A multi-hierarchical approach reveals D-serine as a hidden substrate of sodium-coupled monocarboxylate transporters

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Abstract

Transporter research primarily relies on the canonical substrates of well-established transporters. This approach has limitations when studying transporters for the low-abundant micromolecules, such as micronutrients, and may not reveal physiological functions of the transporters. While D-serine, a trace enantiomer of serine in the circulation, was discovered as an emerging biomarker of kidney function, its transport mechanisms in the periphery remain unknown. Here, using a multi-hierarchical approach from body fluids to molecules, combining multi-omics, cell-free synthetic biochemistry, and *ex vivo* transport analyses, we have identified two types of renal D-serine transport systems. We revealed that the small amino acid transporter ASCT2 serves as a D-serine transporter previously uncharacterized in the kidney and discovered D-serine as a noncanonical substrate of the sodium-coupled monocarboxylate transporters (SMCTs). These two systems are physiologically complementary, but ASCT2 dominates the role in the pathological condition. Our findings not only shed light on renal D-serine transport, but also clarify the importance of non-canonical substrate transport. This study provides a framework for investigating multiple transport systems of various trace micromolecules under physiological conditions and in multifactorial diseases.

41 ## Introduction

42 Membrane transport proteins are key molecules to control the homeostasis of
43 micromolecules. To date, over 450 solute carriers (SLCs), 40 ATP-binding cassettes (ABCs),
44 60 ATPase pumps, and 300 ion channels have been identified in mammals (Ranjan et al., 2011;
45 César-Razquin et al., 2015; Perland and Fredriksson, 2017; The UniProt Consortium et al.,
46 2021). Approximately 30% of membrane transport proteins remain uncharacterized. While
47 some membrane transport proteins can be classified based on sequence similarities and
48 transport substrates of well-studied family members, this approach is limited when it comes to
49 discovering transporters for some micromolecules, such as micronutrients and their metabolites.
50 Many of these micromolecules have not been extensively studied and do not fit into traditional
51 nutrient types. Currently, the importance of micronutrients and gut microbiota-derived
52 metabolites in health and disease has gained a high impact (Shenkin, 2006; Vernocchi et al.,
53 2016; Liu et al., 2022), yet their responsible transporters are not well understood.
54 Understanding the absorption and reabsorption mechanisms of dietary micronutrients and gut
55 microbiota-derived metabolites will provide a key molecular process for controlling their
56 dynamics and molecular activities.

57 This study aims to establish an approach to investigate the physiological function and
58 pathological significance of transporters, particularly transport systems for trace
59 micromolecules. As a paradigm, we investigated renal transport systems for D-amino acids,
60 micronutrients derived mostly from the diet and some from the gut microbiota. Amino acid
61 transport systems have been well studied (Bröer, 2008a), but have focused on L-amino acids,
62 which are macronutrients, leaving open the question of how D-amino acids are transported. We
63 have developed two-dimensional high-performance liquid chromatography (2D-HPLC), which
64 allows the segregation of L- and D-enantiomers in liquid biopsy (Miyoshi et al., 2009; Hamase
65 et al., 2010). The importance of D-amino acids in renal function has received increasing

attention (Sasabe et al., 2014; Kimura et al., 2016; Sasabe and Suzuki, 2018; Nakade et al., 2018; Hesaka et al., 2019; Kimura et al., 2020; Okushima et al., 2021; Iwata et al., 2022; Suzuki et al., 2022). D-Serine in the body fluid was discovered as a promising biomarker for acute kidney injury (AKI) and chronic kidney disease (CKD) due to its clear association with kidney function in rodents and humans (Sasabe et al., 2014; Kimura et al., 2016; Nakade et al., 2018; Hesaka et al., 2019; Okushima et al., 2021). Diseased-associated alterations in plasma and urinary D-/L-serine ratio suggest that renal D-serine transport systems are different and distinct from L-serine transport systems under physiological and pathological conditions. The transport of D-serine has long been found to take place at the proximal tubules (Kragh-Hansen and Sheikh, 1984; Sasabe and Suzuki, 2018; Shimomura et al., 1988; Silbernagl et al., 1999), yet the corresponding transporter has not been clearly elucidated.

D-Serine is found at submillimolar levels in the brain (Hashimoto et al., 1992), where it acts as an obligatory physiological co-agonist of *N*-methyl-D-aspartate receptors (NMDARs) (Mothet et al., 2000; Basu et al., 2009; Papouin et al., 2012; Wolosker, 2018). Extensive studies of D-serine in the brain show that D-serine is transported by four plasma membrane transporters: ASCT1 (SLC1A4), ASCT2 (SLC1A5), Asc-1 (SLC7A10), and SNAT1 (SLC38A1) (Rosenberg et al., 2013; Foster et al., 2016; Kaplan et al., 2018; Bodner et al., 2020). In contrast, D-serine is detected at low micromolar levels in the periphery, and little is known about its dynamics as well as transporting systems. Mammals acquire D-serine by biosynthesis via serine racemase function (Wolosker et al., 1999) and absorption from the diet and gut microbiota presumably via intestinal transport system(s) (Sasabe and Suzuki, 2018; Nakade et al., 2018; Gonda et al., 2023). Similar to the renal epithelia, a D-serine transporter in the intestine is also unknown.

In this study, we integrated multiple analytical methods for the different biological hierarchies. We applied 2D-HPLC to measure plasma and urinary enantiomers of 20 amino

91 acids of ischemia-reperfusion injury (IRI) mice, a model of AKI and AKI-to-CKD transition
92 (Sasabe et al., 2014; Fu et al., 2018). Membrane proteomics of the renal proximal tubules of
93 the IRI mouse models was performed to explore the key molecules responsible for the
94 alterations of D- and L-amino acid transport. With bioinformatics, cell-based screening and
95 cell-free transport analysis, we identified two types of D-serine transport systems in the kidney.
96 Subsequently, *ex vivo* transport analyses from the normal kidney and the IRI model explained
97 the transport mechanism of D-serine as a biomarker in kidney diseases.

98 # Results

99 **Amino acid metabolomics in plasma and urine of the renal IRI model**

100 To understand kidney-associated alteration of enantiomeric amino acids, we measured
101 L- and D-isomers of 20 amino acids in the body fluids of the IRI model (Figure supplements 1-
102 2). Dynamics of L- and D- enantiomers in the plasma were evaluated from the ratios of D-/L-
103 enantiomers (Figure 1A). Among amino acids, only the ratios of plasma D-/L-serine were
104 increased in a time dependent-manner from 4 – 40 hours after the ischemia-reperfusion (Figure
105 1A-B). The elevation of plasma D-/L-serine ratios at early time points (4h – 8h) was due to a
106 sharp decrease of the L-isomer, while the rise of D-/L-serine ratios at late time points (20h –
107 40h) was a result of a continuous acceleration of the D-isomer (Figure 1C-D). The results of
108 plasma D-serine were consistent with previous observations (Sasabe et al., 2014). In addition
109 to D-serine, we detected certain amounts of D-alanine, D-proline and D-methionine in the
110 plasma (Figure supplement 1A). However, their D-/L- enantiomeric ratios were uncorrelated to
111 the pathological conditions (Figure supplement 1A). Our knowledge of traditional amino acid
112 transport suggests that alanine, serine and proline share the same transport systems (Bröer,
113 2008a). The noticeably different profile of plasma D- and L-serine from those of alanine and
114 proline led to the speculation that D-serine transport was possibly mediated by unique transport
115 systems, different from those of D-alanine, D-proline, and the L-isomers. Moreover, such
116 transport systems may be sensitive to the IRI conditions.

117 We measured the enantiomeric amino acid profiles from urine samples of the IRI model
118 (Figure supplement 2). While plasma is a reliable resource of metabolite biomarkers, urine is
119 a meaningful non-invasive diagnostic indicator of kidney function. More importantly, the
120 analysis of urinary metabolites is indispensable for elucidating the unknown reabsorption
121 systems of certain substances in the kidney. From our results, the ratios of urinary D-/L-serine

122 were drastically decreased at early IRI (4h – 8h) (Figure 1E-F). Similar tendencies of D-/L-
123 amino acid ratios were observed in alanine, proline, asparagine, and histidine (Figure 1E-H).
124 These urinary amino acid profiles indicated the impairment in both D- and L-amino acid
125 reabsorption since the early injury. We hypothesized that transporters play a major role in the
126 dynamics of D- and L-serine in the kidney with respect to changes in the D-/L-serine ratio during
127 pathology.

128 **Proteomics for membrane transport proteins from renal brush border membranes of the**
129 **IRI model**

130 We aimed to identify the corresponding molecules for the dynamics of D- and L-serine.
131 To obtain the candidates, we performed proteomic analysis of renal brush border membrane
132 vesicles (BBMVs) (the membrane fraction enriching apical membrane proteins of proximal
133 tubular epithelia) from the IRI model. We analyzed the BBMVs from 4 – 8 hours after IRI, the
134 times of rigorous shift of plasma and urinary D-/L-serine ratio (Figure 1B, F). The proteomes
135 of the IRI samples were calculated as ratios of the negative control group (sham), yielding two
136 groups of comparative proteome data: 4h IRI/sham (4h) and 8h IRI/sham (8h). In total, 4,423
137 proteins were identified, of which 1,187 proteins (27% of the total detected proteins) were
138 categorized as plasma membrane proteins and extracellular matrix proteins (Table supplement
139 1). We observed a significant increase of two well-known early AKI biomarkers: Ccn1 (Cyr61:
140 matrix-associated heparin-binding protein; 4h IRI/sham = 41.4 folds; 8h IRI/sham = 29.9 folds)
141 and Ngal (Lcn2: Neutrophil gelatinase-associated lipocalin; 4h IRI/sham = 1.8 folds; 8h
142 IRI/sham = 9.5 folds). This finding confirmed the reliability of our proteomics for molecular
143 analysis of early AKI (Table supplement 1) (Marx et al., 2018).

144 From the proteome, we detected 398 membrane transport proteins (325 transporters and
145 73 ion channels). Analysis of all membrane transport proteins by Ingenuity Pathway Analysis

146 (IPA) revealed alterations of the biological function in *Molecular Transport*, which are
147 categorized by the types of their canonical substrates (Table supplement 2). Transport of "all
148 micromolecules" was shown to be drastically decreased from 4h IRI (Figure 2A: Top). This
149 decrease was due to the dramatic reduction of transport systems for heavy metals and organic
150 compounds (Figure 2A). Figure 2B shows membrane transport proteins corresponding to the
151 annotation in Figure 2A. This proteomic result unveiled the whole picture of the molecular
152 targets of the injury. Furthermore, the results revealed the key membrane transport proteins
153 behind AKI metabolite biomarkers. It is noted that some transporters were upregulated,
154 suggesting the compensatory mechanism repair processes for cell survival from the injury
155 (Figure 2B).

156 **ASCT2 is one of D-serine transporters at the apical membrane of renal proximal tubules**

157 Given insight into transporters in the proteome, we observed the elevation of mouse
158 Slc1a5/Asct2, a known D-serine transporter in the brain, at both 4h and 8h IRI (Figure 2B:
159 Transport of Amino acids). Asct2 was previously detected in renal brush border membranes,
160 but its precise localization and function in the kidney have not been characterized (Avissar et
161 al., 2001; Scalise et al., 2018). We then examined the localization of Asct2 in the kidney by
162 using affinity-purified Asct2 antibodies that recognized its N-terminus (NT) or C-terminus
163 (CT) (Figure supplement 3A). Asct2 was partially co-immunostained with both Sglt2 (Slc5a2;
164 sodium/glucose cotransporter 2) and Agt1 (Slc7a13, aspartate/glutamate transporter 1), which
165 are apical membrane markers for S1 + S2 and S3 segments, respectively (Figure 3A-B)
166 (Nagamori et al., 2016a; Ghezzi et al., 2018). In contrast, Asct2 did not co-localize with
167 Na^+/K^+-ATPase, a basolateral membrane marker (Figure 3C). The results demonstrated that
168 Asct2 is localized at the apical side in all segments of proximal tubules.

169 To confirm the D-serine transport function of ASCT2 in human cells, we used wild-
170 type (WT) near-haploid human leukemic cancer cell line (HAP1) and HAP1 cells carrying
171 CRISPR/CAS-mediated *ASCT2* knockout (ASCT2-KO). D-serine uptake was significantly
172 decreased in the ASCT2-KO cells (Figure 3D). The function of ASCT2 was also verified in
173 human embryonic kidney HEK293 cells, which endogenously express ASCT2, by using
174 ASCT2-knockdown (Figure 3E).

175 **Screening of candidate molecules for D-serine transporters**

176 While we have shown that ASCT2 is a D-serine transporter at the apical membranes of
177 all proximal tubular segments, ASCT2 transports D-serine with high affinity (K_m of 167 μM in
178 oocyte system) but with weak stereoselectivity (Foster et al., 2016). These properties of ASCT2
179 differ from those of the reported D-serine transport in the kidney. First, D-serine transport
180 systems in S1 + S2 segments are reported to be different from those in the S3 segments and the
181 kinetics of both systems are in mM range (Kragh-Hansen and Sheikh, 1984; Silbernagl et al.,
182 1999). Silbernagl et al. also suggested that ASCT2 is not (or not only) a D-serine transporter at
183 S3 segment (Silbernagl et al., 1999). Second, the plasma and urinary D-/L-serine profiles from
184 CKD and AKI samples indicated the stereoselectivity of the transporter (Figure 1) (Sasabe and
185 Suzuki, 2018). Third, ASCT2 recognizes serine, alanine, and proline, but our enantiomeric
186 amino acid profile in plasma samples showed a distinct D-/L- ratio pattern of serine from alanine
187 and proline (Figure 1B). All data suggest the existence of (an)other D-serine transporter(s).

