximal tubular damage is (partly) due to the absorption of D-serine by SMCTs, in particular by SMCT1 at the S3 segment. Ibuprofen minimized D-serine transport

and attenuated D-serine-induced toxicity in SMCTs-expressing cells (Figure 5C, 6B), implying the efficiency of ibuprofen to alleviate D-serine-induced nephrotoxicity.

What is the physiological importance of D-serine reabsorption? Although ASCT2 transports D-serine with high affinity *in vitro* (Foster et al., 2016), the amount of ASCT2 in the kidney is much lower than those of SMCTs (Table 2: abundance in Sham) indicating the low capacity of D-serine reabsorption. In contrast, the high abundance and low affinity of SMCTs demonstrate a high capacity of D-serine transport. It is most likely that the main contributor for D-serine reabsorption is SMCTs. However, in the physiological conditions where the canonical substrates of SMCTs exist, the amount of reabsorbed D-serine via SMCTs would be relatively low because the luminal D-serine amount is low and D-serine competes with SMCTs' canonical substrates. Possibly, SMCTs take up D-serine by chance, and the reabsorbed D-serine may not be important for carbon-source supplement or particular renal activities as the reabsorbed D-serine is also degraded by DAAO. Despite the inconspicuous merit of reabsorbed D-serine, functional activity of SMCTs to transport D-serine is significant in order to preserve the low-level of serum D-serine.

De novo cells, stem cells, and cancer cells share common characteristics of high proliferative rate and metabolic demands. In the IRI condition where the damaged proximal tubules attempt to recover their function by tissue development and repair, functions of high-affinity transporters would denote great benefit to supply enormous nutrients in a short time. In these regards, ASCT2 is a good candidate for the cellular response mechanism. ASCT2 is found in placenta for fetus development and highly expressed in cancer cells and stem cells (Formisano and Van Winkle, 2016; Kandasamy et al., 2018; Scalise et al., 2018). ASCT2 plays a key role in regulation of mammalian target of rapamycin (mTOR) signaling pathway and glutamine-mediated metabolism (Kandasamy et al., 2018; Scalise et al., 2018). Our proteome

data detected the continued elevation of ASCT2 (Figure 3C, 4A). Conceivably, ASCT2 upregulation is a result of a proliferative pathway for cell growth and repair.

Taken all together, we propose a model of D-serine transport in renal proximal tubules (Figure 8G). In physiological conditions, SMCT1 and SMCT2 mainly contribute to D-serine transport with low affinity. Intracellular D-serine is then catalyzed by DAAO in the proximal tubule. As a consequence, the meager amount of D-serine is detected in serum (Sasabe et al., 2014). In contrast, both SMCTs were decreasing while ASCT2 was upregulating in the IRI model (Table 2, Figure supplement 5). The increase and high affinity of ASCT2 bring about larger D-serine reabsorption. Thus, luminal D-serine is decreased in the pathophysiological conditions (Figure 8G). Recently, SMCT2 was reported as a protein indicator for kidney repair from the injury. Prolonged SMCT2 down-expression indicated the failed repair of proximal tubular cells in IRI (Kirita et al., 2020). The prolonged decrease of SMCT2 during the injury in-turn supports the merit of luminal D-serine as a metabolite biomarker for AKI and CKD.

In this study, we principally focused on the D-serine transport system at the luminal side of proximal tubules and explained the low urinary D-serine in IRI. Combination of ASCT2 induction and DAAO reduction may result in the elevation of serum D-serine. Future study on the D-serine transport system at the basolateral side is necessary to explain the increase of serum D-serine and complete the picture of D-serine transport system in renal proximal tubules.

D-Serine treatment in PAT1- and B⁰AT3-expressing cells showed an increase in cell growth (Figure 5A). PAT1, which is localized at both lysosome and plasma membrane, was reported to transport D-serine while the ability of B⁰AT3 in D-serine uptake is unknown. Both PAT1 and B⁰AT3 may play a role in D-serine secretion in cells. In kidney, PAT1 is located at both apical membranes and inside of the epithelium (maybe also on the basolateral side) of S1 segment (The Human Protein Atlas: <https://www.proteinatlas.org>; updated on Mar 6th, 2020; Thwaites and Anderson, 2011; Vanslambrouck et al., 2010). B⁰AT3 is found at the apical

membranes of S3 segment (Vanslambrouck et al., 2010). The contribution of D-serine transport by PAT1 and B⁰AT3 in the kidney needs to be further examined.

For the detection of membrane transport proteins which require post-translational modification and translocation, proteomics provides a direct association toward the protein function than the transcriptomics does. Although proteomics has been intensively applied for AKI and CKD studies, to our knowledge, none of the studies emphasized the analysis of membrane proteins at the lumen of proximal tubules of the IRI model. Membrane transport proteins are one of the most important targeting groups in the proteomic analysis of AKI because they are directly linked to the metabolites and molecular substances. Our proteomics provides a great hint on membrane transport proteins at hierarchical levels: from individual proteins to systemic scale. The proteome data clearly indicated that homeostasis of inorganic ions is rescued within a short time. On the contrary, the defect of organic compounds is prolonged and requires extensive time to be fully recovered (Figure 3A). These data support the usefulness of the metabolite biomarkers for diagnosis and prognosis of kidney diseases because most metabolite biomarkers are organic compounds which exhibit prolonged defects during IRI. Herein, we predict a new series of membrane transport proteins as biomarkers (Table 2). Since most of them are decreased in the IRI model, their substrates are promising candidates for AKI and CKD metabolite biomarkers, such as substrates of GLUT5, AQP7, OATP-M1, OAT2, THTR2, and PEPT2.

One good example of the usefulness of our proteome is the prediction of transport systems for creatinine, a well-known AKI and CKD biomarker. At the apical membranes, creatinine is excreted by transporters MATE1/SLC47A1 and MATE2-K/SLC47A2 and likely transported by OCTN1/SLC22A4, OCTN2/SLC22A5, and OAT2 (Chu et al., 2016; Tanihara et al., 2007). Our proteome data indicated the reduction of MATE1 (Figure 3B), convincing the importance of MATE1 over MATE2-K in creatinine efflux. Decrease of OCTN1, OCTN2,

and OAT2 in IRI also leads to speculation of their contribution in creatinine transport (Figure 3, Table 1-2). Another example is the glucose transport system. In physiological conditions, SGLTs reabsorb glucose and GLUT5 reabsorbs fructose at the luminal side (Ghezzi et al., 2018; Szablewski, 2017). From the proteome, we found a decrease in SGLT1/SLC5A1 and GLUT5 but an increase in GLUT3/SLC2A3 (Figure 3B). GLUT3 exhibits the highest affinity among GLUTs but very low expression in physiological conditions (Simpson et al., 2008). Accordingly, GLUT3 might be a key transporter for metabolic activities in the repair processes.

Apart from the kidney, D-serine transport in the brain is contributed by multiple transport systems. The affinity of D-serine transport in astrocyte and retinal glia cultures was found to be in the millimolar range (Dun et al., 2007; Foster et al., 2016). SMCT1 is expressed in neurons, astrocytes, and retinal cells, while SMCT2 is rather restricted to retina (Martin et al., 2007, 2006). Meta-data analysis demonstrated the interaction of SMCT1 and five types of NMDARs (<https://amp.pharm.mssm.edu/Harmonizome/>; Rouillard et al., 2016). SMCTs are also found in the colon, intestine, and thyroid organs (Ganapathy et al., 2008; Paroder et al., 2006). It is worthwhile to investigate the contribution of SMCTs on D-serine transport systems in those tissues.

Here, we utilize a multi-hierarchical approach to identify D-serine as a hidden substrate of SMCTs. This finding clarifies the physiological transport system of D-serine at the apical membranes of proximal tubules and proposes the D-serine transport in IRI. Our strategy can be applied to the investigation of any transport systems with hidden physiological substrates.

