

Differential regulation of two sets of mesonephric tubules by WT-1

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SUMMARY

Mammalian renal development undergoes two transient stages, the pronephros and the mesonephros. While the regulation of metanephric differentiation has received considerable attention, very little is known about the mode of differentiation of the mesonephros and its regulation. We have followed mesonephric differentiation to unravel the developmental mechanisms and fates of mesonephric tubules by whole-mount immunohistology using antibodies to laminin, brush border epitopes, cytokeratin-8/18, p75 neurotrophin receptor and some other renal antigens as markers. In rat and mouse embryos, two distinct sets of tubules were observed throughout mesonephric development. Four to six pairs of cranial mesonephric tubules developed as outgrowths from the Wolffian duct. The majority of tubules were caudal tubules which never fused with the Wolffian and differentiated similarly to metanephric nephrons. The murine mesonephric tubules degenerate by apoptosis, except in males where the cranial tubules become the epididymal ducts. These developmen-

tal differences between the cranial and caudal sets of tubules suggested different regulatory systems for each. Targeted disruption of the Wilms' tumour gene product, WT-1, results in renal aplasia, and a reduction in the number of mesonephric tubules (Kreidberg, J. A., Sariola, H., Loring, J., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). *Cell* 74, 679-691). We therefore analysed more closely mesonephric differentiation in WT-1-deficient mice, and showed that they only develop the cranial mesonephric tubules but not the caudal ones. Thus, WT-1 appears to regulate only the development of the caudal mesonephric tubules that conceivably are formed from mesenchymal cells like the metanephric tubules. WT-1 therefore seems to be necessary for the mesenchyme to epithelium transitions at different stages of nephrogenesis.

Key words: whole-mount immunohistochemistry, embryonic development, mesonephric development, WT-1, rat, mouse

INTRODUCTION

The diversity of cell types and the complexity of the structures increase during the urogenital morphogenesis (reviewed by Saxén, 1987; Bard et al., 1996). This is exemplified by the development of the mammalian permanent kidney, the metanephros, that is preceded by two transient and primitive organs, the pronephros and the mesonephros (reviewed by Saxén, 1987). They develop along the caudally growing nephric duct, the Wolffian duct, that is initially formed in the trunk region by fusion of caudal pronephric tubules (Toivonen, 1945). Shortly before the Wolffian duct reaches the cloaca, a ureteric bud is evolved and serves as the inducer of the differentiation of the metanephros (Grobstein, 1953, 1955).

Regulation of mesonephric differentiation has been studied in some mammals, but more thoroughly in amphibians and in birds. In chicken (Boyden, 1927; Gruenwald, 1937) and in the newt, *Triturus pyrrhogaster* (Kotani, 1962), no mesonephric nephrons are formed from the mesonephric mesenchyme after the removal of the Wolffian duct. At least in chicken a segment of the distal tubule is derived from the Wolffian duct (Croisille

et al., 1974; Croisille, 1976). Mammalian mesonephric nephrons consist of a glomerulus-like body, a proximal tubule and a distal tubule (Martino and Zamboni, 1966; Schiller and Tiedemann, 1981; Tiedemann and Egerer, 1984; Smith and MacKay, 1991), but there is a great deal of variation in the size, structure, functional maturity and developmental fate of the mesonephros between species. In pig (Tiedemann and Egerer, 1984) and human (Martino and Zamboni, 1966) the mesonephros is well developed as a secretory organ during embryonic development. In murine species, however, the mesonephros is primitive and non-secretory (Zamboni and Upadhyay, 1981; Smith and MacKay, 1991).

The mesonephric kidney regresses prenatally in mammals. The degradation is completed in rat by embryonic day 17 (E_r17) and in mouse by day 15 (E_m15) (reviewed by Saxén, 1987). The regression in mouse starts from the caudal mesonephric tubules and spreads cranially (Smith and MacKay, 1991). In females the degradation is complete but in males the remaining mesonephric tubules form the epididymal ducts and the Wolffian duct becomes the vas deferens (Moore, 1977; Orgebin-Crist, 1981; Nistal and Paniagua, 1984). The

degradation is due to apoptosis that has been described by morphological criteria in the mesonephric field at early developmental stages (Smith and MacKay, 1991).

