Organic anion and cation transporter expression and function during embryonic kidney development and in organ culture models

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Organic anion and cation transporters (OATs, OCTs, and OCTNs) mediate the proximal tubular secretion of numerous clinically important compounds, including various commonly prescribed pharmaceuticals. Here, we report determination of the ontogeny of these transporters and of *NaPi2* and *SGLT1*, using quantitative polymerase chain reaction (QPCR) to determine expression levels of transporter genes in rat embryonic kidneys on each day of gestation from embryonic day (ed) 13 to ed18, in cultures of induced and uninduced metanephric mesenchyme (MM), and on each day of 1 week of whole embryonic kidney (WEK) culture. We also examined ontogeny of Oat1 protein expression in rat embryonic kidney by immunohistochemistry. Finally, we used uptake of fluorescein (FL) as a novel *in vitro* functional assay of OAT expression in WEK and MM. Developmental induction of OAT and OCT genes does not occur uniformly: some genes are induced early (e.g., *Oat1* and *Oat3*, potential early markers of proximal tubulogenesis), and others after kidney development is relatively advanced (e.g., *Oct1*, a potential marker of terminal differentiation). The ontogeny of transporter genes in WEK and MM is similar to that observed *in vivo*, indicating that these organ culture systems may represent convenient *in vitro* models to study the developmental induction of OATs, OCTs, and OCTNs. Functional transport was evidenced by accumulation of FL in the developing tubule in WEK and MM organ cultures. Our findings on the renal ontogeny of OATs and OCTs could carry implications both for the development of more rational therapeutics for premature infants, as well as for our understanding of proximal tubule differentiation.

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Many organic ions are excreted from the body via secretion mediated by specific transporters in the proximal tubule of the kidney. Such transporters are located on either the basolateral or apical surface of the proximal tubular cell, positioned so as to potentially mediate the successive steps of basolateral uptake and apical efflux that are required for tubular secretion. Individual transporters are also (broadly) specific for either organic anions or organic cations. Accordingly, four functional groups of these transporters might be distinguished, apical or basolateral organic anion transporters (OATs) and apical or basolateral organic cation transporters (OCTs/OCTNs). Notwithstanding these functional distinctions, OATs and OCTs/OCTNs are in fact phylogenetically related (as evidenced by their structural homology), and are accordingly grouped within the same gene family (*Slc22*,[1] in turn grouped within the Major Facilitator Superfamily (MFS) of solute carriers[2]).

These transporters are of considerable pharmacological and physiological interest, because their substrates include numerous commonly used drugs (encompassing antibiotics, antivirals, diuretics, non-steroidal anti-inflammatories, H2-blockers, and antihypertensives, among others). In addition, several physiologically important endogenous compounds are also substrates; these include urate, folate, prostaglandins, choline, and monoamine neurotransmitters (for recent reviews, see Eraly *et al.*,[3] Koepsell[4] and Wright and Dantzler[5]). This is further supported by studies in the Oat1 knockout mouse (see Note added in proof) Thus investigation of the ontogeny of OATs, OCTs and OCTNs will have significant implications for our understanding of the developmental maturation of the body's capacity to 'handle' many clinically important substances.

Following the molecular cloning of the genes encoding OATs and OCTs (reviewed in Eraly *et al.*,[3] Koepsell[4] and Wright and Dantzler[5]), there have been several studies of the developmental changes in expression of these genes in the early post-natal period.[6–8] By contrast, changes in gene expression occurring during embryogenesis have not been studied in detail, although renal developmental changes in Oat1 were described in the initial identification of this gene

as NKT.[9] In addition, we previously investigated the embryonic expression of *Oat1–3* and *Oct1* in mouse.[10] We discovered that renal expression of these genes (as determined by *in situ* hybridization and Northern blotting) is detectable as early as embryonic day (ed) 14, approximately coinciding with the onset of proximal tubular differentiation. We also discovered, unexpectedly, that these genes manifest transient embryonic expression in a variety of disparate tissues including the brain, spinal cord, aorta, heart, lung, liver, and bone, in addition to the kidney. These observations suggest a possible role for the encoded transporters in development, conceivably involving transport of morphogens.

A more careful analysis not only has implications for understanding the basic biology of proximal tubule differentiation, but may also bear on the ability of premature infants to handle a variety of drugs. We have now performed a detailed examination of the prenatal renal ontogeny of OATs and OCTs in rat. The specific genes examined were Oat1 (*slc22a6*;[9,11,12]), Oat2 (*slc22a7*;[13,14]), Oat3 (*slc22a8*;[15,16]), Oct1 (*slc22a1*;[17,18]), Oct2 (*slc22a2*;[19,20]), OCTN1 (*slc22a4*;[21]), and OCTN2 (*slc22a5*;[22–24]). Expression of the apical proximal tubular OATs, OAT4 (*slc22a11*;[25]) and RST/URAT1 (*slc22a12*;[26,27]), could not be examined because the corresponding rat orthologs, if they exist, have not yet been identified. We used quantitative polymerase chain reaction (QPCR) to determine expression levels of the above genes in rat embryonic kidneys on each day of gestation from ed13 to ed18, correlating our findings with expression levels of genes encoding other markers of proximal tubular differentiation, NaP_i2 (Na^+/phosphate co-transporter[28]) and SGLT1 (Na^+/glucose co-transporter[29]). We also examined the expression of OATs and OCTs in two *in vitro* models of kidney development, whole embryonic kidney (WEK) culture[30] and co-culture of metanephric mesenchyme (MM) with spinal cord.[31] We determined transporter expression in these systems both by QPCR as well as functionally, by assessment of transport of the fluorescent compound, fluorescein (FL), which is a specific OAT substrate.[32–34] We found that the ontogeny of transporter genes in either *in vitro* model was similar to that observed *in vivo*. Therefore, WEK and MM cultures may represent convenient *in vitro* models for study of function as well as of expression of OAT and OCT genes.

