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Expression of metanephric nephron-patterning genes in differentiating mesonephric tubules.

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Abstract

The metanephros is the functional organ in adult amniotes while the mesonephros degenerates. However, parallel tubulogenetic events are thought to exist between mesonephros and metanephros. Mesonephric tubules are retained in males and differentiate into efferent ducts of the male reproductive tract. By examining the murine mesonephric expression of markers of distinct stages and regions of metanephric nephrons during tubule formation and patterning, we provide further evidence to support this common morphogenetic mechanism. Renal vesicle, early proximal and distal tubule, loop of Henle and renal corpuscle genes were expressed by mesonephric tubules. *Vip*, *Slc6a20b* and *Slc18a1* were male-specific. In contrast, mining of the GUDMAP database identified candidate late mesonephros-specific genes, 10 of which were restricted to the male. Amongst the male-specific genes are candidates for regulating ion/fluid balance within the efferent ducts, thereby regulating sperm maturation and genes marking tubule-associated neurons potentially critical for normal male reproductive tract function.

Keywords

mesonephros; metanephros; nephron; gene expression; efferent duct; male reproductive tract

Introduction

In vertebrates, three distinct excretory organs can develop from the intermediate mesoderm (IM). The most basic of these, the pronephros, functions during development in some species but is replaced by the mesonephros as the functional postnatal excretory organ of amphibians and fish. Higher organisms develop a third pair of excretory organs, the metanephros. While the pronephros and mesonephros form in amniotes, both regress leaving the metanephros as the functional postnatal kidney. The induction of these organs involves two paired nephric ducts (ND; Wolffian ducts) which arise from the IM posterior to the forelimb bud (8.0 dpc, days post coitum, in mice). In mice, the mesonephros arises from the rostral IM around 9.0 dpc in response to signals from the ND, while the metanephros forms at the most caudal end of the ND adjacent to the hindlimb at 10.5 dpc (Saxen, 1987; Sainio et al., 1997; Sainio and Raatikainen-Ahokas, 1999). Between 9.0 and 10.5 dpc, mesenchymal condensations parallel with the ND develop within the mesonephros, representing the first signs of mesonephric tubule formation (Smith et al., 1991; Sainio et al.,

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1997; see Figure 1 A). These epithelial tubules have been classified into cranial tubules (Figure 1 A, MTcr), which have a connection with the ND, and caudal tubules (Figure 1 A, MTca) located within the mesenchyme and not connected to the ND (Smith et al., 1991; Sainio et al., 1997). While it was initially proposed that the cranial tubules developed as outgrowths from the ND, fate-mapping has shown that both cranial and caudal tubules develop via a mesenchyme-to-epithelial transition (MET) within the mesonephric mesenchyme (Mugford et al., 2008). This suggests a very similar process of tubule formation to the metanephric nephrons.

While the mesonephros degenerates from 13.5 dpc, some elements of this organ persist. In the male, the cranial mesonephric tubules develop into the efferent ducts, the anterior ND develops into the epididymis and vas deferens and the caudal ND into the seminal vesicles. The tubule-derived efferent ducts play a role in sperm maturation and are essential for male fertility. In both sexes, the ND is also involved in the induction of the paramesonephric duct (PMD) or Müllerian duct, which arises at the rostral tip of each mesonephros at 12.5 dpc and extends caudally in parallel to the ND (reveiewed by Yin and Ma, 2005; Klattig and Englert, 2007; Massé et al., 2009) (Figure 1 A). In males, the PMD degenerates. In contrast, in females, the ND degenerates and the PMD gives rise to the oviducts, uterine horns and upper part of the vagina (Saxen, 1987; Sainio et al., 1997; Sainio and Raatikainen-Ahokas, 1999; Tilmann and Capel, 2002; Vize et al., 2002). While the development of the metanephric kidney has been extensively studied, little is known about the molecular mechanisms involved in the formation of the mesonephric tubules and the subsequent regulation of efferent duct differentiation and maturation in the male reproductive tract.

Our current understanding of the molecular basis of mesonephric development is drawn from studies on metanephric development. Mutant mice displaying metanephric defects frequently show mesonephric defects due to the significant overlap in the pathways involved. For example, induction of both the metanephric and mesonephric tubules is dependent on the ND (Saxen, 1987; Grobstein, 1953; Carroll et al., 2005) with Wnt signaling responsible for the initial induction of MET in both tubule types. Wnt9b is secreted by the ND (Carroll et al., 2005) and initiates the tubulogenic program involving Fgf8 (Perantoni et al., 2005; Grieshammer et al., 2005) and Wnt4 (Stark et al., 1994; Vainio et al., 1999) in the nascent tubules of both organs (see Figure 1 B). Hence, loss of *Wnt9b* results in a complete loss of all mesonephric tubules and the loss of metanephric MET. Other genes common to the formation of both tubule types include *Pax2* (Torres et al., 1995; Bouchard et al., 2002; Grote et al., 2006; Narlis et al., 2007), *Lhx1* (Kobayashi et al., 2004; Shawlot and Behringer, 1995), *Osr1* (Wang et al., 2005; James et al., 2006), *Sall1* (Nishinakamura et al., 2001; Ott et al., 2001), *Six1* (Xu et al., 2003; Kobayashi et al., 2007) and *Wt1* (Sainio et al., 1997). Despite this congruence, the mature tubules that develop in each organ are distinctly different, with only the metanephric tubules of the kidney forming functional excretory nephrons in the mouse.

One obvious divergence in structure between the metanephros and mesonephros lies in the branching collecting duct of the metanephros, a structure that does not exist within the mesonephros. Indeed, expression of *Gdnf* is restricted to the metanephric mesenchyme (MM) and it is this ligand that induces the upregulation of *Ret* and its co-receptor *Gfra1* in

the adjacent ND to initiate ureteric bud outgrowth. This distinction in developmental patterning between metanephric and mesonephric development is in part due to the expression of the Hox11 paralogs, *Hoxa11*, *Hoxc11* and *Hoxd11*. Loss of these transcription factors within the IM results in failure to express crucial factors in the caudal IM, including *Gdnf* and *Six2*. The result is renal agenesis without affecting the mesonephros (Wellik et al., 2002; Mugford et al., 2008). Conversely, when *Hoxd11* is ectopically expressed in the mesonephric mesenchyme, *Six2* is ectopically activated, inducing additional tubule formation in the mesonephros without induction of *Gdnf* expression (Mugford et al., 2008).

While it has been proposed that there is considerable overlap in the processes of tubule formation and patterning between mesonephros and metanephros, there is debate on how similar these processes are. Some studies have suggested that mesonephric tubules lack a juxtaglomerular apparatus and segments of the loop of Henle (Vize et al., 2002), while glomerulus-like structures are rare and restricted to MTcr (Smith et al., 1991; Sainio et al., 1997). However, other studies revealed mesonephric tubular expression of some but not all markers of differentiated loop of Henle segments with markers of more distal segments absent (Mugford et al., 2008), leaving open the question of parallels in developmental programs between the two tissues. In this study, we have investigated this question further taking advantage of our recent studies defining an atlas of gene expression during metanephric development (Brunskill et al., 2008). From that atlas, we have previously identified and validated genes enriched in the renal vesicle (RV), the first stage of nephron development (Georgas et al., 2009), and defined a set of anchor genes specifically expressed in distinct developmental stages or compartments of the metanephric nephrons (Thiagarajan et al., 2011). Here we reexamine the expression of these RV-enriched and tubular compartment anchor genes during murine mesonephric development from 10.5 to 13.5 dpc using wholemount *in situ* hybridization. Our results extend the number of genes expressed during both mesonephric and metanephric tubule formation and patterning. The strong congruence between RV and early mesonephric tubule expression supports the existence of a common pathway for tubule initiation and primary patterning between the two organs. A review of the Genitourinary Development Molecular Atlas Project (GUDMAP; www.gudmap.org; McMahon et al., 2008) database looking for evidence of differences between these pathways identified 9 early mesonephric-specific genes and 4 metanephric-specific genes. While these 9 genes were differentially expressed in mesonephric and not metanephric tubules, they were expressed in other metanephric structures, including UB and MM. The metanephric-specific genes were not expressed in any stage of mesonephric tubule development. Of note, a subset of 3 mesonephric/metanephric tubule anchor genes showed expression specific to male mesonephric tubules at 13.5 dpc. A further 10 genes expressed specifically in male mesonephric tubules were also identified via a review of GUDMAP database. This suggests a sexually dimorphic divergence in the differentiation of mesonephric tubular structures, presumably reflecting specific requirements for efferent duct function.