188 To search for the D-serine transporters, we chose the ratios of mouse Asct2 as the cutoff
189 values of our membrane proteomic data at both 4h and 8h IRI (Figure 4A). Membrane transport
190 proteins showing more alteration than Asct2 at both increased and decreased values were
191 selected because some transporters are known to switch roles upon pathology. Further, we
192 focused on the luminal transporters and omitted transporters that are reported to be located at

193 basolateral membranes and organelles as the BBMVs are enriched apical membrane fractions.
194 Membrane transport proteins that recognize only inorganic ions were also excluded. Finally,
195 ten candidates were selected (Table 1). Slc36a1/Pat1 and Slc6a18/B^0at3, the known small
196 amino acid transporters, were included in the list, although their expressions only passed the
197 cutoff at only one-time point (either 4h or 8h IRI). Slc5a12/Smct2 was also included because
198 Smct1, another member of sodium-coupled monocarboxylate transporter (SMCT) family, was
199 selected as a candidate. Smct2 has a comparable role with Smct1, but both transporters are
200 localized at different segments of the proximal tubules.

201 To analyze the function of the candidate transporters, we selected HEK293 cell line for
202 the screening method because of its high transfection efficiency. Similar to other several
203 common cell lines, HEK293 cells express ASCT2 endogenously. However, we examined other
204 possible D-serine transport systems in HEK293 cells, aiming to reduce the transport
205 background. Proteomic analysis of the membrane fractions from HEK293 cells identified
206 amino acid transporters as shown in Table supplement 3. Transport activity of D-serine in
207 HEK293 cells showed Na^+ dependency and ASCT2-substrate mediated inhibition (L-Ser, L-
208 Thr, and L-Met) (Figure supplement 3B-C). Ben-Cys (*S*-benzyl-L-cysteine) and GPNA (L-γ-
209 glutamyl-*p*-nitroanilide) inhibited D-serine transport in HEK293 cells similar to that of ASCT2-
210 expressing cells (Bröer et al., 2016), but SLC38 inhibitor MeAIB (2-(methylamino)isobutyric
211 acid) had no effect (Figure supplement 3C). These results indicated that ASCT2 is the main D-
212 serine transporter in HEK293 cells.

213 Due to the absence of any ASCT2-specific inhibitor and the compensatory transport
214 mechanism that occurred in the ASCT2-deletion condition (Figure supplement 3E) (Bröer et
215 al., 2016), we avoided using the traditional cell-based transport assay for screening. Instead,
216 we established a new screening method based on cell growth. Apart from the studies on D-
217 serine biomarkers in kidney diseases, a previous study found that high concentrations (10 – 20

218 mM) of D-serine impaired the growth of a human proximal tubular cell line (Okada et al., 2017).
219 We then tested whether high concentrations of D-serine affect HEK293 cell growth. Unlike L-
220 serine which had no effect, D-serine reduced cell growth in a concentration-dependent manner
221 with IC_{50} of 17.4 ± 1.05 mM (Figure supplement 3D). In the ASCT2-knockdown cells, the
222 growth inhibition by D-serine was attenuated (Figure 4B), indicating that D-serine suppressing
223 cell growth was caused by ASCT2-mediated D-serine transport.

224 Based on this finding, we utilized cell growth determination assay as the screening
225 method even without ASCT2 knockdown. HEK293 cells were transfected with human
226 candidate genes. The transfected cells were treated with either 15 mM (near IC_{50} concentration)
227 or 25 mM (high concentration) D-serine for 2 days, and cell growth was determined. The
228 positive control Asc-1 transfected cells showed a reduction in cell growth, confirming the
229 effectiveness of this assay (Figure 4C-D). Among ten candidates, PAT1 (SLC36A1) and
230 B^0AT3 (SLC6A18) showed higher cell growth than the mock cells, suggesting that PAT1 and
231 B^0AT3 may reduce D-serine influx mediated by endogenous ASCT2. In contrast, SMCT1
232 (SLC5A8) and SMCT2 (SLC5A12) extended the growth suppression after treatment with both
233 15 and 25 mM D-serine (Figure 4C-D and supplement 4A-B). Thus, with the hypothesis that
234 the candidate molecule influxes D-serine and brings about growth reduction, we selected both
235 SMCT1 and SMCT2 for further analysis.

236 **Characterization of D-serine transport in SMCTs**

237 SMCTs are known to transport monocarboxylates such as lactate, propionate, and
238 nicotinate in a Na^+-dependent manner (Ganapathy et al., 2008). To characterize D-serine
239 transport in both SMCT1 and SMCT2, we generated Flp-In T-REx 293 cells stably expressing
240 human SMCT1 (FlpInTR-SMCT1) and SMCT2 (FlpInTR-SMCT2). Expression of SMCTs in
241 FlpInTR-SMCT1 and FlpInTR-SMCT2 was verified by Western blot using anti-FLAG

242 antibody to detect FLAG-tagged SMCTs (Figure supplement 5A). SMCT1 and SMCT2
243 expressions remained unchanged upon ASCT2 knockdown (Figure supplement 5A). The
244 function of SMCTs was confirmed by their canonical substrate [^{14}C]nicotinate transport in the
245 SMCT1- and SMCT2-stable cells (Figure supplement 5B-C).

246 The transport functions of SMCTs are inhibited by non-steroidal anti-inflammatory
247 drugs (NSAIDs; e.g., ibuprofen and acetylsalicylate) (Itagaki et al., 2006; Gopal et al., 2007).
248 To verify the contribution of SMCTs to D-serine-reduced cell growth, we added ibuprofen to
249 the growth assay. In contrast to mock, in which ibuprofen has no effect, the growth suppression
250 by D-serine treatment was gradually attenuated by ibuprofen in both FlpInTR-SMCT1 and
251 FlpInTR-SMCT2 cells, indicating that the cell growth suppression by D-serine treatment is the
252 result of SMCT1 and SMCT2 functions (Figure 5A).

253 We characterized SMCTs mediating D-[^{3}H]serine transport by using SMCT-stable cell
254 lines. Both FlpInTR-SMCT1 and FlpInTR-SMCT2 cells transported D-[^{3}H]serine over Mock
255 (Figure 5B) and the transport was enhanced when ASCT2 was knocked down (Figure 5C),
256 indicating the recognition of D-serine by both SMCT1 and SMCT2. The D-[^{3}H]serine transport
257 was inhibited by ibuprofen and acetylsalicylate, confirming the specific transport by SMCTs
258 (Figure 5D). The excess of non-radioisotope-labeled D-serine also inhibited D-[^{3}H]serine
259 uptake in both SMCTs. Still, its inhibitory effect in SMCT2 was much less than in SMCT1,
260 suggesting the lower affinity of D-serine in SMCT2 than in SMCT1 (Figure 5D).

261 **D-Serine transport properties and kinetics in SMCT1**

262 Kinetics of D-[^{3}H]serine transport in SMCT1 was measured in FlpInTR-SMCT1 cells
263 in the presence of ASCT2 knockdown. SMCT1 transported D-[^{3}H]serine in a concentration-
264 dependent manner and the curve fitted to Michaelis−Menten kinetics with the apparent K_m of
265 3.39 ± 0.79 mM and V_{max} of 18.23 ± 1.73 pmol/μg protein/min (Figure 5E and supplement 5D).

266 The substrate selectivity of SMCT1 was investigated using synthetic biochemistry to
267 avoid the interference of amino acid and carboxylate transports by endogenous transporters in
268 cells. We established a cell-free assay system using proteoliposomes, in which purified proteins
269 are reconstituted into liposomes and all substances in the system are controllable. We purified
270 human SMCT1 by affinity column and reconstituted SMCT1-proteoliposome (SMCT1-PL)
271 (Figure 6A). The function of SMCT1 in SMCT1-PL was verified by the transport of lactate,
272 propionate, and nicotinate but not urate (negative control) (Figure supplement 6A). The
273 kinetics of [^{14}C]nicotinate transport in SMCT1-PL revealed an apparent K_m of 442 ± 94 μM
274 (Figure supplement 6B), which is in a similar range to the K_m previously described by
275 electrophysiological experiments in *Xenopus* oocyte expression systems (K_m 390 μM) (Paroder
276 et al., 2006), confirming the functional properties of SMCT1 in our cell-free transport assay
277 system.

278 The transport of D-[^{3}H]serine in SMCT1-PL was Na^+-dependent and reached the
279 stationary phase at approximately 5 min (Figure 6B). The amount of D-serine transport was
280 slightly lower than lactate and propionate but higher than L-serine and the transport was
281 inhibited by ibuprofen, confirming SMCT1-mediated D-serine transport (Figure 6C). Amino
282 acid selectivity revealed that SMCT1 recognized both L- and D-serine over other small amino
283 acids (L- and D-alanine), acidic amino acids (L- and D-glutamate), and large neutral amino acids
284 (L- and D-tyrosine) (Figure 6D). Therefore, we concluded that serine is the substrate of SMCT1
285 and the recognition is more stereoselective for the D- than the L-isomer.

286 **ASCT2 and SMCTs contribute to D-serine transport in renal proximal tubular epithelia**

287 We next examined the contributions of ASCT2 and SMCTs to D-serine reabsorption in
288 the kidney using an *ex vivo* transport assay. ASCT2 is an antiporter, influx one amino acid with
289 efflux of another, while SMCTs are symporters (Ganapathy et al., 2008; Scalise et al., 2018).

290 Therefore, we measured D-serine transport in the presence or absence of L-Gln preloading and
291 with or without ibuprofen to distinguish the functions of mouse Asct2 and Smcts. The
292 enantiomeric amino acid profile showed D-serine concentrations of 4 – 10 μM in the plasma
293 samples (Figure supplement 1) and 20 – 100 μM in the urine samples (Figure supplement 2).
294 Accordingly, we tested 10 μM D-[^{3}H]serine transport in BBMVs derived from normal mice. In
295 the non-preloading condition, D-[^{3}H]serine uptake in Na^+ dependence gradually but
296 continuously increased and the uptake was remarkably inhibited by ibuprofen, suggesting that
297 the transport was mainly attained from Smcts functions (Figure 7A). In L-Gln preloaded
298 BBMVs, D-[^{3}H]serine uptake in Na^+-dependence arose quickly and reached the saturated point
299 at 1 – 2 min (Figure 7B). The uptake at early time points (≤ 30 sec) was ibuprofen-insensitive
300 but became partly ibuprofen-sensitive from 1 min to the stationary phase (Figure 7B). These
301 results indicate that the D-serine transport was mediated by both Asct2 and Smcts. At early
302 time points, D-[^{3}H]serine was transported by Asct2, which occurred highly and rapidly.
303 Meanwhile, Smcts functions (ibuprofen-sensitive) initiated later but had prolonged functions
304 as seen in the non-preloading condition. Taken altogether, we suggested that, in the normal
305 kidney where renal proximal tubular epithelial cells contain intracellular L-Gln, D-serine
306 reabsorption is derived from combinational functions of both ibuprofen-sensitive (e.g. Smcts)
307 and ibuprofen-insensitive (e.g. Asct2) transporters.

308 The localization of Smct1 in the kidney was unclear. Gopal et al. reported the
309 localization of Smct1 at S3 and Smct2 in all segments, while single-nucleus RNA sequencing
310 (snRNA-Seq) showed Smct1 at S2 – S3 and Smct2 at S1 segment (Figure supplement 7:
311 Controls) (Gopal et al., 2007; Kirita et al., 2020). We generated an anti-Smct1 antibody and
312 examined the localization of Smct1. The result showed that Smct1 was mainly localized at the
313 S3 and slightly at the S1 + S2 segments (Figure supplement 8).