RESULTS

Transporter expression during embryonic kidney development

The expression profiles of 10 genes (*Oat1*, *Oat2*, *Oat3*, *Oct1*, *Oct2*, *OCTN1*, *OCTN2*, *SGLT1*, NaP_i2, and *β-actin*) during rat embryonic kidney development from ed13 to ed18 were examined by QPCR. Standard curves were generated for each gene of interest using a dilution series of reverse transcribed adult rat kidney RNA as template. Profiles were then generated for each gene of interest in the various tissue samples and values for their respective levels of expression extrapolated from the standard curves. These values were normalized to glyceraldehyde-3-phosphate dehydrogenase, and are presented as the ratio of the level of expression in the indicated tissue sample to the level of expression in adult kidney (Figure 1). For example, the value of $\sim 1 \times 10^{-1}$ for *Oat1* in ed18 kidney (Figure 1) means that the expression level of *Oat1* at ed18 is approximately one-tenth the level of *Oat1* expression in adult kidney; similarly, a value of 4×10^{-2} for *Oat3* at ed18 indicates its mRNA expression at this developmental time point is 25-fold lower (i.e., 1/25th) than in adult kidney (Figure 1).

Of all the genes examined, only *Oat2* expression was never detected in embryonic kidney. As *Oat2* message was readily detectable in newborn and adult rat kidney (not shown), its expression is likely to be upregulated between ed18 and birth. The expression patterns of the other genes fell into three groups: Group 1 genes manifested a generally progressive increase between ed13 and ed18; these included the OATs *Oat1* and *Oat3*, and the proximal tubular markers *SGLT1* and NaP_i2. Group 2 genes manifested by and large similar expression over the course of embryonic development. These genes included the organic cation transporter, *Oct2*, and the

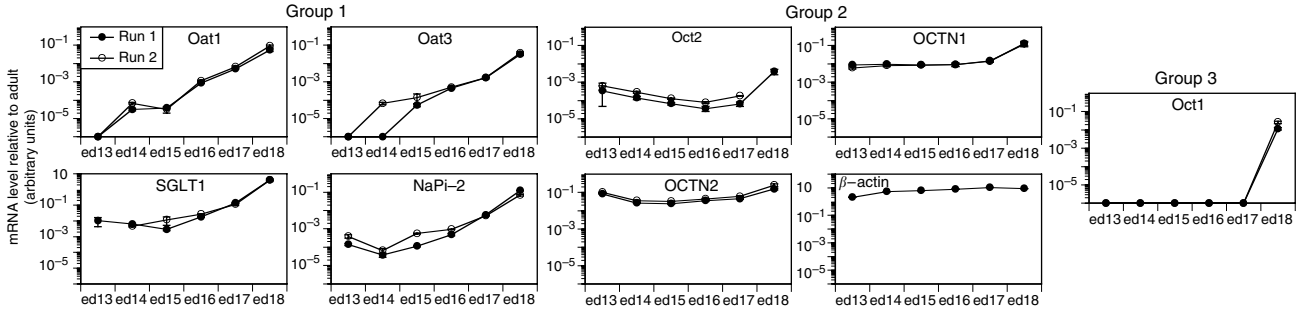

Figure 1 | Relative gene expression levels of OAT and OCT genes in embryonic kidney as determined by QPCR. RNA was prepared from embryonic kidneys at the indicated days of gestation, reverse-transcribed, and analyzed by QPCR (please refer to Materials and Methods for details). With the exception of *β-actin*, the expression of each gene at each time point was determined in samples prepared from two independent experiments (i.e., run 1 and run 2) and each sample was analyzed in triplicate. Data were normalized to the level of expression of glyceraldehyde-3-phosphate dehydrogenase in the same sample, and are reported as the ratio of the level of normalized gene expression in the sample of interest to the level of gene expression in adult kidney (mean values ± s.d.). *Oat2* was also examined; however, expression in embryonic kidney was never observed.

organic cation/carnitine transporters, *OCTN1* and *OCTN2*, and, as expected, the housekeeping gene, *β-actin*. Finally, *Oct1* was grouped separately as Group 3 as it manifested a sharp rise in expression relatively late in kidney development, around ed18.

We also examined the expression of Oat1 protein in rat embryonic kidney by immunohistochemical analysis of frozen kidney sections from ed15, ed17, and ed19 rat embryos. Distinct tubular staining was noted in ed19 kidneys, but not at the earlier gestational stages (Figure 2). This staining is essentially confined to the inner cortex (Figure 2a and b), which is consistent with observation that the most differentiated nephrons in the developing kidney are located near the medulla, while less mature nephrons predominate in the area just below the renal capsule, the so-termed nephrogenic zone.[35] Absence of detectable immunostaining in ed15 and ed17 rat embryonic kidneys is not unexpected, given that the QPCR data indicate that *Oat1* mRNA levels at these stages correspond, respectively, to one-ten thousandth and one-hundredth of the level in adult kidney (Figure 1).