Results

Metanephric renal vesicle genes are expressed in early mesonephric tubules but not retained in mature mesonephric tubules

Using a set of genes up-regulated in the renal vesicle (RV) of the developing nephron at 15.5 dpc (Georgas et al., 2009), we reexamined the expression of these genes using wholemount *in situ* hybridization (WISH) at two earlier stages in both the metanephros and mesonephros (10.5 dpc and 12.5 and/or 13.5 dpc). At 10.5 dpc, early mesonephric tubules are visible and the metanephric mesenchyme (MM) is first observed flanking the caudal ND. From 12.5 to 13.5 dpc, early nephrons are readily identifiable within the metanephros and more mature mesonephric tubules are present within the mesonephroi of both sexes. The resulting expression patterns were annotated using the anatomical ontology generated for the mouse urogenital system (Little et al., 2007). Of the RV-enriched genes expressed in early nephrons at 15.5 dpc (Georgas et al., 2009) 21 genes were identified in the early nephrons of the 12.5–13.5 dpc kidney, and all of these were also expressed in 10.5 dpc mesonephric tubules (Table 1). Representative images are shown in Figure 2. These included genes previously shown to distinguish distal RV (*Dkk1*, *Greb1*, *Lhx1*, *Papss2*, *Pcsk9*, *Pou3f3*, *Jag1*, *Wnt4*), proximal RV markers (*Tmem100*, *Wt1*) and markers of the early connecting segment of the RV (*Lhx1*, *Ltbp1*, *Pax8*, *Pou3f3*). Where possible, the mesonephric tubules were subdivided into MTcr and MTca based on the presence (MTcr) or absence (MTca) of a connecting segment joining the MT to the ND. The majority of genes showed expression in both types, with the exception of *Npy* which appeared to be specific to MTca. This may simply reflect the earlier stage of development of MTca with respect to MTcr. Of note, *Pou3f3* (*Brn1*), an early marker of the connecting segment in the metanephric kidney, was more strongly expressed in MTcr, again possibly reflecting a more advanced stage of development and / or a connection with the ND. While MTcr and MTca differ in the appearance of a direct connection with the ND, the question of whether these represent distinct tubular populations or a temporal developmental series (cranial more advanced than caudal) has not been determined. However, it has been stated that MTca regress leaving the cranial MTcr to differentiate into the efferent ducts of the epididymis, thereby providing a connection with the testis via the rete testis (Sainio et al., 1997). *Dkk1*, *Lhx1* and *Pdgfa* were the only genes expressed in the ND/mesenchyme-derived connecting tubule component of the cranial MT connected to the ND and all three were also expressed in the ND itself. Several other genes also showed expression in the ND (*Pax8*, *Pcsk9*, *Pou3f3*, *Ano1*, *Wnt4*) or in the mesonephric mesenchyme (*Greb1*, *Ltbp1*, *Wnt4*, *Wt1*). At this early stage in metanephric development, only the MM is present, the ureteric bud has not invaded and there are no nephrons. Several genes were present in the MM at 10.5 dpc including *Greb1*, *Papss2*, *Tmem100* and *Wt1*, however only *Wt1* was maintained in the cap mesenchyme later in metanephric development (Georgas et al., 2009). In contrast *Cdh4* and *Pax8*, expressed in the cap mesenchyme at 15.5 dpc (Georgas et al., 2009), were absent in the MM at 10.5 dpc.

RV-enriched genes were also examined at 12.5–13.5 dpc to see if their expression was maintained in more developed mesonephric tubules (Table 1). Only 5/18 genes examined at these stages (*Cpb2*, *Ctxn3*, *Dkk1*, *Lhx1*, *Pdgfa*) were expressed in the more mature mesonephric tubules (Figure 3). While the majority of RV genes showed continued

expression in more developed nephron tubules of the kidney, most of these genes did not persist in the mesonephric tubules. As well as expression in the mesonephric tubules, these RV-enriched genes were also expressed in PMD (12 genes), ND (3 genes) or mesonephric mesenchyme (5 genes) (Table 1).

Early proximal tubule metanephric genes are expressed in more mature mesonephric tubules

To investigate congruence in proximal tubular patterning in mesonephric versus metanephric tubules, we then examined the expression pattern of a set of 13 genes previously identified as specific to the early proximal tubule (EPT) segment of the developing kidney (Thiagarajan et al., 2011). In addition, expression of *Scnn1b*, a marker of early distal tubule, and one marker of renal corpuscle (*Vip*) were also examined. While most genes examined (11/13) were also expressed in the 13.5 dpc mesonephric tubules, only *Acaa1b* was expressed in the early mesonephric tubules (Table 2, Figure 4). Our previous analysis suggested that none of these genes were expressed before stage III nephron (Thiagarjan et al., 2011). As anticipated, no expression was seen in the 13.5 dpc metanephros. By 13.5 dpc, gene expression was detected within the mesonephric tubules for markers from all three proximal tubular segments (S1–S3). Of note, expression of two proximal tubule genes (*Slc6a20b*, *Slc18a1*) was restricted to the male mesonephros at 13.5 dpc (Table 2, Figure 4).

Scnn1b has previously been described in the medullary collecting duct, however it is also expressed in the cortical collecting duct and early distal tubules of stage IV nephrons at 15.5 dpc (Thiagarajan et al., 2011). Due to its late onset in the distal tubule, it was not present in the kidney tubules at 13.5 dpc, however it was detected in the ureteric tree. *Scnn1b* was also expressed in both early and late mesonephric tubules, the connecting segment of MTcr and in the ND (Table 2, Figure 4). In the kidney, *Vip* is specifically expressed in the juxtaglomerular arterioles at 15.5 dpc (Thiagarajan et al., 2011). In the mesonephros, expression was restricted to the male mesonephric tubules at 13.5 dpc (Table 2, Figure 4).

Identification of additional markers of male efferent tubules

In order to identify further examples of genes synexpressed in the mesonephros and metanephros and identify potential mesonephric-specific genes, we mined the GUDMAP database using a series of Boolean anatomy searches. First we searched for genes common to the early and late mesonephric tubules as well as kidney. This identified a further 14 synexpressed genes (*Cd24a*, *Clp1*, *Enpep*, *Epha4*, *Itga4*, *Lama1*, *Lamb1–1*, *Npnt*, *Osr2*, *Pcnt*, *Pepd*, *Rhoa*, *Tgfbr1* and *Tmem147*; Supplementary Table 1, Supplementary Figure 1). Five genes (*Lama1*, *Lamb1–1*, *Rhoa*, *Tgfbr1*, *Clp1*) were expressed in the connecting tubule segment of the mesonephric tubules from 10.5 dpc at both stages, whereas *Pcnt* expression in this structure was restricted to 10.5 dpc (Supplementary Figure 1). Again using Boolean anatomy queries we identified another set of 14 genes specifically expressed in more mature MT at 12.5–13.5 dpc and absent from kidney tubules at the same stage (Table 3). Interestingly, these mesonephric-specific candidates included 10 genes displaying male-specific gene expression within the mesonephros (Figure 5). Such genes may be involved in the early development of the male epididymis and / or efferent tubules.