Materials and Methods

Materials, animals and graphical analysis

General chemicals and cell culture media were purchased from Wako Fujifilm. Chemicals used in mass spectrometry were HPLC or MS grades. Flp-In T-Rex 293 cells, Expi293F cells, Expi293 Expression medium, Lipofectamine 3000, fluorescent-labeled secondary antibodies, and Tyramide SuperBoost kit were from Thermo. Amino acids, fetal bovine serum (FBS), anti-FLAG antibody and anti-FLAG M2 column were from Sigma. Secondary antibodies with HRP conjugated were from Jackson ImmunoResearch. L-[³H]Serine, D-[³H]serine, L-[³H]alanine, and D-[³H]alanine were from Moravek. L-[³H]Glutamic acid, D-[³H]glutamic acid, L-[¹⁴C]tyrosine, D-[¹⁴C]tyrosine, DL-[³H]lactic acid, [³H]propionic acid, [¹⁴C]nicotinic acid and [¹⁴C]uric acid were from American Radiolabeled Chemicals. Anti-SGLT2 and anti-Na⁺/K⁺-ATPase antibodies were obtained from Santa Cruz Biotechnology.

All animal experiments were carried out following institutional guidelines under approval by the Animal Experiment Committees of Nara Medical University and Keio University, Japan.

Unless otherwise indicated, data shown in all figures are mean ± standard error of the representative data from three independent experiments. Statistical differences and p-values were determined using the unpaired Student *t* test. Graphs, statistical significance, and kinetics were analyzed and plotted by GraphPad Prism 8.3.

Plasmid construction

In this study, we used the cDNA of human SLC5A8/SMCT1 clone “NM_145913”. We generated the clone NM_145913 from the clone AK313788 (NBRC, NITE, Kisarazu, Japan). At first, SMCT1 from AK313788 was subcloned into p3XFLAG-CMV14 (Sigma) via *Hind*III

and *Bam*HI sites. The clone “pCMV14-SMCT1” NM_145913 was subsequently generated by mutagenesis of p3XFLAG-CMV14-SMCT1_AK313788 at I193V, T201A and I490M (variants between NM_145913 and AK313788) by HiFi DNA Assembly Cloning (NEB) and site-directed mutagenesis. Human SLC5A12/SMCT2 cDNA (NM_178498; Sino Biological Inc.) was amplified by PCR and cloned into p3XFLAG-CMV14 via *Kpn*I and *Bam*HI sites to generate “pCMV14-SMCT2”. Human expression clones of SLC7A10/Asc-1 (NM_019849), SLC7A1/CAT1 (NM_003045), SLC36A1/PAT1 (NM_078483), SLC2A5/GLUT5 (NM_003039), SLCO4C1/OATP-M1 (NM_180991), SLC22A13/OAT10 (NM_004256), SLC22A7/OAT2 (NM_153320), SLC6A18/B⁰AT3 (NM_182632), SLC15A2/PEPT2 (NM_021082), and TMEM27/Collectrin (NM_0202665) were obtained from GenScript and RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. Mouse Asct2 (mAsct2, NM_009201) TrueORF clone was obtained from OriGene. p3XFLAG-CMV14 empty vector was used for Mock production. pcDNA5-SMCT1 and pcDNA5-SMCT2 used for generation of SMCT1- and SMCT2-stable cell lines, respectively, were constructed by assembling the PCR products of SMCT1 or SMCT2 into pcDNA5/FRT/TO (Thermo) by HiFi DNA Assembly Cloning kit.

Antibody (Ab) production

Anti-human ASCT2 (hASCT2) and anti-mAsct2 Abs were self-produced. First, we generated pET47b(+)-GST. The fragment encoding GST was amplified by using pET49b(+) as the template. The GST fragment was cloned into pET47b(+) via *Not*I and *Xho*I restriction sites. To obtain the antigen for anti-hASCT2 Ab, the PCR products corresponding to amino acid residues 7-20 of hASCT2 were amplified and cloned into pET47b(+)-GST to obtain GST-fusion protein. For antigens from mAsct2, both N-terminal (mAsct2(NT), amino acid residues 1 – 38) and C-terminal (mAsct2(CT), amino acid residues 521 – 553) fragments were fused with GST by cloning into pET47b(+)-GST or pET49b(+), respectively (Cosmo Bio Co., Ltd).

The GST-fusion antigens were expressed in *E. coli* BL21(DE3) and purified by Glutathione Sepharose 4B (GE Healthcare) as described previously (Nagamori et al., 2016a). The antigens were used to immunize rabbits to obtain anti-sera were obtained (Cosmo Bio).

Anti-hASCT2 Ab was purified from the anti-sera using HiTrap Protein G HP (GE Healthcare) following the manufacturer's protocol. After purification, the antibody was dialyzed against PBS pH 7.4 and adjusted concentration to 1 mg/mL.

Anti-mAsct2(NT) and anti-mAsct2(CT) Abs were purified by using two-step purifications. First, affinity columns of GST-fused mAsct2(NT)-antigen and GST-fused mAsct2(CT)-antigen were made by conjugated the antigens with HiTrap NHS-activated HP (GE Healthcare) following the manufacturer's protocol. To purify anti-mAsct2(NT) Ab, the anti-sera was subjected to the first purification by using the GST-fused mAsct2(NT)-antigen column. The elution fraction was dialyzed and subsequently subjected to the second column of GST-fused mAsct2(CT)-antigen column. The flowthrough fraction corresponding to the affinity-purified anti-mAsct2(NT) Ab was obtained. Purification of anti-mAsct2(CT) Ab was performed in the same way as mAsct2(NT) Ab but used GST-fused mAsct2(CT)-antigen column followed by GST-fused mAsct2(NT)-antigen column.

Ischemia-reperfusion injury (IRI) model and isolation of brush border membrane vesicles (BBMVs) for mass spectrometry analysis

C57BL/6J (CLEA Japan) male mice between 12 – 16 weeks old underwent experimental procedures for the IRI model. Ischemia-reperfusion was performed as previously described (Sasabe et al., 2014). Before IRI induction, right kidney was removed. After 12 days, the mice were grouped by randomization. Ischemia was operated for 45 min by clamping the vessel under anesthesia with pentobarbital. After that, the vessel clamp was removed, and the abdomen was closed. Sham-operated control mice were treated identically except for clamping.

At 4 and 8 hours after reperfusion, mice were anesthetized with isoflurane and euthanized by perfusion with PBS pH 7.4. The kidney was removed and stored at -80 °C until use.

BBMVs were prepared by calcium precipitation method (Modified from Biber et al., 2007). Frozen kidneys were minced into fine powders by using a polytron-type homogenizer (Physcotron, Microtec) in the homogenization buffer containing 20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 1 mM EDTA and cOmplete EDTA-free protease inhibitor cocktail (Roche). After low-speed centrifugation at 1,000 ×g and 3,000 ×g, the supernatant was collected and incubated with 11 mM CaCl₂ for 20 min on ice with mild shaking. The supernatant was ultra-centrifuged at 463,000 ×g for 15 min at 4 °C. The pellet was resuspended in the homogenization buffer and repeated the steps of CaCl₂ precipitation. Finally, the pellet of BBMVs was resuspended in 20 mM Tris-HCl pH 7.6 and 250 mM sucrose. Membrane proteins of BBMVs were enriched by the urea wash method (Uetsuka et al., 2015). The urea-washed BBMV samples were subjected to sample preparation for mass spectrometry.

Cell culture, transfection, and generation of stable cell lines

HEK293 and Flp-In T-Rex 293 cells were cultured in DMEM supplemented with 10 % (v/v) FBS, 100 units/mL penicillin G, and 100 µg/mL streptomycin (P/S), and routinely maintained at 37 °C, 5 % CO₂ and humidity. For transfection experiments, the cells were seeded in antibiotic-free media for one day prior to transfection to obtain approximately 40% confluence. DNA transient transfection and ASCT2-siRNA (ID#s12916; Ambion) transfection were performed by using Lipofectamine 3000 following the manufacturer's protocol. The ratio of DNA : P3000 : Lipofectamine 3000 is 1.0 µg : 2.0 µL : 1.5 µL. The ratio of siRNA : Lipofectamine 3000 is 10 pmol : 1 µL. The cells were further maintained in the same media for 2 days and used for the experiments.