Transporter expression in the induced MM culture model

Culture of the MM (the embryonic tissue which is destined to give rise to the nephrons of the kidney) in the presence of a suitable inducer has long been used to model nephrogenesis *in vitro*.[30,31] For example, following induction by embryonic spinal cord, cultured MM forms proximal tubule-like structures. We cultured spinal cord-induced and uninduced MM (Figure 3; see Materials and Methods for description of culture and induction of MM), and determined the expression of OAT and OCT genes (as above, *Oat1*, *Oat2*, *Oat3*, *Oct1*, *Oct2*, *OCTN1*, *OCTN2*, *SGLT1*, and *NaP$_i$2*) and of the control gene, *β-actin*, in the two culture systems (Figure 4). The expression of all the transporter genes, with the sole exception of *OCTN2*, was substantially upregulated by spinal cord induction (Group A in Figure 4). Expression of *OCTN2* was similar in the induced and uninduced cultures, as was that of the control *β-actin* gene (Group B).

OAT function in the induced MM culture model

We demonstrated the presence of functional OAT activity in induced MM by observing the inhibitor-sensitive uptake of the OAT substrate FL, which appears to be a specific substrate for Oat1 and Oat3[32–34] (Figure 5). FL uptake was completely blocked by 2 mM probenecid (Figure 5j–l) or 10 mM *p*-aminohippurate (data not shown). Moreover, FL accumulation occurred in a highly specific pattern being associated only with the induced tubule-like structures (Figure 5a–i). Observation at higher magnification revealed that the induced tubule-like structures appeared to consist of a single layer of cells arranged around a central fluid-filled space or lumen (Figure 5g). In correlation with this interpretation, FL was observed to be concentrated within this space (Figure 5h and i).

Transporter expression in the cultured kidney model

Culture of the WEK (derived from ed13 rat embryos) is a second *in vitro* system that is commonly used to model kidney development (Figure 6). We used QPCR to assess expression of *Oat1*, *Oat2*, *Oat3*, *Oct1*, *Oct2*, *OCTN1*, *OCTN2*, *SGLT1*, *NaP$_i$2*, and *β-actin* in WEK cultures (Figure 7). Two major patterns of gene expression could be discriminated: *Oat1*, *Oat3*, *Oct1*, *SGLT1*, and *NaP$_i$2* manifested a progressive

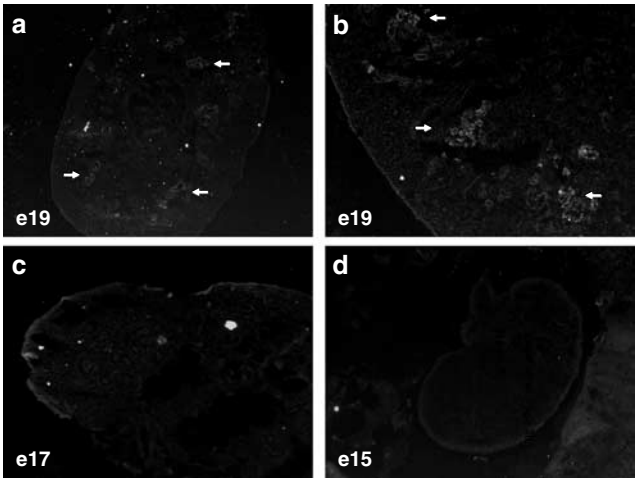

Figure 2 | Oat1 immunoreactivity in rat embryonic kidney. Frozen sections of kidneys from ed15, ed17, and ed19 rat embryos were incubated with an Oat1-specific antibody followed by binding to an Alex-fluor 568-conjugated secondary antibody. Images presented are representative of analysis of 8–12 sections at each embryonic stage. Specific staining is noted in tubular structures of the inner cortex of ed19 kidneys (**a** and **b**; arrows), but not in ed17 or ed15 kidneys (**c** and **d**). (**a**, **c**, **d**) Low-power images, original magnification × 4. (**b**) Higher power view, original magnification × 20.

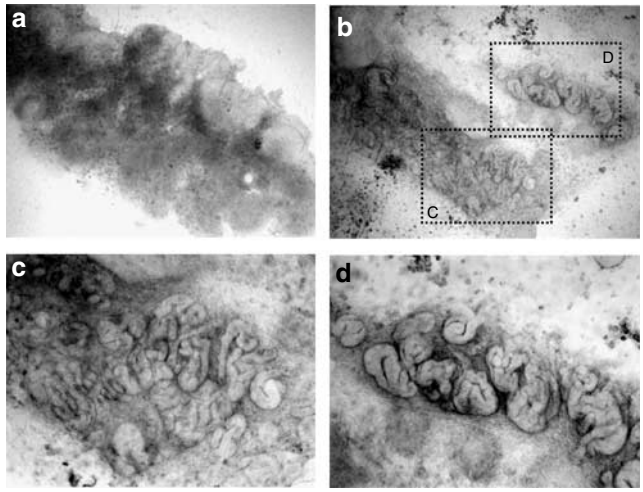

Figure 3 | Transmitted light micrographs of induced MM. Twenty-four hours of co-culture of ed13 MM with ed13 spinal cord induces the subsequent formation of renal tubule-like structures. (**a**) Rat ed13 MM tissue on Transwell filter membrane after 24 h of co-culture (spinal cord tissue has been removed). (**b**) Induced tubule-like structures 5 days after spinal cord-induction. (**c** and **d**): Higher magnification images of the regions indicated by dashed rectangles in panel b.