314 **D-Serine transport in renal proximal tubular epithelia of IRI model**

315 Under IRI conditions, we observed low urinary but high plasma levels of D-serine,
316 suggesting high D-serine reabsorption (Figure supplements 1A, 2A). Our membrane
317 proteomics revealed that Asct2 was increased while and Smct1 and Smct2 were decreased in
318 both 4h and 8h IRI (Table 1). We then examined the functional contributions of Asct2 and
319 Smcts on the D-serine reabsorption during the pathology. Similar to those of the normal
320 BBMVs (Figure 7A-B), D-[^{3}H]serine transport in Sham operation showed ibuprofen-sensitive
321 in non-preloading and partly ibuprofen-sensitive in L-Gln preloading conditions (Figure 7C:
322 Sham). D-[^{3}H]Serine influx reduced sharply in the BBMVs of 4h IRI model, and the transport
323 was ibuprofen-insensitive in both with and without L-Gln preloading (Figure 7C: 4h IRI).
324 Likely, this feature was due to the slight increase of Asct2 and the dramatic decrease of Smcts
325 as observed in the proteome data (Asct2: 4h IRI/sham = 1.29 fold, Smct1: 4h IRI/sham = 0.59
326 fold; Smct2: 4h IRI/sham = 0.86 fold; Table supplement 1). We verified that BBMVs from all
327 conditions contain functional proteins by measuring L-[^{3}H]aspartate transport, which
328 represented the function of Slc1a1/Eaac1 (Kanai and Hediger, 1992; Bailey et al., 2011). The
329 assay confirmed that BBMVs derived from 4h IRI model were not fully damaged, although the
330 low influx was likely from the loss of protein expression during the injury (Figure 2B: Amino
331 acids, Figure supplement 9). In the BBMVs of 8h IRI model, D-[^{3}H]serine transport was
332 exceedingly high in L-Gln preloading and it was ibuprofen-insensitive (Figure 7C: L-Gln
333 preloaded). These results could be explained by the continuing increase of Asct2 and decrease
334 of Smcts as revealed by our proteomics (Asct2: 8h IRI/sham = 1.64 fold; Smct1: 8h IRI/sham
335 = 0.62 fold; Smct2: 8h IRI/sham = 0.78 fold) (Table 1, Table supplement 1). The slight increase
336 in D-[^{3}H]serine transport in non-preloaded 8h IRI BBMVs may be due to an unknown
337 transporter (Figure 7C: No preload). Our amino acid profiling showed that the amount of
338 plasma and urinary L-Gln were not largely altered during 4h – 8h IRI (Figures 1B, 2B),

339 suggesting that renal epithelial cells maintain intracellular L-Gln. Most likely, the high
340 reabsorption of D-serine during IRI resulted from the increase of Asct2, despite the decreases
341 of Smct1 and Smct2.

342 For comparison with our proteome data, we analyzed the mRNA expressions of Asct2,
343 Smct1, and Smct2 in proximal tubule clusters at the early IRI from the open-source snRNA-
344 seq dataset (Kirita et al., 2020). Consistent with our proteome data, Smct1 and Smct2 mRNA
345 expressions were dramatically decreased, whereas Asct2 was increased since the early IRI
346 (Figure supplement 7). The correlation of proteomics and transcriptomics suggested that the
347 protein alterations of Asct2 and Smcts during the pathology emerged from transcriptional
348 mechanisms rather than post-translational protein processing or protein degradation.

349 ## Discussion

350 In this study, we presented an approach to investigate the transport systems for
351 micronutrients and metabolites under physiological conditions and in multifactorial diseases.
352 We selected AKI as a model study because AKI is known to target renal proximal epithelia
353 and disrupt absorption/reabsorption systems that are the results of cooperative functions of
354 multiple transporters. We utilized amino acid metabolomics to reveal a unique feature of the
355 enantiomeric dynamics of serine in the AKI and analyzed membrane proteomes to obtain the
356 D-serine transporter candidates. We then applied cell-based and cell-free transport assays to
357 identify two D-serine transport systems; one was ASCT2 and the other consisted of
358 unanticipated SMCTs (SMCT1 and SMCT2). Using *ex vivo* analysis of apical membrane-
359 enriched renal proximal tubules, we showed that both transport systems contributed
360 comparably to D-serine handling in the normal kidney, but ASCT2 became dominant in AKI,
361 leading to the increase of D-serine in the blood. The alteration of two transport systems
362 explained the dynamics of D-serine and suggested the transport mechanism behind the D-serine
363 biomarker.

364 Several studies have reported the non-canonical substrates of membrane transport
365 proteins, such as the recognition between amino acids, carboxylates and amines among SLCs
366 (Metzner et al., 2005; Matsuo et al., 2008; Schweikhard and Ziegler, 2012; Wei et al., 2016).
367 Serine consists of a hydroxypropionic acid and an amino group at the α-carbon. Possibly, the
368 hydroxypropionic acid on serine is the main part to interact with SMCTs because
369 hydroxypropionic acid shares similar moieties to lactic acid and propionic acid which are the
370 high-affinity substrates of SMCTs. The apparent K_m of D-serine transport in SMCT1 is 3.39
371 mM (Figure 5E), which is within the range of known SMCT1 substrates (0.07 – 6.5 mM)
372 (Ganapathy et al., 2008), suggesting that SMCT1 accepts D-serine in the same manner as other
373 monocarboxylate substrates. Inhibition of D-[^{3}H]serine transport by non-radioisotope labeled

374 D-serine in SMCT2 was less effective than in SMCT1 (Figure 5D). Together with the previous
375 report that SMCT2 had lower inhibition affinities for nicotinate, lactate and butyrate than
376 SMCT1 (Srinivas et al., 2005), we suggest that SMCT2 recognizes D-serine with lower affinity
377 than SMCT1.

378 Kinetics analysis of D-serine transport revealed the high affinity by ASCT2 (K_m 167
379 μM) (Foster et al., 2016) and low affinity by SMCT1 (K_m 3.39 mM; Figure 5E). Besides
380 transport affinity, the expression level is another important factor in evaluating the transport
381 capacity. Regarding chromatogram intensities in the proteomics data, we found that the
382 intensities of Smct1 (2.9 x 10^9 AU) and Smct2 (1.6 x 10^8 AU) were remarkably higher than
383 Asct2 (1.5 x 10^7 AU) in the control mice (Table 1: abundance in Sham). Although it is not
384 accurate to compare intensities between different proteins in a mass spectrometry analysis, the
385 comparison may roughly reflect the protein amounts. Together with our proteome data, this
386 observation is consistent with the snRNA-seq result (Figure supplement 7: Controls),
387 suggesting that Asct2 is only expressed in minor cell populations, while Smct1 and Smct2
388 expressions are high and ubiquitous in the normal conditions. Thus, we proposed that ASCT2
389 transports D-serine with low capacity due to its low expression level, while SMCTs have high
390 transport capacity due to their abundance. Consequently, both ASCT2 and SMCTs are likely
391 to contribute to D-serine reabsorption in the normal kidney as seen in the *ex vivo* assay (Figure
392 7B).

393 Several pieces of evidence support SMCT1 at S3 segment and SMCT2 at S1 + S2
394 segments on renal D-serine handling. First, our enantiomeric amino acid profile from the IRI
395 model, which agrees with the previous reports in CKD patients and AKI animal models,
396 suggested the existence of a stereoselective transport system for serine (Figure 1) (Hesaka et
397 al., 2019; Kimura et al., 2020, 2016; Sasabe et al., 2014; Sasabe and Suzuki, 2018). The system
398 seems distinct from the known classical small amino acid transport systems and ASCT2.

399 Second, previous studies indicated renal D-serine transport takes place at the proximal tubules
400 by the distinct transport systems between S1 + S2 and S3 segments. Both systems exhibited
401 the characteristics of Na^+ dependency, electrogenicity, low affinity (mM range), and partial
402 stereoselectivity (Kragh-Hansen and Sheikh, 1984; Shimomura et al., 1988; Silbernagl et al.,
403 1999). These properties of the D-serine transport suit well to SMCTs and convince us of the
404 contribution of SMCTs to renal D-serine transport in addition to ASCT2. Moreover, continuous
405 administration of high doses of D-serine induces nephrotoxicity at the S3 segment of proximal
406 tubules in normal animals (Silbernagl et al., 1999; Morehead et al., 1945; Hasegawa et al.,
407 2019). It is most likely that the D-serine-induced proximal tubular damage is partly due to the
408 absorption of D-serine by SMCTs, in particular by SMCT1 at the S3 segment. It is noted that
409 SMCT2 is reported as a protein indicator for the kidney repair during the injury. Prolonged
410 SMCT2 down-expression indicates the failed repair of proximal tubular cells in IRI (Kirita et
411 al., 2020). The significance of SMCT2, in turn, supports the merit of D-serine usage in AKI
412 and CKD diagnosis.

413 Combining all the results, we propose the model of D-serine transport systems in renal
414 proximal tubules (Figure 8). In the normal kidneys, the expression levels of SMCT2 at S1 + S2
415 segments and SMCT1 at S3 segment are high, whereas ASCT2 is low. Both SMCTs and
416 ASCT2 are involved in D-serine reabsorption. Nonetheless, the net reabsorption levels appear
417 to be relatively low due to the low affinities of SMCTs. It is likely that reabsorption at the S3
418 segment is dominated by SMCT1 function. The reabsorbed D-serine in proximal tubular cells
419 is degraded into OH-pyruvate by D-amino acid oxidase (DAAO), thereby maintaining the low
420 plasma D-serine but high urinary D-serine (Figure 8: Normal). In pathological conditions such
421 as AKI and CKD, the expression levels of SMCT1 and SMCT2 decrease dramatically while
422 ASCT2 increases. The high affinity and ubiquitous expression of ASCT2 suggest the increased
423 D-serine reabsorption in all proximal tubular segments. Together with the genetic inactivation

424 of DAAO during the pathology (Sasabe et al., 2014), the excess reabsorbed D-serine may lead
425 to a high plasma D-serine level (Figure 8: IRI).

426 Enantiomeric profiles of serine showed low plasma D/L-serine ratios in the normal
427 control but high D/L-serine ratios in IRI regardless of the weak stereoselectivity of ASCT2
428 (Figure 1B). This data suggested the different renal handling between D-serine and the L-serine.
429 L-Serine reabsorption has been reported to be mediated by B^0AT3 (Singer et al., 2009), and we
430 showed SMCTs-mediated D-serine reabsorption in this study. Thus, we propose that the low
431 plasma D/L-serine ratio in the normal kidney is due to the high L-serine reabsorption by B^0AT3,
432 together with the DAAO function that degrades D-serine in the cells. Our enantiomeric amino
433 acid profile detected low plasma L-serine and high urinary L-serine in IRI (Figure supplements
434 1B, 2B) and the proteomics revealed B^0AT3 decrease (4h IRI/sham = 0.56 fold; 8h IRI/sham
435 = 0.65 fold; Table S1). It is possible that low L-serine reabsorption in IRI is a result of B^0AT3
436 reduction.

437 In proteomics, some transporters including ASCT2 were found to be increased in the
438 AKI conditions (Figure 2B), yet the mechanism behind this change is still unclear. snRNA-seq
439 study revealed that the cell clusters in all proximal tubular segments during early AKI were
440 drastically different from the healthy kidney suggesting that the new distinct cell clusters in
441 AKI were *de novo* synthesized for the repair process, as early AKI is a reversible condition
442 (Kirita et al., 2020). ASCT2 was found to be only one type of small amino acid transporter
443 which was increased during AKI conditions (Figure 2B). ASCT2 was reported to be highly
444 expressed in proliferative cells and stem cells and played a key role in the regulation of
445 mammalian target of rapamycin (mTOR) signaling pathway and glutamine-mediated
446 metabolism (Scalise et al., 2018; Formisano and Van Winkle, 2016; Kandasamy et al., 2018).
447 Thus, we postulate that ASCT2 is a nascent transporter to provide small amino acids and

448 glutamine for cell growth, as a molecular mechanism in *de novo* cellular synthesis for the
449 recovery processes.

450 Besides ASCT2 and SMCTs, other transporters may contribute to D-serine handling in
451 the kidney. Influxes of D-[^{3}H]serine in the Na^+-free condition of the normal mouse BBMVs
452 accounted for ~ 30% of total uptake (Figure 7A-B). These are results of the experimental
453 background of non-specific accumulation of radiotracers and probably also Na^+-independent
454 transporter(s). In BBMVs from 8h IRI model, D-[^{3}H]serine uptake without preloading
455 suggested the existence of Na^+-dependent transporters during the injury. In the D-serine
456 transporter screening, we found that PAT1 and B^0AT3 attenuated D-serine-mediated growth
457 inhibition (Figure 4C-D). PAT1 is reported to transport D-serine in the transfected cell and
458 oocyte models while the recognition of D-serine by B^0AT3 is unknown (Boll et al., 2002; Chen
459 et al., 2003). PAT1, a low-affinity proton-coupled amino acid transporter, was found at both
460 sub-apical membranes at the S1 segment and inside of the epithelia (The Human Protein Atlas:
461 https://www.proteinatlas.org; updated on Dec 7th, 2022) (Sagné et al., 2001; Vanslambrouck et
462 al., 2010). It is suggested that PAT1 is less active at the apical membrane where the luminal
463 pH is neutral (Miyauchi et al., 2005; Bröer, 2008b). However, a future experiment is required
464 to address the significance of PAT1 on D-serine transport in pathological conditions where the
465 pH homeostasis could be disrupted (Nakanishi et al., 2012; Bouchard and Mehta, 2022; Imenez
466 Silva and Mohebbi, 2022). Despite the D-serine transport systems at the apical side, other
467 transport systems at the basolateral side are also necessary to complete the picture of the D-
468 serine transport systems in renal proximal tubules.