Identification of candidate mesonephric- and metanephric-specific tubular genes

A search of the database analysing genes examined in the urogenital tract at 10.5 dpc was performed to identify genes congruent or discordant in expression between the early mesonephros and metanephros. A further 9 genes expressed in the mesonephric tubules of the 10.5 dpc mesonephros were identified (*Adamts5*, *Ccne2*, *Ccdc91*, *Col9a1*, *Crym*, *Mfap3*, *Mtap*, *Rps6ka6*, *Vcan*, Supplementary Table 2, Supplementary Figure 2). However, these genes were also expressed in early metanephric structures, including the UB and MM at 10.5dpc and later at 12.5–13.5 dpc in non-nephron structures within the metanephros, including the interstitium, cap mesenchyme and ureteric tree (Supplementary Table 2).

Conversely, 4 candidate metanephric-specific genes (*Cdh11*, *Gcnt1*, *Hapln1*, *Hoxa10*) were identified, whose expression was absent from both early and late mesonephric tubules (Supplementary Table 3, Supplementary Figure 3). Of particular note, *Hoxa10* was strongly MM-specifc at 10.5dpc and showed expression in early tubules as well as in CM and renal intersititum at 13.5dpc and was validated at 15.5dpc. *Cdh11* was also strongly expressed in the MM but was also detected in the mesonephric mesenchyme. This gene has previously been identified as specific to the developing metanephros at 10.5 dpc (Challen et al., 2004). In contrast, *Gcnt1* and *Hapln1* were restricted to the more mature tubules of metanephric nephrons.

Discussion

Although both excretory organs are composed of epithelial tubules, the molecular distinctions between the development of the mesonephros and metanephros are poorly understood. The work presented in this study represents the most thorough comparison of tubule formation in both organs performed to date. We have examined gene expression in the mesonephric tubules just after they first appear at 10.5 dpc and later at 12.5–13.5 dpc in both males and females. Markers of early nephrogenesis expressed in renal vesicles of the metanephros were also expressed in early mesonephric tubules at 10.5 dpc. In concordance with Mugford et al. (2008), we found that specific markers of the more differentiated metanephric nephron segment, the early proximal tubule were also found in 12.5–13.5 dpc mesonephric tubules. This included markers of the proximal convoluted tubules (S1 and S2) and proximal straight tubules (S3). Metanephric markers of the immature loop of Henle, early distal tubule and juxtaglomerular arteriole were also expressed in mature mesonephric tubules.

Further evidence supporting a shared early morphogenetic pathway was the discovery of very few genes restricted to the tubules of either the mesonephros or metanephros. Of the candidate organ specific genes identified in this study, some were expressed in non-tubular structures within both organs, suggesting congruence in developmental processes outside of tubular patterning. A small number of genes were identified that showed metanephric tubule specificity, including *Cdh11* and *Hoxa10*. *Cdh11* has been identified as such before in an expression profiling comparison between 10.5 dpc metanephric mesenchyme and adjacent intermediate mesodermal structures (mesonephros and early gonad of the same timepoint) (Challen et al., 2004). Similarly, prior studies have noted the metanephric-specific expression of the Hox11 transcription factors, *Hoxa11*, *Hoxc11* and *Hoxd11* (Wellik et al.,

2002; Mugford et al., 2008). The overlapping expression patterns of 28 Hox genes, including *Hoxa10* and the Hox11 paralogs, have previously been described in the mouse metanephros (Patterson and Potter, 2004) suggesting potentially redundant roles for these genes in nephrogenesis and patterning in the metanephric kidney.

During embryogenesis the mesonephros regresses, however in males the cranial mesonephric tubules persist and differentiate into the efferent ducts of the male epididymis, connecting it to testis via the rete testis. In the adult male, the major role of the mature efferent ducts is the concentration of sperm via the reabsorption of testicular fluid. This process, essential for the maturation of sperm, involves ion transporters and aquaporin water channel proteins, the expression of which is controlled by oestrogen and androgen (Hess, 2002; Hess et al., 2002; Oliveira et al., 2005). When genes involved in the reabsorption process or its control are mutated, such as estrogen receptor 1 α (*Esr1*) or Na+/H+ exchanger *NHE3* (*Slc9a3*), tissue swelling occurs as a result of a defect in fluid reabsoprtion at the efferent ducts, leading to liquid and sperm accumulation and male sterility (Zhou et al., 2001; Schultheis et al., 1998a; Hess et al., 1997). A similar outcome results from the disruption of *Lgr4* in male knockout mice. These mice display a postnatal developmental defect of the epididymis and efferent ducts, which are less convoluted and severely hypoplastic (Mendive et al., 2006). Disruption of estrogen receptor 1 α function leads to the altered expression of ion transporters and aquaporin 1 (*Aqp1*) in the efferent ducts (Lee et al., 2000; Zhou et al., 2001; Oliveira et al., 2002). Of note, *Slc9a3* and *Aqp1* are also expressed by the renal proximal convoluted tubule and function in fluid homeostasis in the kidney, where reaborption defects also occur when these genes are mutated (Schultheis et al., 1998b; Ma et al., 1998). *Lgr4* is also expressed in the kidney and null mutations result in renal hypoplasia with a reduced number of functional nephrons and polycystic kidneys, which occur as a result of a defect in renal development (Kato et al., 2006). In this study, we have shown the continued expression of a number of nephron segment-specific genes in the mesonephric tubules forming the male efferent ducts. It is possible that many of the proximal tubule genes expressed in the efferent ducts also play functional roles in the reabsorption of fluid. We observed the male specific expression of *Slc6a20b*, *Slc18a1* and vasoactive intestinal peptide (*Vip*). The neurotransmitter transporter *Slc6a20b*, when expressed in the proximal tubule, acts as an amino acid transporter critical for the reclamation of proline and hydroxyproline (Broer et al., 2009). *Slc6a20b*, along with other transporters and ion channels, has been shown to be downregulated in humans with non-obstructive azoospermia (Dubé et al., 2008). *Vip* is known to function as an erectile neurotransmitter, with receptors for this peptide in the corpus cavernosum of the penis (Zhang et al., 2010). While previously isolated from epididymis (Osterhoff et al., 1997), the onset of expression of this gene in the efferent tubules has not previously been reported. Of note, *Slc18a1* plays a role in vesicular monoamine transport. Its role in kidney or efferent duct is unclear, but it is known to play a role in presynaptic neurotransmitter packaging in the nervous system (Eiden et al., 2004; Erickson et al., 1996).

In addition to the continued expression of nephron segment marker genes in the mesonephric tubules, we have also identified a set of genes specific to the 12.5–13.5 dpc male mesonephric tubules and possibly involved in the formation and/or function of the efferent ducts. Male-specific genes *Spdya* and *Usp9x* are both involved in the cell cycle and

