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## Functional Transport of Organic Anions and Cations in the Murine Mesonephros

**Citation for published version:**

Lawrence, L, Smith, J & Davies, J 2018, 'Functional Transport of Organic Anions and Cations in the Murine Mesonephros', *Journal of the American Society of Nephrology*. <https://doi.org/10.1152/ajprenal.00021.2018>

**Digital Object Identifier (DOI):**

[10.1152/ajprenal.00021.2018](https://doi.org/10.1152/ajprenal.00021.2018)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

Journal of the American Society of Nephrology

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1     **Functional Transport of Organic Anions and Cations in the Murine**  
2                                    **Mesonephros**

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6  
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12  
13  **Key Words:** Mesonephros, Transport, Efflux, OAT, OCT, Mate1, Mate2, Mate2K,  
14  BCRP, MRP, Kidney, SLC22A1, SLC22A2, SLC22A6, SLC22A8, SLC47A1, SLC47A2

15  
16  **Running title:** Functional transport in the murine mesonephros

17  
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26

27 **Abstract**

28

29 The mesonephros of mammals is a transient renal structure that contributes to  
30 various aspects of mammalian fetal development including the male reproductive  
31 system, hematopoietic stem cells and vascular endothelial cells. The mesonephros  
32 develops from the intermediate mesoderm and forms tubules that are segmented in  
33 a similar way to the nephrons of the permanent kidney (but lacking loops of Henle).  
34 Early studies have suggested that the mesonephros in marsupials and some  
35 placental mammals may perform an excretory function, but these studies have not  
36 directly shown active transport of organic anions and cations. Excretory function in  
37 the rodent mesonephros has not been investigated. Functional characterisation of  
38 the earliest stages of mammalian renal development is important for our  
39 understanding of congenital disease and may help to inform the growing field of  
40 renal tissue engineering. Here, we use live uptake and efflux assays *in vitro* to show  
41 that the murine mesonephros is able to transport organic anions and cations through  
42 specific transporters from early in its development. Transcript analysis suggests that  
43 there are subtle differences between the transporters involved in uptake and efflux  
44 by the murine permanent, metanephric tubules and by the mesonephric tubules.  
45 These data suggest that the mammalian mesonephros can provide an excretory  
46 function for the early developing embryo, in addition to the excretory function  
47 provided by the placenta.

48

## 49 Introduction

50

51 In lower vertebrates the mesonephros is the final and definitive renal structure,  
52 performing an excretory function for the organism. In mammals, the mesonephros is  
53 a temporary structure that is eventually replaced by the metanephros, the permanent  
54 kidney. The mammalian mesonephros develops parallel to the nephric duct (formed  
55 from condensed intermediate mesoderm) as the duct elongates along either side of  
56 the midline in a cranial to caudal direction.(21) This primitive kidney consists of  
57 segmented tubules that connect to the adjacent nephric duct, making an arcade of  
58 tubules draining into a common duct that initially drains into the cloaca and, later,  
59 into the urogenital sinus. In mice, the mesonephros begins to develop from around  
60 embryonic day 9.5 (E9.5), with tubules continuing to form caudally alongside the  
61 nephric duct. The most cranial 4-6 tubules of the mesonephros are attached to the  
62 nephric duct and a further 14-20 will remain unattached whereas, in humans, all the  
63 mesonephric tubules will connect to the nephric duct. As the permanent kidney  
64 develops, the mammalian mesonephros regresses and will disappear by E14 in  
65 mouse, with some tubules being modified in males to contribute to the male  
66 reproductive system.

