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Temporo-spacial micoranatomical distribution of the murine sodium-dependent ascorbic acid transporters Slc23a1 and Slc23a2 in the kidney throughout development

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1 Temporo-spacial micoranatomical distribution of the murine sodium-dependent ascorbic acid
2 transporters *Slc23a1* and *Slc23a2* in the kidney throughout development

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35 Running Title: murine renal *Slc23a1* and *Slc23a2*

36 Key words: *Slc23a1*, *Slc23a2*, kidney, murine, development
37

38 **Abstract**

39 The two membrane transporters Slc23a1 and Slc23a2 mediate ascorbic acid uptake into cells.
 40 We recently determined the key role of Slc23a1 in renal re-absorption of ascorbic acid in a
 41 knockout mouse model. However, the renal spatial and temporal expression patterns of
 42 murine *Slc23a1* and *Slc23a2* are not defined. This study utilizes database evidence combined
 43 with experimental confirmation via *in situ hybridization* to define spatial and temporal
 44 expression of *Slc23a1* in the murine kidney. *Slc23a1* is expressed in the early proximal
 45 tubule, but not in its precursors during embryonic development, and exclusive proximal
 46 tubular expression persists throughout the animal's lifetime. In contrast, *Slc23a2* is uniformly
 47 expressed in metabolic cell types such as stromal cells. The expression patterns appear to be
 48 conserved from rodent lineages to humans.

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50 Introduction

51 Ascorbic acid (ascorbate, Vitamin C) is an indispensable metabolite essential for survival.
52 Ascorbate cannot be synthesized in humans due to a loss of functional gulonolactone oxidase
53 (EC 1.1.3.8), but is synthesized in murine species, such as mouse and rat (Padayatty et al.
54 2003; Nishikimi et al. 1988). The kidney is a central organ for ascorbic acid homeostasis.
55 Slc23a1 plays a key role in ascorbate homeostasis and pharmacokinetics, as demonstrated by
56 the facts that *Slc23a1*^{-/-} knockout mice have very high urinary losses leading to low systemic
57 concentrations (Corpe et al. 2010). Slc23a1 is known to mediate ascorbic acid uptake into
58 epithelial cells of the small intestine, liver and kidney (Boyer et al. 2005; Lee et al. 2006; Luo
59 et al. 2008; Maulen et al. 2003; Varma et al. 2008), and is also expressed in some epithelia of
60 the reproductive system and the brain (Tsukaguchi et al. 1999). Slc23a1 transports ascorbic
61 acid with comparatively low affinity ($K_m \approx 250 \mu\text{M}$) but high capacity, reflecting its role in
62 intestinal absorption and renal re-absorption (Daruwala et al. 1999; Tsukaguchi et al. 1999).
63 Compared to wildtype controls, the *Slc23a1*^{-/-} mouse has up to an 18-fold increase in
64 fractional ascorbate excretion. The consequence is that ascorbate renal reabsorption is lost,
65 resulting in low plasma and tissue concentrations and high perinatal loss of offspring (Corpe
66 et al. 2010).

67 A second ascorbic acid transporter, *Slc23a2*, is found in almost all cell types, mediates
68 ascorbic acid transport across cell membranes with high affinity (K_m 15 μM), and requires
69 cations (Na^+ , Ca^{2+} , or Mg^{2+}) for optimal activity (Daruwala et al. 1999; Godoy et al. 2007;
70 Tsukaguchi et al. 1999). The global elimination of *Slc23a2* results in undetectable ascorbate
71 tissue concentrations, and *Slc23a2*^{-/-} mice died within minutes of birth (Sotiriou et al. 2002).
72 Due to its expression in a wide range of tissues it is generally accepted that Slc23a2 is
73 responsible for ascorbate tissue accumulation needed for survival (Sotiriou et al. 2002).

74 Despite the importance of both sodium-dependent ascorbic acid transporters and the fact that
75 both genes have been eliminated in mouse models, their spatial expression in defined
76 anatomical compartments of the nephron and the temporal expression during embryonic
77 development are not described for murine species. This study reports the temporal-spatial
78 distribution of the murine *Slc23a1* and *Slc23a2* transcripts throughout kidney developmental
79 stages by comparing *in situ hybridization* (ISH), microarray and quantitative PCR data of
80 nephron compartments.

81

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82 **Materials and Methods**

83 In Situ Hybridizations

84 *Organ samples:* Samples were obtained from embryonic mice and adult rats as part of animal
85 protocols approved by the Eunice Kennedy Shriver National Institute of Child Health and
86 Human Development and the National Institute of Diabetes and Digestive and Kidney
87 Diseases Animal Care and Use Committees. Specimens were harvested, snap frozen in dry
88 ice and stored at -70°C. Serial sections of 10 µm thickness were cut at -15°C and thaw-
89 mounted onto poly-L-lysine-coated slides for in situ hybridization or immunohistochemistry.

