Nuclear localization of the protein encoded by the Wilms' tumor gene *WT1* in embryonic and adult tissues

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SUMMARY

The human Wilms' tumor gene *WT1* encodes a putative transcription factor implicated in tumorigenesis and in specifying normal urogenital development. We have studied the distribution of WT1 protein and mRNA using immunohistochemistry and in situ hybridization.

Monoclonal antibodies were raised against a peptide specific to the first alternative splice site of WT1. Two antibodies specifically reacted on Western blot to this WT1 isoform. Immunofluorescence localized WT1 protein to podocytes during mesonephric and metanephric development. In situ hybridization revealed a similar pattern of expression except that WT1 mRNA was also present in metanephric blastema and renal vesicles. Messenger RNA expression was most pronounced in the kidneys during early fetal development and declined thereafter. In contrast, WT1 protein was readily detectable in glomerular podocytes throughout adulthood. WT1 protein in Wilms' tumor was present in blastema and glomeruloid structures.

Expression in the female gonad was linked to the different stages of granulosa cell development. In the male gonad, expression was restricted to Sertoli cells and their precursors, the embryonic tunica albuginea and the rete testis.

The intracellular distribution of the WT1 protein was investigated by confocal laser microscopy and was demonstrated to be exclusively nuclear.

The nuclear distribution and the selective pattern of expression support the proposed role of WT1 as a transcription factor active during urogenital development. The persistence of WT1 expression in the adult kidney suggests a role in homeostasis of the podocyte.

Key words: Wilms' tumor, genitourinary development, podocyte, Sertoli, granulosa

INTRODUCTION

Wilms' tumor (WT) is an embryonal neoplasm of the kidney arising from the metanephric blastema, affecting approximately 1 in 10,000 children. This tumor occurs both as sporadic and inherited forms, affecting one or both kidneys. Many hereditary cases of Wilms' tumor show an association with congenital developmental abnormalities such as aniridia, genitourinary malformations and mental retardation. This constellation of abnormalities (the WAGR complex) is associated with cytogenetically visible deletions on chromosome 11p13 affecting genes that are important for urogenital, eye and brain development.

A Wilms' tumor susceptibility gene, *WT1*, from 11p13 has recently been cloned and characterized (Call et al., 1990; Gessler et al., 1990). This gene is homozygously deleted in a small informative set of sporadic Wilms' tumors (Lewis et al., 1988; Gessler et al., 1990; Davis et al., 1991; Royer-

Pokora et al., 1991). In addition, *WT1* is specifically altered in several sporadic and hereditary WTs, providing evidence for the involvement of this gene in WT etiology (Haber et al., 1990; Huff et al., 1991; Pelletier et al., 1991a,c; Ton et al., 1991).

The *WT1* gene contains ten exons, which encode four distinct mRNA species, reflecting the presence or absence of two alternatively spliced transcripts (Haber et al., 1990; 1991). The transcript containing both alternative splices is the most prevalent variant, whereas the least common is the one missing both alternatively spliced exons. The relative level of each isoform is constant during development, as well as in WTs (Haber et al., 1991). Two of the four WT1 isoforms differ in DNA-binding capacity (Rauscher et al., 1990). The predicted WT1 protein shows several features of a transcription factor, including four Cys2-His2 zinc fingers at the carboxy terminus (Call et al., 1990; Gessler et al., 1990). WT1 can bind to DNA sequences similar to those

recognized by the early growth response genes (*EGR*) (Rauscher et al., 1990) and there is evidence that WT1 regulates the expression of insulin-like growth factor II (IFG2) (Drummond et al., 1992) and platelet-derived growth factor alpha (PDGFA) (Gashler et al., 1992) and thus may directly be involved in regulating cellular differentiation and proliferation.

Genetic data concerning the role of *WT1* in differentiation and proliferation of the urogenital system were obtained from the identification of the molecular defect in Denys-Drash syndrome. Patients with Denys-Drash syndrome have a constellation of phenotypes, which include Wilms' tumor, intersex disorders and nephropathy (Habib et al., 1985). The structures affected in these individuals correspond to the sites of highest WT1 expression. Recently, a number of mutations, clustering within the third zinc finger of the *WT1* gene, were identified in Denys-Drash patients (Pelletier et al., 1991c; Bruening et al., 1992; Baird et al., 1992).

In situ hybridization studies demonstrated that WT1 is selectively expressed in metanephric blastema, S-shaped bodies and glomerular epithelium during embryonic and fetal kidney development (Pritchard-Jones et al., 1990). In addition, WT1 expression is present in the human undifferentiated gonadal ridge (Pritchard-Jones et al., 1990) and in the prenatal and postnatal testis and ovary of the mouse (Pelletier et al., 1991b; Armstrong et al., 1992). The expression of WT1 in the gonads, as well as germ-line mutations in individuals with urogenital abnormalities and WT suggests a central role for WT1 in development of the genital system (Pelletier et al., 1991a). However, to date, WT1 expression in the developing human urogenital system has not been extensively characterized. In addition, no data exist defining expression of the WT1 protein in the human urogenital system. These studies are the key to elucidating a role of WT1 in development and understanding the molecular basis for differences in phenotype between the human WAGR syndrome, and the corresponding mouse model system Sey$^{Dey/+}$ (Glaser et al., 1990).

In the present study, in situ hybridization and immunohistochemistry was used to address these issues. WT1 mRNA expression was compared with the localization of the WT1 gene product using two newly developed monoclonal antibodies directed against the first alternatively spliced exon of WT1. We demonstrate that WT1 is expressed in podocyte cells during mesonephric and metanephric development and is present in these cells in adulthood. Expression in male and female gonads is restricted to Sertoli cells, granulosa cells, as well as their precursors. Intracellular localization of the WT1 gene product was carried out with confocal laser microscopy and revealed an exclusively nuclear localization, supporting the proposed role of WT1 as a transcription factor.

MATERIALS AND METHODS

Embryology and tissue samples

Intact human embryos of 7 to 10 weeks gestational age were obtained after therapeutic abortions. Embryonic stage was deter-mined by morphological appearance, weight and crown-rump length. Kidney tissue was obtained from macroscopically and histologically normal sections surgically removed for renal carcinoma. The ages of the patients ranged from 4 to 80 years. Samples of WT tissue were obtained from surgical specimens of patients operated at the Children's Hospital in Mainz, Germany. Four samples from patients who had not been treated with chemotherapy were investigated for WT1 expression.

Antibody preparation

A peptide specific to the first alternative splice site of WT1 (VAAGSSSSVKWTEGQSN) was coupled to maleimide-activated keyhole limpet hemocyanin (Pierce) according to the manufacturer's instructions. Monoclonal antibodies were generated as follows: spleen cells of Balb/c mice immunized with conjugated peptides were fused to SP2/0 myeloma cells (Bazin and Lemieux, 1989). Antibody-secreting hybridomas were screened and selected by ELISA twice by limiting dilutions. Two monoclonals, 1B6 and 13B5, that showed a high reactivity on ELISA were cloned for further experiments. These antibodies were purified on protein G agarose (Gibco/BRL) as specified by the supplier.

Western blot analysis

COS-1 cells were transfected as previously described (Pelletier at al., 1991b) with CMV-based expression vectors which produced WT1 isoforms (+1) or lacking (−1) the first alternative splice site. Total cell proteins were prepared 2 days after transfection as follows: Cells (10 cm^2 dish: ca. 10^6 cells) were washed 2× in PBS, lysed in 0.5 ml of 2% SDS/50 mM Tris 7.5. The mixture was heated in a boiling water bath for 10 minutes, DPA sheared by passage through a 25-gauge needle 15 times and centrifuged at 10.000 *g* for 10 minutes. 40 μl of supernatant were electrophoresed on a SDS/10% polyacrylamide gel, transferred on to nitrocellulose, and subjected to immunoblot analysis as described in the figure legend. Blots were exposed to Kodak film (XAR-5) at room temperature for 2 minutes.