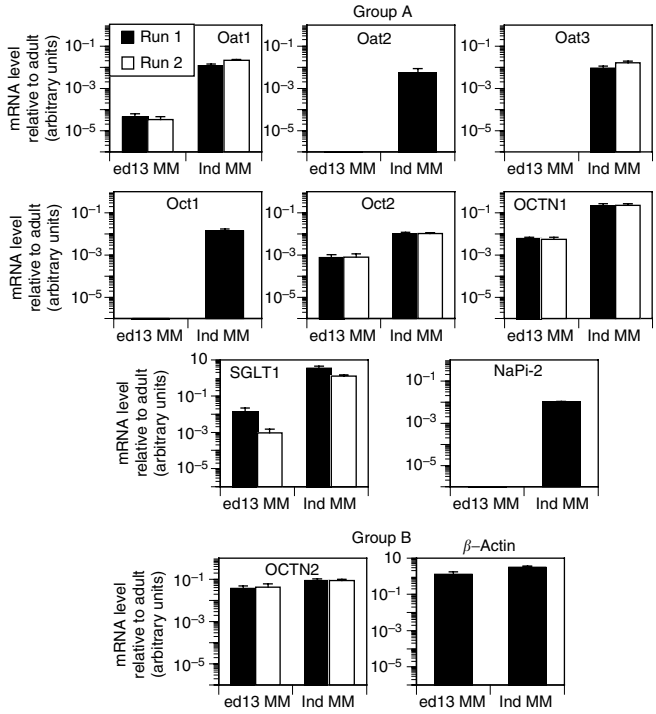

Figure 4 | Relative gene expression levels of the indicated transporter genes in induced and uninduced MM as determined by QPCR. RNA was prepared from cultures of induced and uninduced MM (refer to Materials and Methods for details), and QPCR analysis was performed as described in the legend for Figure 1. Gene expression of the examined transporter genes could be divided into two general groups, those substantially upregulated by spinal cord induction (Group A) and those essentially unchanged between the induced and uninduced cultures (Group B).

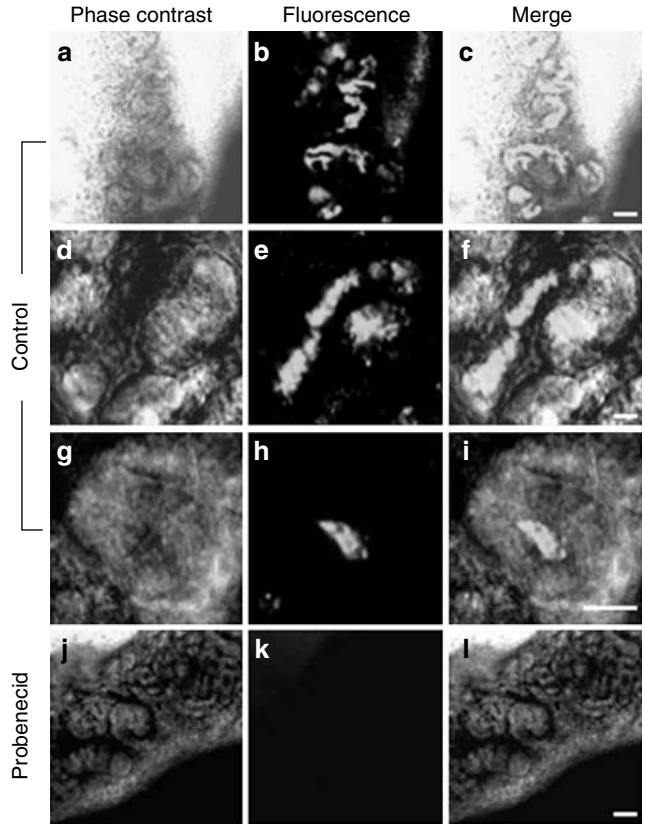

Figure 5 | Confocal microscopic images showing FL accumulation in induced MM. (**a–l**) MM tissue isolated from ed13 rat kidneys was co-cultured with ed13 spinal cord tissue for 24 h and then cultured for an additional 5 days following removal of the spinal cord tissue (refer to Materials and Methods for details). Confocal microscopic images were acquired 30 min after exposure to 2 μM FL in the culture medium in either the absence (Control; **a–i**) or presence (probenecid; **j–l**) of 2 mM probenecid. (**a**, **d**, **g**, **j**) Phase contrast photomicrographs of the induced epithelial tubular structures. (**b**, **e**, **h**, **k**) Corresponding fluorescence photomicrograph of the same tissue shown in previous panel. (**c**, **f**, **i**, **l**) Merged image of the two previous photomicrographs indicating the accumulation of FL in the induced MM. (**a–c**) Low magnification examination of a group of tubular structures in the induced mesenchyme in the absence of probenecid; Bar = 100 μM. (**d–f**) High magnification examination of tubular structures in the induced mesenchyme in the absence of probenecid; Bar = 20 μM. (**g–i**) Higher magnification examination of a tubular structure in the induced mesenchyme in the absence of probenecid; Bar = 20 μM. Note the accumulation of FL, to a concentration greater than the medium, in what appears to be a fluid filled space (presumptive lumen). (**j–l**) Low magnification examination of a group of tubular structures in the induced mesenchyme in the presence of 2 mM probenecid; Bar = 100 μM. Note the absence of concentrative FL accumulation.

increase in expression over the 7 days of WEK culture (Group A), whereas *Oct2*, *OCTN1*, *OCTN2*, and *β-actin* expression did not substantially change over the duration of culture (Group B). *Oat2*, which was not detected in embryonic kidneys *in vivo* (Figure 1), was also undetectable in WEK culture.

OAT function in the cultured kidney model

As with induced MM culture, we established the presence of OAT in WEK (cultured for 1 week) by demonstrating accumulation of FL in convoluted tubule-like structures within the organ culture (Figure 8a–f). These structures appeared to be continuous with a more deeply situated network of broader and straighter passages, presumably part of the collecting duct system (Figure 8g–i). As observed with MM, accumulation of FL in WEK was completely inhibited by probenecid (Figure 8j–l) or *p*-aminohippurate (Figure 9). Furthermore, we examined the time course of acquisition of transport function in WEK culture. Specific uptake of FL (*p*-aminohippurate- or probenecid-inhibitable uptake) was consistently noted following approximately 7 days of culture, with a further increase at 9 days of culture (Figure 9). This finding of increasing capacity for FL uptake is consistent with the progressive increase in *Oat1* and *Oat3* mRNA levels that occurs during organ culture (Figure 7).