Unlike metanephric development, regulatory molecules involved in mesonephric induction, differentiation or regression are not well known. The transcription factor *pax-2* (Deutsch and Gruss, 1991) is expressed in the pronephric and mesonephric tubules, in the Wolffian duct, in the early metanephric condensates and in the ureter bud, and it is down-regulated in the mature metanephric epithelia (Dressler et al., 1990; Dressler and Douglass, 1992; Phelps and Dressler, 1993). *Pax-2* is necessary for the development of the excretory system, since targeted disruption of the *pax-2* gene results in lack of the meso- and metanephric kidneys, ureters and genital tract in homozygous transgenic mice, and in the reduction in the size of the metanephros in adult heterozygotes (Torres et al., 1995). The tumour suppressor gene *WT1* is expressed in the developing urogenital system (Pritchard-Jones et al., 1990) including mesonephric glomerulus-like structures in mouse and human embryos (Armstrong et al., 1993). Targeted disruption of *WT1* (Kreidberg et al., 1993) leads to embryonic lethality in homozygous mice. In these mice the ureteric bud does not develop and the metanephric mesenchyme dies through apoptosis. The mesonephros in the homozygous embryos develops, although mesonephric tubules are not as numerous as in wild-type littermates (Kreidberg et al., 1993). This raises the possibility that metanephric and mesonephric morphogenesis may be regulated by different mechanisms.

Mesonephric development in mammals has mainly been studied by serial histological sections that do not necessarily give a clear picture of the anatomical relationships between the Wolffian duct and the developing tubular structures. We therefore employed whole-mount immunocytochemistry to follow the murine mesonephric development in order to gain insight into the morphogenesis of mesonephric tubules, and show that both rat and mouse mesonephros contain two developmentally distinct sets of tubules. Finally, we analysed the mesonephros of *WT-1*-deficient mice and show that the homozygotes lack the caudal

mesonephric tubules, but develop the cranial ones. Thus, *WT-1* appears to regulate the development of only caudal mesonephric tubules and is necessary for the mesenchyme to epithelium transition of the renal mesenchyme that form these tubules.

MATERIALS AND METHODS

Animals

For the structural analysis, Sprague-Dawley rat and CBA×NMRI F₁ mouse embryos were used throughout the study. *WT-1*-deficient transgenic mice we generated as described by Kreidberg et al. (1993). In mice, the appearance of vaginal plug was considered as the day 0 of pregnancy and in rats the animals were mated overnight. The pregnant females were anaesthetised by CO₂ and killed by cervical dislocation (rats) or by cervical dislocation only (mice). The stage of the embryos was further estimated by morphological criteria according to Theiler (1989). In rats the embryos were taken at E_r11 to E_r18. In mice the embryos were taken at E_m11 to E_m16.

Tissue separation and organ culture

Whole urogenital blocks including mesonephros, Wolffian duct, genital ridge and metanephros were dissected in Dulbecco's phosphate-buffered saline. Tissue blocks were cultured in Trowell-type organ cultures on 1.0 or 0.05 µm Nuclepore polycarbonate filters (Costar) and fixed as described below. Some kidneys were fixed in ice-cold methanol immediately after dissection for the whole-mount immunohistochemistry as described below. Tissue separation and organ culture methods are described by Saxén and Lehtonen (1987).

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as described (Sariola et al., 1988; Sainio et al., 1994a,b). Briefly, the urogenital blocks were fixed in ice-cold methanol for 5 minutes, washed 3 times for 5 minutes each in phosphate-buffered saline (PBS), pH 7.4, containing 1% of bovine serum albumin and 11% sucrose. The samples were incubated with primary antibodies overnight at +4°C, washed at least 3 times for 2 hours each in PBS, incubated with fluorescein- or rhodamine-conjugated secondary antibodies (Jackson Immuno.) overnight at +4°C, washed again at least 3 times for 2 hours each in PBS, mounted in either Elvanol or Immumount (Shandon) and analysed using a Zeiss Axiophot fluorescence microscope with epifluorescence. The primary and secondary antibodies used are listed in Table 1.