Probes for in situ hybridization

For in situ hybridizations a subclone (WT-H) of WT33 (Call et al., 1990) was used. This probe is a 610 bp *Hinc*II/*Eco*RI fragment cloned into pKSII+. The probe is derived from the 3 untranslated region of WT33, to avoid cross-hybridization of the WT1 zinc fingers with similar sequences in other transcripts. The cDNA was transcribed with high efficiency from the T7 or T3 promoter in the presence of [^{35}S]UTP (800 μCi/mmol, Amersham, Poole, UK) to generate labeled sense and antisense cRNA, respectively (specific activity: 2-3×10^8 disints/minute/μg). Labeled RNA was separated from unincorporated nucleotide triphosphates by precipitation with ethanol.

In situ hybridization and immunofluorescence

The techniques employed for in situ hybridization were as previously described (Mundlos et al., 1991). The tissue was fixed in 4% buffered formalin, immersed in 0.5 M sucrose and snap frozen in liquid nitrogen. Frozen sections were mounted on triethoxyethanolamine-treated microscope slides. The slides were immersed in 4% paraformaldehyde in PBS for 15 minutes, followed by digestion with pronase (Boehringer) at 0.3 mg/ml for 8 minutes at room temperature, fixation in paraformaldehyde for 10 minutes, and dehydration in a graded series of ethanol steps. Probes were diluted to a specific activity of 10^5 disints/minute/μl in hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris, pH 7, 10 mM NaHPO$_4$, pH 5, 5.5 mM EDTA, 0.02% Denhardts' and tRNA [0.5 mg/ml]). Hybridization was performed overnight at 42°C. The slides were washed in 50% formamide/2× SSC at 45°C for 2 hours followed by incubation with 20 μg/ml RNAse A (Sigma, München, FRG) for 15 minutes

and again washed three times in 2× SSC for 15 minutes at room temperature. After dehydration, slides were dipped in Kodak NTB-2 photoemulsion diluted 1:1 with water and air dried. Exposure time varied from 10-20 days. The exposed slides were developed, fixed, stained and examined using the Zeiss system for epipolarization.

Immunofluorescence was carried out using unfixed, snap frozen material. Frozen sections were cut and mounted on tri-ethoxyethanolamine-treated slides. Tissue was fixed for 1 hour in methanol/EDTA (0.02%), air dried and hydrated in PBS, pH 7.4. The first antibody was applied at a dilution of 1:100 to 1:300 and incubated at room temperature in a moist chamber for 1 hour. A CY3-coupled anti-mouse antibody (Daco; dilution 1:200) was used for detection. Parallel experiments with positive and negative controls (minus first antibody) were always performed. The slides were examined on a Zeiss fluorescence microscope. Investigation of the intracellular localization of the WT1-protein was performed on a Zeiss laser scanning microscope.

Reverse transcription (RT)-PCR analysis

RT-PCR analysis of WT1 in fetal and adult kidney was done as previously described (Pelletier et al., 1991c). For amplification of -2 microglobulin, the two oligonucleotides (Rosa et al., 1983) used were:

   2-S-(5 -GCTGTGCTCGCGCTACTCTCTC-3),
   2-AS-(5 -CTTCAACCTCCATGATGCTGC-3).

For amplification of WT1, two oligonucleotides flanking zinc fingers 2, 3 and 4 (Call et al., 1990) were used:

   RG16-(5 -CCCGGTCCGACCACCTG-3),
   RG01-(5 -GTGAGGAGGAGTGGAGAG-3).

PCR products were resolved on a 3% Nusieve (FMC) agarose gel followed by Southern blot analysis using the human WT1 cDNA as probe.

RESULTS

Characterization of anti-WT1 monoclonal antibodies

To define the sites of expression of the WT1 isoforms that contain the first alternative splice site, we used two monoclonal antibodies. WT1 protein isoforms were transiently produced in COS-1 cells by transfecting CMV-based expression vectors. The two monoclonal antibodies react on a Western blot with the WT1 isoform containing the first alternative splice site [+/+] but not with the WT1 isoform lacking this splice site [−/+] (Fig. 1, compare lanes 5 and 8 to lanes 6 and 9, respectively). Neither monoclonal antibodies reacted with cellular proteins from COS-1 cells, a kidney-derived cell line (Fig. 1, lanes 4 and 7).

Intracellular distribution of WT1 protein

When 1B6 and 13B5 antibodies were used to stain fetal and adult kidneys, the +1 WT1 isoforms were detected exclusively in the cell nucleus (Fig. 2). Using confocal laser microscopy, no significant amount of WT1 protein was detectable in the cytoplasm. The nuclear staining showed a coarse speckled pattern. This pattern was maintained throughout the different stages of development and in all the tissues investigated, including Wilms' tumors. No differences were observed between the two antibodies in tissue distribution or intensity (data not shown). The immunoflu-
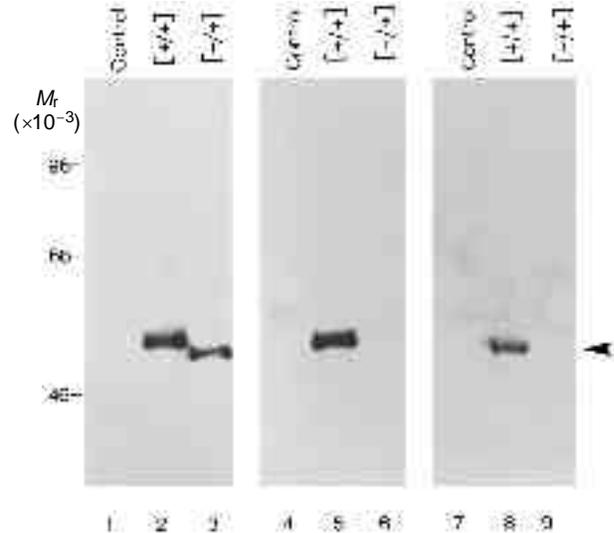
Fig. 1. Characterization of anti-WT1 monoclonal antibodies by Western blot. Cell extracts (40 µl) from COS-1 cells (lanes 1, 4 and 7), COS-1 cells transfected with a CMV-based expression vector expressing the WT1 [+/+] isoform (lanes 2, 5 and 8) or the WT1 [−/+] isoform (lanes 3, 6 and 9) were subjected to SDS/PAGE. Proteins were transferred onto nitrocellulose filter followed by incubation with anti-WT1 polyclonal sera (diluted 1:200) (lanes 1-3) or with anti-WT1 monoclonal antibodies 1B6 (diluted 1:500) (lanes 4-6) or 13B5 (diluted 1:500) (lanes 7-9). The protein was visualized using the ECL Western blot detection kit (Amersham). Molecular mass of marker proteins are indicated on the left. The arrowhead indicates the position of the WT1 [+/+] isoform.

orescent signal obtained with 1B6 and 13B5 could be specifically blocked by a 10-fold molar excess of peptide against which these antibodies had been raised, but not by an equivalent amount of peptide representing the WT1 isoform lacking the first alternatively spliced exon (data not shown).

