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# YAC complementation shows a requirement for *Wt1* in the development of epicardium, adrenal gland and throughout nephrogenesis

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## SUMMARY

**The Wilms' Tumour gene *WT1* has important functions during development. Knock-out mice were shown to have defects in the urogenital system and to die at embryonic day E13.5, probably due to heart failure. Using a *lacZ* reporter gene inserted into a YAC construct, we demonstrate that *WT1* is expressed in the early proepicardium, the epicardium and the subepicardial mesenchymal cells (SEMC). Lack of WT1 leads to severe defects in the epicardial layer and a concomitant absence of SEMCs, which explains the pericardial bleeding and subsequent embryonic death observed in Wt1 null embryos. We further show that a human-derived *WT1* YAC construct is able to completely rescue heart defects, but only partially rescues defects in the urogenital system. Analysis of the observed hypoplastic kidneys demonstrate a continuous requirement for WT1 during nephrogenesis, in particular, in the formation of mature glomeruli. Finally, we show that the development of adrenal glands is also severely affected in partially rescued embryos. These data demonstrate a variety of new functions for WT1 and suggest a general requirement for this protein in the formation of organs derived from the intermediate mesoderm.**

Key words: Adrenal, Epicardium, Nephron, Transgenics, WT1, YAC, Mouse, Human, Wilms' Tumour

## INTRODUCTION

The *Wilms' Tumour 1* gene (*WT1*) was originally identified on the basis of its disruption or deletion in a significant proportion (10-15%) of Wilms' tumours (Hastie, 1994 and references therein). (Where we refer to the *Wilms' Tumour 1* gene generically, we use the human abbreviation (*WT1*).) Wilms' tumour affects around 1 in 10,000 children and is a good example of tumourigenesis caused by normal developmental processes gone awry. The tumour arises from a single kidney precursor (metanephric blastema) cell in which developmental control has been lost.

The *WT1* gene consists of 10 exons spanning 50 kilobases of genomic sequence. It encodes a protein with four zinc fingers of the $C_2H_2$ type. Alternative splicing of *WT1* generates four different isoforms (Hastie, 1994), which may have dual roles both as a transcription factor and in RNA processing (Larsson et al., 1995). During mammalian development, *WT1* is expressed in the kidneys, gonads, coelomic epithelium and subcoelomic epithelial mesenchyme (Pritchard Jones et al., 1990; Pelletier et al., 1991b; Moore et al., 1998). The domains in which *WT1* is expressed suggest that one of the roles it may play is in enabling cells to flip between mesenchymal and epithelial cell states. Within the developing kidney, the expression pattern of *WT1* indicates that it may play different roles at several stages of nephrogenesis. It is expressed at low levels in the metanephric blastema. Following invasion, the ureteric bud divides forming branches within the metanephric blastema and induces the blastema to condense at the tip of each. This condensed mesenchyme undergoes a burst of proliferation and then differentiates into the renal vesicle, a hollow ball of epithelial cells which continue to express *WT1*. The renal vesicle now undergoes a series of morphological changes; it first forms a comma-shaped then an S-shaped body. During this process, *WT1* expression becomes localised to the cells at the proximal part of the S-shaped body. These cells flatten to form the glomerular podocytes. After birth, expression of WT1 in these cells continues implying that the gene has a role in the maintenance of their differentiated state.

Confirmation that *WT1* is required for correct urogenital development came from the discovery that *WT1* mutation is responsible for developmental abnormalities of the gonad and kidney in three human congenital syndromes. WAGR, Denys-Drash Syndrome (DDS) and Frasier syndrome (van Heyningen et al., 1990; Pelletier et al., 1991a; Barbaux et al., 1997) show slightly different phenotypes in the urogenital system, which is reflected in the different nature of mutations found in *WT1*.

The kidneys and gonads of *Wt1* homozygous null mice fail to develop. The metanephric blastema forms, but the ureteric bud does not branch from the mesonephric duct and, as no

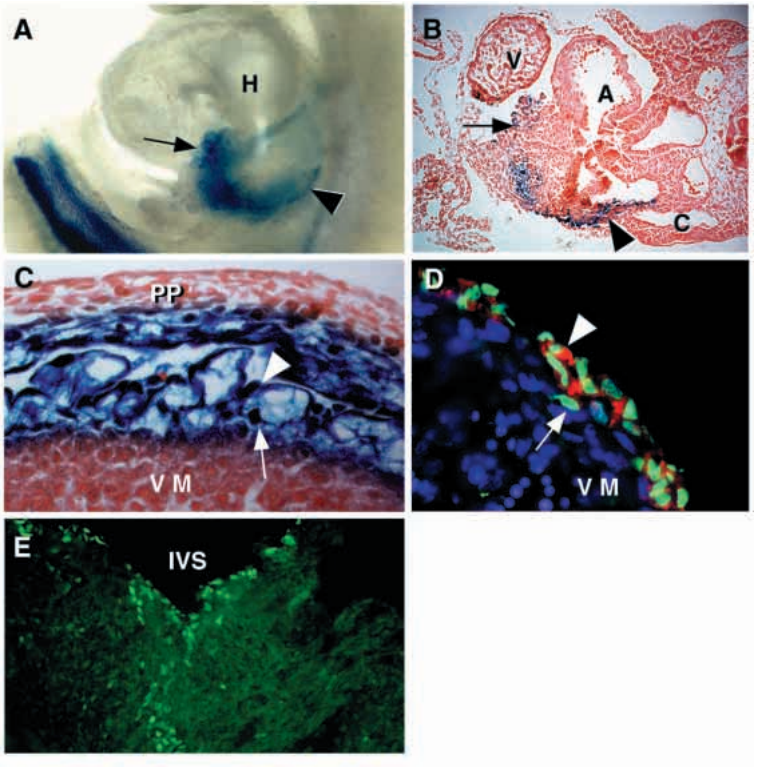


**Fig. 1.** Expression of Wt1 in the forming epicardium and migrating subepicardial mesenchymal cells. (A) Expression of the WT470LZ$^{H}$ transgene in an E9.5 embryo marks cells on the cranial surface of the septum transversum, which make up the proepicardial organ (arrow). A further domain of expression expands to meet the surface of the atria embedded within the septum transversum (arrowhead). H, heart; ventral, left; cranial, up. (B) Transverse section of an E9.5 embryo showing expression of W470LZ$^{H}$ in the proepicardial domain of the transversum (arrow) and the atrial surface (arrowhead). V, ventricle; A, atrium; C, pleuroperitoneal canal; ventral, left). (C) Expression of WT470LZ$^{H}$ in an E12.5 mouse embryo. WT470LZ$^{H}$ is expressed in the epicardium (arrowhead), subepicardial mesenchyme cells (arrow). WT470LZ$^{H}$ is also expressed in the coelomic epithelium and subcoelomic epithelial cells of the parietal pericardium (PP). VM, myocardium of ventricle. (D) Co-expression of Wt1 (green) and cytokeratins (red) in the epicardium (arrowhead) and subepicardial mesenchyme (arrow) of a ventricle of an E13.5 mouse heart. Blue staining (DAPI) marks nuclei. Labels as in C. (E) Migration of Wt1-positive cells (green) into the intraventricular sulcus (IVS) of an E13.5 mouse heart.

induction occurs, these cells die. Isolated and cultured *Wt1* null metanephric blastema is unable to condense and begin to proliferate even if it is given proper induction. Hence *WT1* plays two roles at this early stage of kidney development. It is required for both the formation of the ureteric bud and the ability of the metanephric blastema to be induced by the bud. Mice homozygous for the *Wt1* null allele die in utero around halfway through gestation (E12-14). The heart of these *Wt1* null embryos is malformed and the ventricular myocardium thin, the embryos are edemic and have pericardial bleeding, both of which are symptomatic of heart failure (Kreidberg et al., 1993).

It is, at present, unclear both how disruption of the *Wt1* gene leads to heart malformation and whether this is indeed responsible for the midgestational death. In addition, the dynamic expression pattern of *Wt1* within the developing kidney and the glomerular phenotypes of DDS and Frasiers syndrome patients imply that the gene may play a role at more than the initial stages of kidney development illustrated in the null mice. To study the expression of *Wt1* in the developing heart both in wild-type and *Wt1* null embryos, we used *WT1*lacZ fusion YAC transgenic lines. We then used human-derived, YAC transgenic constructs spanning the *WT1* locus to complement *Wt1* null mice. The use of YACs as constructs allowed us to introduce the transgene in an almost natural chromosomal context and hence give correct gene regulation and isoform ratios. We used a human-derived YAC in this study for several reasons. Firstly, we have previously shown that transgene expression from this YAC mimics the endogenous expression of the *Wt1* gene (Moore et al., 1998). Secondly, the human and mouse WT1 proteins have an amino acid sequence identity of over 96% and ourselves and others have shown that human-derived YAC transgenes can be used to fully complement other null phenotypes in mice (Schedl et al., 1996). Finally, the use of a human construct facilitates the distinction between endogenous and transgenic gene copies.