90 *Probes Hybridization and Visualization:* The human *SLC23A1* and *SLC23A2* complete
91 coding sequences subcloned into pGEM®-T Easy Vectors (Promega) were used to prepare
92 RNA probes for in situ hybridizations (Daruwala et al. 1999). The synthesis of ³⁵S-labeled
93 cRNA probes, and the hybridization and visualization procedures have been previously
94 described in detail (Bondy et al. 1993).

95 Renal gene expression analysis in adult rats

96 *Microdissection:* Male Sprague-Dawley rats (250-300g) were anesthetized by an
97 intraperitoneal injection of 120 mg/kg thiobarbital. After interruption of the aortic blood
98 flow to the kidney it was perfused with 30 ml cold PBS (Sigma Chemical, St Louis, MO)
99 followed by 30 ml culture medium [Dubecco's modified Eagle's Medium (DMEM), Sigma
100 Chemical, St Louis, MO] containing 1 mg/ml collagenase (*Clostridium histolyticum*, Sigma
101 Chemical). The kidney was then removed, cut into slices, and incubated in the
102 DMEM/collagenase solution for 22 minutes at 37°C. Microdissection was performed with
103 sharpened forceps at 4°C under a stereomicroscope. The lengths of dissected segments were
104 determined with an eyepiece micrometer. In general, 6 to 10 mm of tubule segments were
105 dissected and pooled to constitute one sample. The following specimens were dissected:
106 glomeruli (Glm), proximal straight tubules (PST), proximal convoluted tubules (PCT),

107 medullary thick ascending limb (mTAL), cortical thick ascending limb (cTAL), macula densa
108 containing segment (MDCS), cortical collecting duct (CCD), outer medullary collecting duct
109 (OMCD), inner medullary collecting duct (IMCD). Segments of the proximal convoluted
110 tubule (PCT) may have contained S1 and S2 segments, but not S3 segments. Samples were
111 placed in 100 μ l guanidine isothiocyanate buffer (GITC buffer: 4 M guanidine isothiocyanate,
112 25 mM sodium acetate, 0.8% β -mercaptoethanol, pH 6.0), snap frozen in liquid nitrogen and
113 stored at -80°C .

114 *RNA isolation:* RNA from the renal samples were thawed in ice slurry and sonicated for 15
115 seconds. Twenty micrograms of E-coli ribosomal RNA (Boehringer, Minneapolis) was added
116 as carrier. The 100ul sample in GITC buffer was layered onto a gradient of cesium chloride
117 (100 μ l of 97%, 20 μ l of 40% cesium chloride in 25 mM sodium acetate buffer) in a 250 μ l
118 polycarbonate ultracentrifuge tube. Samples were centrifuged for two hours at 100000 g in a
119 Beckman TLA 100 ultracentrifuge (Beckman Instruments, Fullerton) with a fixed angled
120 rotor. The resulting RNA pellet was dissolved in 0.3 M sodium acetate and precipitated with
121 ethanol. The purified RNA was dissolved in diethyl pyrocarbonate treated water containing
122 20 U RNAsin.

123 *Reverse transcription:* Reverse transcription was performed in the presence of 100IU
124 Moloney murine leukemia virus reverse transcriptase (Superscript BRL, Gaithersburg), 0.5
125 μ g oligo(dT)₁₂₋₁₈ (Pharmacia, Piscataway), 20 IU RNAsin (Promega, Madison), 10 mM
126 dithiothreitol, 0.5 mM dNTP (Pharmacia), and 1% bovine serum albumin (Boehringer) in the
127 buffer provided by the manufacturer in 20 μ l. Prior to the addition of reverse transcriptase the
128 reaction mixture was heated to 65°C for 5 minutes to allow annealing of the primers. cDNA
129 was synthesized at 42°C for one hour and precipitated with 1 μ l of linear acrylamide, 4 M
130 ammonium acetate in 100% ethanol. The pellet was redissolved in Tris-EDTA buffer
131 adjusted so that each 2 μ l of cDNA corresponded to 1 mm of segment dissected.