Flp-In T-REx 293 stably expressing SMCT1 (FlpInTR-SMCT1) and SMCT2 (FlpInTR-SMCT2) were generated by co-transfection of pOG44 and pcDNA5-SMCT1 or pcDNA5-SMCT2 and subsequently cultured in the media containing 5 mg/L blasticidin and 150 mg/L hygromycin B for positive clone selection. Mock cells were generated in the same way using the empty plasmid. Expressions of SMCT1 in FlpInTR-SMCT1 and SMCT2 in FlpInTR-SMCT2 were induced by adding 1 mg/L doxycycline hyclate (Dox; Tet-ON system) one day after seeding. The cells were further cultured for two days prior to performing experiments.

Wild-type and *ASCT2*-knockout HAP1 cells (Horizon Discovery) were cultured in IMDM supplemented with 10 % (v/v) FBS, P/S, and routinely maintained at 37 °C, 5 % CO₂ and humidity.

Expi293F cells were cultured in Expi293 Expression medium at 37 °C, 8 % CO₂, and humidity. To express SMCT1 transiently, the cells were transfected with pCMV14-SMCT1 using PEI MAX pH 6.9 (MW 40,000; Polysciences) and cultured for two days.

Mass spectrometry

Mass spectrometry of mouse BBMVs was performed as described previously with some modification (Uetsuka et al., 2015). After preparing urea-washed BBMVs, the samples were fractionated into four fractions by SDB-XC StageTips and desalted by C18-StageTips. Mass spectrometry was performed using the Q Exactive (Thermo) coupled with Advance UHPLC (Michrom Bioresources). The HPLC apparatus was equipped with a trap column (L-column ODS, 0.3 x 5 mm, CERI) and a C18 packed tip column (100 µm ID; Nikkyo Technos). Raw data from four fractions were analyzed using Proteome Discoverer 2.2 (Thermo) and Mascot 2.6.2 (Matrix Science). Data from four fractions were combined and searched for identified proteins from the UniProt mouse database (released in March 2019). The maximum number of missed cleavages, precursor mass tolerance, and fragment mass tolerance were set

to 3, 10 ppm and 0.01 Da, respectively. The carbamidomethylation Cys was set as a fixed modification. Oxidation of Met and deamidation of Asn and Gln were set as variable modifications. A filter (false discovery rate < 1%) was applied to the resulting data. For each mouse sample, the analysis was conducted twice and the average was used. One data set was composed of 3 samples from each operation condition (n = 3).

Mass spectrometry of HEK293 cells was analyzed from crude membrane fractions. Membrane fractions from HEK293 cells were prepared from 3-day cultured cells as described (Nagamori et al., 2016b). Membrane proteins were enriched by the urea wash method, and tryptic peptides were subject for analysis as described above.

Proteome data analysis and availability

Protein localization and functional categories were determined by Ingenuity pathway analysis (IPA, Qiagen). The prediction of transmembrane regions was acquired using the transmembrane hidden Markov model (TMHMM) Server v. 2.0. Localization in kidney segments was evaluated based on related literature and The Human Protein Atlas (<http://www.proteinatlas.org>; updated Dec 19, 2019).

The biological functions, the toxicity functions (diseases and organ pathologies), and the biological networks were analyzed using IPA. Molecules from the dataset that met the cutoff of the Ingenuity Knowledge Base were considered for the analysis. A right-tailed Fisher's Exact Test was used to calculate the p-value determining the probability (z-scores) of the biological function or the toxicity function assignment (IPA). The networks derived from IPA were simplified by omitting the proteins in canonical pathways that were not detected and not key regulators. Networks with the shared regulators were merged and displayed in the Figures.

All proteomics data have been deposited in Japan Proteome Standard Repository/Database: JPST000929 and JPST000931.

Effect of cell D-serine on cell growth

HEK293 cells were seeded into 96-well-plate at 10,000 cells/well. Transient transfection was performed 12 hours after seeding followed by L- or D-serine treatment 12 hours after that. In the case of FlpInTR-stable cell lines, ASCT2 siRNA was transfected 12 hours after seeding, if needed. Dox was added one day after seeding followed by treatment with L- or D-serine (in the presence or absence of ibuprofen as indicated) 10 hours after adding Dox. The cells were further maintained for 2 days. Cell growth was examined by XTT assay. In one reaction, 50 μ L of 1 mg/mL XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (Biotium) was mixed with 5 μ L of 1.5 mg/mL phenazine methosulfate. The mixture was applied to the cells and incubated for 4 hours at 37 °C in the cell culture incubator. Cell viability was evaluated by measuring the absorbance at 450 nm. Cell growth in serine treatment samples was compared to the control (without serine treatment). For transporter screening, the growth of the transfected cells at a specific D-serine concentration was compared to that of Mock after normalization with no treatment.

Transport assay in cultured cells

Transport assay in cells was performed as described previously with some modifications (Nagamori et al., 2016b). Briefly, for D-[³H]serine transport in HEK293 cells, the cells were seeded into poly-D-lysine-coated 24-well plates at 1.2×10^5 cells/well and cultured for three days. Uptake of 10 μ M (100 Ci/mol) or 100 μ M (10 Ci/mol) D-[³H]serine was measured in PBS pH 7.4 at 37 °C at the indicated time points. After termination of the assay, the cells were lysed. An aliquot was subjected to measure protein concentration, and the remaining lysate was mixed with Optiphase HiSafe 3 (PerkinElmer). The radioactivity was monitored using a β -scintillation counter (LSC-8000, Hitachi). In the transport assay with or without Na⁺, Na⁺-HBSS, or Na⁺-free HBSS (choline-Cl substitution) were used instead of PBS.

Transport assay in FlpInTR-stable cell lines was performed in a similar way to HEK293 cells. After cell seeding for one day, SMCT1 and SMCT2 expression were induced by adding Dox for two days. For the ASCT2 knockdown experiment, ASCT2-siRNA was transfected 12 hours prior to Dox induction. The time course of 100 μ M D-[³H]serine transport (10 Ci/mol) was measured 37 °C for 5 – 20 min. Inhibition assay was performed by adding the test inhibitors at the same time with D-[³H]serine substrate. Kinetics of D-[³H]serine transport were examined by the uptake of D-[³H]serine at the concentration of 0.5 – 8 mM (0.125 – 2 Ci/mol) for 10 min at 37 °C.

Transport of D-[³H]serine in wild-type and *ASCT2*-knockout HAP1 was performed in a similar way to HEK293 and FlpInTR-stable cells but without the process of transfection.

hSMCT1 purification, proteoliposome reconstitution, and transport assay

hSMCT1 was purified from SMCT1-expressing Expi293F cells. Membrane fraction and purification processes were performed as previously described with small modifications (Nagamori et al., 2016a). First, cell pellets were resuspended in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, and protease inhibitor cocktail (Roche). The crude membrane fraction was derived from sonication and ultracentrifugation (sonication method). Membrane proteins were extracted from the crude membrane fraction with 2% (w/v) DDM and ultracentrifugation. hSMCT1 was purified by anti-FLAG M2 affinity column. Unbound proteins were washed out by 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, and 0.05% (w/v) DDM then hSMCT1 was eluted by 3xFLAG peptide in the washing buffer. Purified hSMCT1 was concentrated by Amicon Ultra Centrifugal Filters 30K (Millipore).

The reconstitution of proteoliposomes was performed as described with minor modifications (Lee et al., 2019). The purified hSMCT1 was reconstituted in liposomes (made from 5:1 (w/w) of type II-S PC : brain total lipid) at a protein-to-lipid ratio of 1:100 (w/w) in 20 mM MOPS-Tris pH 7.0 and 100 mM KCl.