67

68 The degree to which the mammalian mesonephros is functional, during its brief  
69 existence, remains unclear. Previous studies in rabbits and pigs have shown that  
70 their mesonephroi may be capable of producing urine, although attempts to  
71 demonstrate filtration by analysing the composition of the allantoic fluid(3, 4, 14) or  
72 bladder contents(24) were inconclusive. One very early study in the opossum (a  
73 marsupial in which the mesonephros persists for at least 10 days after birth),  
74 involving injection of ferrocyanide and phenol red followed by investigation of  
75 mesonephric fluids, suggested that some glomerular and tubular transport may have  
76 occurred.(6) In addition, *in vitro* microperfusion procedures suggest that volume  
77 transport rates of tritiated water per unit area are similar between mesonephric  
78 tubules and metanephric proximal tubules in rabbits(27), and transepithelial ion  
79 transport in perfused *in vitro* rabbit mesonephric tubules has been demonstrated by  
80 examining transmembrane electrical cell potential difference(26). However, these  
81 studies have not directly shown active transport; in particular, the uptake and efflux  
82 of organic anions and cations in the rodent mesonephros has not been studied.

83

84 The nephron is the functional unit of the metanephric (permanent) kidney.  
85 Transepithelial transport across nephron tubules is an important mechanism for  
86 reabsorption and excretion of endogenous and xenobiotic compounds. Anions in the  
87 mouse metanephric kidney are transported into the tubular cells by the organic anion  
88 transporters (OATs) of the SLC22A family, including the basolateral Oat1 and Oat3,  
89 the apical Oat2, Oat5 and Urat1, as well as some members of the selective SLCO  
90 family(1, 8, 19). Hydrophilic anion efflux into the lumen of the tubules is primarily  
91 mediated by the apically-located MRP (ABCC) family of efflux proteins and to some  
92 extent the efflux transporter Breast Cancer Resistance Protein (BCRP).(17) Another  
93 important extrusion protein at the apical brush border is P-glycoprotein (MDR1) but  
94 this efflux pump mainly mediates extrusion of hydrophobic or neutral  
95 compounds.(28) In addition to these anion transporters, uptake of organic cations in  
96 mouse metanephric kidneys is mediated mainly by Oct1 and Oct2 (also part of the  
97 SLC22A family). The H<sup>+</sup> antiporter Mate1 is another cation transporter in the murine  
98 metanephros, acting as an extrusion or uptake transporter depending on the  
99 concentration of H<sup>+</sup> in the intracellular compartment relative to the external  
100 environment.(25)

101

102 When used with specific inhibitors of individual transporters or families of  
103 transporters, fluorescent anions or cations such as 6-carboxyfluorescein (6-CF,  
104 6FAM) and DAPI can be employed as tracers to examine transporter activity in live  
105 cultures *in vitro*. Due to morphological similarities between mesonephric and  
106 metanephric tubules, we hypothesised that murine mesonephric kidney tubules  
107 would also be capable of transepithelial transport through apical and basolateral  
108 uptake and efflux proteins.

109

110 Here, we show that organic anion and cation transporters are expressed in the  
111 murine mesonephros at least from E10.5. We also show expression of two efflux  
112 pumps in the murine mesonephros that are known to be important extrusion proteins  
113 in the tubules of the metanephros. Using a combination of published uptake assays  
114 and an efflux assay,(12) we show that the murine mesonephros is capable of  
115 inhibitable uptake and efflux of organic anions, and uptake of cations in culture.  
116 These data add to previous work showing accumulation of fluid from the

117 mesonephros in other mammalian species, and suggest that the mammalian  
118 mesonephric kidney functions in a similar way to the metanephric kidney, providing  
119 an early excretory system for the mammalian embryo and acting in parallel with the  
120 placenta.

121

## 122 **Materials and Methods**

123

### 124 *Dissection and culture of mouse mesonephroi*

125 Embryos from timed mated CD1 females were removed from the uterus and the  
126 embryonic mesonephroi were dissected using standard techniques.(16) Gonads  
127 were carefully removed, leaving as many mesonephric tubules as possible intact.  
128 Mesonephroi were placed in culture dishes using the Sebinger method, for optimal  
129 imaging,(22) and then assayed directly in the dishes.

130

### 131 *Chemicals and Reagents*

132 Metformin hydrochloride (PHR1084) and cimetidine (C4522) were purchased from  
133 Sigma Aldrich. 6-carboxyfluorescein (C1360) was purchased from Invitrogen.  
134 Probenecid (P36400) was purchased from Life Technologies. Probenecid,  
135 metformin, cimetidine and 6-carboxyfluorescein were dissolved in water to make  
136 stock concentrations as follows: probenecid 250mM, metformin 100mM, cimetidine  
137 25mM, 6-carboxyfluorescein 1mM. Dilutions for live assays were diluted in kidney  
138 culture medium: see below.