Expression of WT1 in the developing and adult kidney

The development of the human permanent kidney or metanephros is preceded by two temporary organs, the non-functional pronephroi and the mesonephroi, which serves as a temporary excretory organ. Differentiation of the human metanephros is initiated with the interaction of the ureteric bud with the surrounding metanephric blastema with subsequent conversion of mesenchymal cells into epithelial structures. The different phases of glomerular development were defined according to Reeves et al. (1980): metanephric mesenchyme, renal vesicle, S-shaped body, developing capillary loop and maturing stages.

Mesonephros

WT1 expression patterns were similar in the mesonephros when compared to the metanephros (Fig. 3). The glomerular structures of the mesonephros stain for WT1 expression. Visualization by immunofluorescence includes nuclear staining in the presumptive podocytes at the outer surface of the glomerulus (Fig. 3C).
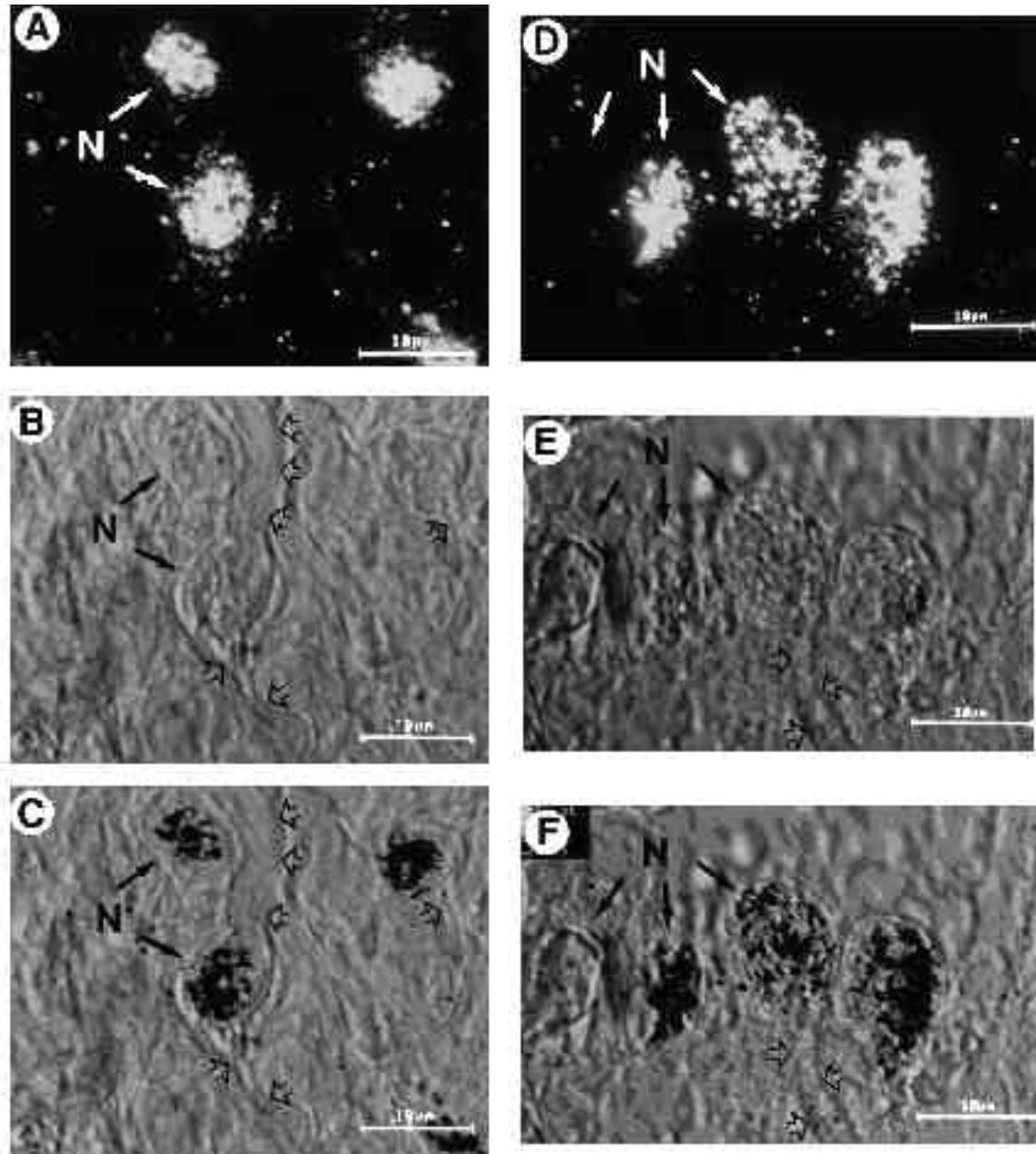
Fig. 2. Confocal laser microscopy demonstrates nuclear localization of the WT1 protein. Staining with antibodies 1B6 and 13B5 gave a nuclear distribution and a coarse speckled pattern in adult (A-C) and fetal (D-F) podocytes. Phase contrast of A and D given in B and E and corresponding overlay in C and F. Note selective staining of nuclei (*N*). Positive cells show long curving processes that are not stained (open arrows). Bars, 10 μm.

Metanephric mesenchyme and renal vesicle

Following induction, an aggregation of mesenchymal cells around the ampullar extremity of the ureteric bud branches can be seen. These aggregates will soon form pretubular vesicles at the tip of the ureteric bud. In situ hybridization revealed low levels of expression in the condensed metanephric mesenchyme and in the outer cell layer of renal vesicles (Fig. 4A,B). However, immunofluorescence failed to detect the +1 WT1 isoforms during this stage (Fig. 5). Some immunoreactivity was noted in early stages of nephro-genesis (stage 20) in metanephric blastema, but not there-after. No expression was present in the ureteric bud.

S-shaped body

Following development of the renal vesicle, two indenta-tions appear, converting the vesicle into a comma and then the S-shaped body. Lumina of both, the presumptive tubules and of Bowman's space (capsule), separating parietal from visceral epithelium can be differentiated. Following the indentation of the vesicle, WT1 mRNA expression is mainly
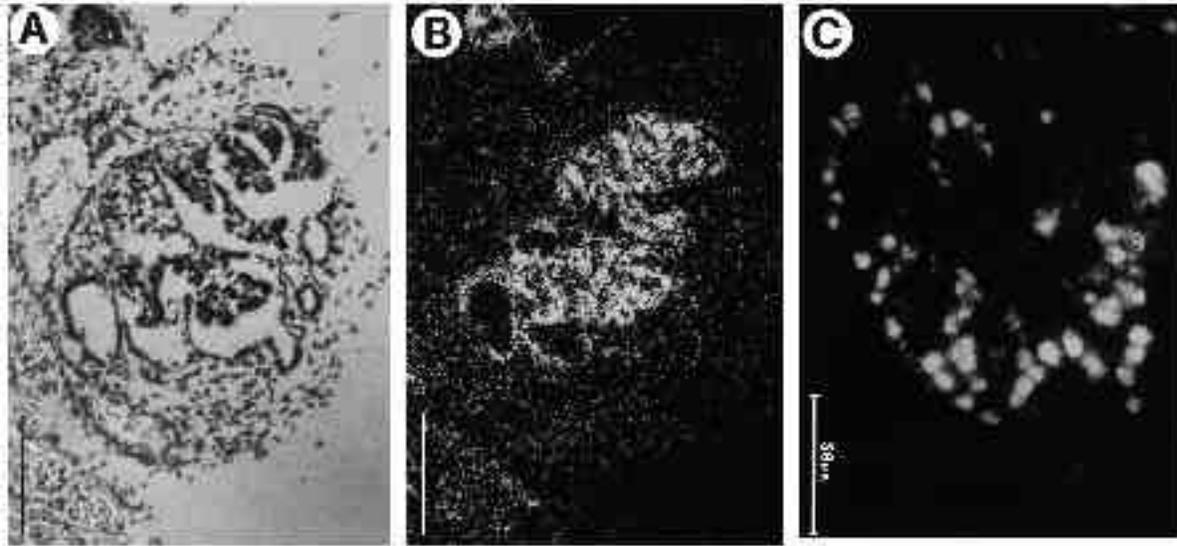
Fig. 3. Expression of WT1 mRNA and protein in the mesonephros. In situ hybridization of a stage 23 mesonephros showing signal within the glomerulus (B). The bright-light image of B is given in A. (C) Immunofluorescence of a stage 20 mesonephros showing nuclear staining of the outer cell layer.

localized in the lower cell layer of the inferior cleft (Fig. 4D). These cells are considered to be the precursors of the podocytes. At this stage, strong immunoreactivity was noted within the nuclei of podocyte precursors (Fig. 5B).