DISCUSSION

We have closely examined the ontogeny of OAT and OCT expression during rat embryonic kidney development *in vivo*, using the highly sensitive method of QPCR. We find that *Oat1* and *Oat3*, the principal basolateral OATs of the proximal tubule,[9,11,12,15,16,36] as well as the proximal tubular markers, *SGLT1* and *NaP$_i$2*,[28,29] manifest a progressive increase in prenatal gene expression over the period examined, ed13–18 (Figure 1). Thus, these genes might be

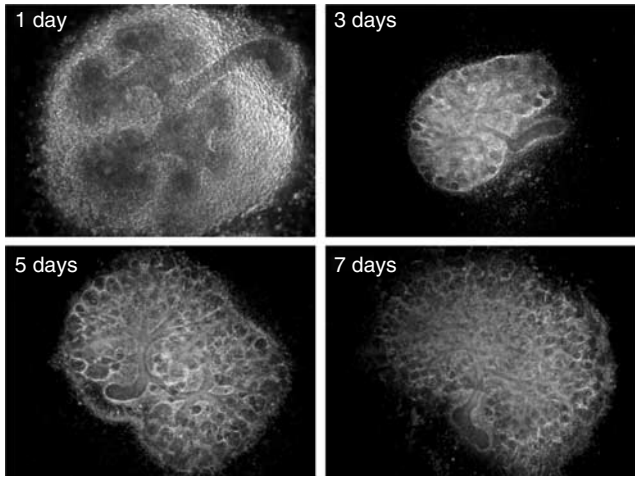

Figure 6 | Dark-field images of WEK. Culture of ed13 renal masses on transwell filter inserts for several days leads to the formation of small organ-like structures that resemble kidneys. The micrograph depicting WEK at 1 day of culture is at twice the magnification of the other micrographs.

considered relatively early markers of nephrogenesis (provided a sufficiently sensitive method of detection is employed). Of note, *Oat1* and *Oat3* are chromosomally paired and have similar expression patterns in adult tissue.[1,37] Therefore, their approximately co-temporal activation of expression during embryonic kidney development may represent an instance of coordinate transcriptional regulation of linked genes. Immunohistochemical analysis indicated that a detectable level of Oat1 protein expression occurs sometime between ed17 and ed19 (Figure 2). This corresponds to a period in embryonic kidney development where the expression level of *Oat1* mRNA exhibits at least a 10-fold increase from one-one hundredth (ed17) to one-tenth (ed18) of the level in adult kidney (Figure 1).

In contrast to the basolateral OATs, prenatal expression of the basolateral organic cation transporter, *Oct1*,[38,39] is undetectable till kidney development is fairly advanced, and therefore appears to be a marker of terminal differentiation. *Oct2*, the other basolateral organic cation transporter,[40,41] and *OCTN1* and *OCTN2*, apically located organic cation and carnitine transporters,[42,43] do not manifest substantial changes in expression level over the period of embryonic development studied. Finally, *Oat2* message, while expressed in adult rat and mouse kidney, was undetectable at any embryonic time point examined in this study (ed13–ed18). These findings are consistent with a recent study examining OAT mRNA expression levels in rat kidney starting at birth, in which no appreciable expression of renal *Oat2* mRNA was observed as late as 35 days of age.[7]

Just as the prior studies of transporter expression in the neonatal period[6–8] could have implications for drug dosing in pediatric patients, our results could ultimately contribute to the development of more rational therapeutics for premature infants. Moreover, identification of individual OATs and OCTs as potential early (*Oat1*, *Oat3*) or late (*Oct1*) markers