469 Transport of a micromolecule is contributed by multiple transporters that orchestrate
470 together to maintain homeostasis and to control transport compensation in an aberrant
471 condition (Bröer et al., 2016; Wiriyasermkul et al., 2020; Gauthier-Coles et al., 2021). The
472 molecular characteristics and physiological functions of transporters are largely dependent on

473 their microenvironments, such as electrochemical gradients and the presence of other
474 transporters for coordinating functions. In our proteomic analysis, a high population of
475 membrane transport proteins was obtained in the dataset by applying urea treatment to the
476 membrane preparation process (Figure 2, Table supplement 1) (Kongpracha et al., 2022). By
477 integrating data focused on specific targets, such as transporters or amino acids, from the
478 protein molecule level to the metabolome or proteome, we were able to reveal the subtle
479 changes in the body and identify the causes of those small changes. This integrative and multi-
480 hierarchical approach, emphasizing the coordinate functions of multiple transporters, holds the
481 potential for investigating the dynamics of a broad range of micromolecules under diverse or
482 intricate conditions within any tissues or organs.

483 # Materials and Methods

484 **Materials, animals, and graphical analysis**

485 General chemicals and cell culture media were purchased from Wako Fujifilm and
486 Nacalai tesque. Chemicals used in mass spectrometry were HPLC or MS grades. Flp-In T-Rex
487 293 cells, Expi293F cells, Expi293 Expression medium, Lipofectamine 3000, fluorescent-
488 labeled secondary antibodies, and Tyramide SuperBoost kit were from Thermo. Amino acids,
489 fetal bovine serum (FBS), anti-FLAG antibody, and anti-FLAG M2 column were from Sigma.
490 Secondary antibodies with HRP conjugated were from Jackson ImmunoResearch. L-[^{3}H]Serine,
491 D-[^{3}H]serine, L-[^{3}H]alanine, and D-[^{3}H]alanine were from Moravek. L-[^{3}H]Glutamic acid, D-
492 [^{3}H]glutamic acid, L-[^{14}C]tyrosine, D-[^{14}C]tyrosine, DL-[^{3}H]lactic acid, [^{3}H]propionic acid,
493 [^{14}C]nicotinic acid, [^{14}C]uric acid, and L-[^{3}H]aspartic acid were from American Radiolabeled
494 Chemicals. Anti-SGLT2 (SC-393350) and anti-Na^{+}/K^{+}-ATPase (SC-21712) antibodies were
495 obtained from Santa Cruz Biotechnology.

496 All animal experiments were carried out following institutional guidelines under the
497 approval of the Animal Experiment Committees of The Jikei University School of Medicine,
498 Nara Medical University, and Keio University, Japan. Eight weeks old C57BL/6J mice were
499 purchased from Japan SLC and CLEA Japan. Frozen kidneys of the 8 weeks old C57BL/6J
500 mice were purchased from Sankyo Labo Service.

501 Human genes/proteins were defined with all letters in uppercase while those of the
502 mouse molecules were defined with the first letter in uppercase. Unless otherwise indicated,
503 data shown in all figures are mean ± SEM of the representative data from 3 reproducible
504 experiments. Statistical differences and p-values were determined using the unpaired Student *t*
505 test. Graphs, statistical significance, and kinetics were analyzed and plotted by GraphPad Prism
506 8.4.

507 **Plasmid construction**

508 In this study, we used the cDNA of human SLC5A8/SMCT1 clone "NM_145913". We
509 generated the clone NM_145913 from the clone AK313788 (NBRC, NITE, Kisarazu, Japan).
510 At first, SMCT1 from AK313788 was subcloned into p3XFLAG-CMV14 (Sigma) via *Hind*III
511 and *Bam*HI sites. The clone "pCMV14-SMCT1" NM_145913 was subsequently generated by
512 mutagenesis of p3XFLAG-CMV14-SMCT1_AK313788 at I193V, T201A and I490M
513 (variants between NM_145913 and AK313788) by HiFi DNA Assembly Cloning (NEB) and
514 site-directed mutagenesis. Human SLC5A12/SMCT2 cDNA (NM_178498; Sino Biological
515 Inc.) was amplified by PCR and cloned into p3XFLAG-CMV14 via *Kpn*I and *Bam*HI sites to
516 generate "pCMV14-SMCT2". Human expression clones of SLC7A10/Asc-1 (NM_019849),
517 SLC7A1/CAT1 (NM_003045), SLC36A1/PAT1 (NM_078483), SLC2A5/GLUT5
518 (NM_003039), SLCO4C1/OATP-H (NM_180991), SLC22A13/OAT10 (NM_004256),
519 SLC22A7/OAT2 (NM_153320), SLC6A18/B^0AT3 (NM_182632), SLC15A2/PEPT2
520 (NM_021082), and TMEM27/Collectrin (NM_020665) were obtained from GenScript and
521 RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. Mouse
522 Asct2 (NM_009201) TrueORF clone was obtained from OriGene. p3XFLAG-CMV14 empty
523 vector was used for Mock production. pcDNA5-SMCT1 and pcDNA5-SMCT2 used for
524 generation of SMCT1- and SMCT2-stable cell lines, respectively, were constructed by
525 assembling the PCR products of SMCT1 or SMCT2 into pcDNA5/FRT/TO (Thermo) by HiFi
526 DNA Assembly Cloning.

527 **Antibody (Ab) production**

528 Anti-mouse Smct1 Ab was custom-produced. Peptide antigen corresponding to the
529 amino acid residues 596 – 611 of Smct1 and the corresponding anti-sera from the immunized
530 rabbit was produced by Cosmo Bio. Anti-Smct1 Ab was purified using HiTrap Protein G HP

531 (GE Healthcare) following the manufacturer's protocol. After purification, the Ab was dialyzed
532 against PBS pH 7.4 and adjusted concentration to 1 mg/mL.

533 Anti-human ASCT2 and anti-mouse Asct2 Abs were custom-produced. First, we
534 generated pET47b(+)-GST by subcloning the GST from pET49b(+) template into pET47b(+)
535 via *Not*I and *Xho*I restriction sites. Then, human ASCT2 antigen (amino acid residues 7 – 20)
536 was amplified and cloned into pET47b(+)-GST to obtain GST-fused ASCT2 antigen. For the
537 Asct2 antigen, both N-terminal (Asct2(NT), amino acid residues 1 – 38) and C-terminal
538 (Asct2(CT), amino acid residues 521 – 553) fragments were fused with GST by cloning into
539 pET47b(+)-GST and pET49b(+), respectively (Cosmo Bio). The GST-fusion antigens were
540 expressed in *E. coli* BL21(DE3) and purified by Glutathione Sepharose 4B (GE Healthcare) as
541 described previously (Nagamori et al., 2016a). The antigens were used to immunize rabbits to
542 obtain anti-sera (Cosmo Bio).

543 Anti-ASCT2 Ab was purified from the anti-sera using HiTrap Protein G HP (GE
544 Healthcare) following the manufacturer's protocol. After purification, the Ab was dialyzed
545 against PBS pH 7.4 and adjusted concentration to 1 mg/mL.

546 Anti-Asct2(NT) and anti-Asct2(CT) Abs were purified by using two-step purifications.
547 First, affinity columns of GST-fused Asct2(NT)-antigen and GST-fused Asct2(CT)-antigen
548 were produced by conjugating the antigens with HiTrap NHS-activated HP (GE Healthcare).
549 To purify anti-Asct2(NT) Ab, the anti-sera was subjected to the first purification by using the
550 GST-fused Asct2(NT)-antigen column. The elution fraction was dialyzed and subsequently
551 subjected to the second column of GST-fused Asct2(CT)-antigen column. The flowthrough
552 fraction corresponding to the affinity-purified anti-Asct2(NT) Ab was obtained. Purification of
553 anti-Asct2(CT) Ab was performed in the same way as Asct2(NT) Ab but used GST-fused
554 Asct2(CT)-antigen column followed by GST-fused Asct2(NT)-antigen column.

555 **Ischemia-reperfusion injury (IRI) model**

556 C57BL/6J (CLEA Japan) male mice between 12 – 16 weeks old underwent
557 experimental procedures for the IRI model. Ischemia-reperfusion was performed as previously
558 described (Sasabe et al., 2014). Before IRI induction, right kidney was removed. After twelve
559 days, the mice were grouped by randomization. Ischemia was operated for 45 min by clamping
560 the vessel under anesthesia. After that, the vessel clamp was removed, and the abdomen was
561 closed. Sham-operated control mice were treated identically except for clamping. Urine and
562 blood samples were collected at 4, 8, 20 and 40 hours after reperfusion. The mice were
563 anesthetized with isoflurane and euthanized by perfusion with PBS pH 7.4. The kidney was
564 removed and stored at -80 °C until use.

565 **Measurement of amino acid enantiomers by 2D-HPLC**

566 Plasma and urine samples collected from IRI mice were prepared as described with
567 modifications (Miyoshi et al., 2009; Hamase et al., 2010). Briefly, 20-fold volumes of methanol
568 were added to the samples and an aliquot was placed in a brown shading tube and used for
569 NBD derivatization (1 μL of the plasma was used for the reaction). After drying the solution
570 under reduced pressure, 20 μL of 200 mM sodium borate buffer (pH 8.0) and 5 μL of
571 fluorescent-labeling reagent [40 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) in
572 anhydrous acetonitrile (MeCN)] were added, and then heated at 60 °C for 2 min. An aqueous
573 0.1% (v/v) TFA solution (375 μL) was added, and 20 μL of the reaction mixture was applied
574 to 2D-HPLC.

575 Enantiomers of amino acids were quantified using the 2D-HPLC platform (Miyoshi et
576 al., 2009; Hamase et al., 2010). The NBD-derivatives of the amino acids were separated using
577 a reversed-phase column (Singularity RP column, 1.0 mm i.d. × 50 mm; designed by Kyushu
578 University and KAGAMI INC.) with the gradient elution using aqueous mobile phases
579 containing MeCN and formic acid. To determine D- and L-amino acids, the fractions of amino

580 acids were automatically collected using a multi-loop valve and transferred to the
581 enantioselective column (Singularity CSP-001S, 1.5 mm i.d. × 75 mm; designed by Kyushu
582 University and KAGAMI INC.). Then, D- and L-amino acids were separated in the second
583 dimension by the enantioselective column. The mobile phases are the mixed solutions of
584 MeOH–MeCN containing formic acid, and the fluorescence detection of the NBD-amino acids
585 was carried out at 530 nm with excitation at 470 nm. Target peaks were quantified by scaling
586 the standard peak shapes (Hamase et al., 2018).

587 **Isolation of brush border membrane vesicles (BBMVs) for mass spectrometry analysis**

588 BBMVs were prepared by the calcium precipitation method (Biber et al., 2007;
589 Kongpracha et al., 2022). Frozen kidneys were minced into fine powders by using a polytron-
590 type homogenizer (Physcotron, Microtec) in the homogenization buffer containing 20 mM
591 Tris-HCl, pH 7.6, 250 mM sucrose, 1 mM EDTA and cOmplete EDTA-free protease inhibitor
592 cocktail (Roche). After low-speed centrifugation at 1,000 ×g and 3,000 ×g, the supernatant was
593 collected and incubated with 11 mM $CaCl_2$ for 20 min on ice with mild shaking. The
594 supernatant was ultra-centrifuged at 463,000 ×g for 15 min at 4 °C. The pellet was resuspended
595 in the homogenization buffer and repeated the steps of $CaCl_2$ precipitation. Finally, the pellet
596 of BBMVs was resuspended in 20 mM Tris-HCl pH 7.6, and 250 mM sucrose. Membrane
597 proteins of BBMVs were enriched by the Urea Wash method (Kongpracha et al., 2022). The
598 urea-washed BBMV samples were subjected to sample preparation for mass spectrometry.

599 **Cell culture, transfection, and generation of stable cell lines**

600 HEK293 and Flp-In T-Rex 293 cells were cultured in DMEM supplemented with 10 %
601 (v/v) FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (P/S), and routinely
602 maintained at 37 °C, 5 % CO_2 and humidity. For transfection experiments, the cells were
603 seeded in antibiotic-free media for one day prior to transfection to obtain approximately 40%

604 confluence. DNA transient transfection and ASCT2-siRNA (ID#s12916; Ambion) transfection
605 were performed by using Lipofectamine 3000 following the manufacturer's protocol. The ratio
606 of DNA : P3000 : Lipofectamine 3000 is 1.0 μg : 2.0 μL : 1.5 μL. The ratio of siRNA :
607 Lipofectamine 3000 is 10 pmol : 1 μL. The cells were further maintained in the same media
608 for two days prior to the assays.

609 Flp-In T-REx 293 stably expressing SMCT1 (FlpInTR-SMCT1) and SMCT2
610 (FlpInTR-SMCT2) were generated by co-transfection of pOG44 and pcDNA5-SMCT1 or
611 pcDNA5-SMCT2 and subsequently cultured in the media containing 5 mg/L blasticidin and
612 150 mg/L hygromycin B for positive clone selection. Mock cells were generated in the same
613 way using the empty plasmid. Expressions of SMCT1 in FlpInTR-SMCT1 and SMCT2 in
614 FlpInTR-SMCT2 were induced by adding 1 mg/L doxycycline hyclate (Dox; Tet-ON system)
615 at one day after seeding. The cells were further cultured for two days prior to performing
616 experiments.

617 Wild-type and *ASCT2*-knockout HAP1 cells (Human near-haploid cell line; Horizon
618 Discovery) were cultured in IMDM supplemented with 10 % (v/v) FBS, P/S, and routinely
619 maintained at 37 °C, 5 % CO_2 and humidity.