## MATERIALS AND METHODS

### Generation and molecular analysis of transgenic mice

Line H (WT470LZ$^{H}$) mice and *Wt1* knockout mice were generated and described elsewhere (Moore et al., 1998). WT280 transgenic mice (lines WA, WB, WC and WW) were generated by pronuclear injection (Schedl et al., 1996) of amplified WT280 (Menke et al., 1998) YAC DNA. Positives were identified by Southern blot analysis using WT33 as a probe. Molecular probes used in this study: WT33-*WT1* cDNA, SVA-YAC short vector arm probe (1.4 kb *Sal*I-*Stu*I fragment isolated from pYAC4), LVA-YAC long vector arm probe (1.1 kb *Eco*RI-*Pst*1 fragment isolated from pBR322). (Note that due to technical error, line WB could not be analysed in detail. However, preliminary results showed that WB gave rescue of epicardial defects and midgestational lethality but failure of complete urogenital rescue when crossed onto a *Wt1* null background.)

Line H, WA, WC and WW were genotyped using human-specific primers (intron 9-exon10: 5′ACTTCACTCGGGCCTTGATAG3′ and 5′GTGGAGAGTCAGACTTGAAAG3′). Heterozygous *Wt1* knockout mice were genotyped using primers for the bacterial neo$^{R}$ gene (5′GCGATGCCTGCTTGCCGA3′ and 5′GAAGGCGATAGA-AGGCGA3′). *Wt1* homozygous null embryo were detected by assaying for the absence of the *Wt1* 5′ proximal promoter (370 bp) (5′GGAGATAAGCCCCAAAGTTA3′ and 5′CCTAGCCTAGCTCA-GCAAAA3′) using *Pax6* primers as an internal positive control (150 bp) (5′TATCGAAGACACACTCTACC3′ and 5′TAGTTTATGG-ACTGATGTTCC3′).

RNA was isolated from E15.5, E17.5 and adult snap frozen tissues using the RNeasy kit (Quiagen) and reverse transcription carried out as detailed in Schedl et al. (1996). PCR was carried out using

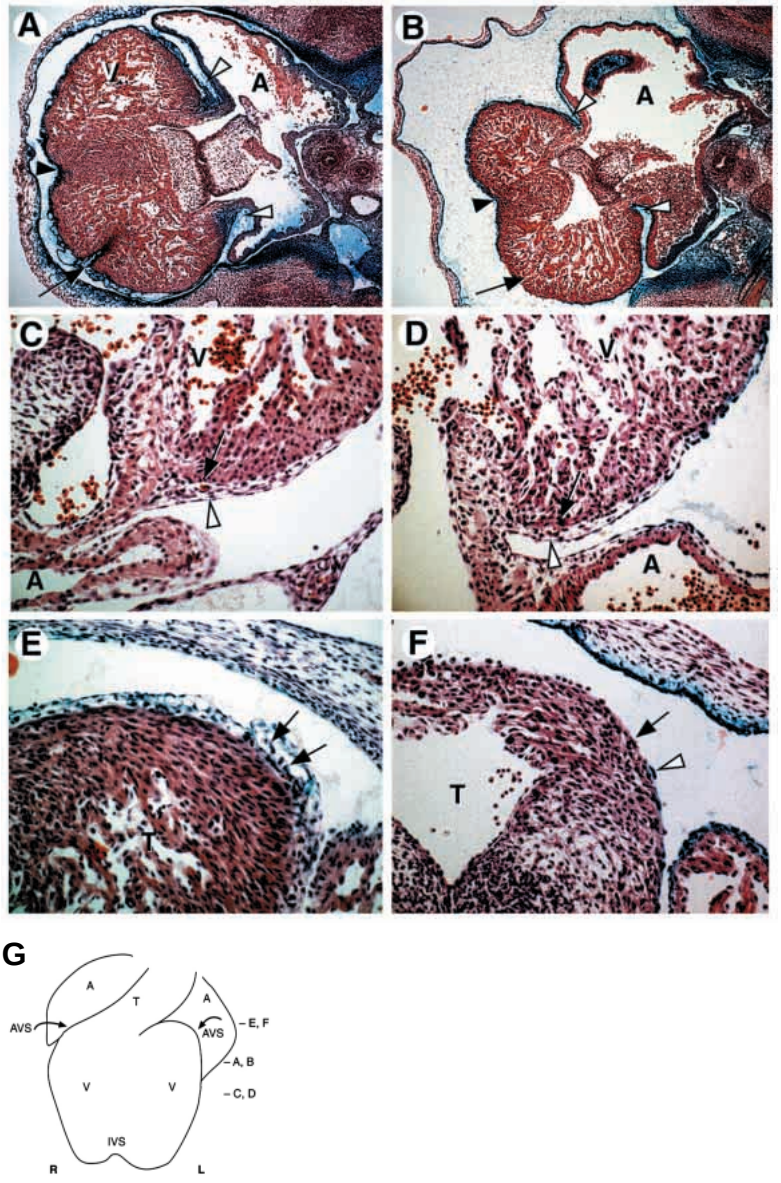


**Fig. 2.** Analysis of heart defects in *Wt1* null mice. Transverse sections through the heart of E12.5 (A,B) and E13.5 (C-F) embryos. Embryos in A, B and D-F carry the WT470LZ$^{H}$ transgene and were stained for β-galactosidase activity. The approximate position of each section in A-F is given in G. (A) Wild-type embryo. Normal formation of the epicardium and SEMCs (arrow). β-galactosidase-positive SEMCs can be seen migrating into the myocardium at the IVS (black arrowhead). Note the development of large pockets of SEMCs in the AVS (white arrowhead). In A and B, dorsal is right, ventral is left. A, atria; V, ventricles. (B) *Wt1* null embryo. Note the malformed atria (A) and ventricles (V), gaps in the epicardium (arrow) and reduction in the formation of SEMCs under the ventricular epicardium and in the AVS (white arrowheads). There is no migration of β-galactosidase-positive SEMCs at the IVS (black arrowhead). (C) Wild-type embryo, transverse close up of AVS (arrowhead). Note the presence of a capillary in a thick SEMC layer (arrow). In C-F, V,ventricle; A, atrium; dorsal is down and ventral up. (D) *Wt1* null embryo. Transverse close up of AVS (arrowhead). Note the presence of a capillary in a thin SEMC layer (arrow). The epicardium is complete. (E) Transverse close up of outflow tract (T) of a wild-type embryo. Note the presence of an extensive capillary network running through the β-galactosidase-positive SEMCs (arrows) beneath an intact epicardial layer. (F) Transverse close up of outflow tract (T) of a *Wt1* null embryo. Note only a few epicardial cells are present (arrowhead). The epicardium is nearly absent, with a subsequent loss of SEMCs and coronary vasculature (arrow). (G) Cartoon of a E13.5 mouse heart viewed from the front. Labels as above; additionally R, right; L, left; AVS, atroventricular sulcus; IVS, intraventricular sulcus.

the following primer pairs: for the +/–17aa isoforms (5′GACGCCCTACAGCAGTGACAA3′ and 5′CGCACATCCTGA-ATGCCTC3′) and for the +/– KTS isoforms: (5′CCACACCAGG-ACTCATACAG3′ and 5′TGCATGTTGTGATGGCGGAC3′). To distinguish between human- and mouse-specific PCR products, the samples were digested with either *Mae*I, for +/– exon 5, or *Msp*I, for +/– KTS, separated on a 10% non-denaturing polyacrylamide gel (1× TBE) and the bands visualised by silver staining. (Restriction digestion of PCR products from these reaction will fail to digest hybrid molecules created by the pairing of one human-derived DNA strand to one mouse-derived. Hence the signal derived from the human in the +/–17aa RT-PCR where the mouse-derived bands are the product of restriction digest will be at a slightly higher ratio in relation to the mouse-derived bands than in the +/-KTS RT-PCR where the human-derived products are product of restriction digest.)

### Histology, staining for β-galactosidase activity, mRNA in situ hybridisation and protein in situ immunohistochemistry

Staged embryos (morning of vaginal plug was designated E0.5) were dissected in PBS. For histological analysis and radioactive in situ hybridisation, they were fixed overnight in 4% paraformaldehyde then wax embedded. Radioactive in situ mRNA hybridisation was carried out as described by Monaghan et al. (1991). Whole-mount in situ hybridisation was carried as described in Wilkinson (1992). RNA probes were synthesised using *P450scc* cDNA (Keeney et al., 1995), *c-ret* which was a gift of V. Pachnis; PSK2 is a 610 bp fragment of *Wt1* 3′ UTR used as a template for probe synthesis. Whole mounts and cryosections were stained for β-galactosidase activity as described in Moore et al. (1998). Embryos were cut on a Reichert-Jung 2030 standard microtome (7-8 μm sections) or a JUNG CM3000 cryostat (Leica) (10 μm). Counter staining was carried out via standard methods and sections analysed on a Zeiss Axioplan2 microscope using a Xillix CCD camera and Viewpoint software (Vysis).