139

### 140 *Transport assays*

141 Assays were carried out on live cultures in the Sebinger culture system using kidney  
142 culture medium (KCM: Eagle's MEM with Earle's salts [Sigma, M5650]), 10% FCS  
143 [Invitrogen, 10108165] and 1% Penicillin/Streptomycin stock [Sigma, P4333]). For  
144 imaging purposes, all uptake and efflux assays were carried out in the presence of  
145 rhodamine-conjugated peanut agglutinin (20µg/ml, Vector Laboratories, RL-1072)  
146 which highlights morphological structures. Anion uptake assays were performed by  
147 treating the mesonephroi in Sebinger culture dishes for 1 hour at 37°C with the  
148 fluorescent anion 6-carboxyfluorecein (1 µM) either with probenecid (2.5 mM in  
149 KCM) or with only culture medium. Cultures were washed once in PBS and then

150 incubated with probenecid (10mM) for 15 min at 37°C to trap any intracellular  
151 fluorophore before imaging. Efflux of anions was assayed by pre-loading all the  
152 cultured mesonephroi with 6-carboxyfluorescein (1  $\mu$ M) in KCM for 1 hour at 37°C,  
153 washing once in PBS, imaging briefly to confirm uptake, then incubating with either  
154 probenecid to block anion efflux (10mM, in KCM) or plain KCM culture medium for  
155 30-60 minutes at 37°C. Cultures were washed in PBS before imaging. Uptake of  
156 cations by the tubules of the mesonephroi was assayed by incubating live cultures  
157 with the fluorescent cation 4',6-Diamidino-2- phenylindole (DAPI) (1  $\mu$ M in KCM) for 30  
158 minutes either alone or in the presence of the cation transporter inhibitors cimetidine  
159 (200  $\mu$ M) and metformin (5mM). DAPI is bound stably to DNA once inside the cell  
160 nucleus and therefore the cultured mesonephroi could be imaged directly without the  
161 need to block efflux.

162

### 163 *Annexin V staining*

164 An Annexin V assay kit was used (BioVision; K103) for staining apoptotic nuclei.  
165 E11.5 mesonephroi were placed in Sebinger culture and incubated with DAPI  
166 without inhibitor as described above. The cultures were washed 3 times for 10 min at  
167 room temperature before incubating with Annexin V binding buffer for 10 min  
168 followed by incubation with Annexin V 1:100 in binding buffer for 5 min at 37°C.  
169 Cultures were kept in the dark and imaged immediately, directly in the culture dish,  
170 using a Nikon A1R inverted microscope (using TIRF attachment). Images were  
171 analysed with FIJI data analysis software.

172

### 173 *RT-PCR analysis*

174 Mesonephroi were dissected from the embryos of timed mated CD1 females at  
175 embryonic day E10.5 (20 pooled mesonephroi), E11.5 (20 pooled mesonephroi) or  
176 E12.5 (15 pooled mesonephroi) taking care to discard the gonads during dissection.  
177 Metanephroi were dissected from embryos of timed mated CD1 females at E13.5 (15  
178 pooled metanephroi) or E15.5 (10 pooled metanephroi). Adult mouse kidney RNA was  
179 extracted from a single adult male CD1 mouse. RNA was extracted using the  
180 RNeasy mini kit (Qiagen, Cat no. 74104) and RNA concentrations were determined  
181 using the Nanodrop system. cDNA was made using 2  $\mu$ g of RNA. Purity of RNA and  
182 absence of genomic DNA was confirmed by PCR for  $\beta$ -actin using primers that

183 span an intron (Table 1) and using cDNA preparations made with or without addition  
 184 of reverse transcriptase. PCRs for genes of interest were carried out in multiplex  
 185 reactions with primers for  $\beta$ -actin acting as loading controls. Less cDNA was used in  
 186 the positive control reactions in the gene-specific PCRs (from adult male) due to  
 187 relatively large amount of transporter transcript in adult kidneys (0.5  $\mu$ l instead of 2  $\mu$ l  
 188 of cDNA, equivalent to 33 ng and 133 ng of RNA respectively).