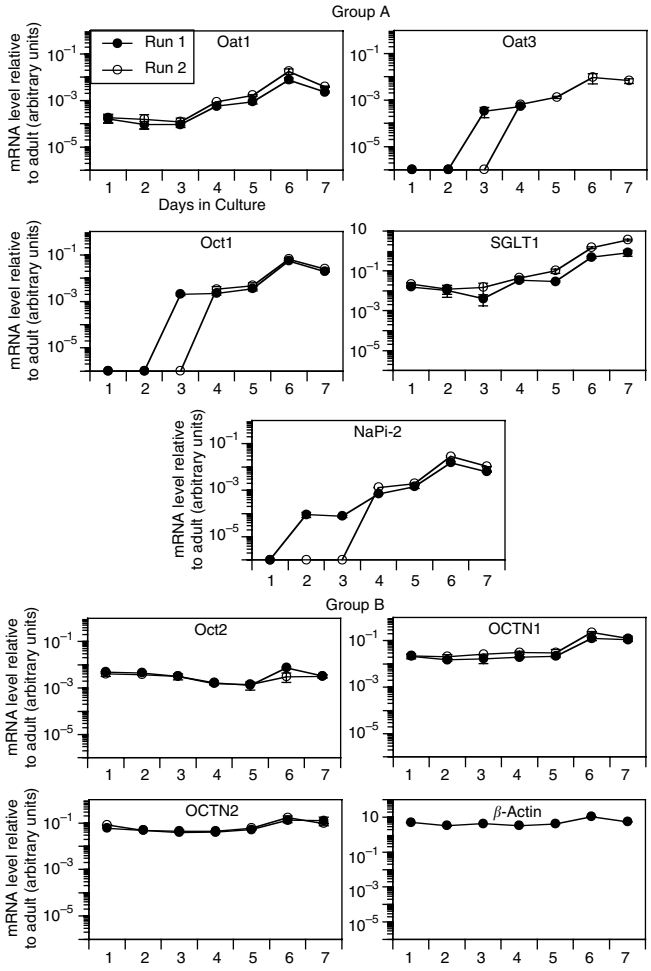

Figure 7 | Relative gene expression levels of the indicated transporter genes in WEK culture as determined by QPCR. RNA was prepared from cultures of WEK (please refer to Materials and Methods for details) and QPCR analysis performed as described in the legend for Figure 1, with the exception that each sample was analyzed in duplicate. Two major patterns of gene expression could be discriminated: *Oat1*, *Oat3*, *Oct1*, *SGLT1*, and *NaP$_i$2* manifested a progressive increase in expression over the seven days of WEK culture (Group A), whereas *Oct2*, *OCTN1*, *OCTN2*, and *β-actin* expression did not substantially change over the duration of culture (Group B). *Oat2*, which was not detected in embryonic kidneys *in vivo*, was also undetectable in WEK culture.

of proximal tubular differentiation could have significant implications for our understanding of the molecular mechanisms underlying this process. For example, expression of 'early' genes could be under the direct control of 'master' factors that are key regulators of proximal tubulogenesis. Indeed, analysis of the upstream regions of the *Oat1* and *Oat3* genes has revealed the presence of evolutionarily conserved binding sites for several transcription factors of demonstrated importance to kidney development, including PAX1, WT1, and HNF1, among others.[37] Of these factors, HNF1 seems particularly likely to be significantly involved in the maturation of the proximal tubule; it has been shown to induce the transcription of several proximal tubular transporters (including, of the ones examined in this study, SGLT1),[44–46]

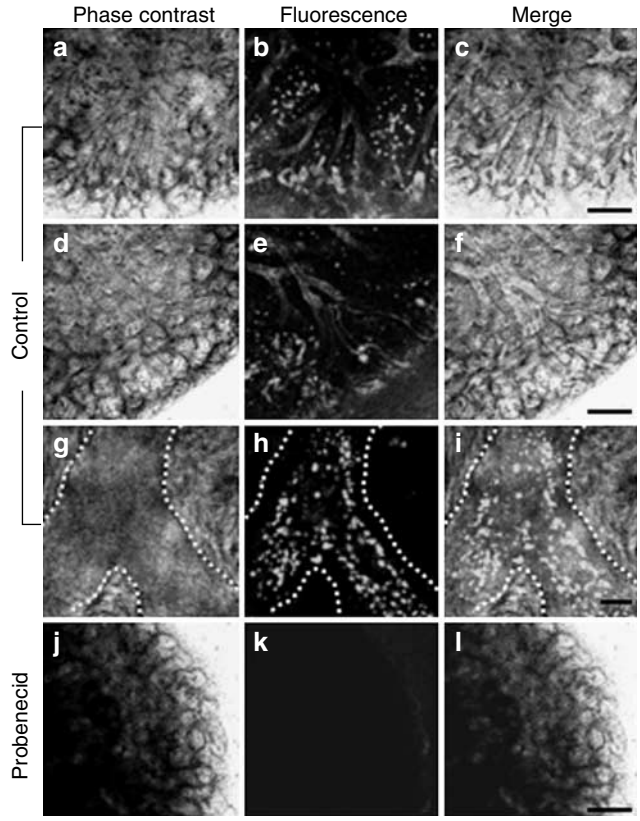

Figure 8 | Confocal images showing FL accumulation in cultured rat kidney. WEKs were isolated from ed13 rat fetuses and cultured for 7 days. Confocal images were acquired 30 min after exposure to 2 μM FL in the culture medium in either the absence (control; **a**–**i**) or presence (probenecid; **j**–**l**) of 2 mM probenecid. (**a**, **d**, **g**, **j**) Phase contrast photomicrographs of the cultured kidneys showing the tissue structure. (**b**, **e**, **h**, **k**) Corresponding fluorescence photomicrograph of the same tissue shown in previous panel. (**c**, **f**, **i**, **l**) Merged image of the two previous photomicrographs indicating the accumulation of FL in the cultured kidney. Note that the FL concentration exceeds the medium concentration in specific compartments of the renal tissue. There is an intense fluorescent signal within underlying convoluted tubule-like structures (presumptive proximal tubules) near the surface of the organ, and a less intense signal present in larger, straighter branching structures (presumptive collecting ducts connected to the rest of the nephron) situated more deeply. (**a**–**f**) Low magnification examination of kidneys cultured in the absence of probenecid; Bar = 200 μM. (**g**–**i**) High magnification examination of collecting ducts found in kidneys cultured in the absence of probenecid. Basolateral surface of collecting duct is indicated by dashed white line; Bar = 20 μM. (**j**–**l**) Low magnification examination of a kidney cultured in the presence of 2 mM probenecid; Bar = 200 μM.

and its knockout results in a renal Fanconi syndrome with urinary loss of glucose, amino acids, and phosphates.[47]

We have also examined transporter expression and function in two prominent *in vitro* models of nephrogenesis: WEK culture, and culture of induced MM (Figures 3–9). The 'organs' in these model systems grow and apparently differentiate during culture, with formation of tubules that bear a remarkable structural resemblance to developing nephrons *in vivo*.[30,31] We now find that expression patterns of OATs, OCTs, and OCTNs in these organ cultures also closely resemble those observed *in vivo*.