Protein in situ immunohistochemistry was carried out according to Larsson et al. (1995) with modifications (details on request). The primary antibodies used in this study were: Tenascin clone Mtn-12 (Sigma) diluted 1/50, CD31/PECAM-1 clone MEC13.3 (PharMingen) diluted 1/50, Synaptopodin clone G1D4 (Progen) undiluted, WT1 product no. either C-19 or H-2 (Santa Cruz

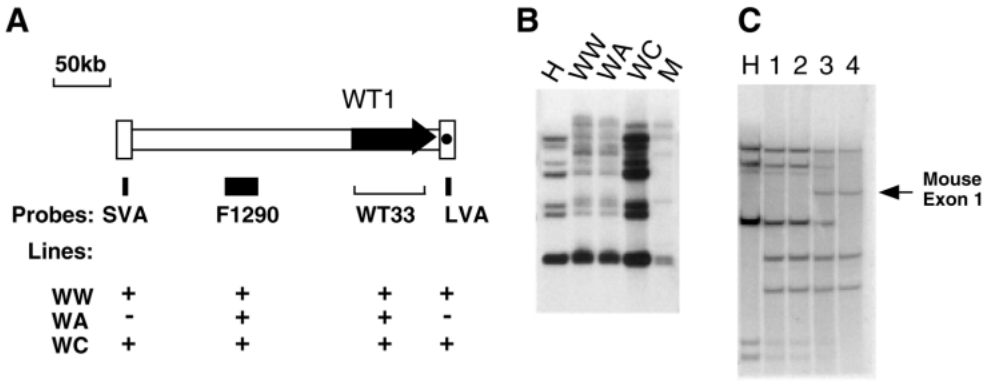


**Fig. 3.** Generation of YAC transgenic mice. (A) Cartoon of the WT280 YAC construct showing the position of the molecular probes used for Southern analysis and FISH (see Materials and methods for probe details). The *WT1* gene is represented by a black arrow pointing in the direction of gene transcription. The boxes represent the vector arms with a dot representing centromeric sequences. (+) indicates sequence integration in each of the lines. (B) *Eco*RI-digested genomic DNA from lines WA, WC and WW were hybrised with human *WT1* cDNA (WT33). In all lines, all human-specific fragments are detected suggesting that at least the integration of the *WT1* locus has occurred without rearrangement. Abbreviations: H, human; M, mouse control. (C) Southern analysis of newborn mice from a $WT280^{WA}$+/–,*WT1*+/– × *WT1*+/– cross. Genomic DNA was digested with *Bgl*II and hybridised with human *WT1* cDNA (WT33). Lanes 1&2, $WT280^{WA}$-complemented *WT1* null mice; lane 3, wild-type mouse carrying $WT280^{WA}$; lane 4, wild-type mouse carrying no transgene.

Biotechnology) diluted 1/100, Pax2 product no. PRB-276P (BAbCO) diluted 1/100, Pan-Cytokeratin product no. C-2562 (Sigma) diluted 1/100, Desmin product no. D-8281 (Sigma) diluted 1/20 and α-Smooth Muscle Actin clone 1A4 (Sigma) diluted 1/100. The secondary antibodies used were FITC or Texas Red-conjugated donkey anti-mouse, rat, goat or rabbit (Jackson Immunolabs) all diluted 1/200. Detection of apoptotic cells (TUNEL analysis) was carried out using an In Situ Cell Death Detection Kit, POD (Boehringer Mannheim) in accordance with the manufacturers instructions. Sections were analysed using a Zeiss Axioplan microscope with a Photometrics CCD camera and IP lab Spectrum software (Digital Scientific).

## RESULTS

### Expression of the *Wt1* gene during heart development

*Wt1* null mice die at midgestation with edema and a malformed heart. The stage at which these embryos die suggests that it is malformation of the heart which is leading to embryonic death (Kreidberg et al., 1993). To define what role *Wt1* plays in the formation of this organ we undertook a detailed analysis of the expression pattern of the *Wt1* gene during heart development. We made use of a YAC transgenic mouse line carrying a *lacZ* reporter gene under control of the *WT1* locus (line $WT470LZ^H$). Previous analysis has demonstrated that *lacZ* expression in line $WT470LZ^H$ completely mimics the endogenous expression pattern of *Wt1* (Moore et al., 1998).

The first expression of $WT470LZ^H$ in the region of the developing heart occurs at around E9, within mesenchymal villi on the cranial surface of the septum transversum (Fig. 1A,B). These villi are the proepicardial organ. They migrate across the pericardial cavity to touch the caudal surface of the heart ventricular myocardium. Once these cells reach the surface of the heart, they spread out and migrate over the myocardium to form the epicardium. The epicardium of the atria is formed in part by cells lying on the lateral surface of the sinus horns at the pleuro-peritoneal canal and in part from the proepicardial organ (Viragh and Challice, 1981). Both of these structures expressed $WT470LZ^H$ (Fig. 1A,B). Between E9.5 and E10, $WT470LZ^H$-positive cells progressively enveloped the caudal surface of the ventricular myocardium and, by E10.5, the epicardial covering of the ventricle was complete. The surface of the aorta was not covered until E11.5 (data not shown).

To confirm that these results reflect the expression of the endogenous WT1 gene, we carried out *Wt1* mRNA in situ hybridisation on mouse and chick embryos (data not shown). As with the transgene, *Wt1* expression in E9.5 mouse embryos was seen in the mesenchymal villi on the cranial surface of the septum transversum and a crescent of tissue running from this point as far as the sinus horns. In Hamburger-Hamilton (HH) stage 16 chick embryos, *WT1* was expressed in the proepicardial organ. Whilst in stage 17 embryos *WT1*-positive cell cords had grown from the proepicardial organ to touch the caudal surface of the heart over which *WT1*-expressing cells were now migrating.

In addition to cells forming the epicardium, we also identified $WT470LZ^H$-*positive* cells on the inner surface of the parietal pericardium between E9.5 and E10. The inner surface of the parietal pericardium will form a sheet of $WT470LZ^H$-positive coelomic epithelium by E10.5. These observations suggest that some cells of the parietal pericardium arise from the *Wt1*-positive cells on the cranial surface of the septum transversum. These data are also in agreement with a recent study noting that cells derived from a quail proepicardium and grafted into a chick embryo could become incorporated into the host parietal pericardium (Perez-Pomares et al., 1998).

Between E11.5 and E12.5 cells derived from the epicardium begin to delaminate into the subepicardial zone to form subepicardial mesenchymal cells (SEMCs). These cells form via an epithelial-to-mesenchymal cell type transition within the epicardium and are characterised by expressing both epithelial-specific cytokeratins and mesenchymal vimentin (Perez-Pomares et al., 1997). $WT470LZ^H$ is expressed in the SEMCs (Fig. 1C), a finding that agrees with our earlier observation that *Wt1* is expressed in mesenchymal cells derived via the proliferation of the coelomic epithelium (Moore et al., 1998).

Cells derived from the SEMC have recently been shown to migrate into the myocardium to give rise to coronary vascular smooth muscle, perivascular fibroblasts and intermyocardial fibroblasts (Mikawa and Gourdie, 1996; Dettman et al., 1998; Perez-Pomares et al., 1998). Analysis of $WT470LZ^H$

**Table 1. Spectra of different kidney structures found in *Wt1* null embryos partially complemented with WT280$^{WA,WC,WW}$**

| Type | WA | WC | WW | Kidney (E17.5) | Kidney histology (E17.5) |
|---|---|---|---|---|---|
| I | 6 | 10 | 11 | No ureters or kidneys. | – |
| II | 1* | – | – | Blind ending ureters. | – |
| IIIa | – | – | 1 | Ureters end in hypodyplastic kidneys. | No growth or branching of ureteric bud within the metanephric blastema tissue. No evidence of nephrogenesis, i.e. no condensation, comma, S-shaped bodies or glomeruli. |
| IIIb | 12 | 2 | – | Ureters end in hypodyplastic kidneys. | Growth and branching of the ureter within the metanephric blastema. Attenuated nephrogenic zone showing condensation but very few (or no) comma-shaped bodies, extremely rare (or no) S-shaped bodies and no mature glomeruli. |
| Wild-type | – | – | – | – | Growth and branching of the ureter within the metanephric blastema. Nephrogenic zone showing all stages of nephrogenesis, condensation of blastema around tips of the uretric branches, comma- and S-shaped bodies and mature glomeruli. |

The above data represents the culmination of the isolation of 344 embryos. Embryos of the genotype *Wt1*–/–,WT280$^{WA,WC,WW}$+/– occurred in the correct Mendelian ratio of 1/7, (*Wt1*–/–, WT280$^{WA,WC,WW}$–/– are midgestational lethal).