Developing capillary loop and maturing glomerulus

Capillaries of the kidneys grow into the lower indentation of the S-shaped body, which subsequently widens and forms a U-shaped structure. At this stage, in situ hybridization and immunofluorescence gave a strong signal in cuboid cells at the outer surface of the presumptive glomerulus (Figs 4F, 5C,F). These cells were identified as developing podocytes. Upon further development, capillaries proliferate and podocytes separate from each other with closure of the U-shaped structure. High levels of WT1 mRNA and protein were observed at this stage in podocytes (Figs 4H, 5D,G,H). Within this complex process of nephrogenesis, WT1 expression is limited to one cell type, the podocyte. Expression is always limited to the outer cell layer of the glomerulus, no expression was observed within the capillary loop, the mesangial cells, or the parietal squamal cells of Bowman's capsule. No expression was found in tubular structures, or the surrounding mesenchyme. Thus, during nephrogenesis, WT1 expression is linked to the development of glomerular epithelial cells (podocytes).

Postnatal stage

Nephrogenesis comes to a halt five weeks before birth. The kidney increases in size after birth mainly due to hypertrophy of the nephrons. Several kidney specimens ranging from newborn to 80 years of age, were investigated for WT1 expression (Fig. 6). In situ hybridization revealed faint labeling in the glomeruli of a 6-year-old kidney, but no signal was obtained in the later age groups (data not shown). However, by immunofluorescence, WT1 protein was present in glomeruli of kidneys from all the age groups

analyzed. In kidneys of newborn and early childhood (6 years), positive nuclei were identified mainly at the outer cell layer of the glomerulus, with some immunoreactive cells also present in the more central part of the glomerulus. In the adult kidney, WT1-expressing cells were randomly scattered throughout the glomerulus. During childhood, as well as in the adult, a relatively constant number of 40-50 positive cells could be identified per glomerulus. The phase contrast with confocal laser microscopy demonstrated that positive nuclei belonged to cells with long curving processes, thus identified as podocytes (Fig. 2).

To confirm the presence of WT1 mRNA in the adult kidney, we used the polymerase chain reaction (PCR) to amplify WT1 cDNA. Complementary cDNA was prepared from human fetal or adult kidney RNA and used in a PCR with either oligonucleotides directed to the human -2 microglobulin (Fig. 7A, lanes 2 to 6) or the WT1 gene (Fig. 7A, lanes 7 to 11). The expected 360 bp -2 microglobulin PCR product was observed in both fetal and adult samples (Fig. 7A, lanes 4 and 6). A 249 bp PCR product was observed in both fetal and adult samples when priming with WT1 oligonucleotides (Fig. 7A, lanes 9 and 11). This product corresponds in molecular weight to WT1 mRNA. To verify the identity of this product, the DNA was transferred to nitrocellulose and probed with [32P] labeled WT1. Fig. 7B autoradiograph corresponding to Fig. 7A demonstrating the identity of the PCR products as WT1. These results demonstrate that WT1 mRNA is expressed in the adult kidney and that the corresponding protein is also present to substantial amounts.

Expression of WT1 in tumor tissue

Expression of WT1 protein in a Wilms' tumor as revealed by immunofluorescence is shown in Fig. 8. Strong staining was observed over blastemal cells and circular structures that resembled glomeruli. Staining was nuclear only and the
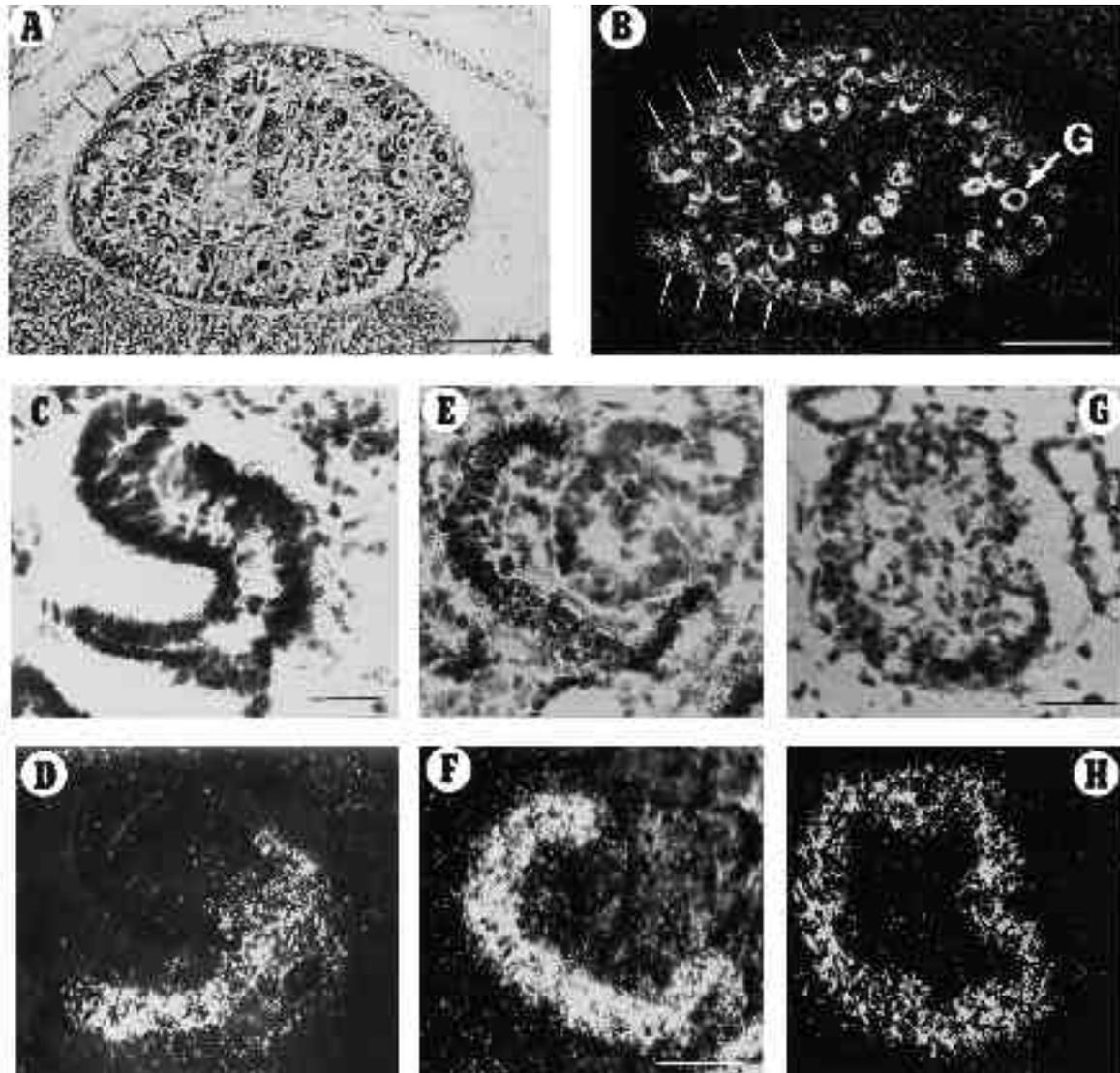
Fig. 4. Expression of WT1 mRNA in the metanephros. In situ hybridization showing expression in the embryonic kidney (stage 23, B) with the bright-light image given in (A). Note expression in metanephric blastema (arrows), S-shaped bodies and maturing glomeruli (**G**). WT1 expression in the fetal kidney during the different stages of nephrogenesis at higher power: (D) S-shaped body, (F) stage of the developing capillary loop (U-shaped structure) and (H) maturing glomerulus. Note the selective expression in the lower indentation of the S-shaped body and in the outer cell layer of the maturing glomerulus. Corresponding bright-light images given in C, E and G. Bars, 400 µm (A,B) and 30 µm (C-H).