In the MM model, we find that all of the genes whose expression increases at some point during *in vivo* nephrogenesis (i.e., *Oat1*, *Oat3*, and *OCT1*) are also upregulated in induced MM (Figures 1 and 4). However, there is not an exact correspondence between induced MM and *in vivo* development, as the gene expression of *Oct2* and *OCTN1* appears to be upregulated in induced MM, but these genes do not manifest a clear trend toward increasing expression *in vivo*. Moreover a unique feature of the induced MM model is the striking upregulation of *Oat2* expression; the only gene examined whose expression was undetectable at any time in either embryonic kidney or WEK culture.

The WEK model recapitulates OAT and OCT gene expression patterns to an even closer degree (Figures 1 and 7). All the genes whose expression increases during *in vivo* nephrogenesis, but none of the genes whose expression does not, manifest increasing expression over the course of WEK culture.

Therefore, our results demonstrate that WEK and MM culture may represent convenient *in vitro* models for study of the developmental induction of OAT and OCT gene expression. In addition, our results demonstrate mediated transport of the OAT-substrate FL in these models. This latter finding suggests that the transporter genes expressed during embryonic development are, at least in some instances, functional. It also establishes an assay for investigation of the maturation of transporter function, as well as, potentially, for analysis of more general aspects of transporter function. In this regard, there are two important advantages of these *in vitro* organ culture systems that bear mentioning. First, in each system there appears to be an open lumen in which substrate accumulates, suggesting that these systems may be used to study not just basolateral uptake, but the luminal efflux step of renal solute transport as well. Second, all aspects of gene expression are under endogenous promoter control and, thus, may more accurately reflect the *in vivo* pathways of gene regulation and expression and *in vivo* transporter protein levels.

MATERIALS AND METHODS

Culture of WEK

Uteri from timed pregnant Sprague–Dawley rats at day 13 of gestation (day 0 of gestation being the day of appearance of the vaginal plug) were removed and the embryos dissected free of any surrounding membranes and tissue. Kidneys were isolated from the embryos and placed on polycarbonate Transwell filters with no separation of males and females. The organ cultures were maintained in DME/F12 (MediaTech, Herndon, VA) supplemented with 10% fetal bovine serum up to 7 days.

Isolation and culture of MM

Embryonic kidneys, isolated as described above, were incubated in L-15 media containing 0.1% trypsin and 0.05 U/μl DNase I for 15 min 37°C (no separation of males and females). Trypsinization was stopped by the addition of fetal bovine serum (final concentration of 10%) and the MM was dissected free of the ureteric bud using minutia pins. Pieces of embryonic spinal cord were also dissected free of surrounding tissues and collected for use

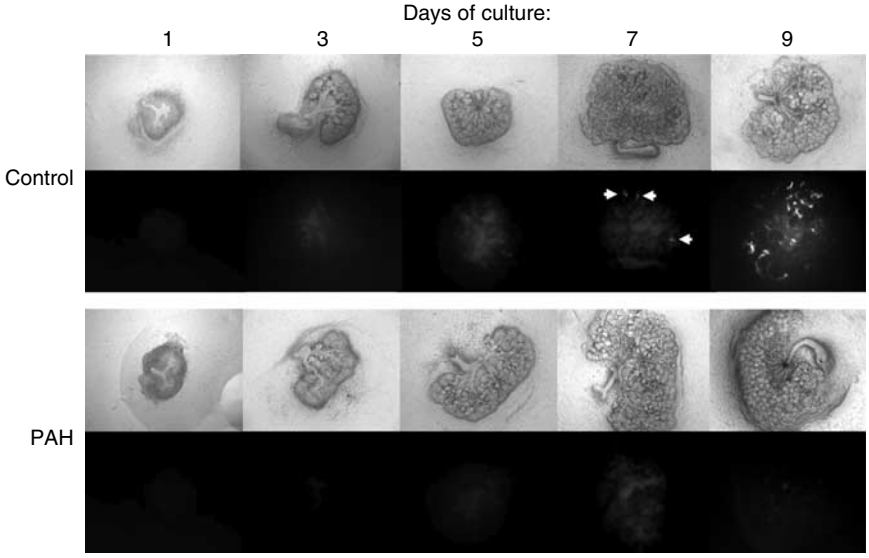

Figure 9 | Maturation of transport function in WEK culture. WEKs were dissected from ed13 rat embryos and 30 min uptake of 2 μM FL (in the presence and absence of inhibitor) was determined by fluorescence microscopy after the indicated number of days in culture (1, 3, 5, 7, and 9). Regions of high fluorescence accumulation consistently appeared after 7 days in culture (indicated by arrows in control day 7). The capacity for FL uptake continued to increase over the 9 days of WEK culture (control; 1–9). Uptake was inhibited by 1 mM *p*-aminohippurate (7 and 9). Images depicted are bright-field micrographs with their corresponding fluorescence micrographs and are representative of multiple replicate experiments. All images were acquired with identical camera and microscope settings.

as the heterologous inducer of the MM. Embryonic spinal cord was applied to the top of a polycarbonate Transwell filter and isolated MM was placed in contact with the embryonic spinal cord. Tissues were maintained in DME/F12 (MediaTech, Herndon, VA) supplemented with 10% fetal bovine serum. After 24 h of induction, the spinal cord tissue was removed and the induced MM cultured for an additional 5 days.