*One *Wt1* null WT280$^{WA}$-complemented embryo had one kidney type II and one type IIIb

expression in E12.5 transgenic embryos shows a number of *lacZ*-positive cells migrating into the heart at the intraventricular sulcus (Fig. 2A). To confirm that this expression of WT470LZ$^{H}$ in the SEMCs is bona fide, we carried out immunohistochemical staining of E13.5 wild-type mouse hearts with anti-Wt1 and Pan-Cytokeratin antibodies. Wt1 was co-expressed with cytokeratins in the SEMCs following their delamination from the epicardium (Fig. 1D). Furthermore, Wt1-positive cells could clearly be seen migrating into the myocardium from the SEMC layer, in particular at the caudal regions of intraventricular sulcus (Fig. 1E). Upon formation of coronary vascular smooth muscle, perivascular fibroblasts and intermyocardial fibroblasts, *Wt1* expression was switched off. Analysis of WT470LZ$^{H}$ and anti-Wt1 antibody expression in 2 days post birth (P2) mouse heart did not show *Wt1* expression in any other structure apart from the epicardial layer (data not shown).

### *Wt1* is required for correct epicardial development

To study the function of Wt1 in heart development, we crossed line WT470LZ$^{H}$ onto the *Wt1* null backgound. (Genetic status: *Wt1*–/–; WT470LZ$^{H}$+/–). Embryos were isolated at E12.5 and E13.5, and genotyped by PCR analysis. Histological analysis of *Wt1*–/–; WT470LZ$^{H}$+/– embryos clearly demonstrated defects in epicardium formation (Fig. 2). An epicardial layer formed at the caudal end of the heart, but was missing over much of the cranial regions of the ventricles, atria and the aorta. Unsurprisingly, no SEMC formation occurred in those areas where the epicardium was absent. However, even where the epicardium did form, SEMCs were not seen. The one exception to this was the atrioventricular (AV) and intraventricular (IV) sulci, where the numbers of SEMCs was still greatly reduced. These results were confirmed by immunohistological analysis of E13.5 *Wt1* null mice with anti-pan-Cytokeratin (data not shown).

Between E12 and E13.5, a large amount of blood appears in the pericardial cavity of *Wt1* null embryos (Kreidberg et al., 1993). We hence investigated whether defects in the epicardial layer of the heart gives rise to this pericardial bleeding. At E12.5 in wild-type embryos, capillaries form in the AV and IV sulci; these vessels proliferate cranially to link up with the coronary arteries at E13.5 (Viragh and Challice, 1981). In contrast, no vessel formation in the IV sulcus was observed in 5/5 *Wt1* null mice analysed. At the caudal end of the AV sulci, vessels formed in the same way as wild-type littermates, although the number of SEMCs in these regions were reduced (Fig. 2C,D). At the cranial region of the AV sulci and over the ventral cranial region of the ventricles, there was no epicardial formation and hence no vessel formation (Fig. 2E,F). The opening of the coronary artery into the pre-existing subepicardial vascular bed occurs at E13.5 in wild-type embryos (Viragh and Challice, 1981). In *Wt1* null embryos, no such bed is formed and hence blood will flow directly into the pericardial cavity, augmenting bleeding already caused by the disruption of the coronary vasculature itself.

### A 280kb YAC transgene spanning the WT1 locus (WT280) rescues epicardial development and midgestational lethality in Wt1 null mice

To express *WT1* in the correct *Wt1*-specific expression pattern, we used a 280 kb YAC (WT280) spanning the *WT1* locus (Menke et al., 1998) as a transgene (Fig. 3A). WT280 can drive the correct *Wt1*-specific expression pattern of a *lacZ* reporter gene inserted into its *WT1* locus (Moore et al., 1998). We generated four transgenic lines, only three of which (WA, WC and WW) could be studied in detail (see Materials and Methods). Southern blot analysis with a *WT1* cDNA (Fig. 3B) demonstrated the presence of all human-specific *Eco*RI fragments in each of these transgenic lines. The absence of any additional bands in lines WA and WW suggests that at least the *WT1* gene has integrated into the mouse genome without major rearrangements. A cosmid probe to the marker D11S87, 87 kb upstream of *WT1*, used for fluorescent in situ hybridisation (FISH) detected signals in each of the lines (data not shown). Probes derived from the YAC vector arms hybridised to DNA of lines WC and WW in Southern analysis of *Eco*RI-digested transgenic genomic DNA. In contrast, line WA seemed to have lost upstream and downstream vector sequences from the WT280 YAC during integration (summarised in Fig. 3A). Semiquantitative PCR using primers in the 3′UTR of *WT1* (Moore et al., 1998), as well as FISH

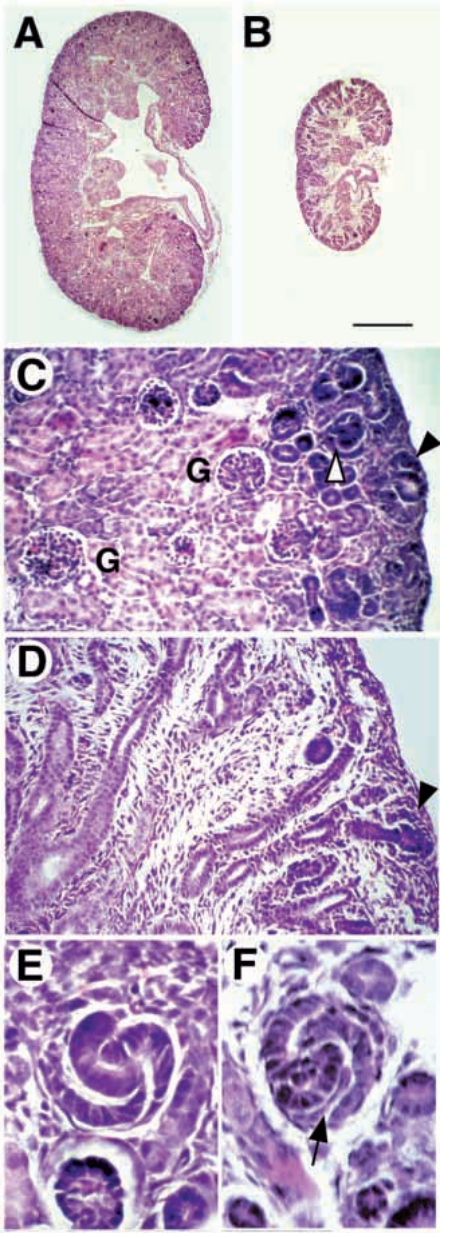


**Fig. 4.** Histological analysis of partially complemented kidneys at E19.5. (A) Wild-type kidney. (B) *Wt1*–/–, WT280$^{WA}$ kidney. Partially complemented kidneys can range in size from that shown to approximately half the size. Scale bar for A and B approximately 550 μm. (C) Wild-type kidney showing ureteric bud branches, condensing mesenchyme (black arrowhead), comma-shaped bodies (white arrowhead) and mature glomeruli (G). (D) Partially complemented kidney with ureteric bud branches and condensing mesenchyme (arrowhead). (E) S-shaped body in wild-type kidney. (F) S-shaped body in *Wt1*–/–, WT280$^{WA}$ kidney. Note the invasion of a capillary (arrow).

analysis, demonstrated the integration of 1-2 copies of WT280 in lines WA and WW and approximately 4-5 copies in line WC (data not shown). Attempts to breed these lines quickly established that lines WC and WW were male sterile, possibly due to the inclusion of a HSV-tk gene on the YAC centromeric vector arm. This vector arm had not integrated in line WA.

All three transgenic lines (WA, WC and WW) were crossed onto the *Wt1* null background. No pups that were *Wt1*–/– but lacking a WT280$^{WA,WC,WW}$ transgene were born. However, newborns with a complemented genotype (*Wt1*–/–;WT280$^{WA,WC,WW}$+/–) were recovered at the expected Mendelian frequency (1/7) indicating that the transgene completely rescues those defects leading to the midgestational death of the nulls (Fig. 3C). Histological analysis of *Wt1*–/–;WT280$^{WA,WC,WW}$+/– embryos at E13.5, E15.5 and E17.5 showed that epicardium formation had occurred over the entire surface of the heart including the aorta. The epicardial layer and subepicardial vasculature of these embryos was indistinguishable from that of wild-type littermates. Additionally, no thinning of the ventricular myocardium or pericardial bleeding was found (data not shown).

We also analysed partially rescued embryos for defects in the diaphragm, a structure that is derived in part from the septum transversum. Whereas *Wt1* null animals showed herniation of the diaphragm (Kreidberg et al., 1993), this tissue developed normally in *Wt1*–/–;WT280$^{WA,WC,WW}$ +/– embryos (data not shown). Taken together, these data indicate that the human WT280$^{WA,WC,WW}$ transgene is able to completely rescue the defects in structures derived from the septum transversum (epicardium and diaphragm) which occur in *Wt1* null embryos.

### *Wt1* is required at multiple steps during urogenital development

Transgene-complemented mice (*Wt1*–/–;WT280$^{WA,WC,WW}$+/–) survived until birth but died within the following 48 hours. Postmortem analysis illustrated a varying degree of kidney development within these animals. This phenotype ranged from a complete absence to the development of hypodysplastic kidneys. In all other littermates, kidney development was normal, indicating that this kidney phenotype was not a dominant effect of the YAC transgene on either a wild-type or Wt1+/– background. This phenotype was also never seen in *Wt1* null embryos isolated before midgestational death (E12.5-E13.5), on the same genetic background (data not shown).