Transport assay in proteoliposomes was conducted by the rapid filtration method (Lee et al., 2019). Uptake reaction was conducted by dilution of 1 μ g SMCT1 proteoliposomes in 100 μ L uptake buffer (20 mM MOPS-Tris pH 7.0, 100 mM NaCl for Na⁺-buffer or KCl for Na⁺-free buffer, 1 mM MgSO₄ and 1 mM CaCl₂) containing radioisotope-labeled substrates. The reaction was incubated at 25 °C for an indicated time. The radioisotope-labeled substrates were used as follows: 5 Ci/mol for [¹⁴C]uric acid, L-[¹⁴C]tyrosine and D-[¹⁴C]tyrosine; 10 Ci/mol for DL-[³H]lactic acid, L-[³H]alanine, D-[³H]alanine, L-[³H]serine, D-[³H]serine, L-[³H]glutamic acid and D-[³H]glutamic acid; and 20 Ci/mol for [³H]propionic acid. In the inhibition assay, 1 mM ibuprofen was applied at the same time with the radioisotope-labeled substrates.

Transport assay in mouse BBMVs

Both left and right kidneys were taken out from the 8 weeks old male wild-type C57BL/6J mice (Japan SLC) after PBS perfusion and frozen until use. After mincing and homogenizing the frozen kidneys (as in the above section) in the buffer containing 20 mM Tris-HCl pH 7.6, 150 mM mannitol, 100 mM KCl, 1 mM EDTA, and protease inhibitor cocktail, the BBMVs were prepared by magnesium precipitation method (Biber et al., 2007). The pellet of BBMVs was resuspended in the suspension buffer (10 mM Tris-HCl, pH 7.6, 100 mM mannitol, and 100 mM KCl). In the L-glutamine preloading experiment, the BBMVs were mixed with 4 mM L-glutamine on ice for 3 hours, centrifuged at 21,000 $\times$ g for 20 min, and resuspended in the suspension buffer.

Transport assay was performed by rapid filtration. Prior to initiating the reaction, 5 μ M valinomycin was added into the BBMV samples and the BBMVs were kept at room temperature for 2 minutes. Transport assay was examined by diluting 100 μ g BBMVs in 100 μ L of the uptake buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl for Na⁺ condition or KCl for

Na⁺-free condition, 50 mM mannitol and 5 μ M valinomycin) containing 10 μ M D-[³H]serine (100 Ci/mol). The reaction was incubated at 30 °C at an indicated time, then terminated by the addition of ice-cold buffer containing 10 mM Tris-HCl pH 7.6 and 200 mM mannitol, and filtered through 0.45 μ m nitrocellulose filter (Millipore), followed by washing with the same buffer once. The membranes were soaked in Clear-sol I (Nacalai Tesque), and the radioactivity on the membrane was monitored. For the inhibition experiments, the tested inhibitors were added into the D-[³H]serine substrate solution at the same time.

Western Blot analysis

Expressions of targeting proteins from membrane fractions were verified by Western blot analysis as described (Nagamori et al., 2016b). Membrane fractions from cultured cell pellets were prepared by sonication. BBMV s were prepared by the magnesium precipitation method. Membrane proteins were dissolved in 1% w/v DDM prior to the addition of the SDS-PAGE sample buffer. Signals of chemiluminescence (Immobilon Forte Western HRP substrate; Millipore) were visualized by ChemiDoc MP Imaging system (Bio-Rad).

Immunofluorescent staining of mouse kidneys

The 8 weeks old male wild-type C57BL/6J mice (Japan SLC) were anesthetized and fixed by anterograde perfusion via the aorta with 4% w/v paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4. The kidneys were dissected, post-fixed in the same buffer for two days, and cryoprotected in 10 %, 20 %, and 30 % w/v sucrose. Frozen kidney sections were cut at 7 μ m thickness in a cryostat (Leica) and mounted on MAS-coated glass slides (Matsunami). The sections were placed in antigen retrieval buffer (10 mM citrate and 10 mM sodium Citrate), autoclaved at 121 °C for 5 min and washed by TBS-T (Tris-buffered saline (TBS) with 0.1 % v/v Tween 20). Immunostaining was done by serial incubation with each antibody as below.

For mAsct2 and mSglt2 co-immunostaining, the samples were incubated with 3 % hydrogen peroxide solution for 10 min, washed with TBS, and incubated in Blocking One Histo (Nacalai tesque) for 15 min. The samples were then incubated with mouse anti-SGLT2 antibody diluted in immunoreaction enhancer B solution (Can Get Signal immunostain, TOYOBO) overnight at 4 °C. Signal was enhanced by Alexa Fluor 568 Tyramide SuperBoost (TSA) kit, goat anti-mouse IgG, following the manufacture's instruction (Thermo). The antibodies were then stripped by citrate/acetate-based buffer, pH 6.0, containing 0.3% w/v SDS at 95 °C for 10 min (Buchwalow et al., 2018), washed by TBS, and incubated with Blocking One Histo. mSglt2 staining was done by conventional staining method (Nagamori et al., 2016b). The samples were incubated with rabbit anti-mAsct2(NT) antibody diluted in immunoreaction enhancer A solution (Can Get Signal immunostain) overnight at 4 °C. After washing with TBS-T, the specimens were incubated with Alexa Fluor 488-labeled donkey anti-rabbit IgG.

For mAsct2 and mAglt1 co-immunostaining, signals of both antibodies were enhanced by TSA kit. First, the specimens were incubated with rabbit anti-mAGT1(G) antibody (Nagamori et al., 2016a) overnight at 4 °C followed by Alexa Fluor 568 TSA kit, goat anti-rabbit. The antibodies were then stripped. The specimens were incubated with rabbit anti-mAsct2(NT) overnight at 4 °C and then repeated the steps of TSA kit using Alexa Fluor 488, goat anti-rabbit.

Staining of mAsct2 and Na⁺/K⁺-ATPase was performed without TSA enhancement. After blocking by Blocking One Histo, the samples were incubated with rabbit anti-mAsct2(NT) antibody diluted in immunoreaction enhancer A solution overnight. The samples were washed with TBS-T, incubated with Alexa Fluor488-labeled donkey anti-rabbit IgG, and washed again. Non-specific staining was blocked by Blocking One Histo and the specimens were then incubated with mouse anti-Na⁺/K⁺-ATPase antibody diluted in immunoreaction

762 enhancer B solution overnight at 4 °C. The specimens were washed with TBS-T, incubated
763 with Alexa Fluor568-labeled goat anti-mouse IgG for 1 hour.

764 All specimens were washed with TBS-T and mounted with Fluoromount (Diagnostic
765 Biosystems). Imaging was detected using a KEYENCE BZ-X710 microscope. Images were
766 processed by using ImageJ ver. 1.51 (NIH).

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Competing interests

A patent has been applied by KAGAMI Inc., Nara Medical University, and NIBIOHN with P.W., S.M., P.K., T.K., M.M., and S.N. as inventors based on this research. KAGAMI Inc. was founded in 2019 to implement the technologies in medicine.

Author contributions

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Pornparn Kongpracha—Investigation, Validation, Formal analysis, Data curation

Nodoka Nakamae—Investigation, Formal analysis

Masataka Suzuki—Investigation, Resources

Tomonori Kimura—Resources, Methodology

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 1121

1122 Figures

Figure 1: Wiriyaermkul, et al.

Figure 1: Proteomics uncovers cellular functions and the relevant diseases altered in the renal IRI model

Proteome of BBMV from mouse kidneys after ischemia operation for 4 hours or 8 hours was normalized to that of sham operation. Proteins with a ratio of 4h IRI/sham (4h) and 8h IRI/sham (8h) passing 1.5-fold change and 0.05 p-value were subjected to analyze cellular functions and the relevant diseases by IPA. Top-10 cellular functions (A) and systemic functions (B) that are significantly altered in both 4h and 8h IRI are shown. dev. = development. C) Hierarchical heatmap of functional pathways corresponding to cellular and systemic functions in A - B. Colors indicate predicted functions. D) Top-10 diseases resulting from altered proteins. E) Area-proportional Venn diagrams (left) of biomarkers predicted by IPA. Thirty-four biomarkers found in both 4h and 8h are shown as a heatmap of protein expression (right). Colors indicated log₂ fold of 4h IRI/sham (4h) or 8h IRI/sham (8h). Proteins highlighted in blue are previously predicted biomarkers for IRI and/or CKD.