Table 1. Primary and secondary antibodies

Antibody	Antigen	Specificity	Source/Ref.	Dilution
Ker-8 mouse monoclonal	Rat cytokeratin-8	Rat cytokeratin-8	Amersham	1:25
4B11 mouse monoclonal	Mouse cytokeratin-18	Mouse and rat cytokeratin-18	Sariola et al., 1991 Dr I. Virtanen	1:1
Laminin rabbit polyclonal	EHS-laminin	Mouse and rat laminin	Virtanen et al., 1985 Gibco, BRL	1:300-1:400
Anti-BB rabbit polyclonal	Rat brush border antigens	Renal proximal tubules	Dr A. Miettinen Ekblom et al., 1980	1:4000
L1 rabbit polyclonal	L1 neural cell adhesion molecule from mouse brain	L1 from rat and mouse	Dr M. Schachner Rathjen and Schachner, 1984; Sainio et al., 1994b	1:200
p75 clone 192 mouse monoclonal	Rat PC12 cells	Rat p75	Boehringer Mannheim Sariola et al., 1991	1:10
p75 rabbit polyclonal	Mouse p75	Mouse and rat p75	Dr M. Chao Sainio et al., 1994a	1:200
Pax-2 rabbit polyclonal	Mouse <i>pax-2</i>	Mouse and rat <i>pax-2</i>	Dr G. Dressler Dressler et al., 1990	1:400
5A mouse monoclonal	Rat glomerulus	Rat podocalyxin	Dr A. Miettinen Miettinen et al., 1990	1:5
Secondary	FITC-donkey IgG	Rabbit antigens	Jackson Immuno.	1:200
Secondary	TRITC-goat IgG	Mouse antigens	Jackson Immuno.	1:100

RESULTS

Structure

The mesonephros is at its early stage of development in E_r11 embryos. Whole-mount immunohistochemistry with antibodies against basement membrane glycoprotein laminin revealed a total of 20–26 mesonephric tubules or pretubular vesicles (Fig. 1), and four to six of the most cranial tubules were budding from the Wolffian duct (Fig. 1A). All other tubules were initially located in close vicinity to the duct, but they were separated from it by a basement membrane (Fig. 1A). At later stages, E_r13 and E_r14, the difference between the cranial and caudal tubules became even more pronounced (Figs 1B,C, 2). The cranial tubules remained connected to the Wolffian duct, whereas the caudal tubules, the majority of the mesonephric nephrons, never formed connections with the Wolffian duct. The caudal tubules also elongated to distinct tubular structures that were more distant from the Wolffian duct (Fig. 2A,C). The two distinct sets of mesonephric tubules were seen both *in vivo* and in cultured mesonephric kidneys. Up to E_r14 and E_m12, no morphological differences between female or male embryos were seen (data not shown). The summary of the renal antigen expression patterns in the developing murine mesonephros at different time points is presented in Table 2.

At E_r11 and E_m10, cytokeratin-8/18 was not expressed in the caudal mesonephric tubules but it was seen in the Wolffian duct and in a short distal segment of the cranial tubules (Table 2). At E_r15, cytokeratin-8/18 was expressed in all tubular structures (Fig. 2B,D). The tubular morphology was similar in the caudal and cranial sets, except for the glomerulus-like structure that was well developed only in the cranial tubules, as shown with antibodies

against the podocyte marker podocalyxin (Schnabel et al., 1989) (Fig. 3B).

At E_r15, the mesonephric tubules, as shown by antibodies against brush border antigens, were well developed in the cranial, but less advanced in the caudal parts (Fig. 2A,C). Similar pattern was found in mouse embryos (Table 2).