In order to determine the nature of the failure of kidney development, a preliminary analysis was carried out on E11.5, E13.5, E15.5 and E18.5 kidneys isolated from WT280$^{WA,WC,WW}$ transgene-rescued *Wt1* null embryos. A more detailed study was carried out at E17.5. A range of different structures that fall into classes similar to those previously described by Schuchardt et al. (1996) in mice homozygous null for the *ret-k* gene were seen (summarised in Table 1).

#### Histological analysis of kidney formation

The majority of the *Wt1*–/–;WT280$^{WA,WC,WW}$+/– embryos showed no sign of the ureteric bud or metanephric blastema at E13.5, E15.5, E17.5 or E18.5. This phenotype is equivalent to that seen in uncomplemented *Wt1* null littermates (type I phenotype; Table 1). It hence represents a failure to release the block on ureteric bud induction caused by a lack of *Wt1* expression in the metanephric blastema.

In one out of 43 embryos ureters had formed and ended in a small piece of tissue, which represented metanephric blastema that had not undergone induction or proliferation. The ureteric bud had failed to branch within this tissue (type IIIa, Table 1).

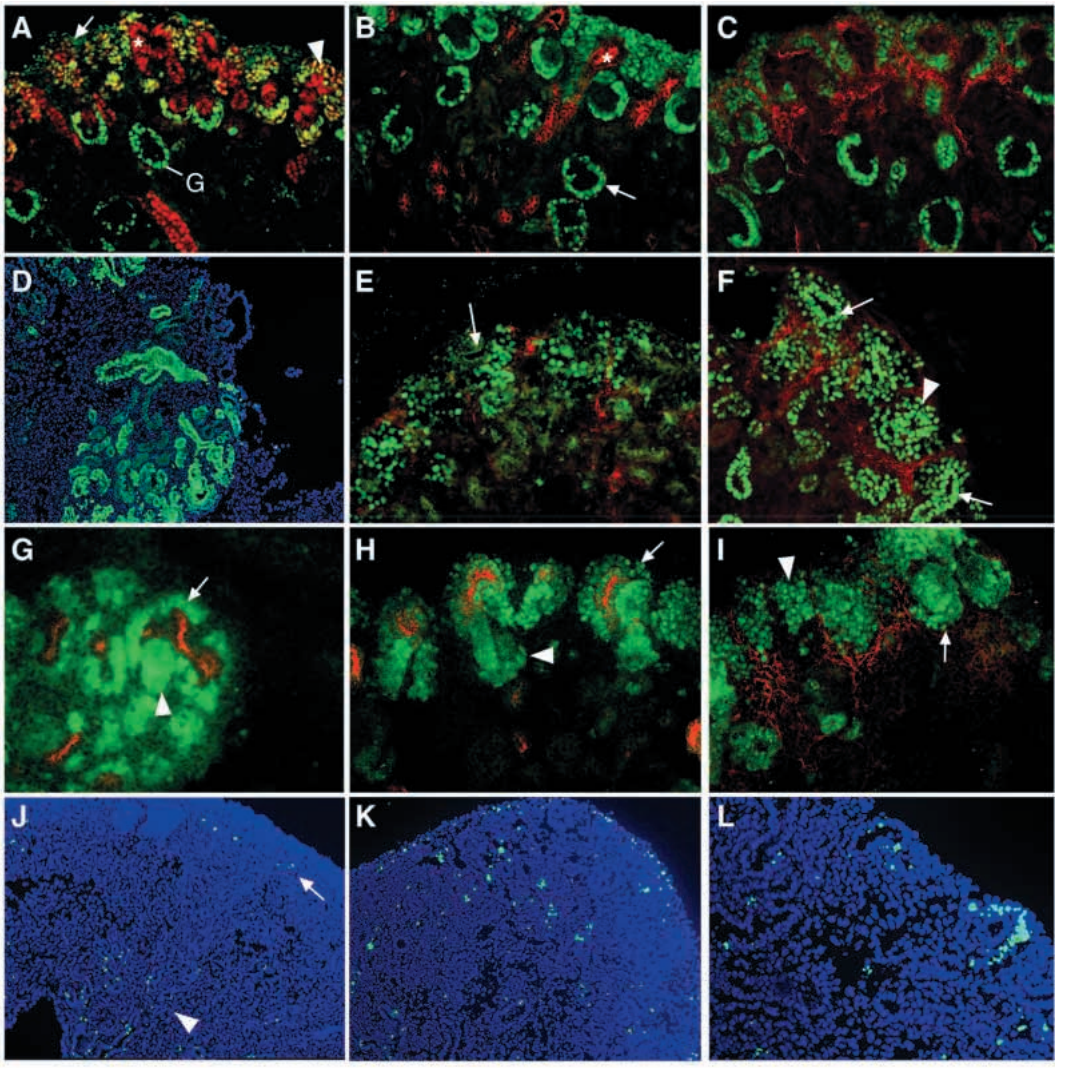


**Fig. 5.** Immunohistochemical analysis of partially complemented kidneys at E17.5. In all panels the kidney periphery is shown either towards the top of the panel or the top and right of the panel. (A-I) Immunostaining for genetic markers for kidney development: (A-C) wild-type; (D-I) *Wt1*–/–, WT280$^{WA}$ kidneys. (A) Wt1 (green), Pax2 (red), overlap between the two expression domains shows up yellow. Wt1 is expressed in the metanephric mesenchyme of the kidney periphery (arrow), the condensing blastema (arrowhead), the proximal part of the comma-shaped bodies and the podocytes of the glomerulus (G). Pax2 is expressed in the ureteric bud branches (asterisk) and the condensing metanephric blastema (arrowhead). It is expressed in the comma-shaped body, but later becomes lost from podocyte precursor cells. (B) Wt1 (green). Pan-cytokeratins (red) mark the branches of the ureteric bud (asterisk) and the cells of the Bowman's capsule (arrow). (C) Wt1 (green), Tenascin (red) marks the stromal tissue differentiating from a subset of metanephric blastema cells. (D) Segmental branching of the ureteric bud in a *Wt1*–/–, WT280$^{WA}$ kidney. Pax2 is in green. Cell nuclei are marked by DAPI (blue). (E) *Wt1*–/–, WT280$^{WA}$ kidney in which nephrogenesis is blocked at the condensation stage. Wt1 (green) is present only in a few cells in the condensation zone, a branch of the ureteric bud is marked with an arrow. Staining for tenascin (red) indicates that differentiation of stromal cells is still occurring between the zone of condensation. (F) Same sample as E. Pax2 (green) marks both the ureteric buds (arrow) and the condensing metanephric blastema cells (arrow head). Note that Pax2 is expressed in all condensing cells and hence is in a wider domain than that of Wt1. Tenascin is in red. (G) *Wt1*–/–, WT280$^{WA}$ kidney in which nephrogenesis has proceeded past the condensation stage. Pan-cytokeratin (red) marks the branches of the ureteric bud. Wt1 (green) is expressed in condensing metanephric blastema (arrow) and a forming renal vesicle (arrowhead). (H) *Wt1*–/–, WT280$^{WA}$ kidney in which nephrogenesis has proceeded past the condensation stage. Pan-cytokertain (red) marks the branches of the ureteric bud; Pax2 (green). Arrow, condensing metanephric blastema; arrowhead, comma-shaped body. (I) *Wt1*–/–, WT280$^{WA}$ kidney in which nephrogenesis has proceeded past the condensation stage. Tenascin (red) marks the differentiation of stromal cells. Pax2 (green); arrow, condensing metanephric blastema; arrowhead, comma-shaped body. (J-L) Analysis for the presence of apoptotic bodies using the TUNEL method. Cell nuclei are indicated by DAPI staining blue; apoptotic bodies are green. (J) Wild-type kidneys show two domains of apoptosis in the medullary papilla (arrowhead) and in the nephrogenic zone (arrow). (K) In a *Wt1*–/–, WT280$^{WA}$ kidney, apoptosis levels are increased and no longer restricted to the medullary and nephrogenic domains. (L) Magnification of the attenuated nephrogenic region of a *Wt1*–/–, WT280$^{WA}$ kidney. Note increased levels of apoptosis in both the kidney mesenchyme and epithelia.

15 out of 43 embryos showed hypodysplastic kidneys of varying sizes ranging from $\frac{1}{4}$ to $\frac{1}{2}$ that of wild-type littermates (Fig. 4B). Histological analysis of these kidneys indicated that the ureter had branched repeatedly and condensation was taking place. However, the majority of the nephrogenic zone of these kidneys was missing. By histological criteria, the degree of nephrogenesis occurring within these kidneys was heterogeneous. Approximately 3/15 showed condensation of the metanephric mesenchyme around the tips of ureteric branches, but no further sign of nephrogenesis (Fig. 4D). In the remaining 12 embryos, however, nephrogenesis up to the stage of comma-shaped bodies occurred in low numbers. The formation of S-shaped bodies, some of which showed invasion by vascular structures (Fig. 4F), was extremely rare. No structures representing mature glomeruli were ever observed.