Figure 2: Wiriyasermkul, et al.

1136 Figure 2: Predicted pathways in response to 4h, and combination of 4h and 8h IRI

1137 Identified proteins from renal BBMV, which are significantly altered more or less than 1.5-
1138 fold change (p -value < 0.05) in 4h and 8h IRI, were curated into networks by IPA and literatures.
1139 Top-3 networks (A - C) with the highest scores and molecule numbers are presented. Protein
1140 expressions, that are upregulated and downregulated, are shown in red and cyan, respectively,
1141 with intensity shading corresponding to the degrees of fold change. Proteins are shaped by
1142 categories, and double cycles indicate protein complexes. Solid and dash lines represent direct
1143 and indirect interactions, respectively. Lines are colored for contrast observation in which blue
1144 and magenta lines imply activation to regulators and targeting proteins, respectively. A)
1145 Network of the *rapid responses* via NF- κ b regulator at 4h IRI. Network is highly involved in
1146 innate immune system, cellular movement, and cell cycle in response to the injury, tissue
1147 disorder, and defect of molecular transport. B) Network of the *rapid responses* at 4h IRI. The
1148 increase of several growth factors from organismal injury and abnormalities apparently
1149 regulate PI3K- and MYC-related pathways. C) Network of the *prolonged responses* during 4h
1150 – 8h IRI via HDL complex and Akt. Significant proteins at 4h and 8h IRI are shown. Proteins
1151 in the network are highly involved in injury-response, cell death and survival, coagulation,
1152 apolipoprotein profile, and antioxidants.

Figure 3: Wiriyaermkul, et al.

Figure 3: Proteome analysis of membrane transport proteins

A) Heatmap of predicted transport function analyzed from membrane transport proteins (319 identified proteins). Transport function is categorized by types of substrates. Ion transport is a group of inorganic ions (lower left) and charged organic compounds (right). Transport of

1157 organic compounds (right) comprises both charged and uncharged compounds (right). Area
 1158 and colors represent $-\log_{10}(\text{p-value})$ and predicted function (z-score), respectively. B) Heatmap
 1159 of membrane transport proteins categorized by types of their canonical substrates. The “Others”
 1160 category includes vitamin and nucleobase transporters. Colors indicate $\log_2$ fold of 4h IRI/sham
 1161 (4h) or 8h IRI/sham (8h).

Figure 4: Wiriyasermkul, et al.

Figure 4: ASCT2 is a D-serine transporter

A) Volcano plots of 319 membrane transport proteins identified from the BBMV proteome of the IRI model. The $\log_2$ fold of 4h IRI/sham (left) or 8h IRI/sham (right) were plotted against $-\log_{10}$ of p-value. The value of Asct2 (red dot) was set as a cut-off value (red lines) to select candidates of D-serine transporters. Candidates with increased or decreased expression were shown in red and cyan areas, respectively. B) Western blot of ASCT2 from the membrane fraction of HEK293 transfected with ASCT2-siRNA indicated the suppression of ASCT2 expression. ASCT2 was detected by using anti-hASCT2 antibody. C) Transport of 100 μ M D- $[^3\text{H}]$ serine was measured in ASCT2-knockdown (ASCT2-siRNA) in comparison to Mock cells. Uptake was measured in PBS (Na^+ -buffer). $n = 3$. D) Cell-growth measurement (XTT assay) of HEK293 cells treated with D-serine. Prior to treatment, the cells were transfected with ASCT2-siRNA or without siRNA (control). After transfection, the cells were treated with D-

1174 serine at the indicated concentration for two days. Data represent percent cell growth compared
1175 to the non-treated cells. The graphs were fitted to inhibition kinetics (Dose-response –
1176 Inhibition). n = 5.

Figure 5: Wiriyasermkul, et al.

Figure 5: Identification of SMCTs as candidates of D-serine transporters

Candidates of D-serine transporters were screened by cell growth determination. HEK293 cells were transfected with various cDNA clones, as indicated. After transfection, the cells were treated with either 15 mM (A) or 25 mM (B) D-serine for two days and cell growth was examined by XTT assay. The growth effect by D-serine treatment in both transfected cells and Mock was normalized with that of no treatment. Subsequently, the normalized growth of the transfected cells was calculated as “fold change” compared to that of Mock at the same D-serine concentration and plotted as log₂fold change. The order of clones was rearranged according to Table 1. **p* < 0.05; ***p* < 0.01; n = 4. C). Inhibition effect of ibuprofen on D-

1186 serine-induced cell growth. FlpInTR-SMCT1 (SMCT1), FlpInTR-SMCT2 (SMCT2) and
 1187 FlpInTR-Mock (Mock) cells were preincubated with 0.5 mM ibuprofen prior to treated with
 1188 D-serine at indicated concentration for 2 days. Cell growth was measured by XTT assay. For
 1189 comparison, the maximum growth inhibition by 25 mM D-serine treatment was set as 100 %
 1190 inhibition and no D-serine treatment was set as 0 % inhibition. The graphs were fitted to
 1191 inhibition kinetics (Dose-response – Inhibition). n = 5.

Figure 6: Wiriyasermkul, et al.

Figure 6: Characterization of SMCT1 and SMCT2 as D-serine transporters using SMCTs-stably expressing cell lines

A) Time course of 100 μ M D-[3 H]serine uptake in FlpInTR-SMCT1 (SMCT1), FlpInTR-SMCT2 (SMCT2) and Mock cells with ASCT2 knockdown. Prior to measure D-[3 H]serine transport, the cells were transfected with ASCT2-siRNA for two days. n = 4. B) Inhibition of 20 μ M D-[3 H]serine uptake by several inhibitors. Transport of D-[3 H]serine by ASCT2-siRNA transfected FlpInTR stable cell lines were measured in the absence (-) or presence of 5 mM inhibitors as indicated. Uptake was incubated for 10 min. Graphs represented the uptake data subtracted with those of Mock cells. n = 3. C) Concentration dependence of D-[3 H]serine transport in FlpInTR-SMCT1 cells. The cells were transfected with ASCT2-siRNA and cultured for two days prior to measuring the transport. Uptake of D-[3 H]serine (0.5 – 8 mM) was measured for 10 min in PBS. The graph represented D-[3 H]serine transport in FlpInTR-SMCT1 subtracted with those of Mock cells. The uptake values were fitted to Michaelis-Menten plot, with apparent K_m of 5.0 mM and V_{max} of 21.6 pmol/ μ g protein/min. n = 3 - 4.

Figure 7: Wiriyasermkul, et al.

Figure 7: Characterization of SMCT1 as a D-serine transporter using SMCT1-reconstituted proteoliposomes

A) Stain-free SDS-PAGE gel (Bio-Rad) shows SMCT1 (SMCT1) purified from pCMV14-SMCT1-transfected Expi293F cells, reconstituted empty liposomes (Liposome) and SMCT1 proteoliposomes (SMCT1-PL). Closed arrowhead indicates SMCT1, whereas opened

1211 arrowhead indicates 3xFLAG peptides, which were used to elute the purified SMCT1. B)
 1212 Ibuprofen effect on the uptake of [^3H]lactate, [^3H]propionate, L-[^3H]serine, and D-[^3H]serine in
 1213 SMCT1 proteoliposomes (SMCT1-PL). Uptakes of 50 μM radiolabeled substrates were
 1214 measured in the presence or absence of 1 mM ibuprofen for 5 min. The graphs represent uptake
 1215 values in Na^+ - buffer (Na^+), which were subtracted with those in Na^+ -free buffer. $n = 3$. C)
 1216 Time course of D-[^3H]serine transport in SMCT1 proteoliposomes. D-[^3H]Serine transport (200
 1217 μM) was measured in the SMCT1 proteoliposomes in Na^+ -buffer. All uptake values were
 1218 subtracted with the uptake in Na^+ -free buffer. $n = 3$. D) Substrate selectivity of SMCT1-PL.
 1219 The uptakes of 50 μM radiolabeled substrates were measured in SMCT1 proteoliposomes for
 1220 5 min. The uptake of each substrate in Na^+ - buffer was subtracted with those in Na^+ -free buffer,
 1221 and calculated as % lactate uptake. Bar graphs represent mean $\pm$ standard error from 3
 1222 independent experiments. $n = 2 - 4$ in each experiment.