Regression stage

At E_r16, the mesonephros was degrading in female rat

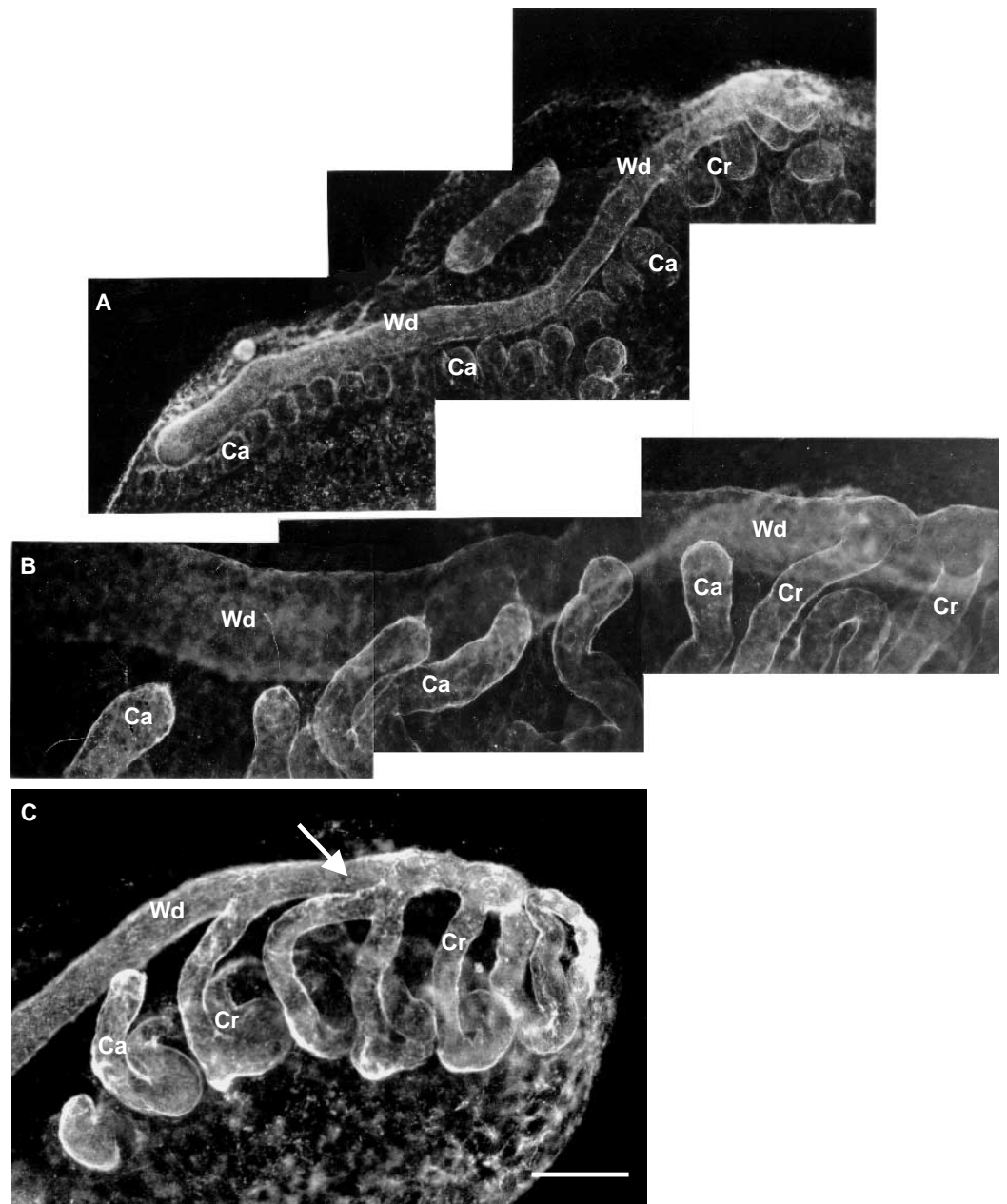


Fig. 1. Characteristics of the two sets of murine mesonephric tubules at early developmental stages. Whole-mount immunohistochemistry with antibodies to laminin. (A) Rat E_r11 mesonephros after 24 hours in culture. Four cranial tubules are budding from the Wolffian duct, and the caudal tubules are in close proximity to the duct. (B,C) Higher magnification of rat E_r13 mesonephros. (B) Two cranial tubules are connected to the Wolffian duct, and six caudal tubules are further away from the Wolffian duct and remain unconnected. (C) Some of the cranial tubules form branches from the distal segment (arrow). Wd, Wolffian duct; Cr, cranial mesonephric tubule; Ca, caudal mesonephric tubule. Bar (A) 250 μ m; (B) 80 μ m; (C) 100 μ m.

Table 2. Expression patterns of renal antigens in the developing murine (mouse and rat) mesonephros

Structure Stage*	Cranial tubules E11/E14/E17	Caudal tubules E11/E14/E17	Proximal tubules E11/E14/E17‡	Wolffian duct E11/E14/E17	Glomerular body E11/E14/E17‡
Cytokeratin 8/18 (r;m)	+†/+/+	-/+/-	-/+/+	+/+/+	-/+/+
Laminin (r;m)	+/+/+	+/+/-	-/+/+	+/+/+	-/+/+
Brush border (r;m)	-/+/+	-/+/-	-/+/+	+/+/+	-/+/+
L1 (r)	+/+/+	-/+/-	-/+/+	+/+/+	-/+/+
p75 (r;m)	-/+/+	+/-/-	-/-/-	-/-/-	-/+/+
Pax-2 (r;m)	+/+/+	+/+/-	-/+/+	+/+/+	-/+/+
Podocalyxin (r)	-/-/-	-/-/-	-/-/-	-/-/-	-/+/+

*The stage marks the rat embryonic days. The corresponding developmental stages in mouse are approximately two days earlier. Those epitopes that have been analysed in rat are marked by r and those in mouse by m.
†Only a short distal segment.
‡Detected only in cranial tubules, at this stage most of the caudal tubules have already been deteriorated.

embryos, and at E_r18, the whole mesonephros had degraded (Table 2). In males at E_r16, the cranial tubules had grown close to the anterior part of the developing testis and showed regular secondary branches (Figs 1C, 4A,B,D). These tubules were still expressing epithelial antigens such as cytokeratin-8/18 and brush border epitopes (Fig. 4A,B). At this stage most of the caudal tubules had already deteriorated. At E_r18 all caudal tubules had disappeared and in male embryos the cranial tubules had formed epididymal ducts with characteristic branches (Fig. 4C,D).