620 Expi293F cells were cultured in Expi293 Expression medium at 37 °C, 8 % CO_2, and
621 humidity. To express SMCT1 transiently, the cells were transfected with pCMV14-SMCT1
622 using PEI MAX pH 6.9 (MW 40,000; Polysciences) and cultured for two days.

623 **Mass spectrometry and proteome data analysis**

624 Proteomics of mouse BBMVs was analyzed as described (Kongpracha et al., 2022;
625 Uetsuka et al., 2015). After preparing urea-washed BBMVs, the samples were fractionated into
626 four fractions by SDB-XC StageTips and desalted by C18-StageTips. Mass spectrometry was
627 performed using the Q Exactive (Thermo) coupled with nano-Advance UHPLC (Michrom
628 Bioresources). The UHPLC apparatus was equipped with a trap column (L-column ODS, 0.3

629 x 5 mm, CERI) and a C18 packed tip column (0.1 x 125 mm; Nikkyo Technos). Raw data from
630 four fractions were analyzed using Proteome Discoverer 2.2 (Thermo) and Mascot 2.6.2
631 (Matrix Science). Data from four fractions were combined and searched for identified proteins
632 from the UniProt mouse database (released in March 2019). The maximum number of missed
633 cleavages, precursor mass tolerance, and fragment mass tolerance were set to 3, 10 ppm and
634 0.01 Da, respectively. The carbamidomethylation Cys was set as a fixed modification.
635 Oxidation of Met and deamidation of Asn and Gln were set as variable modifications. A filter
636 (false discovery rate < 1%) was applied to the resulting data. For each mouse sample, the
637 analysis was conducted twice and the average was used. One data set was composed of 3
638 samples from each operation condition (n = 3). Statistical analyses were determined using
639 Proteome Discoverer 2.2 (Thermo). Statistical differences and p-values were determined using
640 the unpaired Student *t* test. Data represented the median ± SEM (Table S1).

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

645 Proteome of BBMVs from mouse kidneys after ischemia operation for 4 (4h) or 8 hours
646 (8h) was normalized to that of sham operation. The identified proteins were then subjected to
647 annotate the biological functions by Ingenuity Pathway Analysis (IPA, Qiagen). Molecules
648 from the dataset that met the cutoff of the Ingenuity Knowledge Base were considered for the
649 analysis. A right-tailed Fisher's Exact Test was used to calculate the p-value determining the
650 probability (z-scores) of the biological function. Protein localization in kidney segments was
651 evaluated based on related literature and The Human Protein Atlas
652 (http://www.proteinatlas.org: updated Dec 7th, 2022).

653 **Effect of D-serine on cell growth**

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

667 **Transport assay in cultured cells**

668 Transport assay in cells was performed as described previously with some
669 modifications (Wiriyasermkul et al., 2012). Briefly, for D-[^{3}H]serine transport in HEK293 cells,
670 the cells were seeded into poly-D-lysine-coated 24-well plates at 1.2 x 10^5 cells/well and
671 cultured for three days. Unless indicated elsewhere, uptake of 20 μM (100 Ci/mol) or 100 μM
672 (10 Ci/mol) D-[^{3}H]serine was measured in PBS pH 7.4 at 37 °C at the indicated time points.
673 After termination of the assay, the cells were lysed. An aliquot was subjected to measure
674 protein concentration, and the remaining lysate was mixed with Optiphase HiSafe 3
675 (PerkinElmer). The radioactivity was monitored using a β-scintillation counter (LSC-8000,
676 Hitachi). In the transport assay with or without Na^+, Na^+-HBSS, or Na^+-free HBSS (choline-
677 Cl substitution) were used instead of PBS.

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

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

688 **SMCT1 purification, proteoliposome reconstitution, and transport assay**

689 Human SMCT1 was purified from SMCT1-expressing Expi293F cells. Membrane
690 fraction and purification processes were performed as previously described with small
691 modifications (Nagamori et al., 2016a). First, cell pellets were resuspended in 20 mM Tris-HCl
692 pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, and protease inhibitor cocktail (Roche). The crude
693 membrane fraction was derived from sonication and ultracentrifugation (sonication method).
694 Membrane proteins were extracted from the crude membrane fraction with 2% (w/v) DDM
695 and ultracentrifugation. SMCT1 was purified by anti-FLAG M2 affinity column. Unbound
696 proteins were washed out by 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10% (v/v) glycerol, and
697 0.05% (w/v) DDM. Then SMCT1 was eluted by 3xFLAG peptide in the washing buffer.
698 Purified SMCT1 was concentrated by Amicon Ultra Centrifugal Filters 30K (Millipore).

699 The reconstitution of proteoliposomes was performed as described with minor
700 modifications (Lee et al., 2019). The purified SMCT1 was reconstituted in liposomes (made
701 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
702 20 mM MOPS-Tris pH 7.0 and 100 mM KCl.

703 Transport assay in proteoliposomes was conducted by the rapid filtration method (Lee
704 et al., 2019). Uptake reaction was conducted by dilution of 1 μg SMCT1 proteoliposomes in
705 100 μL uptake buffer (20 mM MOPS-Tris pH 7.0, 100 mM NaCl for Na^+-buffer or KCl for
706 Na^+-free buffer, 1 mM $MgSO_4$ and 1 mM $CaCl_2$) containing radioisotope-labeled substrates.
707 The reaction was incubated at 25 °C for an indicated time. The radioisotope-labeled substrates
708 were used as follows: 5 Ci/mol for [^{14}C]uric acid, L-[^{14}C]tyrosine and D-[^{14}C]tyrosine; 10
709 Ci/mol for DL-[^{3}H]lactic acid, L-[^{3}H]alanine, D-[^{3}H]alanine, L-[^{3}H]serine, D-[^{3}H]serine, L-
710 [^{3}H]glutamic acid and D-[^{3}H]glutamic acid; and 20 Ci/mol for [^{3}H]propionic acid. In the
711 inhibition assay, 1 mM ibuprofen was applied at the same time with the radioisotope-labeled
712 substrates. Kinetics of nicotinate transport were measured for 3 min using 0.01 – 2 mM
713 [^{14}C]nicotinate (0.2 – 50 Ci/mol) in Na^+-containing buffer, and then subtracted from the uptake
714 in Na^+-free buffer.

715 **Transport assay in mouse BBMVs**

716 Both left and right kidneys from eight weeks old male normal control (Japan SLC;
717 Sankyo Labo Service) or IRI model (sham, 4h IRI, and 8h IRI) were taken out after PBS
718 perfusion and frozen until use. After mincing and homogenizing the frozen kidneys in the
719 buffer containing 20 mM Tris-HCl pH 7.6, 150 mM mannitol, 100 mM KCl, 1 mM EDTA,
720 and protease inhibitor cocktail, the BBMVs were prepared as described in the above section
721 but using magnesium instead of calcium for BBMV precipitation. The pellet of BBMVs was
722 resuspended in the suspension buffer (10 mM Tris-HCl, pH 7.6, 100 mM mannitol, and 100
723 mM KCl). In the L-glutamine preloading experiment, the BBMVs were incubated with 4 mM
724 L-glutamine (L-Gln) or only buffer (no preload) on ice for 3 hours. External buffers were then
725 removed by centrifugation at 21,000 ×*g* for 20 min and the BBMVs were resuspended in the
726 suspension buffer.

727 Transport assay was performed by rapid filtration. Prior to initiating the reaction, 5 μM
728 valinomycin was added to the BBMV samples. Transport assay was examined by diluting 100
729 μg BBMVs in 100 μL of the uptake buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl for Na^+
730 condition or KCl for Na^+-free condition, 50 mM mannitol and 5 μM valinomycin) containing
731 radioisotope substrates, 10 μM D-[^{3}H]serine (100 Ci/mol) or 10 μM L-[^{3}H]aspartic acid (100
732 Ci/mol), as described in the figures. The reaction was incubated at 30 °C at an indicated time,
733 then terminated by the addition of ice-cold buffer containing 10 mM Tris-HCl pH 7.6 and 200
734 mM mannitol and filtered through 0.45 μm nitrocellulose filter (Millipore), followed by
735 washing with the same buffer once. The membranes were soaked in Clear-sol I (Nacalai
736 Tesque), and the radioactivity on the membrane was monitored. For the inhibition experiments,
737 the tested inhibitors were added into the D-[^{3}H]serine substrate solution at the same time.

738 **Western Blot analysis**

739 Expressions of targeting proteins from membrane fractions were verified by Western
740 blot analysis as described (Nagamori et al., 2016b). Membrane fractions were dissolved in 1%
741 w/v DDM prior to the addition of the SDS-PAGE sample buffer. Signals of chemiluminescence
742 (Immobilon Forte Western HRP substrate; Millipore) were visualized by ChemiDoc MP
743 Imaging system (Bio-Rad).

744 **Immunofluorescence staining of mouse kidneys**

745 The 8 weeks old male C57BL/6J mice (Japan SLC) were anesthetized and fixed by
746 anterograde perfusion via the aorta with 4% w/v paraformaldehyde in 0.1 M sodium phosphate
747 buffer pH 7.4. The kidneys were dissected, post-fixed in the same buffer for two days, and
748 cryoprotected in 10 %, 20 %, and 30 % w/v sucrose. Frozen kidney sections were cut at 7 μm
749 thickness in a cryostat (Leica) and mounted on MAS-coated glass slides (Matsunami). The
750 sections were placed in antigen retrieval buffer (10 mM citrate and 10 mM sodium Citrate),

751 autoclaved at 121 °C for 5 min and washed by TBS-T (Tris-buffered saline (TBS) with 0.1 %
752 v/v Tween 20). Immunostaining was done by serial incubation with each antibody as below.

753 For Asct2 and Sglt2 co-immunostaining, the samples were incubated with 3 %
754 hydrogen peroxide solution for 10 min, washed with TBS, and incubated in Blocking One
755 Histo (Nacalai tesque) for 15 min. The samples were then incubated with mouse anti-SGLT2
756 antibody diluted in immunoreaction enhancer B solution (Can Get Signal immunostain,
757 TOYOBO) overnight at 4 °C. Signal was enhanced by Alexa Fluor 568 Tyramide SuperBoost
758 (TSA) kit, goat anti-mouse IgG, following the manufacturer's instruction (Thermo). The
759 antibodies were then stripped by citrate/acetate-based buffer, pH 6.0, containing 0.3% w/v SDS
760 at 95 °C for 10 min (Buchwalow et al., 2018), washed by TBS, and incubated with Blocking
761 One Histo. Asct2 staining was done as described (Nagamori et al., 2016a). Briefly, the samples
762 were incubated with rabbit anti-Asct2(NT) antibody diluted in immunoreaction enhancer A
763 solution (Can Get Signal immunostain) overnight at 4 °C. After washing with TBS-T, the
764 specimens were incubated with Alexa Fluor 488-labeled donkey anti-rabbit IgG.

765 For Asct2 and Agt1 co-immunostaining, signals of both antibodies were enhanced by
766 TSA kit. First, the specimens were incubated with rabbit anti-Agt1(G) antibody (Nagamori et
767 al., 2016a) overnight at 4 °C followed by Alexa Fluor 568 TSA kit with goat anti-rabbit. The
768 antibodies were then stripped. The specimens were incubated with rabbit anti-Asct2(NT)
769 overnight at 4 °C and then repeated the steps of TSA kit using Alexa Fluor 488, goat anti-rabbit.

770 Staining of Asct2 and Na^+/K^+-ATPase was performed without TSA enhancement. After
771 blocking by Blocking One Histo, the samples were incubated with rabbit anti-Asct2(NT)
772 antibody diluted in immunoreaction enhancer A solution overnight. The samples were washed
773 with TBS-T, incubated with Alexa Fluor488-labeled donkey anti-rabbit IgG, and washed again.
774 Non-specific staining was blocked by Blocking One Histo and the specimens were then
775 incubated with mouse anti-Na^+/K^+-ATPase antibody diluted in immunoreaction enhancer B

776 solution overnight at 4 °C. The specimens were washed with TBS-T and incubated with Alexa
777 Fluor568-labeled goat anti-mouse IgG for 1 hour.

778 Smct1 and Sglt2 were co-stained. After blocking by Blocking One Histo, the samples
779 were incubated with rabbit anti-Smct1 antibody and mouse anti-SGLT2 diluted in
780 immunoreaction enhancer B solution overnight at 4 °C. The specimens were washed with TBS-
781 T and incubated with Alexa Fluor488-labeled donkey anti-rabbit IgG and Alexa Fluor568-
782 labeled goat anti-mouse IgG for 1 hour.

783 Prior to the co-staining of Smct1 and Agt1, anti-Agt1 antibody was conjugated with
784 Alexa Fluor 568 dye via succinimidyl ester reaction. The antibody was adjusted to pH 8.3 and
785 mixed with Alexa Fluor 568 NHS Ester (Thermo) for 2 hours. The reaction was stopped by
786 incubation with 1 M ethanolamine for 1 hour. The unconjugated dye was removed by size
787 exclusion using Bio-Spin P-30 gel columns (Bio-rad) equilibrated in PBS pH 7.4. For Smct1
788 and Agt1 co-staining, after the blocking step, the samples were firstly stained with rabbit anti-
789 Smct1 antibody diluted in immunoreaction enhancer A solution. Signal was enhanced by Alexa
790 Fluor 488 TSA kit with goat anti-rabbit IgG. Subsequently, the specimen was washed with
791 TBS-T and non-specific staining was blocked by Blocking One Histo. The samples were then
792 incubated with Alexa Fluor 568-conjugated anti-Smct1 antibody diluted in Blocking One Histo
793 overnight at 4 °C.