132 *Polymerase chain reaction:* In each experiment, all tubes were assayed for expression of
133 *Slc23a1* and *Slc23a2*. Rat specific primer were designed and optimized from the known
134 sequences NM017315 and NM017315, respectively. The following primer pair was chosen
135 and optimized to amplify a 719 bp piece in between base 1021 and base 1740 of the
136 published r-Slc23a1 cDNA open reading frame sequence. Sense: 5`-
137 TCATCGAGTCCATCGGTG-3`, antisense: 5`-AGAATCCTCTGAAGACTG-3`. A 779 bp
138 fragment of r-Slc23a2 was amplified with the sense primer 5`-GACGTCTTCCCTTCCAAC-
139 3` and the antisense primer 5`-CTTGTTTCCTTTGCTCAC-3` between base 1201 and 1980
140 of the published r-Slc23a2 open reading frame sequence. The primers for both amplifications
141 are located near or in part in the 3` end of the coding region to ensure optimum detection of
142 the cDNA. Amplification conditions included the following: 0.5 μ M of each primer, 50 mM
143 KCL, 10 mM Tris- HCl, 2,5 mM MgCl, 200 μ M of dATP, dCTP and dTTP, 50 μ M dGTP
144 and 150 μ M 7-deaza-2`GTP, as well as Taq DNA polymerase (Life Technologies). The
145 amplification conditions were as follow: 5 min denaturation at 94 C followed by 35 cycles of
146 94 for 30 s, 55 for 30 s and 72 for 30 s. A final extension at 72 for 5 min was performed.
147 Amplification products were confirmed by gel electrophoresis and compared to known DNA
148 standards for estimation of the fragment size. The relative intensity of each PCR product
149 band on the ethidium bromide gel was assessed by optical density. The identities of the PCR
150 products were confirmed by sequencing using the dideoxynucleotide chain termination
151 method on an automatic sequencer (ABI PRISM 377, Perkin Elmer, Foster City, CA) using
152 the manufacturer's supplies. Sequence data were analyzed using Sequencher 4.1 (Genes
153 Codes Corporation, Ann Arbor, MI).

154 Renal gene expression analysis in the developing mouse

155 We re-analyzed the entire kidney development dataset generated with Affymetrix MOE430
156 version 2 microarray, which is available for the GenitoUrinary Development Molecular

157 Anatomy Project (GUDMAP.org) (Harding et al. 2011; McMahon et al. 2008). Gene
 158 expression data from 54 microarrays were analyzed for the expression of *Slc23a1* after
 159 downloading the individual excel files for different nephron segments at different
 160 developmental stages. Data were transformed into relative fluorescent units and graphically
 161 displayed. The protocols for the generation of the tissue samples can be found at the
 162 GUDMAP protocol web site: <http://www.gudmap.org/Research/Protocols>. Expression data
 163 for the medullary collecting duct, cortical collecting duct and collecting duct distal to the last
 164 branch point, S-shaped body, urothelium of the ureter, medullary interstitium, forming
 165 muscle layer surrounding the urothelium (ureteral mesenchyme), loop of Henle (including
 166 cortical anlage of the loop of Henle and medullary immature loop of Henle), and the proximal
 167 tubule were obtained and processed.

168 Corresponding ISH images were obtained from the GUDMAP web server, the protocols used
 169 for tissue isolation, sectioning, in situ hybridization and imaging are available at the protocols
 170 web site: <http://www.gudmap.org/Research/Protocols>.

171

172 **Results**

173 Murine *Slc23a1* is exclusively expressed in the renal proximal tubule

174 Earliest embryonic *Slc23a1* expression can be detected 14 days post conception in the
175 developing kidney (Figure 1A) and emerging gene expression is confirmed in the developing
176 early proximal tubule at embryonic day E15.5 (Figures 1B, 1D). *Slc23a1* is not expressed in
177 the precursors of the early proximal tubule, such as the capping mesenchymal, renal vesicle,
178 and S-shaped body (Figure 1D). The nephron is derived from capping mesenchymal cells,
179 which undergo a mesenchymal-to-epithelial transition to form the renal vesicle. The cells of
180 the renal vesicle differentiate, elongate, and convolute to form an S-shaped body, from which
181 the glomerulus, proximal tube, loop of Henle and distal tubule are derived.

182 In the late stage embryo, at day 18 post conception, the *Slc23a1* transcript is strongly
183 expressed in the maturing renal cortex, and outside the kidney significant expression can be
184 confirmed in the small intestinal loops and the liver (Figure 1C). The proximal tubule is the
185 only renal anatomic compartment where *Slc23a1* is expressed in significant amounts in late
186 embryos, starting at day 14 (Figure 1A) and clearly distinguishable from all other parts of the
187 nephron at day 15.5 (Figure 2A). This expression pattern remains stable in the juvenile and
188 adult kidney (Figures 2B, 2C, 2D). *In situ* hybridizations of the adult murine kidney shows
189 the *Slc23a1* message in a ray-like pattern beginning at the border of the outer medulla and
190 extending into the renal cortex (Figure 2D), consistent with a typical proximal tubule
191 anatomic distribution (Chin et al. 1997). This spatial distribution is confirmed by the more
192 sensitive and detailed dark field micrography (Figures 3A, 3B), and RT-PCR on micro-
193 dissected nephron compartments (Figures 3C), mapping *Slc23a1* mRNA expression to the
194 proximal convoluted and straight tubules of the adult murine kidney.