### Analysis of marker expression in partially complemented kidneys

We wished to determine whether the phenotype of the type IIIb partially complemented kidneys represented blocks of the normal nephrogenic pathway or the formation of aberrant nephron-like structures. To do this, the expression of several markers specific for different stages of kidney development was analysed in wild-type and type IIIb kidneys. We used antibodies against the following proteins: Wt1; Pax2, which is expressed in the branching ureter as well as the condensing metanephric blastema; Synaptopodin, a marker for mature podocytes (Mundel et al., 1997); PECAM1 (CD31), a marker of vascular cell types (Vega et al., 1996); Tenascin, a marker for the stromal component of the kidney (Aufderheide et al., 1987); Desmin, a marker for metanephric blastema stem cells (Koseki et al., 1992); Cytokeratins, which are markers for epithelial cell types and hence mark the ureteric bud and the developing nephron with low expression in the renal vesicle and comma-shaped body and high expression in the Bowman's capsule and the mature nephron tubule (Hölthofer et al., 1984) and α-Smooth Muscle Actin, a marker for smooth muscle and perivascular cells. c-ret expression, normally present at the tip of the branching ureter, was investigated by in situ hybridisation (Schuchardt et al., 1996).

Branching of the ureter varied in the type IIIb kidneys, as determined by staining with pan-Cytokeratin and Pax2 antibodies. Some kidneys had branches running throughout the kidney whilst others had only segments of the metanephric mesenchyme into which branching had occurred (Fig. 5D). In regions where no branching had occurred, we saw no formation of nephric structures. Segments devoid of branching contained Tenascin-positive stromal cells and PECAM1-positive capillaries, but no expression of Wt1, Pax2, Cytokeratins or Synaptopodin.

Where ureteric branches ran throughout the metanephric mesenchyme Wt1 expression correlated with the degree of nephrogenesis occurring. In kidneys showing a block at the condensation stage Wt1 was expressed only in scattered cells at the kidney perimeter and at a higher level in those cells undergoing condensation (Fig. 5E). In the same samples, however, Pax2 showed a wider expression pattern in all the metanephric mesenchyme that was condensing around the end of the ureter branches (Fig. 5F). In partially complemented kidneys, which had bypassed a block at the condensation stage, Wt1 expression was also found in renal vesicles, comma-shaped bodies and, very rarely, S-shaped bodies; however, this expression was often very weak (Fig. 5G). In contrast, in the same samples, Pax2 was correctly expressed in these structures at levels similar to wild type (Fig. 5H,I). Similarly all other markers were expressed in the correct structures in the partially rescued kidneys where these were present. The podocyte-specific marker Synaptopodin was absent from all partially rescued kidneys, in correlation with our histological findings. In summary, in the kidneys of these partially rescued null mice, of those markers investigated, only Wt1 expression deviated from that expected in the nephric structures that were present. Furthermore, the comma- and S-shaped bodies present represented true stages of the nephrogenic pathway rather than the formation of aberrant nephron-like structures.

### Apoptosis in partially complemented kidneys

As the developing kidney is modelled by extensive apoptosis (Coles et al., 1993), we analysed the pattern and number of cells undergoing apoptosis. In wild-type E17.5 kidneys, apoptosis was restricted to the medullary papilla and the nephrogenic region in particular in the mesenchymal cell component adjacent to the forming nephrons (Fig. 5J). In type IIIb kidneys, the amount of apoptosis was increased in both the mesenchyme and epithelial compartments of the kidney and no longer restricted to the medullary papilla and the nephrogenic region (Fig. 5K). Furthermore, we frequently saw focal domains of mesenchyme undergoing extensive apoptosis and apoptosis in epithelial ureteric bud branches even at the kidney periphery (Fig. 5L). By birth, the levels of apoptosis in the metanephric mesenchyme had reduced to levels more similar to wild type, but the levels in the epithelium remained significantly higher than that of wild-type littermates

### Adrenal formation in partially complemented UGS

In addition to showing defects in the formation of the urogenital system, initial macroscopic analysis of $WT280^{WA,WC,WW}$-complemented null mice at birth also suggested the lack of adrenal glands. To distinguish between a mere displacement or complete absence of adrenal glands, we prepared serial sections of E15.5 line WW embryos and wild-type littermates and hybridised them with a probe for *P450scc*, a steroid hydroxylase expressed at high levels within the adrenal cortex and the testis (Keeney et al., 1995). A specific hybridisation signal was observed just below the forming diaphragm. However, this adrenal-like structure was greatly reduced in both size and hybridisation signal when compared to wild-type mice (Fig. 6). *Wt1* is therefore required for correct adrenal formation.

## Analysis of *WT1* mRNA expression from the $WT280^{WA,WC,WW}$ transgenes

Why is *WT1* expression unable to rescue the *Wt1* null phenotype in the urogenital system? Human patients in which the ratio of +KTS:–KTS isoforms of the WT1 protein have been disrupted suffer from failure of gonad development and glomerular sclerosis (Barbaux et al., 1997). These two structures fail to develop correctly in the $WT280^{WA,WC,WW}$-complemented *Wt1* null mice. Therefore, one possibility is that the WT280 transgene does not express *WT1* with all the correct splice isoforms or these isoforms in the proper ratios. A second possibility is that the level of *WT1* transcription derived from the WT280 transgene is not enough to fully rescue the lack of endogenous *Wt1*.

We used a semiquantitative RT-PCR approach to analyse the levels of transgene expression and the ratio of different *WT1* mRNA splice isoforms derived from the $WT280^{WA,WC,WW}$ transgenes. Primers identical for the mouse and human *WT1* gene were designed flanking alternative exon5 and the alternative splice donor site at the end of exon 9 (to detect the +/– KTS isoforms). Both sets of primers span a restriction site difference between mouse and human. Therefore, it is possible to distinguish between human-template-derived and mouse-template-derived PCR products following their restriction. Initial analysis was performed on adult kidneys and testis carrying the hemizygous human transgene on a *Wt1*+/–

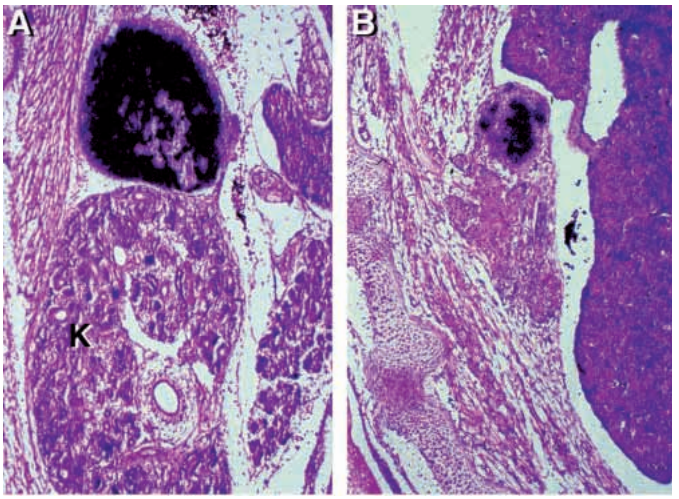


**Fig. 6.** Wt1 is required for adrenal gland development. (A,B) Sections of E15.5 littermates were hybridised with a probe for the P450 steroidhydroxylase gene, which is normally expressed within the adrenal cortex. (B) WT280$^{WW}$ transgene-complemented animal showed only the rudiment of the developing adrenal gland when compared to (A) a wild-type littermate. K, kidney.

background (*Wt1*+/–;WT280$^{WA,WC,WW}$+/–). Analysis of this genotype allowed the direct comparison between levels of gene expression derived from one transgenic locus, versus one endogenous *Wt1* allele. Only one endogenous allele is required for correct *Wt1* gene function in mice (Kreidberg et al., 1993). All four alternatively spliced isoforms were produced from the transgene in lines WA, WC and WW with the ratios of these isoforms similar to those derived from the endogenous *Wt1* locus (see details in Materials and Methods). mRNAs derived from the WT280$^{WA}$ and WT280$^{WW}$ transgenes showed similar levels of expression. In contrast, the WT280$^{WC}$ showed significantly higher mRNA expression levels suggesting that transgene expression is copy number dependent. However, even in line WC carrying 4-5 copies of the transgene, mRNA expression from WT280$^{WC}$ was lower than from a single endogenous locus (Fig. 7A).

To investigate if transgene expression levels and splice ratios vary during kidney development, we analysed isolated mRNA from E15.5 and 17.5 kidneys from the WA line. All four alternatively spliced isoforms of the *WT1* gene were produced from WT280$^{WA}$ in the correct ratios. Expression levels were 3 to 5 times lower than that of a single endogenous *Wt1* locus (Fig. 7B,C). The same results were also seen in the developing testis of E15.5 and 17.5 line WA embryos (data not shown). Therefore, neither the splice ratios of the endogenous gene nor the transgene have changed within the limits of detection over this period of development.