pattern was not different from the coarse speckled pattern observed in kidney or gonads. No expression was found in the stroma or in tubular-like structures. Similar results were obtained using in situ hybridization (data not shown).

### Expression of WT1 in the developing ovary

In the absence of the Sry gene on the Y chromosome, gonadal development occurs more slowly and proceeds along the ovarian pathway. At a time when testicular structures can first be identified, the ovary cannot be differentiated from the earlier, indifferent form. From the 8th week on, a cortex and medulla can be identified. By the 10th week, secondary sex cords extend from the surface epithelium into the underlying mesenchyme. In contrast to the testis, there

is no separation from the surface epithelium. From the 16th week on, primordial follicles develop in large numbers, consisting of an oogonium surrounded by a single layer of flattened epithelial cells. These epithelial cells derive from the ovarian cortex and eventually develop into granulosa cells (Byskov, 1986).

At stage 20/21, WT1 protein expression is present at different levels throughout the ovary (Fig. 9A). The strongest expression was observed in the epithelial cell layer surrounding the ovary. This was most pronounced in areas of close proximity to the paramesonephric duct. Phase contrast showed that these positive cells surround negative cells which were less numerous in amount (Fig. 9B,C). During early fetal development (10th week), this distribution
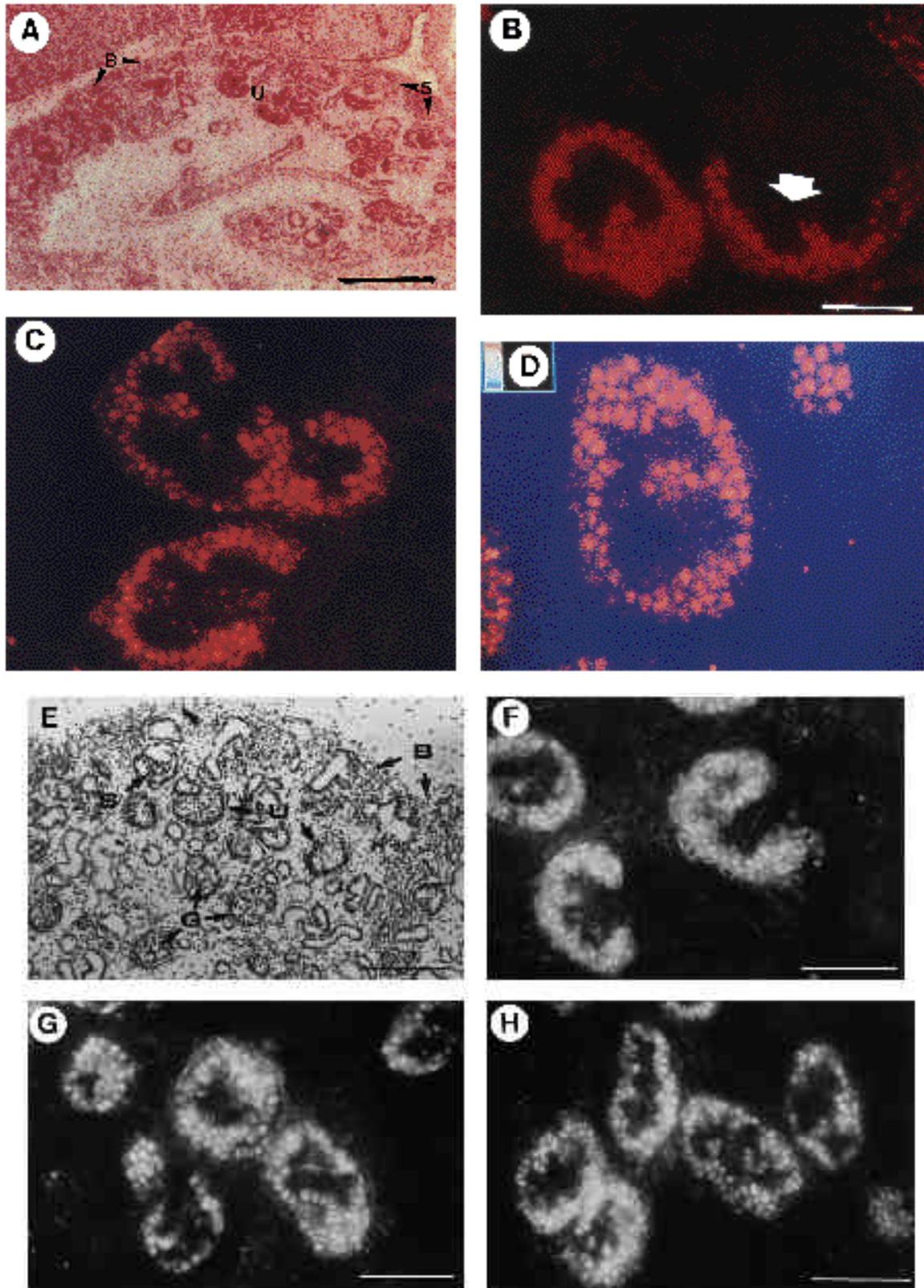
Fig. 5. Expression of WT1 protein in the embryonic (stage 20; A-D) and fetal (E-H) metanephros. Immunofluorescence revealed a nuclear staining of developing and mature podocytes during nephrogenesis with a similar pattern in embryonic and fetal tissue. S-shaped body (B, arrow), U-shaped structure (C,F) and maturing glomeruli (D,G,H). Confocal laser microscopy phase-contrast overlay (D). Bright-light images of embryonic and fetal kidney given in A and E. Blastema (***B***), U-shaped structures (***U***), S-shaped structures (***S***) and glomeruli (***G***). Bars, 50 µm (B-D), 100 µm (F-H), 300 µm (A,E).
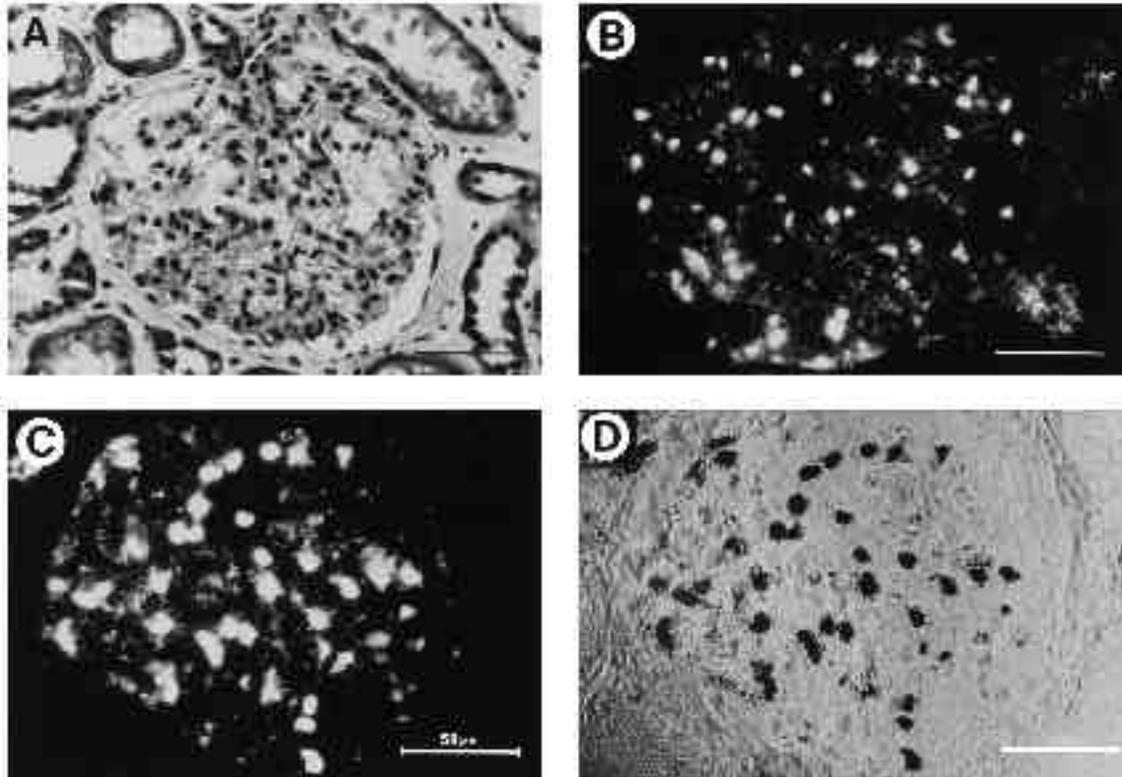
Fig. 6. WT1 protein in the adult kidney. Immunofluorescence with antibody 1B6 and 13B5 shows nuclear staining of selective cells within the glomerulus (B,C). Phase-contrast overlay of C is shown in D. Bright-light image of an adult glomerulus shown in A. Bars, 50 μm.