Mesonephros of the *WT1* mutant mice

Wilms' tumour gene *WT1* is expressed in the mesonephros and metanephros, in particular in the developing nephric tubules, but not in the Wolffian duct or its derivatives (Pritchard-Jones et al., 1990; Armstrong et al., 1993). Transgenic mice deficient for WT-1 fail to develop metanephric kidneys (Kreidberg et al., 1993). In these mutant mice the mesonephros, as analysed by serial histological sections, seemed to develop less tubules than those of wild-type littermates (Kreidberg et al., 1993). A total of 18 embryos from three separate breedings of *WT1*^{+/-} mice were used to analyse the mesonephrogenesis of the WT-1-deficient mice. The urogenital blocks were dissected at E_m11, cultured for 24 hours, and stained as whole-mounts with antibodies against brush border antigens or Pax-2, both of which are expressed in all mesonephric nephrons and in the Wolffian

duct. In the *WT1* heterozygous and wild-type embryos the mesonephros developed normally (Fig. 5A). In *WT1*^{-/-} mice, only the cranial but not caudal mesonephric tubules were formed (Fig. 5B,C). As reported previously (Kreidberg et al. 1993), the mutant embryos also lack the ureteric bud, but the Wolffian duct grows normally (Fig. 5B,C). The number of cranial tubules in all homozygous mice was three to four, while in the heterozygous or wild-type mice it was normal, four to six.

DISCUSSION

Until now, it has been suggested that the mesonephros in murine species consists of uniform nephrons that are all fused to the Wolffian duct. The mesonephric nephrons and the Wolffian duct in females regress, whereas in males, some of the mesonephric tubules develop into epididymal ducts and the Wolffian duct becomes vas deferens. Here we show that the cranial (epididymis precursors) and caudal tubules have a different mode of differentiation and that WT-1 regulates the differentiation of the caudal set of tubules.

We studied mesonephric development in murine species by whole-mount immunohistology technique, which provides a reliable 'three-dimensional' visualisation of developing structures. The maximal number of mesonephric tubules in both

Fig. 2. Markers of metanephric secretory nephrons (brush border epitopes and cytokeratin-8) and collecting ducts (cytokeratin-8) in rat mesonephric nephrons and Wolffian duct. Antibodies to brush-border epitopes (A,C), and to cytokeratin-8 (B,D). (A,B) E_r14 mesonephros. Four cranial tubules are connected to the duct (arrows), but the caudal ones are not connected (arrowheads). Brush border antigens and cytokeratin-8 are faintly expressed in all the epithelial structures. Brush border epitopes are more strongly visible in the proximal-tubule-like segment of the mesonephric nephrons. (C,D) At E_r15, caudal nephrons are further separated from the Wolffian duct. G, developing gonad. Bar 350 µm.

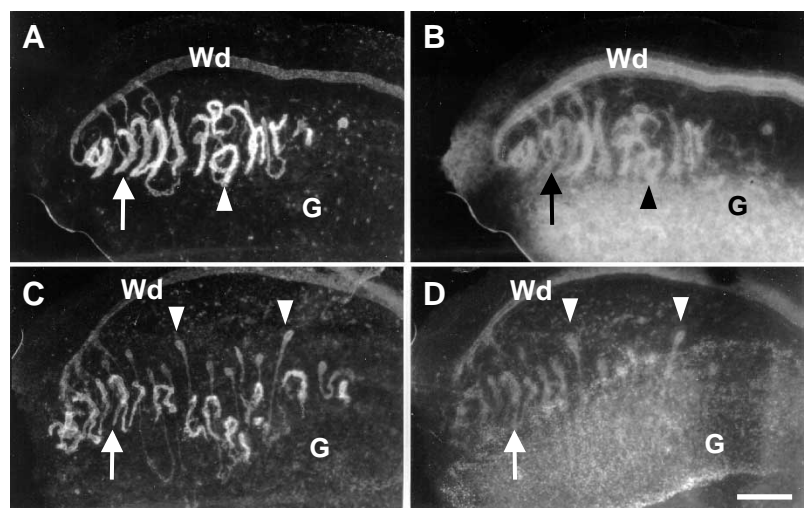
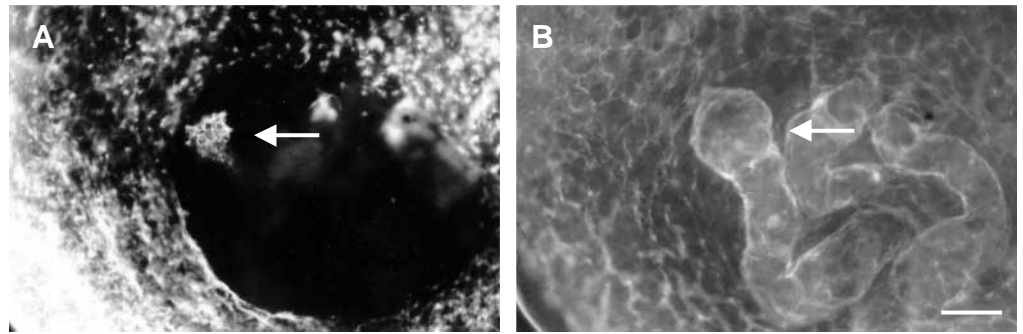