195 Murine *Slc23a2* is uniformly expressed throughout the murine kidney

196 *Slc23a2* mRNA is uniformly expressed throughout the murine late stage embryonic body
 197 without signs of defined organ specific patterns (Figures 4A, 4B) and it does not show a
 198 distinct distribution in the adult kidney (Figure 4C, 4D, 4E). In renal microdissected segments
 199 *Slc23a2* is found in moderate levels, in a relative uniform distribution (Figure 4F), except that
 200 levels are close to the detection limit in the proximal convoluted and straight tubule. This
 201 likely indicates a lack of expression in the proximal convoluted and straight tubules, where
 202 *Slc23a1* is expressed. Highest *Slc23a2* expression is located in the inner medullary collecting
 203 duct, indicating expression by stromal fibroblasts (Chin et al. 1997).

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205 **Discussion**

206 In this report we resolve the temporal and spatial expression of the *Slc23a1* and
207 *Slc23a2* transcripts in the developing and mature murine kidney. The presented data derived
208 from ISH, microarray and RT-PCR analysis consistently demonstrate that *Slc23a1* expression
209 is exclusively limited to the proximal tubule during renal development and in the adult
210 kidney. Thus, *Slc23a1* is one of the rare genes displaying extreme component specific
211 expression (Brunskill et al. 2009). Remarkably, the overwhelming majority of genes with a
212 similar expression pattern encode either known membrane transporters or membrane
213 associated proteins of unknown functions (Brunskill et al. 2009), demonstrating the essential
214 function of the proximal tubule in re-absorption of vitamins and organic
215 compounds/metabolites.

216 These data of exclusive *Slc23a1* expression in the murine renal proximal convoluted
217 and straight tubules are consistent with the expression pattern reported for the human
218 transcript (Corpe et al. 2010; Eck et al. 2013). This is also consistent with the assumption that
219 gene expression throughout renal development is highly conserved between mammalian
220 species (Thiagarajan et al. 2011). Twenty-five distinct renal cell types are known, which are
221 derived from two intermediate mesoderm-derived cell populations: the metanephric
222 mesenchyme and the ureteric bud. The ureteric bud forms a dichotomously branching
223 epithelial tree, giving rise to the cell types that make up the collecting ducts of the kidney and
224 the ureter that connects the kidney with the bladder (Thiagarajan et al. 2011). Before its
225 appearance in the early proximal tubule, *Slc23a1* is not expressed in any precursor tissues,
226 such as the capping mesenchyme surrounding the ureteric bud, which gives rise to the renal
227 vesicle. The renal vesicle then elongates and convolutes to form the S-shaped body, which
228 subsequently gives rise to the glomerulus, proximal tubule, loop of Henle, and distal tubule
229 (Brunskill et al. 2009). *Slc23a1* is not expressed in the ureteric bud derivatives, forming the

tip and non-tip sections in the cortex, and the medullary region, to shape the collecting duct system. Expression is also absent in the cortical and medullary interstitium or stroma.

The key role of the ascorbic acid uptake protein Slc23a1 in the maintenance of systemic vitamin C levels was revealed through *Slc23a1*^{-/-} mice (Corpe et al. 2010). For example, female *Slc23a1*^{-/-} mice had an 18 fold increase in fractional excretion of ascorbate in the kidney, and a 70% decrease in circulating vitamin C concentrations compared to wildtypes. This strongly indicated that Slc23a1 is the only ascorbic acid uptake protein on the apical side of the renal proximal epithelial cell (Corpe et al. 2010; Eck et al. 2013). However, these functional data on the *Slc23a1*^{-/-} mouse did not rule out the possible disruption of proper kidney development as a contributing factor to renal ascorbic acid losses. The temporal and spatial expression patterns described above clearly identify *Slc23a1*'s sole function in the cellular transport of ascorbic acid, ruling out a function in development, cell adhesion and cell-cell communication (Brunskill et al. 2009). We therefore rule out a role as an “anlage in statu nascendi” gene, which would be important in the formation of a structure by showing earlier expression in the anlage (Brunskill et al. 2009). The earliest *Slc23a1* expression is detectable in stage IV nephrons, which is the maturing nephron (Thiagarajan et al. 2011), and persists through adulthood. This also confirms that segmentation of the early nephron into proximal, distal and Loop of Henle elements does not occur until the formation of a stage IV nephron (Thiagarajan et al. 2011). These expression patterns in adult murine are identical to the human site of expression (Eck et al. 2013), allowing an extrapolation to the basic biology of the transporter in humans.