189

190

191

192

193 **Table 1**

194 **Primers for RT-PCR**

<b>Oat1</b>
Forward primer: ATGGTGGGAGTGTTACTGGG
Reverse primer: GGAGCCGGAAAATGCAGTAG
<b>Oat3</b>
Forward primer: TACAGTTGTCCGTGTCTGC
Reverse primer: TTCAGCTCCTCCACAGTGAG
<b>Oct1</b>
Forward primer: GTCCTTCGTTTGCAGACCTG
Reverse primer: TATTGGGTAGATGCGGCCA
<b>Oct2</b>
Forward primer: TGGGCATTGGTTACCTAGCA
Reverse primer: TTGCTGACCAGTCCCTGTAG
<b>Mat1</b>
Forward primer: ACTACCTGTCAGACCACGTG
Reverse primer: GGACGGATAGGCAAAGCTTG
<b>Mrp2</b>
Forward primer: ACACCAACCAGAAATGCGTC
Reverse primer: GGACAGAACAAGCCACAG
<b>Bcrp</b>
Forward primer: GAGTGGGTTTCTAGTCCGGA
Reverse primer: GAAATGGGCAGGTTGAGGTG
<b><math>\beta</math>-actin</b>
Forward primer: CTGGGACGACATGGAGGARA*
Reverse primer: AAGGAAGGCTGGAARAGWGC*

195 • R=50:50 A+G, W=50:50 A+T

196

197 *Animals*



198 This project involved no experiments conducted in living animals. Embryonic tissues  
199 used for post-mortem culture were obtained from healthy CD1 mice killed by trained  
200 staff of the UK Home Office-licensed animal house under Schedule 1 of the UK  
201 Animals (Scientific Procedures) Act 1986.

202

## 203 **Results**

204

205 *Uptake and efflux of fluorescent anions in the murine mesonephros and expression*  
206 *of organic anion transporters*

207

208 We have previously shown that murine embryonic kidneys developing *in vitro* are  
209 able to transport organic anions and cations using a series of uptake and efflux  
210 assays, and that they express known transporters both *in vivo* and *in vitro*.<sup>(12)</sup> Using  
211 a similar approach, we investigated the ability of the murine mesonephric tubules to  
212 take up the fluorescent anion 6-carboxyfluorescein (6-CF, 6-FAM). Transport activity  
213 of organic anion uptake transporters can be competitively inhibited by the uricosuric  
214 drug probenecid.<sup>(5, 15, 30)</sup> In this assay, the mesonephros is isolated from the  
215 murine embryo, and is then treated *in vitro* with the fluorescent anion 6-CF either  
216 with or without the inhibitor probenecid. Any accumulated fluorophore is then trapped  
217 in the cells that have taken it up by treating all samples with a high concentration of  
218 probenecid (Fig 1A). Mesonephric tubules took up 6-CF *in vitro* at E10.5, E11.5 and  
219 E12.5, and this uptake was abolished by the presence of probenecid, indicating  
220 specific uptake function in the mesonephric tubules through organic anion  
221 transporters (Fig 1B). Uptake assays were repeated 4 times (E10.5), 7 times (E11.5)  
222 and 2 times (E12.5) with all cultures assayed for each age displaying appropriate  
223 uptake (without probenecid) or blockage of uptake (treated with probenecid).  
224 Transcript analysis by semi-quantitative multiplex RT-PCR indicated that Oat1 and  
225 Oat3 transcripts were detectable from E10.5 to E12.5 (Fig 1C).