Figure 8: Wiriyasermkul, et al.

1225 A) D-[³H]Serine transport in BBMVs preloaded with 4 mM L-glutamine. Time courses of 10
1226 μM D-[³H]serine transport in L-glutamine-preloaded BBMVs were measured in the presence
1227 or absence of 1 mM ibuprofen. The graphs represented uptake data in Na⁺-buffer (Na⁺)
1228 subtracted with those in Na⁺-free buffer. n = 3 – 4. B) D-[³H]Serine transport in BBMVs
1229 without amino acid preloading. Transports of D-[³H]serine were measured in a similar way to
1230 A) but the experiment was carried out in BBMVs without any amino acid preloading. n = 3 –
1231 4. C) Inhibition of D-[³H]serine transport in BBMVs. Uptake of 10 μM D-[³H]serine was
1232 measured in the presence or absence of 1 mM inhibitors for 1 min. n = 3. D - F) Localization
1233 of Asct2 in mouse kidney by immunofluorescent staining. Mouse kidney slide was co-stained
1234 with anti-mAsct2(NT) antibody (Asct2; green) and protein markers for renal proximal tubule
1235 segments: anti-Sglt2 antibody (D: Sglt2, apical membrane marker of S1 - S2 segment), anti-
1236 Agt1 antibody (E: Agt1, apical membrane marker of S3 segment), and anti-Na⁺/K⁺-ATPase
1237 antibody (F: Na⁺/K⁺-ATPase, basolateral membrane marker). Merge image is shown in the
1238 right pictures with DAPI (blue) staining. Scale bar = 20 μm. G) Schematic model of ASCT2
1239 and SMCTs in D-serine transport at different segments of proximal tubules. In physiological
1240 conditions, ASCT2 expression is less while SMCTs are abundant, suggesting that SMCTs
1241 mainly contribute to D-serine transport. In the IRI where SMCTs are reduced but ASCT2 is
1242 increased, a high affinity of D-serine transport reduces luminal D-serine.

1243 **Table 1.** Protein candidates for diagnosis of ischemia-reperfusion kidney injury

Protein	Name	Localization	Function/Roles	Application in kidney diseases ^a	Application in others ^a	4h/ sham	8h/ sham	References
Protein biomarkers previously predicted in kidney diseases								
CCN1 (CYR61)	IGF binding protein 10	Extracellular: ECM protein	Promotes chemotaxis, angiogenesis, cell adhesion, and tissue repair.	Diagnosis of IRI, renal injury, CKD	Tissue repair	41.4	29.9	Marx et al., 2018
LTF	Lactotransferrin	Extracellular: Enzyme	Multifunctional secreted protein involved in immune response to infection and injury.	High RNA expression in AKI transplantation	Immunotherapy, anti-inflammation	6.5	7.7	Famulski et al., 2013; Kruzel et al., 2017
FPR2 (ALX)	Formyl peptide receptor 2	PM: GPCR	Receptor of neutrophil chemotactic factors which promotes neutrophil activation.	Target of diabetic nephropathy treatment	Treatment of wound repair, Influenza	5.7	9.1	Luan et al., 2020
CYBB (NOX2)	Cytochrome B-245 β -chain	PM: Enzyme	Generates superoxide in phagocytes and emerge inflammation. Acts as a modulator of IRI and fibrogenesis.	Target of IRI treatment	Target for treatment of immunodeficiency diseases	2.2	2.7	Djamali et al., 2008; Karim et al., 2015
SERPINF1	Serpin family F member 1	Extracellular: Others	Neurotrophic protein inducing retinoblastoma cell differentiation.	Prognosis of acute rejection, CKD		1.9	1.7	Lu et al., 2016; Makridakis et al., 2020
C5AR1	Complement C5a receptor 1	PM: GPCR	Activates chemotaxis and superoxide production. Promote inflammation and injury.	Indicator and treatment target of renal injury, fibrosis	Pathogenesis of inflammatory diseases	1.6	2.0	Tang et al., 2018; Zheng et al., 2008
Protein biomarkers newly predicted in this study								
DCTN4	Dynactin subunit 4	Nucleus	Component of dynein-dynactin complex.	[DCTN4 mutation associated with infection in Citric Fibrosis]	-	2.6	2.8	Emond et al., 2012
LDLR	LDL receptor	PM: Carriers	Rate-limiting step for cholesterol synthesis. Modulated by PCSK9	[PCSK9 is a diagnostic marker for nephrotic syndrome]	Treatment of dyslipidemia	2.5	4.6	Shen et al., 2020; Shrestha et al., 2019
BTF3	Basic TF 3	Nucleus: Regulator	General transcription factor forming a complex with RNA polymerase II	-	[Associated with several cancer types]	2.4	2.4	Zhang et al., 2019
ARL3	ADP ribosylation factor-like 3	Cytoplasm: Enzyme	Small GTP-binding protein essential for cilia biogenesis and cytokinesis.	[ARL3 involve in kidney development]		2.4	2.3	Schrick et al., 2006
ZFP36L1 (BRF1)	ZFP36-like 1	Nucleus: Regulator	RNA-BP that promotes ARE-mediated RNA decay. Mediate HIF1 α degradation.	-	Tumor suppressor	2.3	2.7	Sanduja et al., 2011
TPT1	Fortilin	Cytoplasm	Promote cell growth and regeneration, and host defense mechanism	-	Marker for cell viability	2.1	2.2	Pinkaew and Fujise, 2017
CDA	Cytidine deaminase	Cytoplasm: Enzyme	Deaminate cytidine to uridine. Highly express in leukocyte and enable TLR8 response.	-	[Associated with TLR8 function]	2.0	2.1	Furusho et al., 2019
PAFAH1B2	PAF-AH subunit β	Cytoplasm: Enzyme	A HIF α targeting gene involved in development, inflammation, cancer.	-	[Target of cancer treatment]	1.9	2.0	Ma et al., 2018
RPSA	Ribosomal protein SA	Cytoplasm: Regulator	Component of 40S ribosome. Functions as laminin receptor for cell adhesion	-	Target of cancer therapy	1.8	1.7	DiGiacomo and Meruelo, 2016
PIEZO1	Piezo-type mechanosensitive ion channel 1	Cytoplasm: Ion channel	Mechano-sensing cation channel activated for vascular remodeling and development.	[Control intraluminal pressure and urine flow]	Mechano-stress sensor	1.8	2.2	Martins et al., 2016
EIF3D	eIF3 subunit D	Cytoplasm: Regulator	Component of eIF3 specifically initiates mRNA involved in cell proliferation		[Highly detected in cancers]	1.8	1.7	Yin et al., 2018
OCLN	Occludin	PM: Tight junction	A tight junction protein, a substrate of MMP9. Associates with metastasis, inflammation, and repair	Loss and repair in tight junction membranes during IRI	-	1.7	1.7	Lee et al., 2011

PM: Plasma membrane
^aSquare brackets: Prediction

1244 **Table 1 (continued).** Protein candidates for diagnosis of ischemia-reperfusion kidney injury