In contrast to the much defined temporal-spatial *Slc23a1* expression in the murine kidney, *Slc23a2* is found in moderate levels and relative uniform distribution pattern, consistent with expression by stromal fibroblasts (Chin et al. 1997). *Slc23a2* is undetectable in the proximal convoluted and straight tubule, ruling out any role in renal ascorbic acid

reabsorption, and confirming the notion that Slc23a2 is responsible for ascorbic acid distribution into metabolically active stromal cells (Sotiriou et al. 2002).

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Carolyn A. Bondy and Jie Wang (NHLBI) contributed some in situ hybridization images.

Jurgen Schnerman (NIDDK) provided the micro-dissected nephron samples.

This study used data from the GUDMAP database <http://www.gudmap.org> [microarray and ISH images downloaded in January 2015]. ISH selected from dataset GUDMAP:9176, principal investigator Melissa H Little, University of Queensland, Brisbane, Australia.

DISCLOSURE

The authors have no competing interest to disclose.

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342

343 **Figure Captions**

344 Figure 1: *Slc23a1* expression in morphological structures of the developing murine and its
345 kidney assed through *in situ* hybridisation (dark areas indicate expression) and microarray
346 analysis. (A) *Slc23a1* expression in the mouse embryo 14.5 days post conception (E14.5)
347 emerging in the kidney (Ki). (B) *Slc23a1* expression in kidney of the mouse embryo at 15.5
348 days post conception (E15.5). (C) *Slc23a1* expression in the mouse embryo 18 days post
349 conception (E18) showing well-defined ray-like pattern in the maturing renal cortex (Ki), as
350 well as defined patterns in small intestinal loops (Si) and the liver (Li). (D) Sense *Slc23a1*
351 cRNA hybridized to a slide corresponding with figure 1C. (E) Temporal *Slc23a1* expression
352 in developmental precursors of the proximal tubule assessed by microarray analysis and
353 expressed as relative fluorescent units (RFU).

354 Figure 2: *Slc23a1* expression in micro-anatomical compartments of the late embryonic,
355 postpartum and adult murine kidney assed through microarray analysis and *in situ*
356 hybridisation (dark areas indicate expression). (A) *Slc23a1* expression in segments of the
357 embryonal murine nephron at day 15.5 post conception (E15.5) expressed as Relative
358 Fluorescent Units (RFU) from microarrays. (B, C) *Slc23a1* expression in the kidney of a
359 seven day old mouse. (D) *Slc23a1* expression in the adult murine kidney at the border of the
360 outer medulla (OM).

361 Figure 3: *Slc23a1* expression in micro-anatomical compartments of the adult murine kidney.
362 (A) *Slc23a1* expression in the adult murine kidney using a dark field micrograph shows
363 expression in proximal tubules (PT, light spots), but no signal in vascular bundles (VB, dark
364 areas) or the ascending thick limbs of Henle localized in the medullary rays (MR, dark areas).
365 (B) Haematoxylin and eosin stained (H&E) bright field image corresponding to image 3A.
366 Here, PT, VB and MR were microscopically identified by a pathologist. (C) *Slc23a1*

367 expression in dissected nephron segments from the adult murine kidney assessed with
368 quantitative PCR expressed as Relative Band Fluorescent Units (RBFU).

369 Figure 4: *Slc23a2* expression in the murine kidney assessed by ISH (A, B, C), dark field
370 micrographs (D, E), and quantitative PCR (F). (A) *Slc23a2* expression in the mouse embryo
371 at day 18 (E18). (B) Corresponding image to figure 4A hybridized with sense RNA.
372 (C) *Slc23a2* expression in the adult murine kidney. (D) *Slc23a2* expression in the adult
373 murine kidney using a dark field micrograph shows relatively uniform expression.
374 (E) Haematoxylin and eosin stained (H&E) bright field image corresponding to image 4D.
375 Here, PT, VB and MR were microscopically identified by a pathologist. (F) *Slc23a2*
376 expression in dissected nephron segments from the adult murine kidney assessed with
377 quantitative PCR expressed as Relative Band Fluorescent Units (RBFU).