may play a role in the development and differentiation of the efferent ducts in the male. Of particular interest was the expression of a cluster of neural genes, including *Ascl1*, *Gap43* and *Mtap1b*, in the region of the male mesonephric tubules/efferent ducts. Nothing is known about the innervation of the epididymis / efferent ducts, but all three of these genes showed concurrent expression in the developing ganglionic complex surrounding the primitive bladder and in developing ganglia associated with medial sympathetic innervation along the dorsal aorta (data not shown, see supplementary Table 4). This evidence supports the likelihood that these genes mark the initiation of innervation of the testis / epididymis/ efferent ducts. Showing a similar expression pattern along the dorsal aorta were the male-specific mesonephric tubule genes *Slc9a2*, *D630049N15* and *Casq2* (see supplementary Table 4). However these genes have not previously been associated with the nervous system. *Slc9a2* is a Na+/H+ exchanger (*NHE2*), an isoform of *Slc9a3* (*NHE3*), and also known to be expressed in proximal convoluted tubules, as well as in thick ascending loop of Henle (distal straight tubules) of adult mice (Choi et al., 2000; Sun et al., 1997) and in the distal convoluted tubules of the adult human kidney (Ghishan et al., 1995). Although male mice with knockout mutations in *Slc9a3* are infertile, homozygous mice with null mutations in *Slc9a2* do not breed well, however the cause of their abnormal fertility has not been published (Schultheis et al., 1998a; Schultheis et al., 1998b; Choi et al., 2000). *Slc9a3* knockout mice also show defects in fluid reabsorption in the kidney leading to reduced blood pressure, however *Slc9a2*−/− mice show no obvious defects in renal function (Schultheis et al., 1998a; Schultheis et al., 1998b; Choi et al., 2000). In addition to the Na +/H+ exchangers several other ion transporters are known to be present in the efferent ducts including ATPase Na+/K+ transporting α 1 polypeptide, *Atp1a1* (Ilio and Hess, 1992), Chloride anion exchanger, *Slc26a3* (Lee et al., 2001) and the chloride channel *Cftr* (Lee et al., 2001; Leung et al., 2001). In this study, in addition to identifying genes involved in transmembrane ion transport (*Slc9a2*, *Slc6a20b*, *Slc18a1*) male-specific mesonephric tubule genes showed common gene ontology functions in metal ion binding (*Adam12*, *Casq2*, *Ide*), and may also have functional roles in testicular fluid homeostasis in the male efferent ducts.

In summary, these data provide further information on genes potentially involved in both mesonephric tubule formation as well as male efferent duct development. They serve to support the conclusion that tubular morphogenesis in the mesonephros strongly parallels that defined in the metanephros.

Experimental Procedures

Tissue collection and processing

All animal work contributing to this manuscript was conducted according to all state, national and international guidelines. Animal ethics approval was provided by AEEC3 of The University of Queensland. Embryos at 10.5 dpc (TS17), 12.5 or 13.5 dpc (TS20 or 21) and 15.5 dpc (TS23) were collected from adult pregnant female outbred CD1 mice sacrificed by cervical dislocation. A normal variation of ±0.5 days was observed. The urogenital system was examined at 10.5 dpc by dissecting embryos transversely below the forelimbs and longitudinally down the midline to expose the urogenital system. In all images the caudal end is on the right and the hindlimb is visible. Whole urogenital tracts (including

primitive bladder, mesonephros, gonad, ureter and kidney) were collected from both male and female embryos at 12.5 and/or 13.5 dpc and kidneys were collected at 15.5 dpc. All tissues were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight, dehydrated in methanol/PBTX for wholemount in situ hybridization (WISH) or ethanol/water for section in situ hybridization (SISH) and stored in alcohol (100% methanol at −20°C for WISH; 70% ethanol at 4°C for SISH). Paraffin-embedding and sectioning of kidneys was performed as described previously (Rumballe et al., 2008).

In situ hybridization

Detailed protocols for riboprobe generation, WISH and SISH are available on the GUDMAP website (http://www.gudmap.org/Research/Protocols/Little.html) and have been described previously (Georgas et al., 2008; Little et al., 2007; Rumballe et al., 2008). In brief, digoxigenin (DIG)-labelled antisense riboprobes were generated by PCR and purified using Roche Quick Mini Spin Columns. SISH was performed on 7μm paraffin sectioned and de-waxed 15.5 dpc kidneys using a Tecan Freedom Evo 150 robot and WISH performed using a BioLane HTI Robot. Hybridization of riboprobe (0.2μg/ml WISH; 0.3–0.5μg/ml SISH) was performed at 64ºC for 10 hours for WISH and 65°C overnight for SISH. Chromogenic substrates NBT and BCIP were used for WISH and BM Purple for SISH, with incubations of 2–120 hours at 25ºC. Once the signal had reached optimal intensity the tissues were washed (1% Triton in PBS for WISH; PBS for SISH) and fixed in 4% PFA for 10 mins at 25°C in order to preserve the *in situ* hybridization signal. SISH slides were mounted in aqueous mounting medium and WISH tissues stored in PBS at 4°C.

Photography of WISH and SISH and annotation of expression

Wholemount tissues were photographed using a Nikon SMZ1500 research stereomicroscope system with a Nikon DXM1200f, colour 12 megapixel digital camera and ACT-2U Image Application Software and Adobe Photoshop CS2. Sectioned kidneys were scanned automatically using the semi-automated .slide System from Olympus and Soft Imaging Systems (BX51 microscope, digital CCD camera, motorized scanning stage and workstation, automated slide loader and .slide software) and representative images captured using Olyvia software (Soft Imaging Systems, Olympus) and Adobe Photoshop CS2. Gene expression patterns were annotated following standard procedures developed for the GUDMAP database (http://www.gudmap.org) and using the published text-based anatomical ontology of the mouse urogenital system (Little et al., 2007). Annotated expression patterns, riboprobe details and WISH images are available on the GUDMAP website (www.gudmap.org) and the GUDMAP Accession IDs for each gene are listed in Supplementary Table 4.

Boolean anatomy searches of the GUDMAP database

Boolean queries were used to search for genes expressed in the metanephric and mesonephric tubules. We restricted searches to expression patterns of the same probe sequence (TS17, TS20–21 WISH entries and TS23 and adult SISH entries from the Little laboratory) in order to minimize any discrepancies which may have been seen due to sequence differences. Genes expressed in both tubule types were identified using the example Boolean query string for three anatomical terms below and replacing the

metanephros term in each case; for example genes expressed in early nephron and mesonephric tubule were identified using the Boolean query string; GUDMAP: p{in “early nephron GROUP” TS20..TS21} OR p{in “mesonephric tubule of female” TS20..TS21} OR p{in “mesonephric tubule of male” TS20..TS21}. The following metanephros terms were also searched; “cortical renal tubule” TS23..TS28; “immature loop of Henle” TS23..TS23; “s-shaped body”, “renal vesicle” and “late tubule”. We then looked for genes with entries for both types of tubule when the data returned from the query was sorted by Gene. Genes expressed in only one tubule type utilized the same query strings as those above with not detected for one type of tubule, with the following example; genes not detected in early nephron and present in mesonephric tubules had the Boolean query string GUDMAP: nd{in “early nephron GROUP” TS20..TS21} OR p{in “mesonephric tubule of female” TS20..TS21} OR p{in “mesonephric tubule of male” TS20..TS21}. Not present in the metanephros, GUDMAP: nd{in “metanephros” TS17..TS28} OR p{in “mesonephric tubule of female” TS20..TS21} OR p{in “mesonephric tubule of male” TS20..TS21}. Common genes were also searched using the same metanephros terms above and replacing the mesonephric tubule TS20–21 terms with the early mesonephric tubule at 10.5 dpc, “mesonephric tubule (TS17–TS18)” for example; GUDMAP: p{in “cortical renal tubule” TS23..TS28} OR p{in “mesonephric tubule” TS17..TS17}.