794 All specimens were washed with TBS-T and mounted with Fluoromount (Diagnostic
795 Biosystems). Imaging was detected using a KEYENCE BZ-X710 microscope. Images were
796 color-adjusted using ImageJ ver. 1.51 (NIH). Images in Figure 3B were subjected to
797 deconvolution process in Fiji/ImageJ2 ver. 2.9 prior to color adjustment.

798 **Analysis of Smcts and Asct2 from the open-sourced dataset of single nucleus RNA**
799 **sequencing (snRNA-seq) of the IRI model**

800 The dataset of snRNA-seq used is created from the mice after ischemia-reperfusion
801 injury (IRI) and published by Kirita et al. (Kirita et al., 2020). The snRNA-seq dataset
802 (GSE139107), which has already processed by zUMIs and SoupX, was downloaded from GEO,
803 a public functional genomics data repository (Barrett et al., 2012). The dataset revealed
804 percentage of mitochondrial UMI counts of each cell (calculated by *PercentageFeatureSet*
805 function of Seurat) to be < 0.01 % in more than 94% cells in each cluster. From the
806 "GSE139107_MouseIRI.metadata", we annotated the expression of Smct1, Smct2 and Asct2
807 in proximal tubule segments 1 – 3 (S1, S2, and S3) at the early stages of IRI (4 and 12 hours)
808 compared to the control mice. Downstream analyses including normalization, scaling and
809 visualization were performed by Seurat v3.2, an R package designed for Quality Control,
810 analysis and exploration of single-cell RNA-seq data (Stuart et al., 2019). After selecting the
811 cells in S1, S2, and S3 from the datasets (control, 4-hour IRI, and 12-hour IRI), we merged the
812 data of the cells in the same cluster. The data were then calculated as log-normalized values by
813 *NormalizeData* function and scaled by *ScaleData* function of Seurat. Visualization of the
814 expression data was performed using Seurat plug-in of R software and ggplot2 plug-in of R
815 software (Wickham, 2010). Dot plots of gene expression in each cluster after log-
816 transformation and scaling were created by *Dotplot* function of Seurat.

817 **Data and materials availability**

818 All proteomics data have been deposited in Japan Proteome Standard Repository/Database:
819 JPST000929 and JPST000931. Codes for snRNA sequencing reanalysis are available in
820 GitHub repository at https://github.com/SN-PW/snRNA_seq.git.

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Competing Interest Statement

A patent (WO/2021/132691) has been applied by KAGAMI Inc., Nara Medical University, and NIBIOHN with P.W., S.M., P.K., T.K., M.Mit., and S.N. as inventors based on this research. M.Mit. is a founder and CEO of KAGAMI Inc., a startup company working on chiral amino acids analysis and research for medical applications. The authors declare that nothing potential conflicts of interest.

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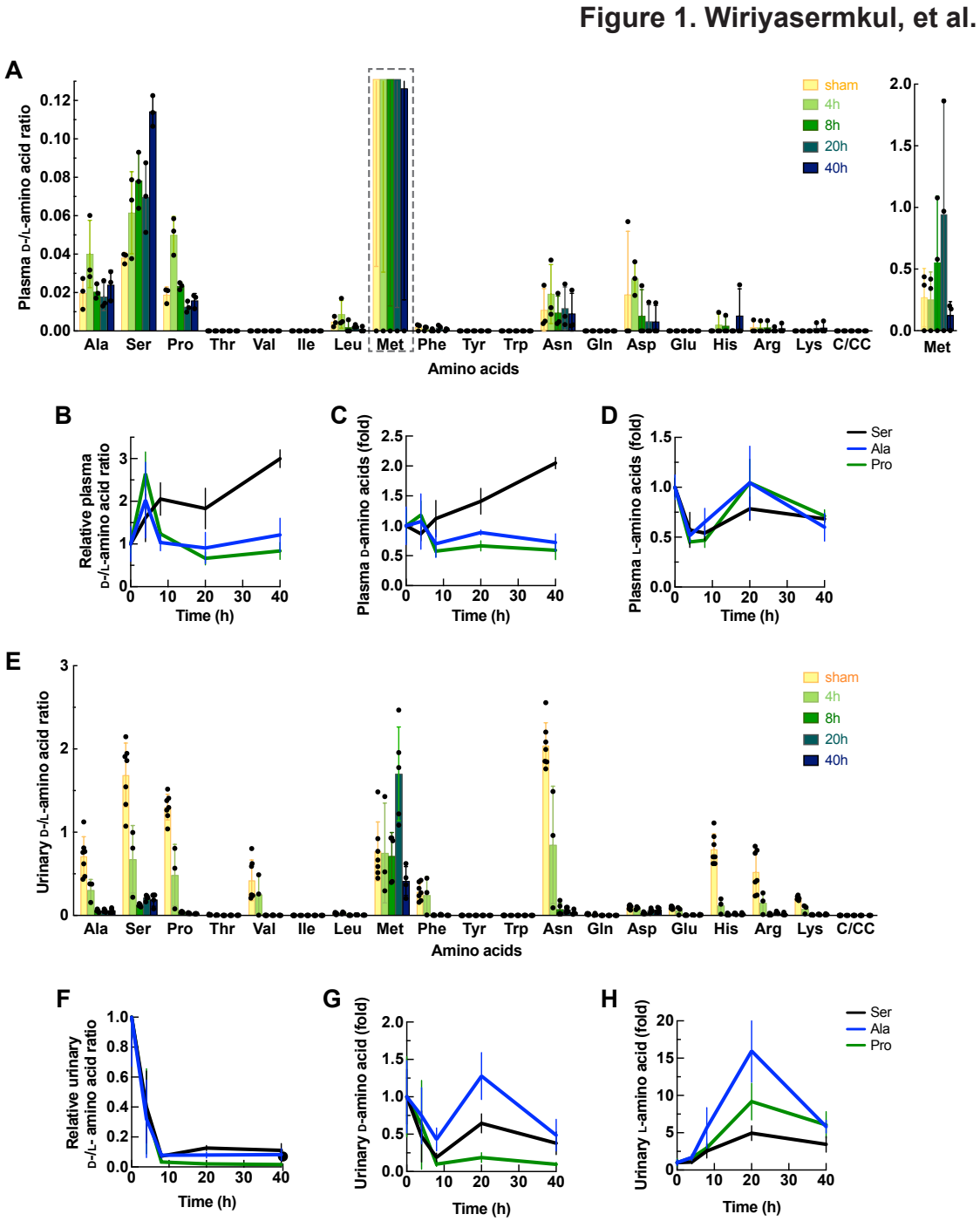
1169 **Figures and Tables**



1170

1171 **Figure 1. Enantiomeric profiles of D- and L-amino acids in plasma and urine of the IRI**

1172 **model**

1173 Plasma and urine were collected from the mice after ischemia operation for 4, 8, 20, and 40 h
1174 or sham operation (0 h). The concentrations of twenties amino acids were measured by 2D-
1175 HPLC and plotted as mean ± SD. (**A**) Ratio of D-/L-amino acids from the plasma of the IRI
1176 model. The graph of Met is shown separately. n = 3. (**B**) – (**D**) Plasma amino acid profiles of
1177 serine, alanine, and proline from (**A**) were normalized with 0 h and plotted as ratios of D-/L-
1178 enantiomers (**B**), relative concentrations of D-isomers (**C**), and relative concentrations of L-
1179 isomers (**D**). (**E**) Ratio of D-/L-amino acids from the urine of the IRI model. n = 3 – 7. (**F**) –
1180 (**H**) Urinary amino acid profiles of serine, alanine, and proline from (**E**) were normalized with
1181 those at 0 h and plotted as ratios of D-/L- enantiomers (**F**), relative concentrations of D-isomers
1182 (**G**), and relative concentrations of L-isomers (**H**). C/CC: cysteine or cystine.

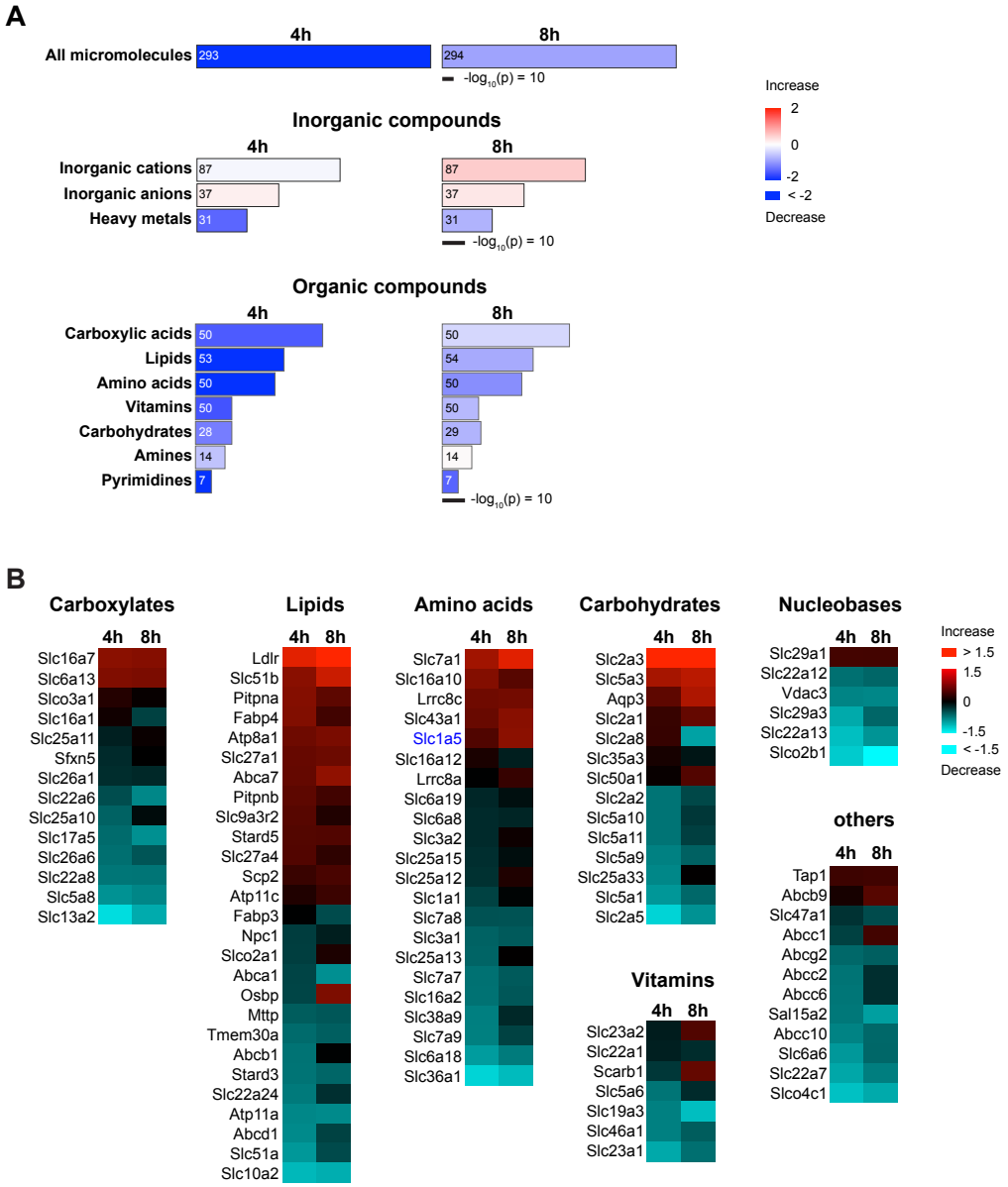
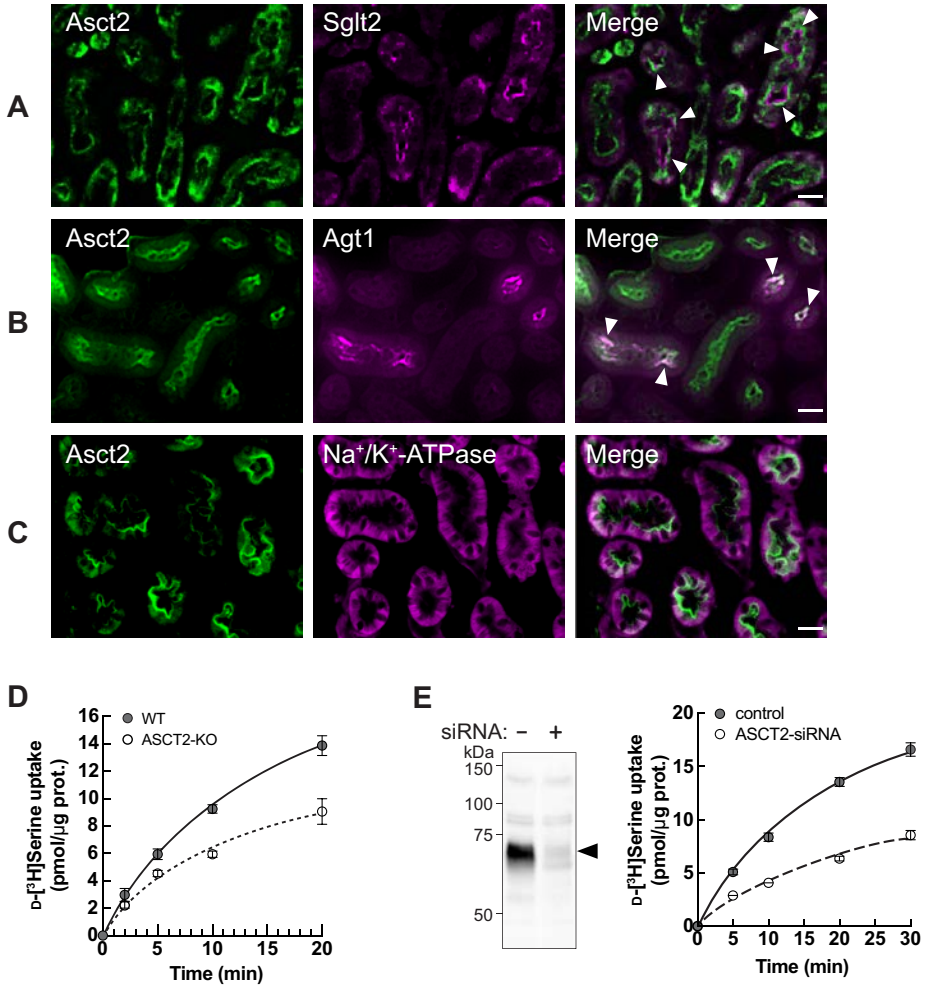