was essentially maintained, but expression of WT1 in the epithelium was less pronounced. Within the medulla, the staining revealed the beginning formation of secondary sex cords (Fig. 9D). Positive cells showed a column-like arrangement in some areas. By birth, expression was limited to the epithelial cells surrounding the oocytes (Fig. 9E). Similar patterns were observed in primordial follicles and in primary follicles. No expression was found in the mesenchyme or the ovarian epithelium.

Expression of WT1 in the developing testis

In the embryonic testis, expression is present in the tunica albuginea, the seminiferous cords and the developing rete testis. Fig. 10A,B shows a section through an 8-week testis (stage 23). The seminiferous cords have just developed and are separated by thick mesenchyme. Expression of WT1 in the fetal and newborn testis was limited to the seminiferous tubules (Fig. 10C,D) and the rete testis (not shown). Using phase-contrast and confocal laser microscopy, two cell types within the seminiferous tubules were identified. Nuclear staining was present in the great majority of cells. However, some negative cells were observed. Cells that did not stain had larger nuclei and were thus identified as spermatogonia. No expression was found in the surrounding mesenchyme or Leydig cells. The cells of the developing rete testis gave a strong signal. The expression in the tunica albuginea observed during the early embryological stages was no longer present in the fetal testis. Thus, expression in the fetal
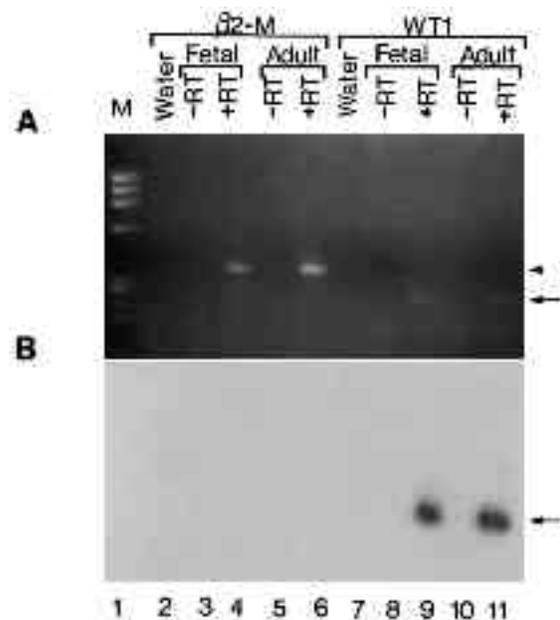
Fig. 7. RT-PCR analysis of WT1 in fetal and adult kidney. The -2 microglobulin (lanes 2-6) or WT1 (lanes 7-11) was amplified by PCR. (A) Agarose gel electrophoresis of PCR products. The arrowhead indicates the position of the -2 microglobulin product, whereas the arrow indicates the WT1 gene product. (B) Southern blot of A hybridized with the human WT1 cDNA probe. M, 174 Rf DNA digested with *Hae*III.
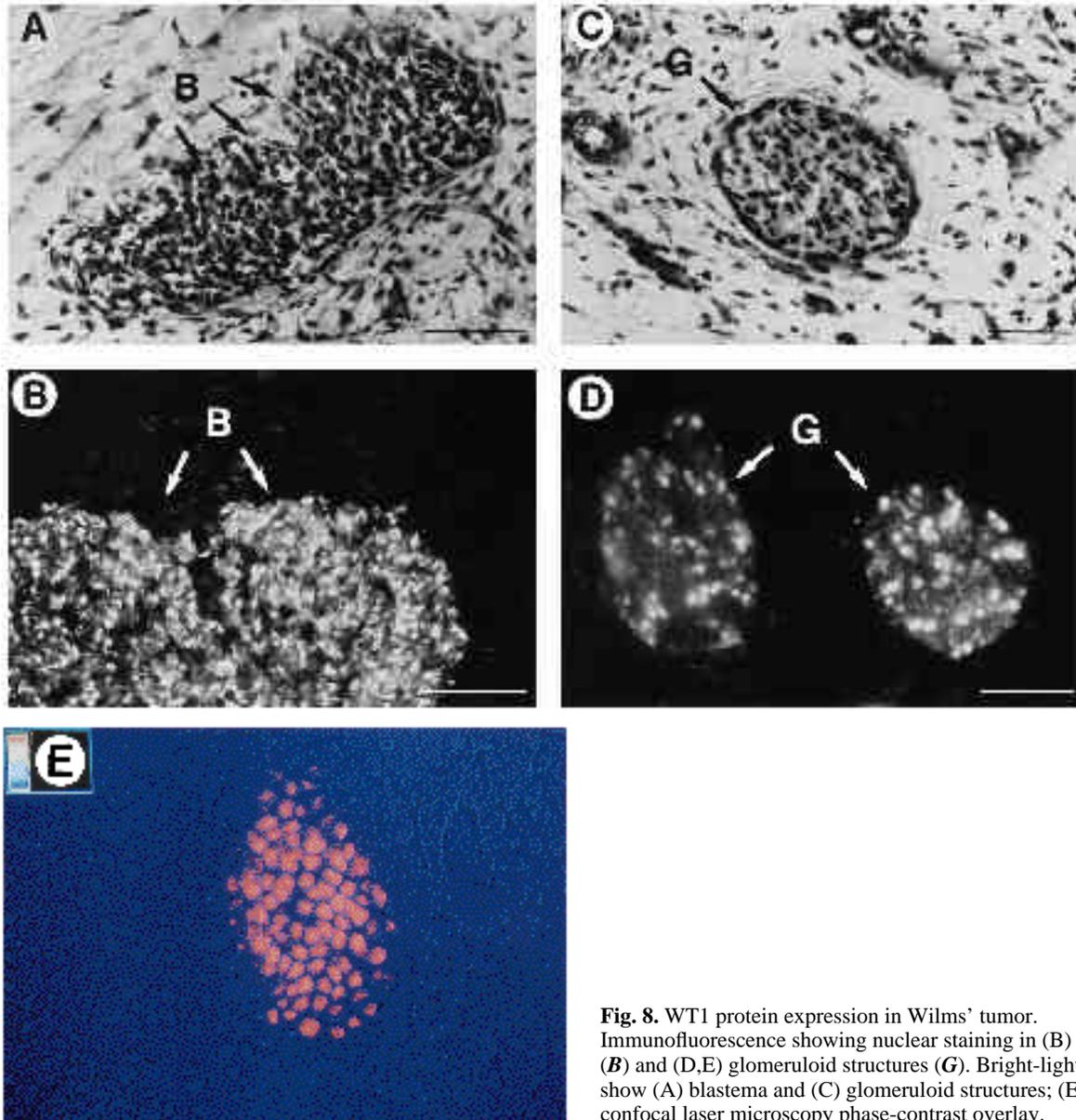
Fig. 8. WT1 protein expression in Wilms' tumor. Immunofluorescence showing nuclear staining in (B) blastema (***B***) and (D,E) glomeruloid structures (***G***). Bright-light images show (A) blastema and (C) glomeruloid structures; (E) confocal laser microscopy phase-contrast overlay.