Supplementary Material

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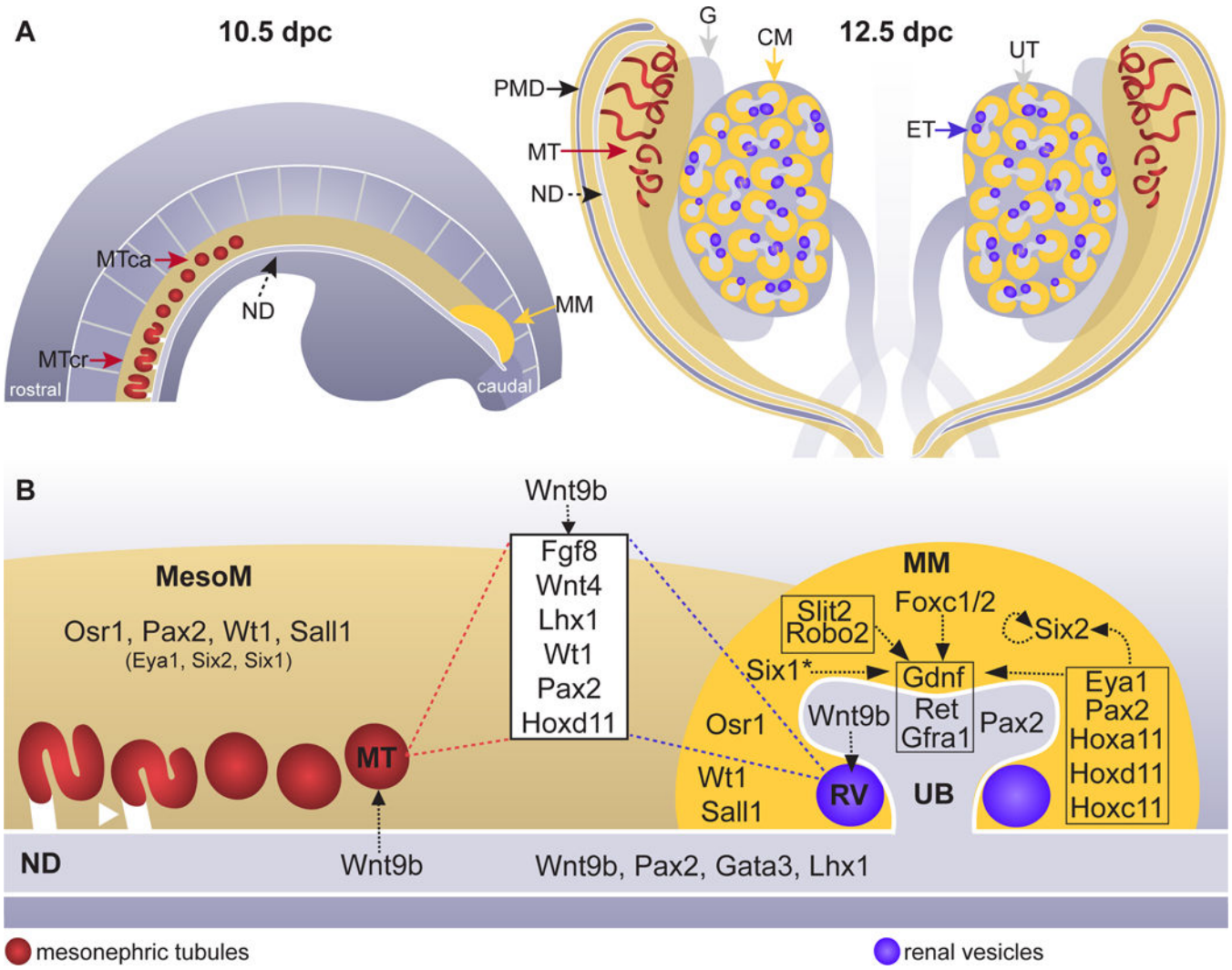

Figure 1. Key molecules in the formation of the mesonephric and metanephric tubules.

A. Schematic (left) illustrating the position of the urogenital tract (UGT) within the 10.5 dpc embryo (hindlimb visible) and the mesonephroi and kidneys of the 12.5–13.5 dpc UGT (right). At 10.5 dpc, S-shaped cranial MT (MTcr) are connected to the nephric duct (ND). Vesicle-shaped caudal MT (MTca) are located within the mesenchyme (Smith et al., 1991). At 12.5–13.5 dpc, the paramesonephric duct (PMD) is present and 18–26 highly convoluted MT extend from the cranial tip to half-way down the gonad (Smith et al., 1991; Sainio et al., 1997). **B.** Enlarged schematic showing the expression of key genes within early mesonephros and metanephros. Wnt9b secreted by the nephric duct (ND) and ureteric bud (UB) into the mesonephric (MesoM) and metanephric (MM) mesenchyme induces a common, tubule promoting developmental program, including expression of *Wnt4*, *Fgf8* and *Lhx1* within both early epithelial mesonephric tubules (MT) and renal vesicles (RV) (boxed region). *Eya1*, *Six2* and *Six1* are transiently expressed in the MesoM at 9.5 dpc and downregulated at 10.5 dpc. G, gonad; CM, cap mesenchyme; ET, early tubules (renal vesicle, S-shaped body); UT, ureteric tip; white arrowhead, MT connecting tubule.

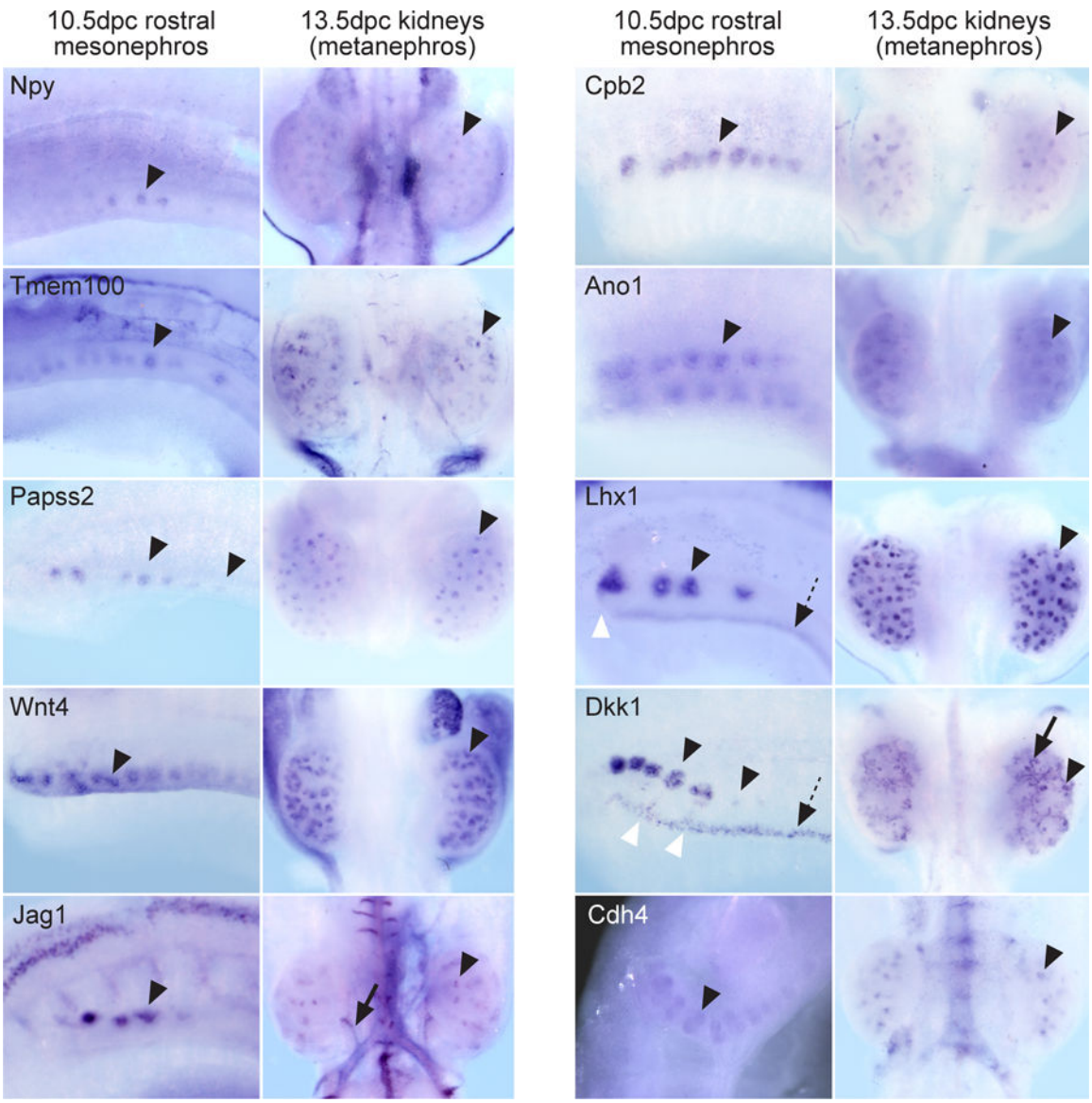

Figure 2. Gene expression patterns of early nephron renal vesicle markers in the kidney and early mesonephros.