226

227 Transepithelial transport is an important mechanism for extrusion of organic ions and  
228 xenobiotics by renal tubules. Having shown that mouse mesonephric tubules can  
229 take up fluorescent anions through specific transporters, we next investigated their  
230 ability to efflux fluorescent anions. In the uptake assay, intracellular accumulation of  
231 the fluorescent anion 6-CF is inhibited by probenecid. In the retention assay, cells

232 are allowed to take up the fluorescent anion and then washed, followed by treatment  
233 with probenecid to block efflux, or with vehicle only to allow efflux through known  
234 extrusion pumps. Retention of the fluorophore when treated with probenecid  
235 indicates specific inhibition of the efflux transporters (Fig 2A). Mesonephric tubules  
236 were able to efflux 6-CF *in vitro* over the course of 15 minutes at E10.5, E11.5 and  
237 E12.5, and retention of the fluorophore in cultures treated with probenecid indicated  
238 that this transport could be inhibited specifically (Fig 2B). Anion efflux assays were  
239 repeated 3 times (E10.5), 4 times (E11.5), and 2 times (E12.5), with all cultures  
240 assayed at each age displaying appropriate efflux (without probenecid) or  
241 fluorophore retention (treated with probenecid). Transcript analysis of Mrp2 (an  
242 important anion efflux protein expressed in the proximal tubules of the developing  
243 murine metanephros)(10) and Bcrp (an efflux pump that can transport some anions)  
244 by multiplex RT-PCR indicated that Mrp2 is expressed from E10.5 to E12.5 with  
245 peak expression at E11.5, and that Bcrp is expressed in the mesonephric tubules of  
246 all ages analysed.

247

#### 248 *Uptake of fluorescent cations in the murine mesonephros and expression of organic* 249 *cation transporters*

250

251 Next, we qualitatively investigated the uptake of cations in the murine mesonephros by  
252 investigating the uptake of the cationic molecule 4',6-Diamidino-2-phenylindole (DAPI) into  
253 the tubules through cation transporters, and probing the specificity of uptake using  
254 competitive inhibitors of the Oct and Mate families, namely, cimetidine and metformin(2, 7,  
255 11, 18, 23) (Fig 3A). The cationic compound DAPI does not easily cross plasma  
256 membranes except via specific cation transporters, and emits blue fluorescence with  
257 higher intensity once intercalated with double-stranded DNA. Accumulation of blue  
258 fluorescence in the cells can therefore be taken to indicate transport across the epithelial  
259 cell membrane through cation transporters. Uptake of DAPI was observed in murine  
260 mesonephroi *in vitro* at E10.5, E11.5 and E12.5, and this accumulation was reduced or  
261 abolished when the cultures were co-treated with cimetidine and metformin (Fig 3B). Each  
262 experiment included both control (no inhibitor) and treated (with inhibitor) mesonephroi. For  
263 E10.5 mesonephroi, appropriate uptake of DAPI (control; without cimetidine and  
264 metformin) or no uptake of DAPI (treated; with cimetidine and metformin) was observed in  
265 5/6 experiments. For E11.5, appropriate uptake of DAPI (control) or no uptake (treated)

266 was observed in 2/3 experiments. For E12.5, appropriate uptake of DAPI (control) or no  
267 uptake (treated) was seen in every experiment. In 1/6 experiments (E10.5) and 1/3  
268 experiments (E11.5) no uptake of DAPI was seen in either control or treated cultures. This  
269 is likely due to a technical issue with the Sebring culture system that is prone to drying,  
270 but we cannot exclude the possibility that in a small proportion of mesonephroi of these  
271 ages, the cation transporters are not being expressed. In cultured mesonephroi some  
272 diffuse fluorescence was seen in all cultures regardless of treatment. This had previously  
273 been observed in metanephric cultures and was shown to be due to DAPI binding to the  
274 nuclei of apoptotic or dead cells with disrupted membranes, since these spots co-localised  
275 with the apoptotic marker Annexin V.(12) We tested this in our mesonephric cultures  
276 (Figure 3D), performing the cation uptake assay (without inhibitors) and then incubating  
277 with Annexin V. No Annexin V staining was seen in the DAPI-positive tubules, and  
278 Annexin V was seen to co-localise with bright, dense DAPI-positive nuclei outside the  
279 tubules indicating that the diffuse DAPI staining seen in these cultures are dead or dying  
280 cells. Using semi-quantitative multiplex RT-PCR we investigated the expression of known  
281 cation transporters in the murine mesonephros. We found that Oct1 and Oct2 transcripts  
282 were not detected, whereas Mate1 transcript was detected at all ages analysed (Fig 3C).