Fig. 3. Development of a glomerulus-like structure in the cranial mesonephric tubules. (A,B) At the tip of the tubule a glomerulus-like structure is formed (arrows). Whole-mount double-immunohistochemistry stainings with antibodies against podocalyxin (A) and laminin (B). Podocalyxin is also detected in endothelial precursors of the mesonephric zone. Bar 80 μm .



mouse and rat was 18-26, and two distinct sets of tubules were observed throughout the development. From the earliest stages of mesonephrogenesis, the cranial tubules were in continuous contact with the Wolffian duct and had a short distal segment that expressed the same markers as the Wolffian duct. This raises the possibility that the cranial tubules were at least partly directly evolved from the Wolffian duct. The caudal tubules that represent the majority of the mesonephric nephrons, were initially seen in close proximity to the Wolffian duct but they were surrounded by a basement membrane already at the early stage of development. Both sets of tubules expressed characteristic markers for metanephric nephrons, i.e. brush border epitopes, cytokeratin-8/18, and p75 neurotrophin receptor even though some stage differences were apparent. At later stages of the mesonephrogenesis, the caudal tubules were located even further away from the Wolffian duct and formed only dead-end tubules. In contrast, the cranial tubules were well differentiated, and developed a distinct glomerulus-like extension in their tip which expressed markers of metanephric glomerulus such as podocalyxin (Table 2; Fig. 3A). It has been reported previously that all mesonephric tubules in mouse embryos are connected to the Wolffian duct (Smith and MacKay, 1991). As shown by the present data, only the cranial tubules are fused with the Wolffian duct, raising the possibility that these tubules may be secretory. Moreover, the cranial mesonephric tubules frequently formed branches (Figs 1C, 4B), which has also been reported in chick (Friebová-Zemanová and Goncharevskaya, 1982). In chick, these 'two-headed' nephrons were branched from the proximal segment but in murine species the branching occurs in the distal segment (Fig. 1C). Branches were found only in the cranial tubules and may reflect the presumptive epididymal branches (Fig. 4C,D).

Elucidation of the molecules regulating metanephric development has been started, but only limited data is available for the mesonephros. Several spontaneous mutations and transgenic mice lineages with disrupted genes show an abnormal metanephric development (reviewed by Sariola, 1996) and mesonephric development has been described in few of them (Kreidberg et al., 1993; Stark et al., 1994; Torres et al., 1995). Mice with a *pax-2* null-mutation lack the meso- and metanephric tubules (Torres et al., 1995), whereas the WT-1-deficient mice lack metanephric tubules but develop a few mesonephric tubules (Kreidberg et al., 1993). After discovering the two sets of mesonephric tubules in normal mice, we analysed mesonephric development in WT-1-deficient mice by whole-mount immunohistochemistry. The *WT1*^{-/-} embryos completely lacked the caudal set of tubules but developed the

cranial ones (Fig. 5B,C). Thus, WT-1 regulates the differentiation of the caudal but not the cranial mesonephric tubules. By whole-mount immunohistochemistry with several markers (Tables 1 and 2) we showed that cranial tubules were always