Examples of genes identified as markers of the renal vesicle in the 15.5 dpc kidney and co-expressed in the early mesonephric tubules (MT) at 10.5 dpc. Wholemount in situ hybridization (WISH) of 10.5 dpc embryos sagitally bisected down the midline to reveal the urogenital system and kidneys at 13.5 dpc are shown. The rostral mesonephros is shown with expression in MT and in the early tubules of kidneys indicated (arrowheads). Additional sites of expression in the kidney were seen for *Dkk1* in a subset of the renal interstitium (arrow) and *Jag1* in the renal arterial system (arrow). *Lhx1* and *Dkk1* also showed expression in the nephric duct (dotted arrows) and the tubular component of the cranial mesonephric tubules which connect these tubules to the duct (white arrowheads). Additional images including the whole urogenital system are available on the GUDMAP website (www.gudmap.org, see Supplementary Table 4).

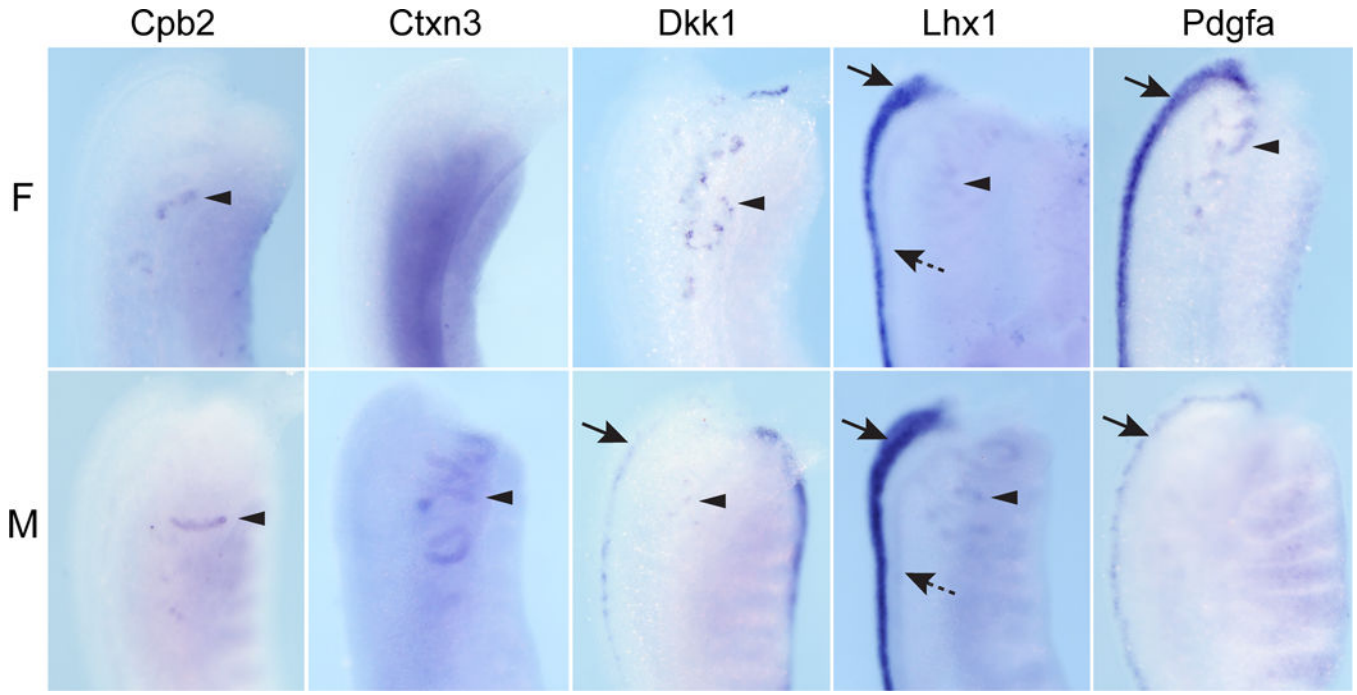

Figure 3. Gene expression patterns of renal vesicle markers co-expressed in mesonephric tubules at 12.5–13.5 dpc.

Examples of genes identified as markers of the renal vesicle in the 15.5 dpc kidney and co-expressed in the mesonephric tubules (MT) at 12.5–13.5 dpc. Wholemount in situ hybridization (WISH) of female (F) and male (M) mesonephros-gonads are shown from the dorsal side and focused on the MT (arrowheads) in the rostral half of the mesonephros. Expression in the nephric duct (ND, dotted arrows) or paramesonephric duct (PND, arrows) is also indicated. Lhx1 also showed expression in the tubular component of the cranial mesonephric tubules which connect these tubules to the duct. Additional images and annotated expression patterns of these genes in the whole urogenital system, including the ovary and testis, are available on the GUDMAP website (www.gudmap.org, see Supplementary Table 4).