Protein	Name	Localization	Function/Roles	Application in kidney diseases ^a	Application in others ^a	4h/ sham	8h/ sham	Refs
<i>Protein biomarkers newly predicted in this study</i>								
PCOLCE	Procollagen C-endopeptidase enhancer	Extracellular: Others	Binds to procollagen and enhances procollagen C-proteinase activity.	highly detected in CKD	[Highly detected in liver fibrosis]	1.6	2.0	Özkan et al., 2019
RPS27A	Ribosomal protein S27a	Cytoplasm	Component of Ubiquitin and 40S ribosome	[Target of MSC for diabetic nephropathy]	[Highly detected in cancers]	1.5	1.6	Yang et al., 2018
KCNAB2	K ⁺ channel subunit β -2	PM: Ion channel	K ⁺ channel regulating nerve signaling. KCNAB2 inhibition attenuates brain hypoxia.	-	Target for treatment of brain hypoxia	1.5	2.6	Wei et al., 2003
ESYT1	Extended synaptotagmin 1	Cytoplasm: Adaptor	Transports lipid between adjacent membranes or exocytosis to compensate for plasma membrane lipid change.	-	-	1.5	1.6	Saheki et al., 2016
APOA2	Apolipoprotein A2	Extracellular: Transporter	Component of HDL. Transports oxidized phospholipids in anti-inflammation.	Altered in kidney diseases	Prognosis of cancer progression	1.5	2.4	Kovacevic et al., 2017; Vaziri, 2016
SLC15A2	Peptide transporter 2	PM: Transporter	Peptide and drug transporter in proximal tubule cells.	[Participate in drug delivery and accumulation of toxicants]	-	0.7	0.6	Kamal et al., 2008
SLC19A3	Thiamine transporter 2	PM: Transporter	Reuptakes Thiamine. Malfunction of THTR2 associated with thiamine deficiency.	[Glucose in diabetes and CKD reduces THTR2 expression]	-	0.6	0.5	Bukhari et al., 2011
GPC4	Glypican 4	PM: Receptor	Heparan sulfate proteoglycan that modulates HGF signal to renal tubule formation	[important for renal tubule formation]	[Tumor suppressor]	0.6	0.6	Karihaloo et al., 2004
SLC5A8	Na ⁺ /mono-carboxylate transporter 1	PM: Transporter	Transports lactate, pyruvate, butyrate, and short chain fatty acids. Target of NSAIDs.	[Lactate is prognosis marker of inflammation]	Tumor suppressor	0.6	0.6	Ganapathy et al., 2008
PLXDC2 (TEM7R)	Plexin domain containing 2	PM: Receptor	Receptor of pigment epithelium derived factor.	-	[Expressed in Ischemic stroke]	0.5	0.6	Cheng et al., 2014
SLC22A7	Organic anion transporter 2	PM: Transporter	Transports multiple substances and creatinine from blood to renal epithelia.	[Creatinine is a biomarker for IRI and kidney diseases]	-	0.5	0.6	Chu et al., 2016; Shen et al., 2017
CYB5B	cytochrome B5 type B	Mitochondria: Enzyme	Membrane-bound hemoprotein in electron transport chain	-	-	0.5	0.6	James et al., 2012
SLCO4C1	Organic anion transporter M1	PM: Transporter	Transports uremic toxin from blood to renal epithelia.	[Increase SLCO4C1 attenuates CKD]	Treatment of hypertension, inflammation	0.5	0.5	Suzuki et al., 2011
AQP7	Aquaporin 7	PM: Transporter	Transports glycol. Activates insulin secretion and regulates glucose and lipid metabolism.	[High urine glycerol is observed in acute renal failure]	-	0.5	0.5	Rodríguez et al., 2011; Sohara et al., 2006
SLC2A5	Glucose transporter 5	PM: Transporter	Fructose transporter. Expression is altered by diabetes and inflammation	-	-	0.4	0.6	Douard and Ferraris, 2008
CMKLR1	Chemerin receptor	PM: GPCR	A GPCR which modulates signals to reduce immune response and enhance angiogenesis	[Chimerin is induced in fibrosis rat model]	Treatment of inflammation	0.4	0.6	Mocker et al., 2019

PM: Plasma membrane
^aSquare brackets: Prediction

Table 2. Candidate transporters from mass spectrometry analysis of the BBMVs from IRI mouse kidneys. The list is arranged by the fold change.

Transporters	Accession	log ₂ fold 4h IRI/ sham	p-value of 4h IRI/ sham	log ₂ fold 8h IRI/ sham	p-value of 8h IRI/ sham	Peptides	Score Mascot	Abundance in sham ^a
<i>Transport proteins found to be increased</i>								
Slc7a1/Cat1	Q09143	0.8	0.12	1.3	0.01	1	131	2.6E+06
Slc1a5/Asct2	P51912	0.4	0.10	0.7	0.09	4	767	1.5E+07
<i>Transport proteins found to be decreased</i>								
Slc36a1/Pat1	Q8K4D3	-1.2	0.08	-1.0	0.12	3	103	5.4E+06
Slc2a5/Glut5	Q9WV38	-1.2	0.01	-0.8	0.04	2	1,140	6.5E+07
Slco4c1/Oatp-m1	Q8BGD4	-1.1	0.00	-0.9	0.00	9	4,011	1.5E+08
Slc22a13/Oat10	Q6A4L0	-1.1	0.03	-0.8	0.03	15	5,948	5.6E+08
Slc22a7/Oat2	Q91WU2	-0.9	0.00	-0.6	0.02	13	6,100	4.3E+08
Slc6a18/B ⁰ at3	O88576	-0.8	0.17	-0.6	0.25	11	4,310	2.1E+08
Slc5a8/Smct1	Q8BYF6	-0.8	0.05	-0.7	0.02	25	26,044	2.9E+09
Slc15a2/Pept2	Q9ES07	-0.7	0.01	-1.1	0.05	9	2,379	4.6E+07
Slc5a12/Smct2	Q49B93	-0.6	0.01	-0.9	0.01	12	4,725	1.6E+08
TMEM27/ Collectrin ^b	Q9ESG4	-0.4	0.01	-0.1	0.17	9	23,686	3.3E+09

^aMedian from n = 3

^bCollectrin is a regulatory protein for B⁰at3 function

1249 Supplement Figures

Figure supplement 1: Wiriyasermkul, et al.

1250 Figure supplement 1: Proteome of BBMV and pathologies of mouse kidney IRI

1251 A) Volcano plots of proteome identified from renal BBMV of the IRI model. The log₂ fold of
 1252 4h IRI/sham (left) or 8h IRI/sham (right) were plotted against -log₁₀ of p-value. Blue lines
 1253 indicate protein fold change of 1.5 (log₂ fold change = 0.58) and red line indicates p-value of
 1254 0.05 (-log₁₀ p-value = 1.3). One protein which is out of range is omitted for resolution purpose.
 1255 B) Top-10 organ pathologies that are presented in both 4h and 8h IRI (left), dominant in 4h IRI
 1256 (middle), and dominant in 8h IRI (right). C) Hierarchical heatmap of organ pathologies
 1257 corresponding to B). Colors indicate predicted pathologies (z-score).

Figure supplement 2: Wiriyasermkul, et al.

1258 Figure supplement 2: Predicted pathways in response to 8h IRI

1259 Identified proteins from renal BBMV, which are significantly altered more or less than 1.5-
1260 fold change (p-value < 0.05) in 8h IRI, were curated into networks by IPA and literature. A –
1261 C networks indicate the proteins which are highly altered at 8h compared to 4h IRI. Protein
1262 expressions that are upregulated and downregulated are shown in red and cyan, respectively,
1263 with intensity shading corresponding to the degree of fold change. Proteins are shaped by
1264 categories and double cycles indicate protein complexes. Solid and dash lines represent direct
1265 and indirect interactions, respectively. Lines are colored for contrast observation in which blue
1266 and magenta lines imply activation to regulators and targeting proteins, respectively. A)
1267 Network involved in connective tissue development and function via ANXA1 and BCL2
1268 regulators. B) Network involved in cell-cell interaction and system development. C) Network
1269 involved in cell morphology, cell-cell interaction, and movement via Integrin signaling.

Figure supplement 3: Wiriyasermkul, et al.

1270 **Figure supplement 3: Hierarchical heatmap of abnormalities from all identified proteins.**
1271 Heatmap of all proteome with no cut-off value compared to data from the cut-off values of 1.5-
1272 fold change and 0.05 p-value (1.5 fold, p < 0.05). The abnormalities highlighted in red are those
1273 involved with kidney. Colors indicate predicted pathologies (z-score).