283

## 284 **Discussion**

285

286 The mammalian mesonephros is thought to confer some excretory function mainly  
287 due to the presence of glomeruli and likely production of urine, with previous studies  
288 focussed on larger mammals due to their larger and more well-developed  
289 mesonephroi. Directional transport across the tubule epithelia has been suggested  
290 by earlier studies, but the functionality of the mesonephros in rodents has not been  
291 studied. The mesonephros contributes to other aspects of mammalian development  
292 including the development of the male reproductive system, hematopoietic stem cells  
293 and vascular endothelial cells,(20) giving evolutionary rationale for the retention of  
294 this transient structure during mammalian development. Excretion and reabsorption  
295 of waste products and endogenous compounds by the mesonephros in the early  
296 mammalian embryo may also be important for development.

297

298 In this study, the tubules of the murine mesonephros was shown to be capable of  
299 both uptake and efflux of a fluorescent organic anion through membrane transporters

300 in an inhibitable manner. Transcripts for organic anion uptake transporters were  
301 detected in all ages investigated. In addition, the cationic compound DAPI was taken  
302 up by the tubules and this uptake could be competitively inhibited by co-treatment  
303 with inhibitors of the Oct and Mate families, cimetidine and metformin. DAPI has been  
304 shown in cultured human kidney cells to be a substrate for the renal organic cation  
305 transporters MATE1, MATE2K and OCT1, but not OCT2, OCT3 or the OCTN family.(29)  
306 As with humans, mice express Mate1 and Oct2 in the metanephric kidneys, and in  
307 contrast to humans, mice also express Oct1 in the kidneys (in humans, Oct1 is expressed  
308 mainly the liver).(9) Murine Mate2 is not expressed in the kidney, with expression highest  
309 in the testis in males and the colon in females(13) underlining species differences. Of the  
310 possible murine renal cation transporters for which DAPI is a substrate, only Mate1  
311 transcript was detected, indicating a different expression pattern than in the murine  
312 metanephros, where Oct1 and Oct2 are also expressed. The absence of Oct1 transcript  
313 suggests that uptake of DAPI in the murine mesonephric tubules is through the  
314 cation transporter Mate1. Mate1 is considered to be an extrusion protein for cations  
315 physiologically, with many compounds transported by Mate1 being exchanged for  
316 luminal H<sup>+</sup>, driving extrusion of cations into the lumen at the apical surface.  
317 However, DAPI has been shown to be taken up by Mate1 in a manner independent  
318 of H<sup>+</sup> concentration or pH,(29) suggesting that at least some cations can be  
319 transported by Mate1 in a facilitative manner. In addition, acid-base regulation in  
320 fetal development is largely carried out by maternal mechanisms, and therefore the  
321 luminal H<sup>+</sup> gradient seen in the post-natal kidney is not likely to be present in the  
322 mesonephros. Access to Mate1 by DAPI at the luminal surface is possible in our  
323 culture system because the Wolffian duct is severed during dissection, leaving the  
324 tubular system open at one end. Another possibility is that Mate1 is basolaterally  
325 located in the rodent mesonephros in contrast to the metanephros. There may also  
326 be an unknown cation transporter not yet identified that mediates cation uptake at  
327 the basolateral surface of the E10.5 to E12.5 rodent mesonephros.

328

329 This study adds to earlier work that suggests the mesonephros in some mammals is  
330 capable of excretory function. The presence of transcripts for known uptake and  
331 efflux transporters suggests that transepithelial reabsorption and excretion of  
332 xenobiotics and endogenous compounds occurs through known basolateral and  
333 apically localised transport proteins in addition to glomerular filtration. Together these

334 data offer evidence that the rodent mesonephros provides an early excretory  
335 mechanism for the developing embryo as well as its contribution to later  
336 developmental structures.