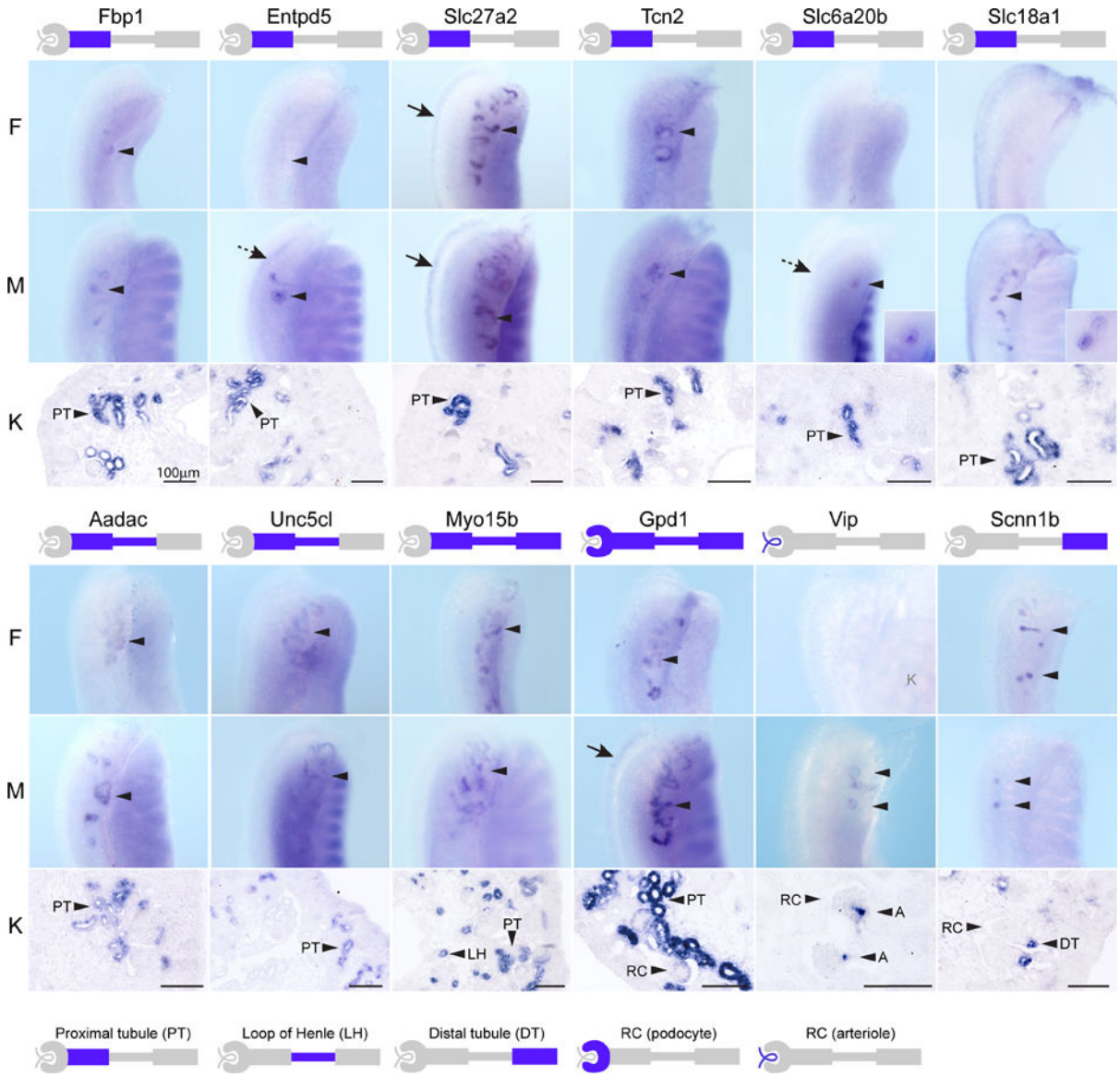

Figure 4. Gene expression patterns of proximal and distal tubule kidney markers in the mesonephros.

Examples of genes identified as markers of the early proximal tubule, early distal tubule or renal corpuscle of the kidney at 15.5 dpc and co-expressed in the mesonephric tubules (MT) at 12.5–13.5 dpc. Wholemount in situ hybridization (WISH) of female (F) and male (M) mesonephros-gonads are shown from the dorsal side and focused on the MT (arrowheads) in the rostral half of the mesonephros. Expression in the nephric duct (ND, dotted arrow) or paramesonephric duct (PND, arrows) is also indicated. Section ISH of 15.5 dpc kidneys are shown below and the expression patterns in the segments of the nephron are indicated in the schematic below each gene symbol. K, kidney; PT, early proximal tubule; LH, immature loop of Henle; DT, early distal tubule; RC, renal corpuscle; A, arteriole. All scale bars = 100μm. Additional images and annotated expression patterns of these genes in the whole urogenital system including the ovary and testis are available on the GUDMAP website (www.gudmap.org, see Supplementary Table 4).

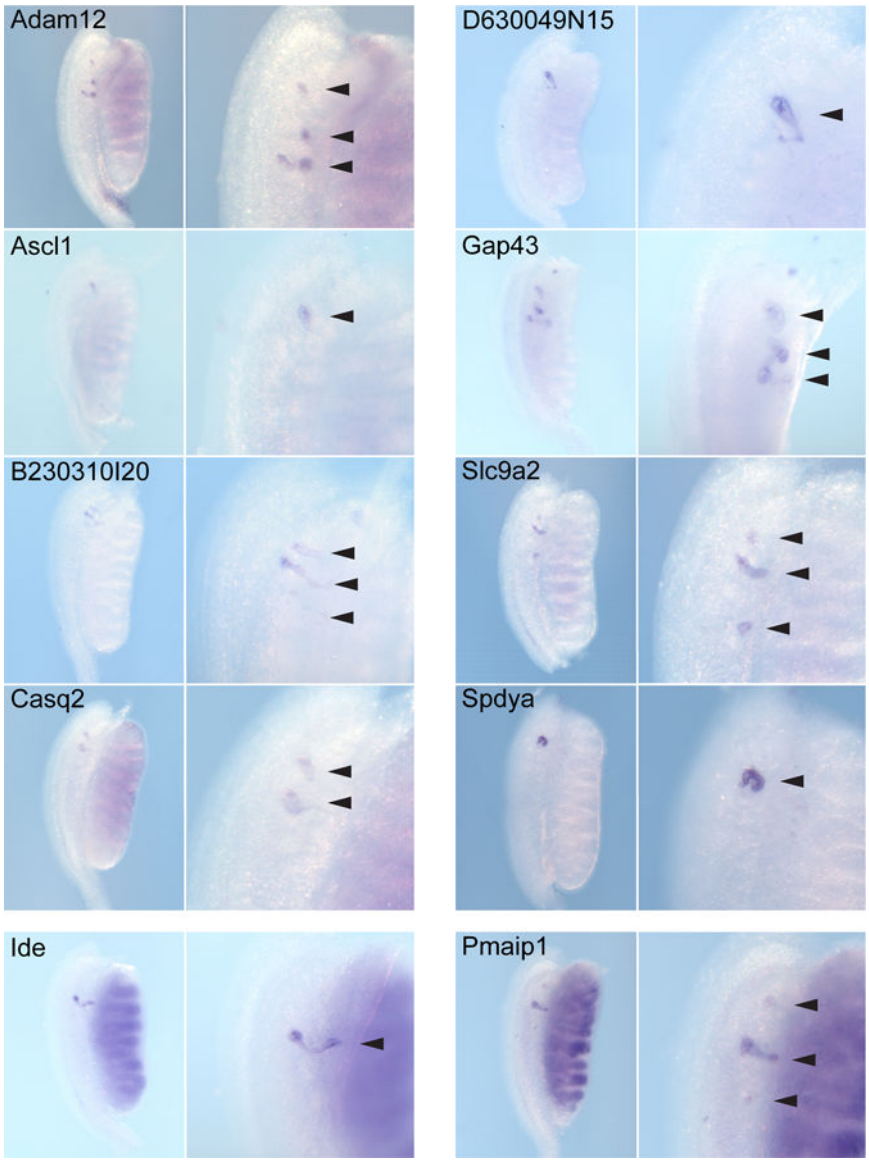

Figure 5. Expression patterns of male-specific mesonephric tubule genes.

Examples of genes identified in a search of the GUDMAP database for mesonephric-specific tubular expression. Genes exclusively expressed in the mesonephric tubules (MT) of the male at 13.5 dpc were identified (*Adam12*, *Ascl1*, *B230310I20*, *Casq2*, *D630049N15*, *Gap43*, *Slc9a2*, *Spdya*) as well as a few genes expressed in male and female MT (*Pmaip1*) or in the male MT and in the kidney (*Ide*). Wholemount in situ hybridization (WISH) images of male mesonephros-gonads are shown from the dorsal side with the rostral mesonephros enlarged to show expression in the MT (arrowheads). Additional images for the male and all female images are available on the GUDMAP website together with the text-annotated expression patterns of these genes in the whole urogenital tract including expression in the ovary and testis (www.gudmap.org, see Supplementary Table 4).

TABLE 1.

Expression Profile of Early Metanephric Tubule Renal Vesicle Markers in the Mesonephros[a]