Figure 2. ***Molecular Transport*** **in renal BBMVs proteome of the IRI model**

(**A**) Ingenuity Pathway Analysis (IPA) shows the heatmaps of *Molecular Transport* in the membrane proteome from BBMVs of the IRI model (4h IRI/sham (4h) or 8h IRI/sham (8h)). Transport functions are categorized by types of substrates. Transport of all micromolecules (top) is derived from the combination of inorganic (middle) and organic compounds (bottom). Area and colors represent -$\log_{10}$(p-value) and annotated functions (z-score), respectively.

1190 Numbers inside the columns indicate the numbers of corresponding proteins. (**B**) Heatmaps of
1191 membrane transport proteins that mediate the transport of organic compounds. The proteins
1192 with ratios of more than 1.1-fold change are shown. The category "others" includes the
1193 transport of peptides, organic cations, organic anions, and drugs. Colors indicate $\log_2$ fold of
1194 4h IRI/sham (4h) or 8h IRI/sham (8h).



1195

1196 **Figure 3. ASCT2 is one of D-serine transporters at the apical membrane of renal proximal**
1197 **tubular epithelia.**

1198 (**A**) – (**C**) Localization of Asct2 in mouse kidney by immunofluorescence staining. Mouse
1199 kidney slides were co-stained with anti-Asct2(NT) antibody (Asct2; green) and protein markers
1200 for renal proximal tubule segments: anti-Sglt2 antibody (**A**: Sglt2, apical membrane marker of
1201 S1 + S2 segments), anti-Agt1 antibody (**B**: Agt1, apical membrane marker of S3 segment), and
1202 anti-Na^+/K^+-ATPase antibody (**C**: Na^+/K^+-ATPase, basolateral membrane marker). Merge
1203 images are shown in the right panel. Arrowheads indicate co-localization of the proteins. Scale
1204 bar = 20 μm. (**D**) Time course of 100 μM D-[^{3}H]serine transport in wild-type (WT) and *ASCT2*-

1205 knockout (ASCT2-KO) HAP1 cells measured in PBS pH 7.4. Dot plot = mean ± SEM; n = 3.

1206 E) Left: Western blot using anti-ASCT2 antibody verified the suppression of ASCT2 in

1207 ASCT2-siRNA transfected HEK293 cells. Right: Transport of 100 μM D-[^{3}H]serine (in PBS

1208 pH 7.4) was measured in ASCT2-knockdown (ASCT2-siRNA) in comparison to the Mock

1209 cells (control). Dot plot = mean ± SEM; n = 3.

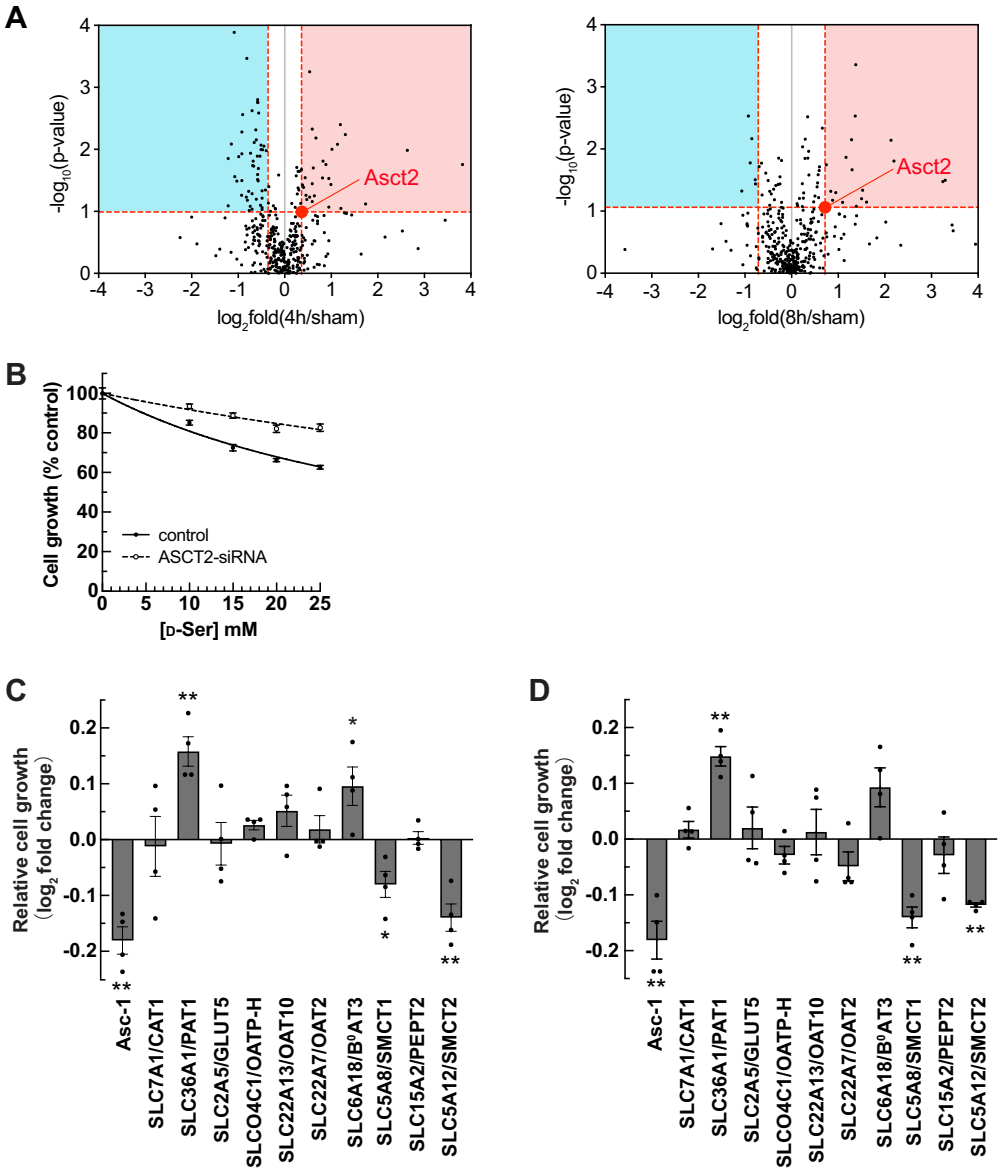
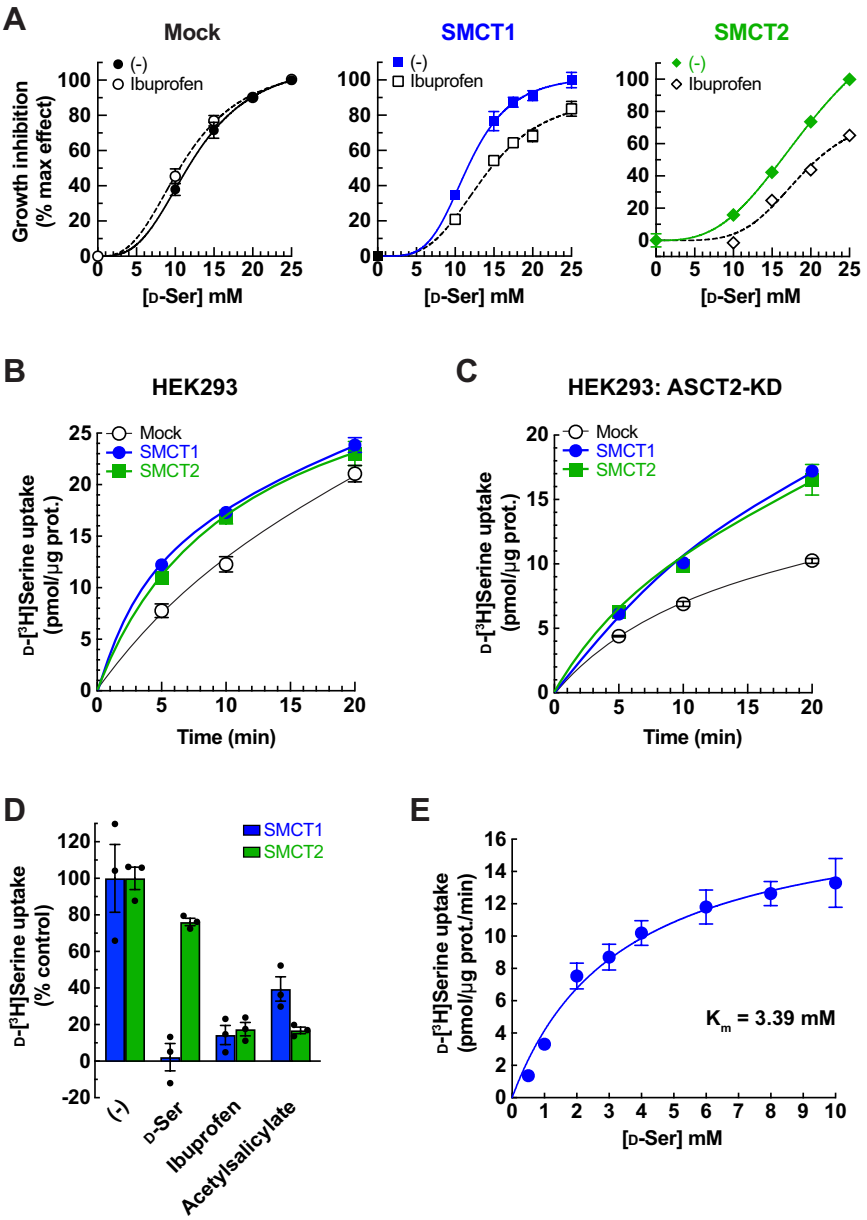


Figure 4. Identification of SMCT1 and SMCT2 as candidates of D-serine transporters

(**A**) Volcano plots of 398 membrane transport proteins identified from the BBMV proteome of the IRI model. The Median of $\log_2$ fold of 4h IRI/sham (left) or 8h IRI/sham (right) were plotted against $-\log_{10}$ of p-value. Three proteins with $\log_2$ fold more than 4.0 (see values in Table S1) are omitted for the better view. The value of Asct2 (red dot) was set as a cut-off value to select D-serine transporters candidates (both increased (red area) and decreased (blue area)

1217 expressions). (**B**) Cell-growth measurement (XTT assay) of ASCT2-siRNA or without siRNA
1218 (control) transfected HEK293 cells treated with D-serine. Data represent percent cell growth
1219 compared to the non-treated cells. The graphs were fitted to inhibition kinetics (Dose-response
1220 – Inhibition). Dot plot = mean ± SEM; n = 5. (**C**) – (**D**) Candidates of D-serine transporters
1221 were screened by cell-growth measurement. HEK293 cells were transfected with various
1222 cDNA clones, as indicated. After transfection, the cells were treated with either 15 mM (**C**) or
1223 25 mM (**D**) D-serine for two days and cell growth was examined. The growth effect by D-serine
1224 treatment was normalized with that of no treatment and then calculated as $\log_2$ fold change of
1225 Mock at the same D-serine concentration. Asc-1 is used as the positive control. Bar graph =
1226 mean ± SEM; n = 4; *$p < 0.05$; **$p < 0.01$.