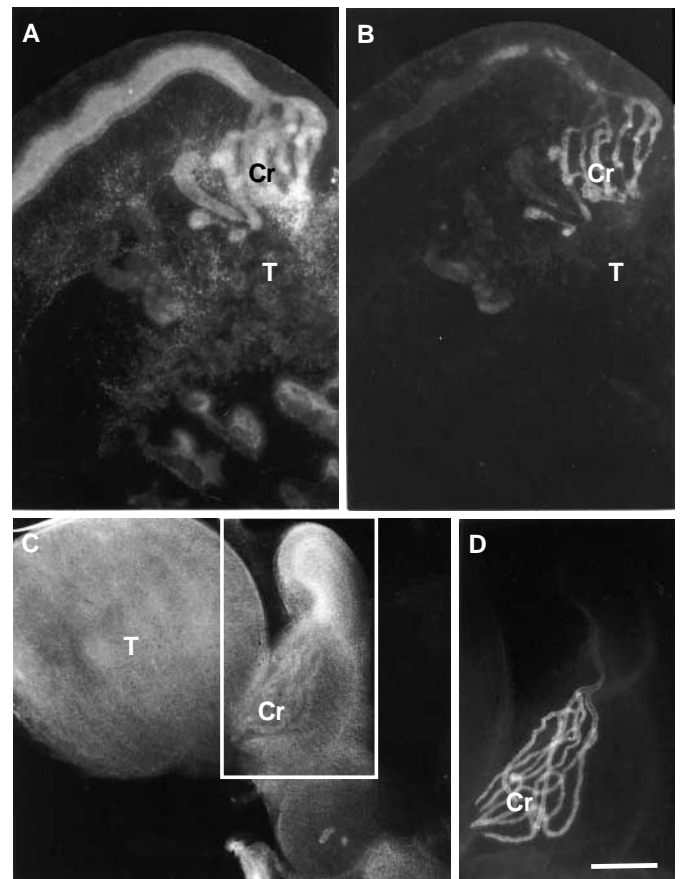


Fig. 4. Male mesonephros during the regression stage. The cranial mesonephric tubules differentiate to the epididymal ducts. Double whole-mount immunohistochemistry with antibodies against cytokeratin-8 (A,C) and brush border antigens (B,D). (A,B) Cranial mesonephric tubules of the E₁₆ male rat embryo show branched tubuli in close proximity to the developing testis. The caudal tubules have regressed almost totally. (C) At E₁₈ the mesonephric nephroi are no longer visible. The cranial tubules, however, have formed the epididymal ducts (framed) fused to the testis. (D) Higher magnification of the framed area shows cranial mesonephric tubules with characteristic branches. T, testis. Bar (A,B) 200 μm ; (C) 450 μm ; (D) 50 μm .