Quantitative polymerase chain reaction

All RNA samples were isolated using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) with inclusion of the optional DNase I digestion step in the protocol. RNA isolated from adult rat kidney was reverse transcribed and used as template for standard curve reactions for each primer pair. Standard curve values were obtained for 0.01, 0.1, 1, and 10 ng dilutions of the original amount of RNA that was reverse transcribed. No-template control reactions for each primer pair were incorporated into each QPCR run. Primer pair sequences used for QPCR analysis are listed in Table 1, final concentration in QPCR reactions was 50 nM. Reactions were set up in 96-well plates using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications and samples were run on an ABI Prism 7700 sequence detection system at the UCSD QPCR core facility. Cycle parameters were as follows: initial 95°C denaturation step for 10 min followed by 40 cycles of 95°C denature for 20 s, 60°C annealing for 20 s, and 72°C extension for 45 s. The data were analyzed using Sequence Detector v1.7 software. Data were normalized to the level of expression of glyceraldehyde-3-phosphate dehydrogenase in the same sample, and are reported as the ratio of the level of normalized gene expression in the sample of interest to the level of gene expression in adult kidney (mean value $\pm$ s.d.; embryonic kidney and MM were triplicate samples; cultured kidney, duplicate samples).

Sample tissues were obtained as follows: embryonic kidneys: renal masses were directly dissected from ed13 to ed18 fetal rats and pooled for RNA isolation; cultured kidneys: renal masses were dissected from ed13 fetal rats, cultured on Transwell support membranes for 1–7 days (as described above in embryonic kidney culture), and RNA isolated from pooled tissue; Induced MM: pooled MM tissue isolated from 13–16 ed13 rat fetuses was co-cultured with ed13 spinal cord for 24 h and then cultured for an additional 5 days after the removal of the spinal cord tissue (as described above in MM culture), RNA from the induced MM was then isolated from pooled tissue.

Each RNA sample was reverse transcribed using oligo-dT and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega

Table 1 | Oligonucleotide primer pairs used for QPCR analysis

Oat1for:	5′-ATGCCTATCCACACCCGTGC-3′
Oat1rev:	5′-GGCAAAGCTAGTGGCAAACC-3′
Oat2for:	5′-TAGCTCCTGCCTCCGATTTG-3′
Oat2rev:	5′-ACCACGGCTCCCACCAGCAC-3′
Oat3for:	5′-TGAGAAGTGTCTCCGCTTCG-3′
Oat3rev:	5′-CTGTAGCCAGCGCCACTGAG-3′
Oct1for:	5′-TGGCTGGGTATACGACACTC-3′
Oct1rev:	5′-TCCTGTAGCCAGAGCCGACA-3′
Oct2for:	5′-CTGGTGCATACCGGAATCTC-3′
Oct2rev:	5′-GAACAGAGCTCGTGAACCAA-3′
OCTN1for:	5′-ACTTCGAACCCTGCCTCGGAG-3′
OCTN1rev:	5′-CTTCTCTGTCCATGGAGACTG-3′
OCTN2for:	5′-CCATATCAGTGGGCTACTTCG-3′
OCTN2rev:	5′-CCCTGAGCATCTGGTCAATGG-3′
SGLT1for:	5′-TATACCTGGCCATTTTCATCC-3′
SGLT1rev:	5′-AAGGAGCTCATGAGGGAAGCC-3′
NaPi2for:	5′-CGACATCCATCATTGTCAGCA-3′
NaPi2rev:	5′-TTTCTCCATGGTGGTGTTTGC-3′
β-actinfor:	5′-CTTCCAGCCTTCCTTCCTGGGTATGG-3′
β-actinrev:	5′-GCGGACTGTTACTGAGCTGCGTTTTA-3′
GAPDHfor:	5′-TGCACCACCAACTGCTTAGC-3′
GAPDHrev:	5′-TGGATGCAGGGATGATGTTC-3′

Corporation, Madison, WI) in reactions containing 2 μg of RNA per 25 μl of reaction volume. Each QPCR reaction contained 1 μl of reverse transcription reaction as template with the exception of the ed18 kidney samples for which the template was diluted 10-fold. Standard curves and all samples to be compared (e.g., ed13–ed18 or kidney cultured for 1–7 days) were analyzed on the same QPCR plate. With the exception of *β-actin* in all systems, and *Oat2*, *Oct1*, and *NaP$_i$2* in the induced MM cultures, the expression of each gene was determined in samples prepared from two independent experiments (i.e., run 1 and run 2).

Immunohistochemistry

Frozen sections were prepared from kidneys dissected from ed15, ed17, and ed19 rat embryos. These sections were rehydrated in phosphate-buffered saline (PBS) for 10 min, then treated with boiling water for 15 min to expose cryptic antigenic sites, followed by washing in PBS for 15 min. Sections were next incubated with 1% bovine serum albumin in PBS for 15 min, to decrease nonspecific binding of antibody, then with the Oat1 antibody (Alpha Diagnostic Inc., San Antonio, TX; diluted 1:500 in PBS) at 4°C overnight. Antibody-treated sections were washed twice in high-salt PBS (containing 2.7% NaCl) and then twice in regular PBS (5 min per wash). Binding of secondary antibody (Alexa-fluor 568 diluted 1:1000; Molecular Probes, Eugene, OR) was performed at room temperature for 2 h, followed by washes as above. Finally, sections were mounted in a fluorescence fading-retardant before visualization by fluorescence microscopy.

FL uptake assays

Embryonic day 13 kidneys cultured for 1–9 days or induced MM cultured for 6 days were exposed to 2 μM FL in the culture medium (with phenol red, antibiotics, and serum omitted) for 30 min in the absence or presence of inhibitors (1–2 mM probenecid; 1 mM *p*-aminohippurate). Following the uptake period, the organ cultures were washed twice with ice-cold PBS before examination by confocal fluorescence microscopy. Before the confocal microscopy of experimental groups, control tissues (no exposure to FL) were examined and microscope levels set such that no background fluorescence was detectable.

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Note added in proof

Eraly SA, Vallon V, Vaughn DA *et al*. Decreased renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knockout mice. *J Biol Chem*; First published December 14, 2005; doi:10.1074/jbc.M508050200.

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