337

338

339 **Acknowledgements and author contributions**

340

341 M.L. Conception and design, acquisition and interpretation of data, drafting and revising

342 the manuscript and figures. J.S. acquisition of data. J.D. Conception and design,

343 interpretation of data, manuscript editing. All authors reviewed the manuscript. We thank

344 Chris Mills for reviewing the manuscript. This work was supported by the Medical

345 Research Council, grant MR/K010735/1 and by Kidney Research UK, grant

346 RP\_002\_20160223.

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439 Figure legends

440

441 Figure 1

442 **Murine mesonephric tubules transport organic anions.** (A) Anion uptake assay  
443 using specific anion transport inhibitor probenecid. (B) Specific tubular uptake of the  
444 anion 6-CF in tubules of E10.5, E11.5 and E12.5 murine mesonephroi. Scale bars  
445 represent 300  $\mu\text{m}$ . (C) RT-PCR analysis of basolateral anion uptake transporters.

446 Meta refers to the embryonic metanephros at the embryonic date specified. PC is  
447 positive control (adult male kidney) and NC is negative control (no template).

448

449 Figure 2

450 **Efflux of anions in murine mesonephric tubules.** (A) Anion efflux assay (retention  
451 assay) using the specific anion transport inhibitor probenecid. (B) Specific tubular  
452 efflux of the anion 6-CF in tubules of E10.5, E11.5 and E12.5 murine mesonephroi.

453 Scale bars represent 200  $\mu\text{m}$ . (C) RT-PCR analysis of apical anion efflux  
454 transporters. Meta refers to the embryonic metanephros at the embryonic date  
455 specified. PC is positive control (adult male kidney) and NC is negative control (no  
456 template).

457

458 Figure 3

459 **Murine mesonephric tubules transport organic cations.** (A) Cation uptake assay  
460 using specific cation transporter inhibitors metformin and cimetidine. (B) Specific  
461 tubular efflux of the cation DAPI in tubules of E10.5, E11.5 and E12.5 murine  
462 mesonephroi. Yellow arrows indicate tubules. Scale bars represent 300  $\mu\text{m}$ . (C) RT-

463 PCR analysis of basolateral and apical cation transporters. Meta refers to the  
464 embryonic metanephros at the embryonic date specified. PC is positive control (adult

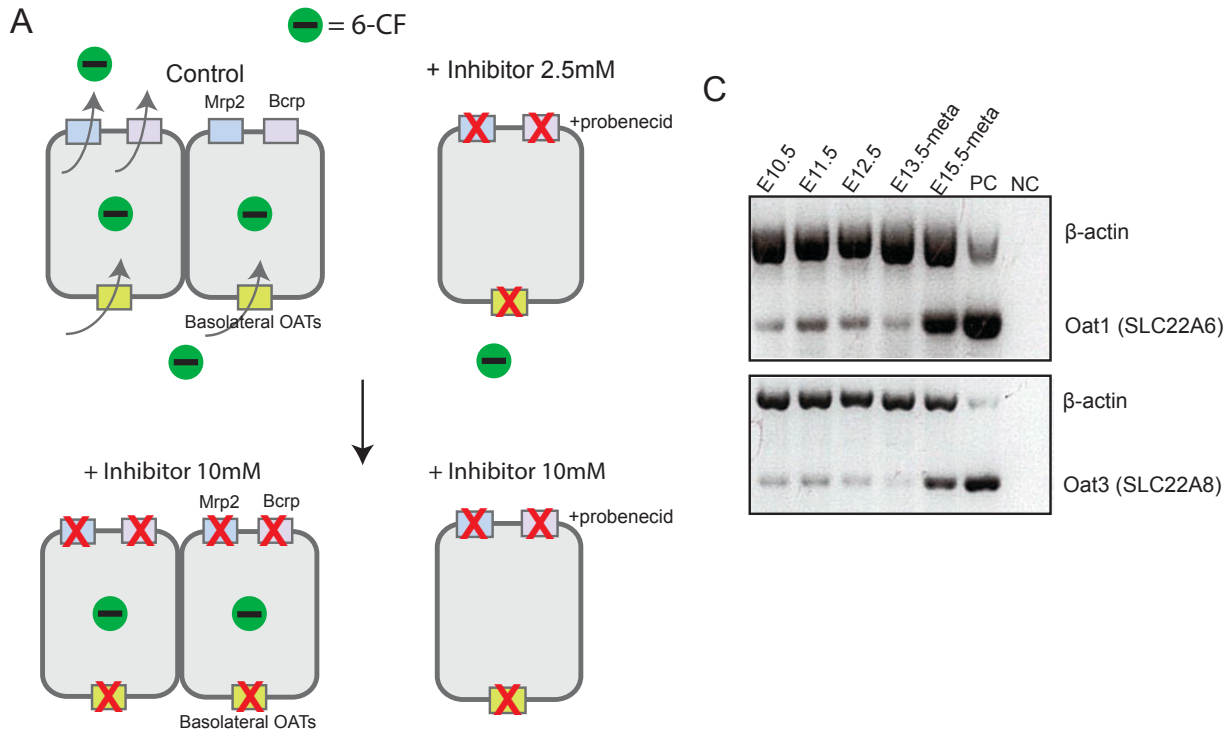
465 male kidney) and NC is negative control (no template). (D) Uptake of DAPI in E11.5