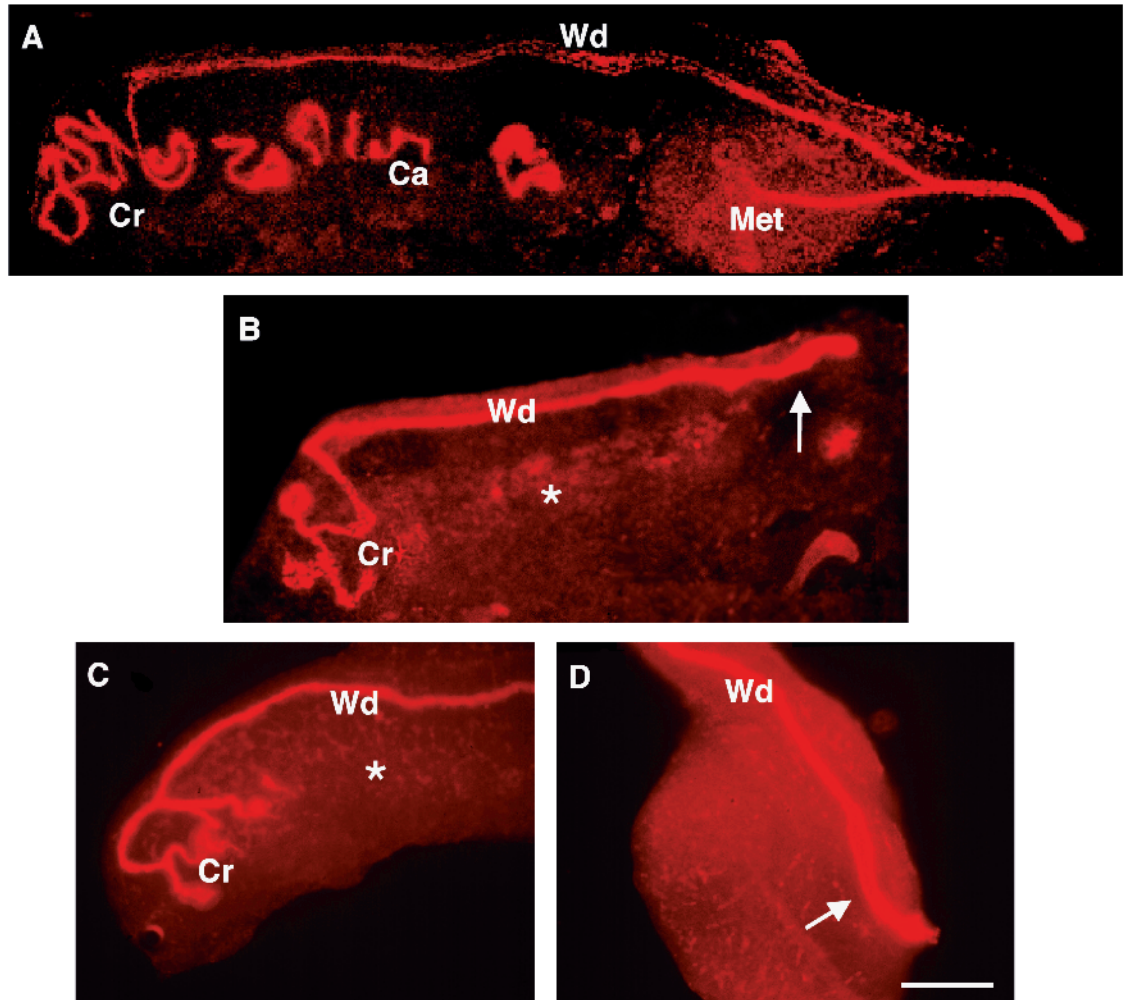


Fig. 5. WT-1-deficient mice lack the caudal mesonephric nephrons. Whole-mount immunohistochemistry with antibodies against brush border epitopes (A,B) and Pax-2 (C,D). (A) Wild-type mouse embryo urogenital block dissected at E_m11 and cultured for 1 day shows normal cranial and caudal mesonephric nephrons, Wolffian duct and developing metanephros. (B-D) Urogenital block from *WT1*^{-/-} embryo. No caudal mesonephric tubules (*) are visible and no ureteric bud develops (arrows). Met, metanephric kidney. Bar 300 μ m.

connected to the Wolffian duct, but the caudal tubules developed renal vesicles (Fig. 1A) and later nephrons (Fig. 2) that did not fuse with the Wolffian duct. This together with the finding of the differential regulation of these two distinct set of mesonephric tubules by WT-1 raises the possibility that the cranial tubules may be derived from the Wolffian duct and only the caudal tubules are derived from the mesonephric mesenchyme. Thus the molecular events during the caudal but not the cranial mesonephric development may be similar to those of metanephric development. This possibility still remains to be investigated.

Recent *in vitro* data suggest (Buehr et al., 1993) that the mesonephros contributes to testis differentiation, since a separated mouse testis fails to develop well differentiated cordal structures without mesonephros. Thus, although mesonephros is a transient organ, it is important for the further morphogenesis of the urogenital field. In accordance, the WT-1-deficient mutants lack not only the metanephros and the caudal tubules of mesonephros, but also the gonads (Kreidberg et al., 1993). However, further mesonephric development in male is dependent on the hormonal induction provided by the developing testis (Cunha et al., 1983). It is possible that the hormone-dependence of mesonephros in mice and rat embryos is only functioning in the cranial set of mesonephric tubules.

Because the cranial mesonephric tubules were not dependent

on WT-1 and the cranial and caudal tubules show different modes of development, the cranial tubules clearly represent a distinct population of mesonephric nephrons in murine species. These tubules are also the only tubules in the mesonephros that could be secretory, because the caudal tubules are never fused with the Wolffian duct. We cannot at the moment, without a reliable marker for murine pronephric tubules, rule out the possibility that the cranial tubules could represent the last caudal tubules of the pronephros, and thus, epididymal ducts would be pronephric rather than mesonephric. This is unlikely, however, because by morphological criteria the cranial tubules of the mesonephros are clearly separated from the more anterior structures, even at the earliest stage of development, and there seems to be no overlapping stage where pro- and mesonephric structures would be fused. Moreover, cranial mesonephric tubules have a different developmental fate from transient pronephric and caudal mesonephric tubules, and they represent the only group of tubules during pro- and mesonephrogenesis, which remain (in male) while all others, earlier and later stages, regress. The caudal tubules of the mesonephros, however, are derivatives of a cell population from the mesonephric mesenchyme and differentiate similarly to metanephric nephrons.

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