Figure supplement 4: Wiriyasermkul, et al.

Figure supplement 4: Characterization of ASCT2 as a D-serine transporter in HEK293 cells

A) Time course of 100 μM D-[³H]serine transport (10 Ci/mol) was measured in wild-type (WT) and *ASCT2*-knockout (*ASCT2*-KO) HAP1 cells. D-[³H]Serine transport in the cells was measured in PBS pH 7.4. n = 3. B) Transport of 10 μM D-[³H]serine was measured for 10 or 20 min in HEK293 cells in the presence or absence of Na⁺. n = 3. C) Inhibition of D-[³H]serine

1280 transport by several compounds. The uptake was measured for 10 min in PBS buffer. Left: 5
 1281 μM D-[^3H]serine uptake was measured in the presence or absence of 2 mM L-amino acids. n =
 1282 4. Right: 2 μM D-[^3H]serine uptake was measured in the presence or absence of 1 mM
 1283 inhibitors; Ben-Cys: *S*-benzyl-L-cysteine, a non-specific inhibitor of ASCT2; GPNA: L- γ -
 1284 glutamyl-*p*-nitroanilide, an inhibitor of ASCT2, SNATs and LATs; MeAIB: 2-
 1285 (methylamino)isobutyric acid, a system A inhibitor. n = 3. D) Cell-growth measurement (XTT
 1286 assay) of HEK293 cells treated with either L-serine (opened circles) or D-serine (closed circles)
 1287 at different concentrations for two days. In contrast to L-serine which did not alter cell viability,
 1288 D-serine reduced cell growth in a concentration-dependent manner. D-Serine inhibition curve
 1289 was fitted to non-linear regression of $\log_{10}[\text{D-Ser}]$ v.s. cell growth, resulting in IC_{50} of 17.4 mM
 1290 and minimum growth at OD 450 nm of 1.15 AU. n = 3 - 5.

Figure supplement 5: Wiriyasermkul, et al.

1291 **Figure supplement 5: SMCTs, PAT1, and B⁰AT3 in the volcano plots of membrane**
 1292 **transport proteins**
 1293 Volcano plot of 319 membrane transport proteins is identical to the plot presented in Figure
 1294 4A. Position of SMCT1, SMCT2, PAT1, and B⁰AT3 are indicated.

Figure supplement 6: Wiriyasermkul, et al.

Figure supplement 6: SMCT1 and SMCT2 function in FlpInTR-SMCT1 and FlpInTR-SMCT2 stable cell lines

A) Western blot of membrane fractions from FlpInTR-Mock, -SMCT1, and -SMCT2 cells with ASCT2-siRNA transfection. Flp-In T-REx 293 stably expressing SMCT1 (SMCT1) and SMCT2 (SMCT2), as well as Mock, were transfected with ASCT2-siRNA for two days. Membrane proteins were extracted from crude membrane fractions and subjected to Western blot analysis. ASCT2 knockdown efficiency was evaluated by anti-hASCT2 antibody. Expression of SMCT1 and SMCT2 were verified by anti-FLAG antibody. B – C) Evaluation of SMCT1 and SMCT2 function by transport assay. Time course of 50 μ M [14 C]nicotinate uptake was measured for 0.5 – 5 min in FlpInTR-SMCT1 (B) and FlpInTR-SMCT2 (C) cells, compared to mock cells (Mock). n = 3. D) Time course of 100 μ M D-[3 H]serine uptake in FlpInTR-SMCT1 (SMCT1), FlpInTR-SMCT2 (SMCT2), and Mock cells. The experiment was carried out in a similar way to experiment in Figure 6A except that the cells were not subjected to ASCT2 knockdown. n = 4. E) Raw data of Figure 6C before subtraction with the uptake data in Mock cells. D-[3 H]serine uptake in FlpIn293TR-SMCT1 compared to FlpIn293TR-Mock cells. The uptake values were fitted to Michaelis-Menten plot. n = 3 – 4.

Figure supplement 7: Wiriyasermkul, et al.

1312 Figure supplement 7: Purification of SMCT1 and functional characterization

1313 A) Western blot of purified SMCT1 from pCMV14-SMCT1-transfected Expi293F cells. The
 1314 protein was detected by anti-FLAG antibody. The closed arrowhead indicates purified SMCT1,
 1315 whereas the opened arrowhead is 3xFLAG peptides for the elution of the purified SMCT1. The
 1316 bands above 250 kDa probably include SMCT1, which is aggregated at the top of SDS-

1317 polyacrylamide gel. B) Raw data of the ibuprofen effect presented in Figure 7B before
 1318 subtraction with the uptake in K^+ -buffer. Uptake of $[^3H]$ lactate, $[^3H]$ propionate, L- $[^3H]$ serine,
 1319 and D- $[^3H]$ serine (all 50 μM) in SMCT1 proteoliposomes (SMCT1-PL). The uptake was
 1320 measured in the Na^+ buffer (Na^+) or Na^+ -free buffer (K^+) in the presence or absence of 1 mM
 1321 ibuprofen. n = 3. C) Raw data of D- $[^3H]$ serine transport in SMCT1-PL presented in Figure 7C
 1322 prior to subtraction with the uptake in K^+ -buffer. Time course of D- $[^3H]$ serine (200 μM)
 1323 transport was measured in the SMCT1 proteoliposomes in Na^+ -buffer (Na^+) or Na^+ -free buffer
 1324 (K^+).

Figure supplement 8: Wiriyasermkul, et al.

Figure supplement 8: D-Serine transport and expression of Asct2 in mouse BBMVVs

A) Raw data of Figure 8A: D-[³H]Serine transport in BBMVVs preloaded with 4 mM L-glutamine. Time course of 10 μM D-[³H]serine transport in L-glutamine-preloaded BBMVVs in Na⁺-buffer (Na⁺) or Na⁺-free (K⁺) buffer in the presence or absence of 1 mM ibuprofen. n = 3 – 4. B) Time course of 10 μM D-[³H]serine transport in BBMVVs without preloading at longer

1330 time points. The transport was measured in Na⁺-buffer (filled circles) or Na⁺-free buffer
 1331 (opened circles). n = 4. C) Western blot of Asct2 in mouse kidney BBMV_s using anti-
 1332 mAsct2(NT) (left) or anti-mAsct2(CT) (right) antibodies.

Table supplement 1. List of proteins identified from proteome analysis of BBMV from IRI mouse kidneys.

Table supplement 1 is provided as an excel sheet which is submitted separately as a supporting file.

Table supplement 2. Plasma membrane amino acid transporters identified from mass spectrometry of HEK293 membrane fraction

Transporter	Accession	Peptides	PSMs	Score Mascot	Abundance
SLC1A3/EAAT1	P43003	1	4	167	1.20E+07
SLC1A5/ASCT2	Q15758	5	15	221	1.68E+08
SLC3A2/4F2hc	J3KPF3	15	46	815	3.62E+08
SLC6A15/B ⁰ AT2	Q9H2J7	2	2	46	1.28E+07
SLC7A1/CAT1	B2R728	2	7	83	4.94E+07
SLC7A6/y ⁺ LAT2	Q92536	1	1	31	7.15E+06
SLC38A2/SNAT2	A8K6H9	1	2	88	2.82E+07
SLC38A9/SNAT9	Q8NBW4	1	1	0	1.33E+07

A**Network of the rapid responses via NF- κ B complex** **B****Network of the rapid responses via MYC and PI3K complex** **C****Network of the prolonged responses via HDL and Akt**

Figure 3: Wiriyasermkul, et al.

Figure 4: Wiriyasermkul, et al.

A **B** **C**

Figure 6: Wiriyasermkul, et al.

A **B** **C** **D**

Figure supplement 2: Wiriyasermkul, et al.

A

B

C

Figure supplement 3: Wiriyasermkul, et al.

A

B

A

B

C

D

E

A

B

C

A

B

C