466 mesonephros in vitro, followed by incubation with Cy-5-conjugated Annexin V (which  
467 stains apoptotic cells). Annexin V colocalises with apoptotic DAPI-positive nuclei

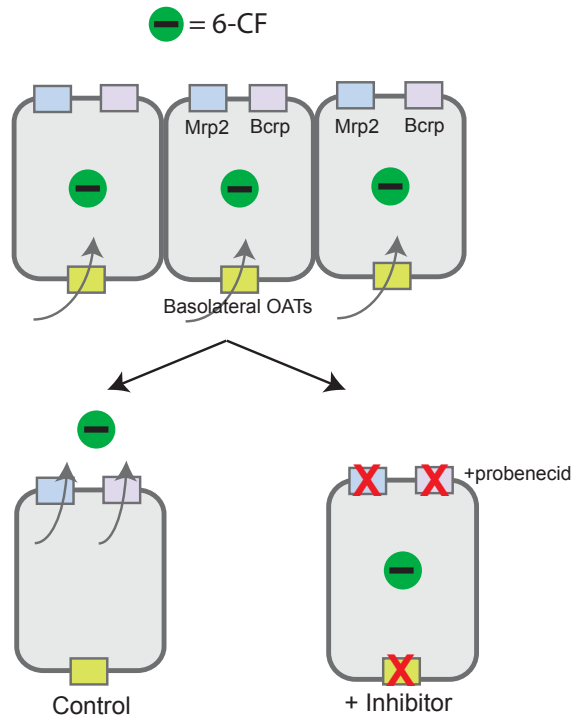
468 outside the tubules (box 1, box 3) but not with DAPI-positive cells within the tubules  
469 (box 2). Scale bars represent 50  $\mu\text{m}$ .

470

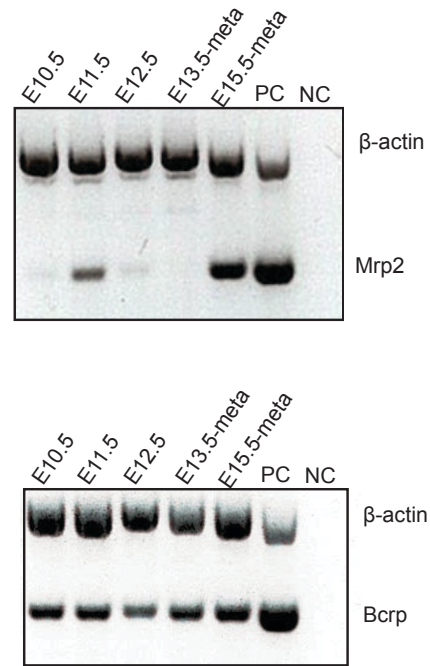




A



C



B