Gene Symbol (MGI)	WISH 10.5 dpc UG system			WISH 12.5–13.5 dpc kidney	SISH 15.5 dpc kidney						Female 12.5–13.5 dpc mesonephros		Male 12.5–13.5 dpc mesonephros	
	MT Cranial	MT Caudal	Other structures	Early tubules	PA	RV	CSB	SSB	III	IV	MT	Other structures	MT	Other structures
Ano1	+	+	ND	+	?	+	+	+	+ RC	RC	–	M	–	M
Cbln4	?	+	G, UB	+	+	+	+	+	+	–	–	PMD	–	PMD
Cdh4	+	+	–	+	+	+	+	+	+	+	–	PMD (12.5)	–	PMD
Cpb2	+	+	–	+	–	+	+	+	+	+	+	–	+	–
Ctxn3	+	+	–	+	*	*	*	*	*	*	–	M	+	–
Dkk1	+ & CT	+	ND, UB	+ & RI	?	+	+	+	+	–	+	–	+	PMD
Gja1	+	+	–	+	+	+	+	+	–	–	–	PMD	–	ND, PMD
Greb1	+	?	Me, MM, UB	+	+	+	+	+	+	–	–	M, ND, PMD	ne	ne
Jag1	?	+	–	+ & V	–	+	+	+	+	+ RC *	–	PMD	–	PMD
Lhx1	+ & CT	+	ND, UB	+	+	+	+	+	+	+	+ & CT	ND, PMD	+ & CT	ND, PMD
Ltbp1	?	+	Me	+	–	+	+	+	+ RC	RC	–	M, PMD	–	M, PMD
Npy	–	+	–	+	–	+	+	–	–	–	–	PMD	–	PMD
Papss2	+	+	MM	+	–	+	+	+	+	+	–	PMD	–	PMD
Pax8	+	+	ND, UB	+	+	+	+	+	+ RC	+	ne	ne	ne	ne
Pcsk9	?	+	ND	+	–	+	+	+	+	+	–	–	–	–
Pdgfa	+ & CT	?	G, ND	+	ne	ne	ne	ne	ne	ne	+	PMD	–	PMD
Pou3f3	+	?	ND, UB	+ & UT	–	+	+	+	+	+	ne	ne	ne	ne
Svopl	+	+	–	+	–	+	+	+	+	+	–	PMD	–	PMD
Tmem100	+	+	UB, MM	+ & V	+	+	+	+	–	–	–	–	–	–
Wnt4	+	+	G, ND, Me	+	+	+	+	+	–	–	–	M	–	M
Wt1	?	?	Me, MM, G	+	+	+	+	+	RC	RC	ne	ne	ne	Ne

[a]Marker genes of the metanephric renal vesicle were examined in the UG system at 10.5 dpc and in the male and female mesonephros and metanephros at 12.5–13.5 dpc by WISH. This table summarizes their whole-mount expression patterns and the previously published SISH expression patterns in the 15.5 dpc kidney (Georgas et al., 2009). MT at 10.5 dpc were divided into cranial and caudal. In whole-mount kidneys at 12.5–13.5 dpc expression in the early tubules may include renal vesicle (RV), comma-shaped body (CSB), and/or S-shaped body (SSB). Published SISH data include metanephric kidney tubule expression across all stages of nephrogenesis including pretubular aggregate (PA), RV, CSB, SSB, stage III capillary loop nephrons, and stage IV maturing nephrons. MT, mesonephric tubule; CT, connecting tubule of mesonephric tubules; G, gonad; ND, nephric duct (mesonephric portion); Me, mesonephric mesenchyme; MM, metanephric mesenchyme; UB, ureteric bud location (ND metanephric portion); RI, renal interstitium; V, vasculature; UT, ureteric tip; RC, renal corpuscle; PMD, paramesonephric duct; ?, uncertain expression; ne, not examined; U, ubiquitous; UG, urogenital; dpc, days post coitum.

*Spotted expression in metanephros for *Ctxn3*.

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* RC indicates expression of *Jag1* in the arterioles.

TABLE 2.

Expression Profile of Proximal and Distal Tubule and Renal Corpuscle Kidney Markers in the Mesonephros[a]

Gene symbol (MGI)	WISH 10.5 dpc urogenital tract		WISH 13.5 dpc female mesonephros		WISH 13.5 dpc male mesonephros		WISH 13.5 dpc Kidney	SISH 15.5 dpc kidney		SISH adult kidney
	MT	Other structures	MT	Other structures	MT	Other structures	Nephron tubules	III	IV	Mature IV
Aadac	−	−	+	−	+	−	−	+	S2–3 EPT, LOH	−
Acaa1b	+ & CT	MesoM, ND, MM	+	M?	+	M?	?	+	EPT, LOH, EDT	S2 PT
Entpd5	P	(ND?)	+*	−	+	ND	−	−	S1–2 EPT	?
Fbp1	−	−	+	−	+	−	−	−	S1–2 EPT	S1–3 PT
Gpd1	?	MesoM, ND, MM	+	−	+	PMD	−	+ RC	EPT, LOH, EDT, RC	S2 PT
Myo15b	−	−	+	−	+	−	−	+	EPT, LOH, EDT	−
Slc18a1	ne	ne	−	−	+	−	−	−	S1–2 EPT	S1–2 PT
Slc27a2	−	−	+	PMD	+	PMD (ND?)	−	−	S1–3 EPT	S2–3 PT
Slc3a1	−	−	?	PMD	?	?	?	−	S2–3 EPT	S2–3 PT
Slc6a20b	?	MesoM (MM?)	−	−	+	ND	−	−	S2 EPT	S2–3 PT
Tcn2	−	MesoM, MM (ND?)	+	M	+	M	?	−	S1–3 EPT	S1–3 PT
Ttr	−	?	−	M ?	−	M ?	−	−	S2–3 EPT, LOH	S3 PT
Unc5cl	−	?	+	M ?	+	M ?	−	+	S1–3 EPT, LOH	−
Scnn1b	+ & CT	ND	+	ND ?	+	−	−	−	EDT (CCD, MCD)	ne
Vip	ne	ne	−	−	+	−	−	−	arterioles stage IV RC	−

[a]Marker genes of the metanephric early proximal and distal tubule and renal corpuscle were examined in the urogenital system at 10.5 dpc and in the male and female mesonephros and metanephros at 13.5dpc by WISH. This table summarizes their whole-mount expression patterns and the previously published SISH expression patterns in the 15.5 dpc and adult kidney Thiagarjan et al., 2011). In whole-mount kidneys at 13.5 dpc, expression in the early tubules may include renal vesicle (RV), comma-shaped body (CSB) and/or S-shaped body (SSB). Published SISH data include metanephric kidney expression in tubules and RC of stage III capillary loop nephrons, segments 1–3 (S1–3) of EPT and other tubule segments of stage IV maturing and stage IV mature nephrons.

*_Entpd5_ female shows only very weak expression in the MT. EPT, early proximal tubule; LOH, immature loop of Henle; EDT, early distal tubule; MT, mesonephric tubule; ND, nephric duct (mesonephric portion); Me, mesonephric mesenchyme; MM, metanephric mesenchyme; RC, renal corpuscle; PMD, paramesonephric duct; ?, uncertain expression; dpc, days post coitum.

TABLE 3.

Expression Profile of Male-Specific Mesonephric Tubule Markers [a]

Gene symbol (MGI)	WISH 12.5–13.5 dpc kidney	Female 12.5–13.5 dpc mesonephros		Male 12.5–13.5 dpc mesonephros	
	Early tubule	MT	Other structures	MT	Other structures
Male-specific MT expression					
Adam12	–	–	–	+	(ND?)
Ascl1	–	–	–	+	–
B230310I20	–	–	–	+	–
Casq2	–	–	–	+	–
D630049N15	–	–	–	+	–
Gap43	–	–	–	+	–
Mtap1b	–	–	–	+	PMD
Slc9a2	–	–	–	+	ND
Spdya	–	–	–	+	–
Usp9x	–	?	–	+	–
Male and female MT expression					
Smoc2	RI	?	ND, M	+	ND, M
Pmaip1	–	+	(M?)	+	(ND?)
Expression in the MT and kidney					
Ide	? (kidney)	–	–	+	ND
Cthrc1	- (Utree)	+ & CT	ND, PMD	+ & CT	ND, PMD

[a]Genes identified from the GUDMAP database as expressed in the mesonephric tubules and absent from the kidney tubules. This table summarizes the expression patterns available on the GUDMAP Web site (www.gudmap.org).

[*]Regional expression in the metanephros likely to include early tubule. MT, mesonephric tubule; CT, connecting tubule; M, mesonephric mesenchyme; ND, nephric duct (mesonephric portion); MM, metanephric mesenchyme; Utree, ureteric tree; RI, renal interstitium; PMD, paramesonephric duct; ?, uncertain expression; ne, not examined; dpc, days post coitum.