## DISCUSSION

### *Wt1* is required for proper formation of the epicardium

In this study, we have shown that *Wt1* is expressed in the proepicardial mesenchymal villi on the cranial surface of the septum transversum in the mouse at E9 and also in the proepicardial organ of the chick. In both of these species, *Wt1* marks the epicardial cells that migrate from the proepicardial organ onto the surface of the myocardium. Later, between E11.5 and E12.5, *Wt1*-positive cells begin to delaminate from the epicardium to form subepicardial mesenchymal cells (SEMCs). Subsequently, some of these SEMCs migrate into the heart as precursors of coronary vascular smooth muscle cells, perivascular fibroblasts and intermyocardial fibroblasts (Mikawa and Gourdie, 1996; Dettman et al., 1998; Perez-Pomares et al., 1998). *Wt1* is then no longer expressed in these cell types following their terminal differentiation.

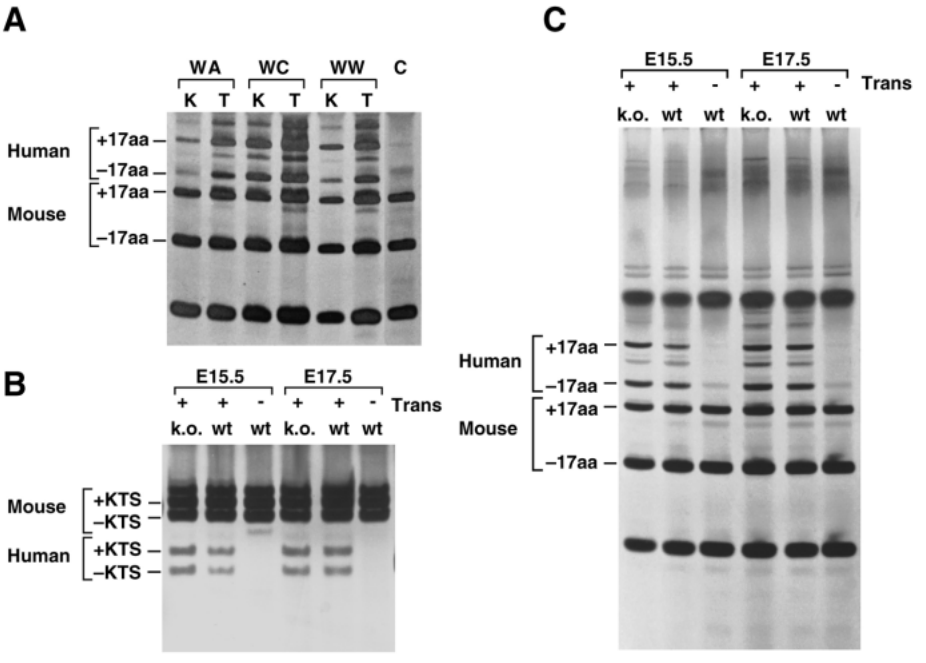


**Fig. 7.** Analysis of WT280$^{WA,WC,WW}$ transgene expression. mRNA from adult *Wt1*+/–, WT280$^{WA,WC,WW}$ kidneys was subjected to RT-PCR analysis. A species-specific digest allowed us to distinguish expression from the transgene and the endogenous gene. Expression in lines WA, WC and WW relates to copy numbers, but is found at a lower level than expression of the endogenous gene even in line WC (4-5 copies). (B,C) Expression analysis of WT280$^{WA}$ in E15.5 and E17.5 kidneys (knockout (ko) +*Wt1*+/–, wild type (wt) *Wt1*+/+). All four alternatively spliced sliced isoforms (B) +/–KTS (C) +/–exon5 are produced. Expression in line WA is 3-5 times lower than that of one copy of the endogenous gene.

In the absence of *Wt1* activity, the epicardium does not form correctly resulting in large gaps at the cranial end of the heart and a complete absence of the epicardium over the ventral surface of the aorta. These areas represent the parts of the epicardium that are last to form (in mice, this study; in chick, Hiruma and Hirakow, 1989). As a result of these epicardial defects, formation of the coronary vasculature is disrupted in turn leading to pericardial bleeding. Concurrent with these heart defects is midgestational death of the embryo. Complementation of *Wt1* null embryos by the $WT280^{WA,WC,WW}$ transgene rescues both embryo heart defects and midgestational death, implying that it is heart failure that causes the *Wt1* null embryo to die.

Failure of epicardium formation along with pericardial bleeding and embryonic death at day 12.5 to 15.5 is seen in mice homozygous null for either the *α4 Integrin* or *VCAM* genes (Yang et al., 1995; Kwee et al., 1995). Given the co-expression of both *Wt1* and *α4 Integrin* in the epicardium, it is tempting to speculate that WT1 is required for *α4 Integrin* activity. However, such a requirement could be at most partial, since *α4 Integrin*-deficient mice show a total failure of epicardium formation in contrast to the partial failure in *Wt1* nulls.

Interestingly, *Wt1* null animals also show a failure in development of the diaphragm. This is a second structure formed, in part, by cells migrating from the surface of the septum transversum. Perhaps, therefore, it is the migratory and/or proliferative capacities of the septum-transversum-derived cells that are affected in the *Wt1* null. Such an association of *Wt1* expression with the ability of cells to proliferate has previously been proposed (Menke et al., 1996; Algar et al., 1996).

In addition to a failure of proper epicardial development, *Wt1* null hearts also show thinning of the myocardium. Myocardial thinning has been reported in mice homozygous null for *VCAM* (Kwee et al., 1995), *N-myc* (Moens et al., 1993), *Tef1* (Chen et al., 1994) amongst others. Whereas many of these molecules are expressed in the myocardium, *Wt1* is not and must therefore cause thinning via its activity in the epicardium. How defects in the epicardium lead to myocardial thinning is unclear. Perhaps the epicardium is required to lend the myocardium mechanical strength. Alternatively, signalling may occur between an intact epicardium and the myocardium during myocardial growth. Given the results described in this study, further possibility is that the reduced thickening of the ventricular myocardium in *Wt1* null mice is due to failure of SEMC formation. Where no epicardium overlays the heart, there is consequently no production of SEMCs. Furthermore, even in regions of the *Wt1* null that had an epicardium, the amount of SEMC production was reduced and no migration of SEMCs into the myocardium was observed.

### Wt1 activity is required for correct adrenal gland development

The introduction of the $WT280^{WA,WC,WW}$ transgene into the *Wt1* null background allowed the embryos to survive past midgestation. Analysis of these surviving animals indicated that the adrenal gland did not develop correctly. Adrenal gland size and *P450scc* in situ hybridisation signal was greatly reduced in the complemented *Wt1* null mice when compared to wild-type animals. *Wt1* expression is not found within the developing adrenal cortex (Nachtigal et al., 1998; Moore et al., 1998) suggesting that the gene is required at an earlier stage of development, probably whilst the adrenal gland and gonad are part of a common primordium. This adreno-gonadal primordium is marked by its expression of steroidogenic factor 1 (SF-1), which is required for the activation of a number of genes involved in steroid synthesis (Hatano et al., 1996). Interestingly, animals homozygous null for *SF-1* also fail to develop gonads and adrenal glands (Luo et al., 1994; Sadovsky et al., 1995). As the *Wt1* gene is still expressed in the *SF-1* null background and the *SF-1* gene in the *Wt1* null background direct regulation of either gene by the other is unlikely (Parker and Schimmer, 1997). However, it is interesting that WT1 and SF-1 are able to interact with each other to transcriptionally activate the *Mullerian Inhibiting Substance (MIS)* gene in vitro (Nachtigal et al., 1998). Potentially a similar interaction may be required for the activation of genes essential for the survival of the adreno-gonadal primordium.

### Why is $WT280^{WA,WC,WW}$ unable to fully rescue urogenital development in *Wt1* null embryos?

Urogenital defects associated with *Wt1* null embryos were only partially rescued by the human $WT280^{WA,WC,WW}$ transgene. Different littermates showed different degrees of kidney development ranging from the failure of ureteric bud outgrowth, to a block in nephrogenesis at the S-shaped body to glomerular transition. Mice homozygous or heterozygous null for a number of genes that affect kidney development also show blocks at a range of different kidney developmental stages among littermates e.g. *ret-k* (Schuchardt et al., 1996), *Gdnf* (Sanchez et al., 1996; Pichel et al., 1996), *Wnt4* (Stark et al., 1994) and *Integrin α8* (Muller et al., 1997). In fact, *Wt1* is rare in showing an invariant null phenotype in this organ.