and newborn testis was limited to Sertoli cells and the rete testis.

In addition to the developing urogenital system, WT1 expression was observed in cell layers of the visceral epithelium. Expression was not confined to the outermost layer but was present in adjacent cells which seemed to penetrate the epithelial layer (data not shown).

DISCUSSION

In this study, we have described the temporal and spatial expression of the WT1 mRNA and protein during development of the human urogenital system. In contrast to other recessive oncogenes such as the retinoblastoma gene or p53, expression of WT1 is restricted to specific cell types during different stages of early and late development. This limited

expression is observed in the kidney as well as in male and female gonads. The expression pattern points to an important role of WT1 not only during early human urogenital development but also during fetal and postnatal life. Two anti-WT1 monoclonal antibodies allowed us to sublocalize the WT1 gene product within cells expressing WT1 mRNA. Previous transfection experiments with COS-1 cells using a fusion of the influenza haemagglutinin epitope with WT1 have demonstrated intense staining localized to the nucleus (Pelletier et al., 1991b). These experiments however were performed in transfected COS-1 cells, a cell type that does not express WT1 mRNA. Our current study is the first to report on the subcellular localization of WT1 protein in cells that endogenously express WT1 mRNA. We have used confocal laser microscopy to sublocalize the WT1 protein within cells expressing WT1 mRNA. In concordance with the proposed function of WT1 as a transcription factor, we
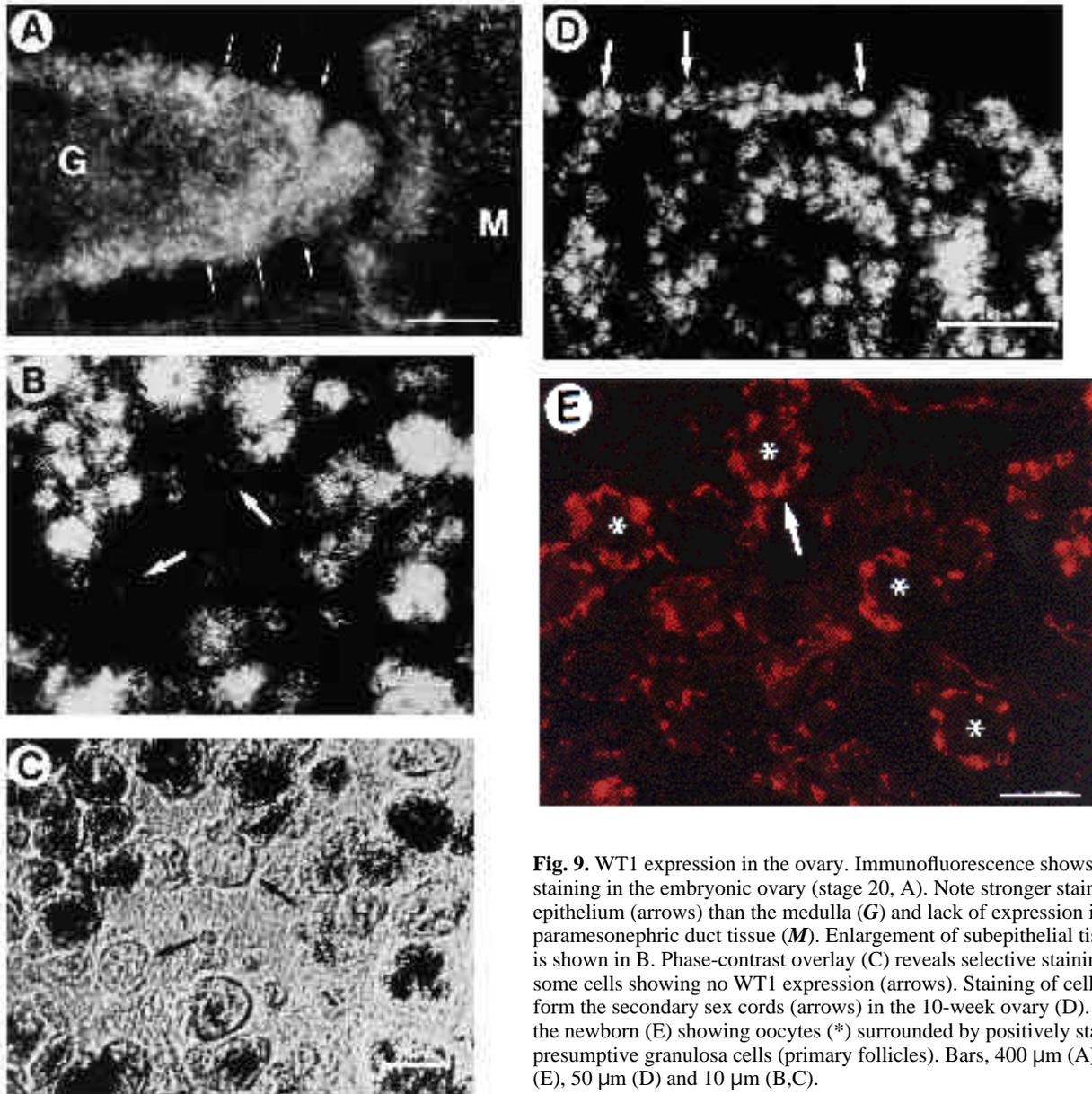
Fig. 9. WT1 expression in the ovary. Immunofluorescence shows nuclear staining in the embryonic ovary (stage 20, A). Note stronger staining of the epithelium (arrows) than the medulla (**G**) and lack of expression in paramesonephric duct tissue (**M**). Enlargement of subepithelial tissue of A is shown in B. Phase-contrast overlay (C) reveals selective staining with some cells showing no WT1 expression (arrows). Staining of cells that form the secondary sex cords (arrows) in the 10-week ovary (D). Ovary of the newborn (E) showing oocytes (*) surrounded by positively stained presumptive granulosa cells (primary follicles). Bars, 400 µm (A), 100 µm (E), 50 µm (D) and 10 µm (B,C).

have defined the expression of WT1 as nuclear. This localization is independent of the cell type and developmental stage investigated. Although the cytoplasm of the embryonic and fetal cells investigated was very small, it was possible to define staining restricted to the nucleus, using confocal laser microscopy and color overlay techniques. The nuclear staining showed a coarse speckled pattern.