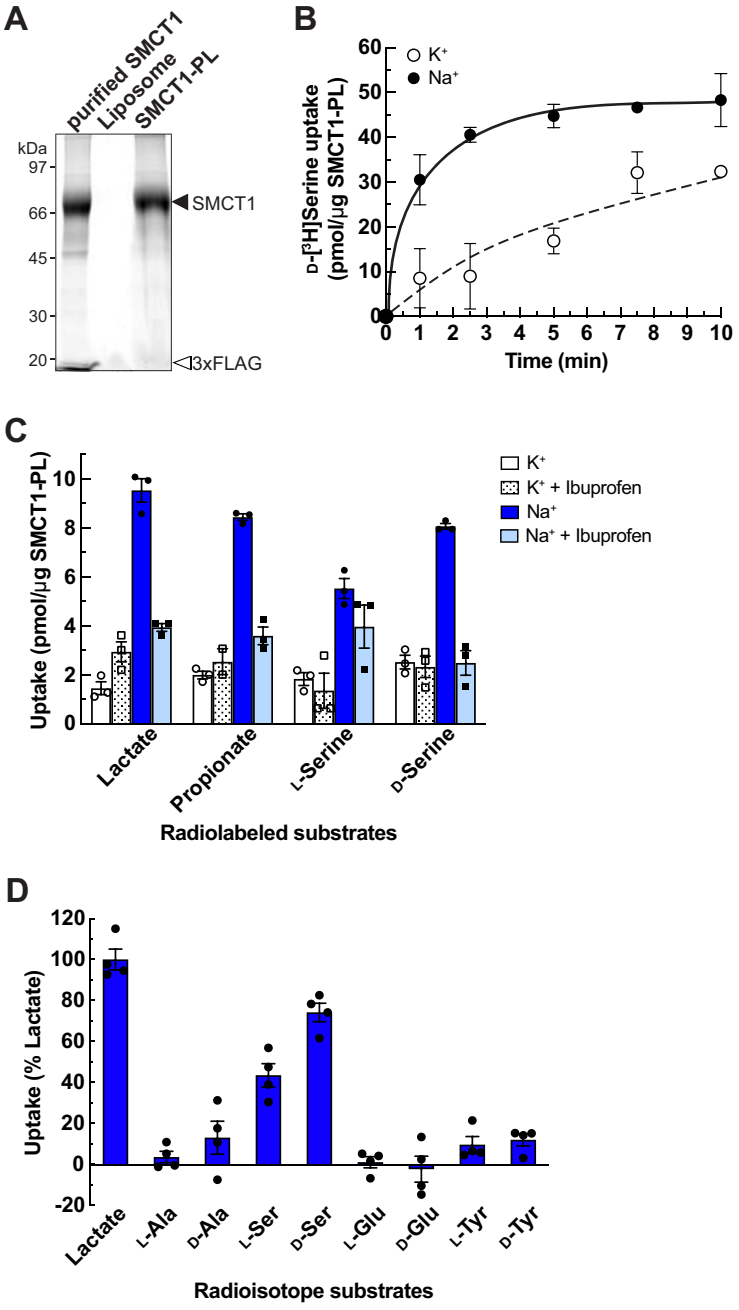


1227

1228 **Figure 5. Characterization of SMCT1 and SMCT2 as D-serine transporters using**
1229 **SMCT1- and SMCT2-stably expressing cells**

1230 (**A**) Inhibition effect of ibuprofen on D-serine-induced cell growth. FlpInTR-Mock (Mock),
1231 FlpInTR-SMCT1 (SMCT1), or FlpInTR-SMCT2 (SMCT2) cells were treated with D-serine
1232 for two days in the presence or absence of 0.5 mM ibuprofen. Cell growth was measured by
1233 XTT assay. For comparison, the maximum growth inhibition by 25 mM D-serine treatment was

1234 set as 100 % inhibition, and no D-serine treatment was set as 0 % inhibition. The graphs were
1235 fitted to inhibition kinetics (Dose-response – Inhibition). Dot plot = mean ± SEM; n = 5. (**B**)
1236 Time course of 100 μM D-[^{3}H]serine uptake in FlpInTR-SMCT1 (SMCT1), FlpInTR-SMCT2
1237 (SMCT2) and Mock cells. D-[^{3}H]Serine transport was measured in PBS pH 7.4. Dot plot =
1238 mean ± SEM; n = 4. (**C**) Time course of 100 μM D-[^{3}H]serine uptake was measured similarly
1239 to B), but the cells were subjected to ASCT2 knockdown (siRNA transfection) two days before
1240 the assay. Dot plot = mean ± SEM; n = 4. (**D**) Transport of 20 μM D-[^{3}H]serine by ASCT2-
1241 siRNA-transfected FlpInTR-SMCT1 or FlpInTR-SMCT2 stable cell lines were measured in
1242 the absence (-) or presence of 5 mM indicated inhibitors. The uptake was measured for 10 min
1243 in PBS pH 7.4. Graphs represented the uptake data subtracted from those of Mock cells. Bar
1244 graph = mean ± SEM; n = 3. IBU, Ibuprofen; ASA, Acetylsalicylic acid. (**E**) Concentration
1245 dependence of D-[^{3}H]serine transport in ASCT2-siRNA-transfected FlpInTR-SMCT1 cells.
1246 Uptake of D-[^{3}H]serine (0.5 – 8 mM) was measured for 10 min in PBS pH 7.4. Raw data was
1247 shown in Figure supplement 5D. The uptake data in FlpInTR-SMCT1 were subtracted from
1248 those of Mock cells and fitted to Michaelis-Menten plot with the apparent K_m of 3.39 ± 0.79
1249 mM and V_{max} of 18.23 ± 1.73 pmol/μg protein/min. Dot plot = mean ± SEM; n = 3 – 4.

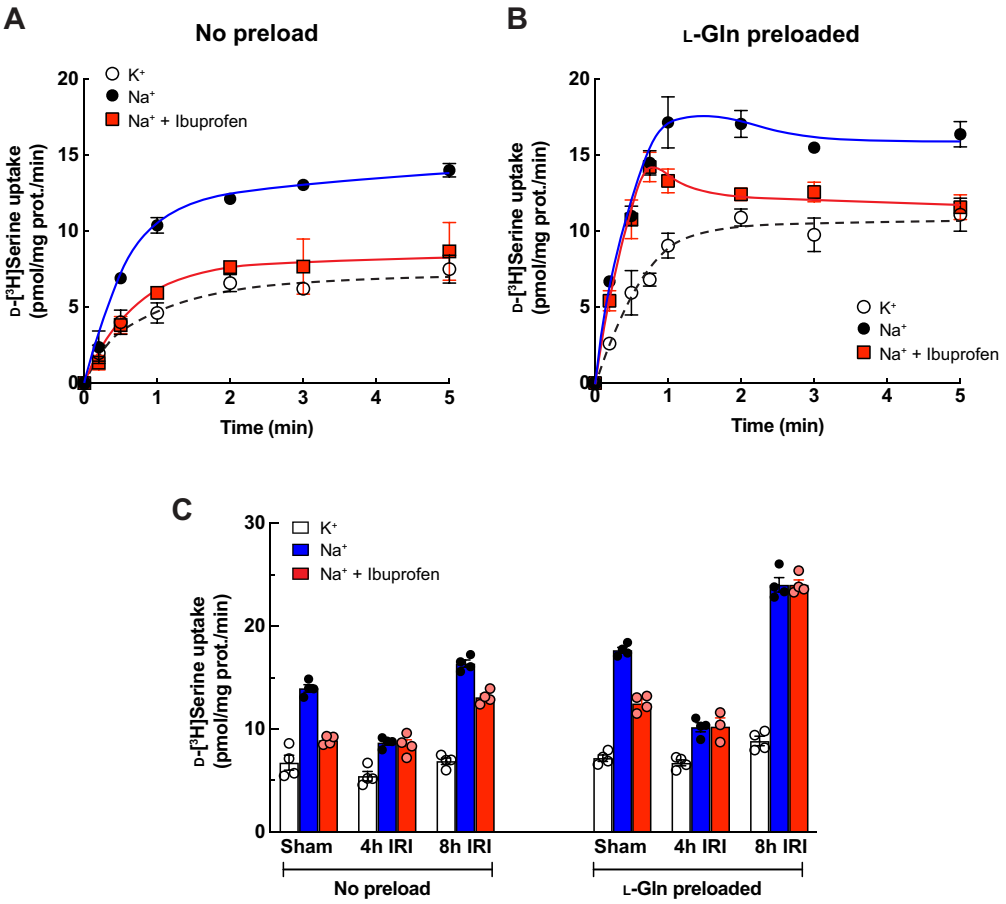


1250

1251 **Figure 6. Characterization of SMCT1 as D-serine transporter using SMCT1-**
1252 **reconstituted proteoliposomes (SMCT1-PL)**

1253 (**A**) Left: Stain-free SDS-PAGE gel shows SMCT1 (purified SMCT1) purified from pCMV14-
1254 SMCT1-transfected Expi293F cells, reconstituted empty liposomes (Liposome) and SMCT1-
1255 reconstituted proteoliposomes (SMCT1-PL). (B) Time course of D-[^{3}H]serine transport in

1256 SMCT1-PL. Uptake of 200 μM D-[^{3}H]serine was measured in Na^+-containing buffer (Na^+)
1257 compared to Na^+-free buffer (K^+). Dot plot = mean ± SEM; n = 3. (**C**) Ibuprofen effect on the
1258 uptake of [^{3}H]lactate, [^{3}H]propionate, L-[^{3}H]serine, and D-[^{3}H]serine in SMCT1-PL. Uptakes
1259 of 50 μM radiolabeled substrates were measured for 5 min in Na^+-containing buffer (Na^+) or
1260 Na^+-free buffer (K^+) in the presence or absence of 1 mM ibuprofen. Bar graph = mean ± SEM;
1261 n = 3. (**D**) Amino acid selectivity of SMCT1-PL. Transport of 50 μM radiolabeled amino acids
1262 was measured in SMCT1-PL for 5 min. The substrate uptake in Na^+-containing buffer was
1263 subtracted from those in Na^+-free buffer and calculated as % lactate uptake. Bar graph = mean
1264 ± SEM; n = 4.



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1266 **Figure 7. Characterization of D-serine transporters in BBMVs of normal mice and the**

1267 **IRI model**

1268 (**A**) Transport of 10 μM D-[^{3}H]serine in renal BBMVs isolated from normal mice. The uptake

1269 was measured in Na^+-free buffer (K^+), Na^+-containing buffer (Na^+), or the presence of 1 mM

1270 ibuprofen (Na^+ + Ibuprofen). Dot plot = mean ± SEM; n = 3. (**B**) Transport of 10 μM D-

1271 [^{3}H]serine in renal BBMVs isolated from the normal mice was performed similarly to (**A**) but

1272 the BBMVs were preloaded with 4 mM L-Gln prior to the measurement. Dot plot = mean ±

1273 SEM; n = 4. (**C**). Transport of 10 μM D-[^{3}H]serine in renal BBMVs isolated from IRI model.

1274 Prior to uptake measurement, the BBMVs were preloaded with 4 mM L-Gln or buffer (no

1275 preload). The uptake was measured for 1 min in Na^+-free buffer (K^+), Na^+-containing buffer

1276 (Na^+), or the presence of 1 mM ibuprofen (Na^+ + Ibuprofen). Bar graph = mean ± SEM; n = 3

1277 – 4.

Figure 8. Wiriyasermkul, et al.

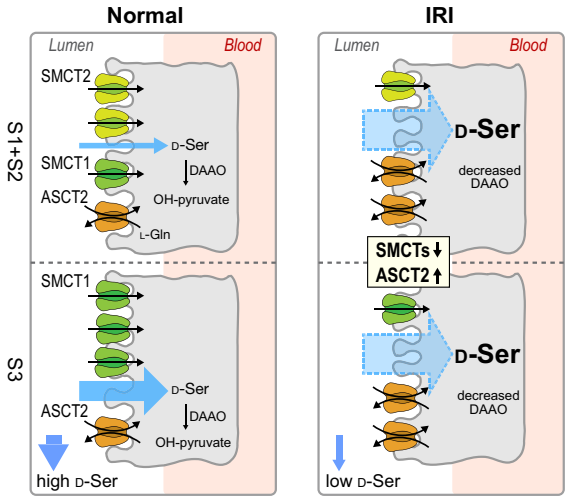

Figure 8. Proposed model of D-serine transport systems in renal proximal tubules

The model summarizes the contributions of ASCT2, SMCT1, and SMCT2 to D-serine transport in renal proximal tubules. Left: the physiological conditions (Normal kidney). SMCT2 at S1 + S2 segments and SMCT1 at segment S3 have high expression levels, whereas the expression of ASCT2 is low. The higher D-serine affinity of SMCT1 compared to SMCT2 supports that the D-serine reabsorption tends to be exceeded at the S3 segment. Right: the pathological conditions (IRI model). expressions of SMCTs decrease while that of ASCT2 increases. ASCT2 expresses ubiquitously and exhibits high D-serine affinity. Blue arrows anticipate total D-serine fluxes in each proximal tubular segment.

1289 **Table 1**. Candidate transporters from proteomics of the BBMVs from the IRI model. The list
1290 is ordered according to 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]
				Increased				
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
				Decreased				
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^0at3	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

[a]Median from n = 3
[b]Collectrin is a regulatory protein for B^0at3 function

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