Interestingly, the degree to which kidney rescue occurs in the two kidneys of a single $WT280^{WA,WC,WW}$-complemented *Wt1* null embryo was almost always found to be comparable. Whilst in embryos null for many genes affecting kidney development e.g. *ret-k*, different phenotypes are found for either side, suggest that the two kidneys of an embryo develop more or less independently. The genetic background of the crosses described in this study is a heterogeneous one. Lines WA,WC and WW were generated on a mixed background of CBAxBl6 mice and subsequently crossed onto the *Wt1* null strain (mixed background for Bl6 and 129). Hence, the degree to which rescue occurs is likely to be due to genetic modifiers. Further crossing experiments should allow us to map these modifiers.

In this study, we were never able to fully rescue urogenital system development in $WT280^{WA,WC,WW}$-complemented null mice. Hence there must be defects in the activity of *WT1* expressed from the transgene with these animals. RT-PCR analysis demonstrated the presence of all different *WT1* splice isoforms, at the same ratios as the mouse, in the kidney and the gonad. Therefore, it is unlikely that it is an imbalance in splice isoforms that leads to the inability of the transgene to rescue urogenital development. However, we cannot discount a functional difference between the mouse and human proteins, even though the WT1 amino acid sequence is 96% conserved between these two species.

Alternatively, the divergence between the human and mouse genes may be at the level of regulation. All three transgenic lines, WA, WW and WC, express the transgene at levels lower

than that of one endogenous *Wt1* copy, which may be insufficient to allow full kidney, gonad and adrenal formation. In this study, however, it is difficult to rationalise why the relatively low expressing line WA consistently rescued kidney development to a further stage, when compared to the highly expressing line WC. Furthermore, preliminary data suggests that crossing *Wt1*+/–,WT280$^{WA}$+/– to *Wt1*+/–,WT280$^{WA}$+/– mice, hence leading to the generation of *Wt1* null mice homozygous for the WT280$^{WA}$ transgene, does not lead to a complete rescue of kidney gonad or adrenal development, nor even a clear extension of the degree to which this development proceeds.

Perhaps the failure in transgene activity is not in the amplitude of its expression, but in its correct spatiotemporal control. A *lacZ* reporter integrated into the *WT1* locus of the WT280 YAC, is capable of driving β-galactosidase expression in all the correct *Wt1*-specific urogenital structures in vivo (Moore et al., 1998). However, in the partially complemented kidneys of this study, Wt1 was not correctly expressed in the nephrogenic structures that were present although all other markers were. Within a structure, it was not necessarily expressed in all the cells in which it should have been nor at levels comparable to wild type. Even so, whilst Wt1 expression in the partially formed nephric structures does not mimic that of the wild-type situation, we cannot distinguish whether this effect is caused either by a failure of gene regulation or as a knock-on effect of incorrect gene function.

## Multiple roles for *Wt1* during kidney development

The spectra of different stages at which kidney development is blocked in this study demonstrate that *Wt1* is required continuously throughout metanephric development. In the following paragraphs, we will discuss each individual step of nephrogenesis and what function WT1 may have in this process.

### Wt1 activity is required in the metanephric mesenchyme for invasion of the ureteric bud and growth of the collecting ducts

In *Wt1* null, and some *Wt1* null mouse embryos partially complemented with the WT280$^{WA,WC,WW}$ transgene, there is no ureteric bud outgrowth. As *Wt1* is not expressed within the ureteric bud, activity within the blastema must be required for a signal inducing ureteric bud outgrowth (Kreidberg et al., 1993). The consequent failure of induction of the metanephric blastema by the ureter leads to apoptosis, thus giving rise to a type I kidney phenotype.

*Wt1* is also required to allow the growth and branching of the ureteric bud into the metanephric blastema. This is demonstrated by the failure of ureteric bud invasion in type IIIa kidneys and the segmental ureteric bud invasion phenotype of some type IIIb kidneys. In these segmental type IIIb kidneys, a low level of WT1 expression is seen solely in the metanephric blastema at the end of the ureteric bud branches. Hence, it is tempting to speculate that *Wt1* expression in the metanephric blastema is continuously required to stimulate growth of the ureteric bud throughout kidney development. Alternatively, the observed expression of WT1 in those segments of type IIIb kidneys that show ureteric bud branch invasion could be a secondary effect, caused by stimulation of *WT1* gene expression within the condensing blastema.

A type I defect in kidney development has also been reported in mice with targeted disruptions of genes involved in the c-ret/Gdnf signalling pathway (Schuchardt et al., 1996). This pathway controls both the outgrowth of the ureteric bud from the mesonephric duct and growth and branching of the bud within the metanephric blastema. As both *Wt1* and *Gdnf* are expressed in the metanephric blastema prior to ureteric bud induction, it is possible that *Gdnf* signalling requires *Wt1* activity (Schuchardt et al., 1996). Similarly, in the blastema, localised sources of Gdnf directing bud branching and growth may depend on WT1 expression. It will be interesting to investigate whether *Gdnf* is expressed correctly in *Wt1* null embryos and whether it is restricted to areas of WT1 transgene activity in segmented typeIIIb kidneys.

### A continual requirement for Wt1 activity in the forming nephron

The metanephric blastema from *Wt1* null mice is incapable of responding to inducing signals (Kreidberg et al., 1993), demonstrating that *Wt1* is required for the initial condensation step. However, *Wt1* is not just expressed in the metanephric blastema as it undergoes condensation but is expressed and upregulated through the comma- and S-shaped body stages of nephrogenesis. It later becomes confined to the forming podocyte cells as they mature within the glomerulus. This expression pattern implies that *Wt1* may additionally play roles at these later stages of nephrogenesis. The data presented in this study confirm this proposal. Shortage of Wt1 activity can block nephrogenesis at both the comma- and S-shaped stage. Furthermore, we never saw the production of podocytes and mature glomerular structures in any WT280$^{WA,WC,WW}$-complemented *Wt1* null kidneys demonstrating that the gene is also required to form mature glomerular structures.

From other studies, there are a number of candidate molecules through or along side which Wt1 could be exerting its effects on nephrogenesis. PAX2 and Wnt4 are both expressed within the condensing blastema and are required for early nephrogenic events in particular condensation of the blastema (Rothenpieler and Dressler, 1993). Pax2 expression in the attenuated nephrogenic zone of typeIIIb kidneys appears to be at wild-type level. Hence it seems unlikely that the failure to undergo nephrogenesis is a direct consequence of a reduction or lack of Pax2 activity. *Wnt4* null mice show a block at the condensation stage. Nephrogenesis in partially rescued kidneys proceed further, which would imply that *Wt1* is required for stages of nephrogenesis after the block seen in *Wnt4* mice.

Other possible candidate for interaction with *Wt1* are *Cadherin6* and *Integrinα8* (Cho et al., 1998; Muller et al., 1997). Both are expressed in the induced condensing mesenchyme at the tips of the ureteric bud branches and then downregulated as the vesicle undergoes epithelialization. Metanephric cultures treated with anti-Cadherin6 antibodies block at the condensation stage. In many *Integrinα8* null kidneys, nephrogenesis is blocked at the condensation stage with the rare occurrence of comma- and very rare occurrence of S-shaped bodies (Muller et al., 1997). The *Integrinα8* null phenotype in particular, is very similar to that observed in our complementation analysis and hints that WT1 could interact with signalling processes mediated by this molecule.

### Does *Wt1* have the same function in both epicardial and metanephric development?

This study demonstrates several roles for the *WT1* gene in the formation of two separate organs, the epicardium and the metanephros. *Wt1* may control cellular proliferation, as the association of Wilms' tumours with *WT1* mutation implies. In the *Wt1* homozygous null mouse, both epicardial and diaphragmatic defects may be due to a failure of cellular proliferation. *WT1* activity is required to enable signals emanating from the metanephric blastema to control both formation of the ureteric bud and also its invasion of the blastema tissue; similarly we could hypothesise that myocardial malformation in *Wt1* null animals may be derived, at least in part, from an inability of the epicardium to signal to the myocardium. Finally, there is strong evidence that *Wt1* activity may be important to allow cells to switch between mesenchymal and epithelial cell states. The process of nephrogenesis is dependent upon continuous *Wt1* activity and consists of a mesenchymal-to-epithelial transition. As nephrogenesis proceeds, *Wt1* activity becomes restricted to the presumptive podocytes, which go from being fully epithelial to partially mesenchymal as they terminally differentiate (Hölthofer et al., 1984). The development of the epicardium and the subepicardial mesenchymal cells shows a similar flipping of cells back and forward between a mesenchymal and epithelial state. The cells of the proepicardial organ are mesenchymal in nature. These cells then become epithelial as they differentiate in the epicardium. The epicardium however retains the ability to retransform into subepicardial mesenchymal cells, these transiently express both epithelial and mesenchymal markers before differentiating terminally into cells of a fully mesenchymal nature (Perez-Pomares et al., 1997). In both forming nephrons and the epicardium, *Wt1* becomes downregulated in those cells that have terminally differentiated as either epithelium or mesenchyme and will not again undergo such a transition e.g. the nephron tubule in the kidney and the coronary vascular smooth muscle cells, perivascular fibroblasts and intermyocardial fibroblasts of the heart.

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