During nephrogenesis, the earliest expression was observed in selective cells of the mesonephric glomerulus. In situ hybridization and immunofluorescence studies showed that WT1 mRNA and protein were present only in the outer cell layer of the primitive glomeruli. Judging from the localization of positive cells and the phase-contrast micrographs showing positive cells having cellular processes, the stained cells were identified as podocytes (de Martino and Zamboni, 1966).

Differentiation of the human metanephros begins at approximately the fifth week of development with the outgrowth of the ureteric bud from the mesonephric duct. The key event involved in nephrogenesis is the interaction of the ureter bud with the surrounding metanephric blastema and the subsequent conversion of mesenchymal cells into epithelial structures. The induction, and following differentiation, is an ongoing process in the fetal kidney, which continues to the 36th week of development (Saxen, 1987). Consistent with previous studies in humans (Pritchard-Jones et al., 1990) in the developing metanephros, WT1 mRNA expression was first noted in the undifferentiated metanephric blastema and in renal vesicles. In the following stages of nephrogenesis, expression was limited to the precursors of podocytes (glomerular epithelial cells). In the S-shaped body these cells can be located to the lower cell layer of the inferior cleft. After the capillary ingrowth, the layer of podocytes folds around the capillary loop and forms the
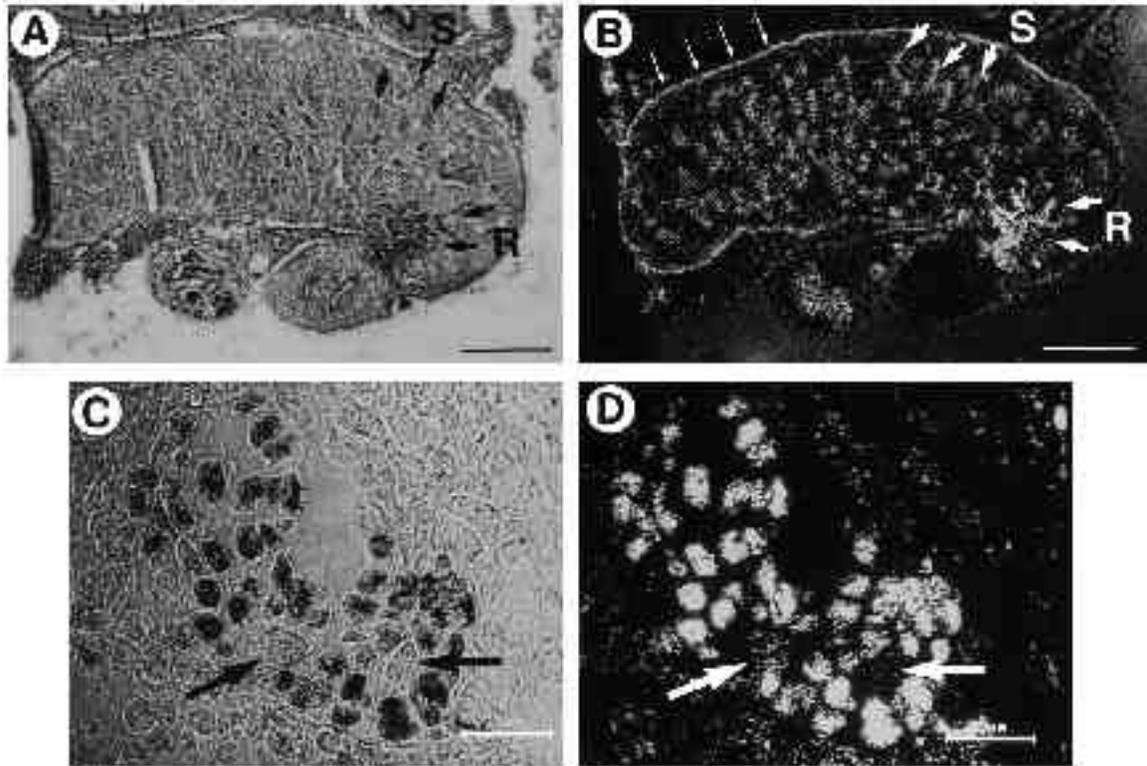
Fig. 10. WT1 expression in the testis. In the embryonic testis, shortly after the development of sex cords (B, stage 23), in situ hybridization shows expression in the tunica albuginea (arrows), seminiferous cords (**S**) and in the developing rete testis (**R**). Corresponding bright-light image is given in A. Immunofluorescence of the fetal testis (20th week) shows nuclear staining of selective cells within the seminiferous tubuli (C,D). (C) Phase-contrast overlay of D. Note lack of expression in spermatogonia (arrows) and surrounding mesenchyme. Bars, 400 μm (A,B) and 25 μm (C,D).

visceral sheet of Bowman's capsule. The long cellular processes reach inside the glomerular capsule and build a common basal membrane with the capillary epithelium. In the maturing glomeruli, expression was present only in the outer cell layer, the visceral sheet of Bowman's capsule. mRNA and protein expression were completely concordant except for the metanephric blastema and the renal vesicle. Here, WT1 mRNA was clearly detectable, but immunoreactivity was not noted before the S-shaped body stage. This indicates posttranscriptional regulation of the *WT1* gene during these early stages of glomerular differentiation.

As judged by the number of grains per cell, expression of WT1 mRNA was highest in the mature, rounded glomeruli of early fetal development (12-18 weeks) and declined thereafter. In the childhood kidney, WT1 mRNA was barely detectable. In contrast, immunofluorescence showed a consistently strong staining of podocyte nuclei during prenatal and postnatal life. The oldest specimen investigated was from an 80-year-old patient. Haber et al. (1990) investigated WT1 expression in adult human kidney by Northern blot and was not able to detect WT1 mRNA upon prolonged exposure. However, in the adult mouse kidney, WT1 mRNA is clearly present, although at substantially lower levels (approx. 12-fold) than in fetal kidney (Buckler et al., 1991; Armstrong et al., 1992). We have detected WT1 mRNA in adult human kidney RNA by the more sensitive method of RT-PCR. From the data presented here, we concluded that

WT1 expression is developmentally regulated, but present throughout life. These results suggest a role for WT1 in homeostasis of the mature glomeruli. A role for homeostasis of the podocytes by WT1 is supported by the identification of mutations in the *WT1* gene of patients with Denys-Drash-syndrome. In the majority of patients, point mutations in the *WT1* gene could be identified, affecting the third zinc finger and consequently DNA binding (Pelletier et al., 1991c). Such mutations apparently affect podocyte function, leading to a severely damaged glomerulus with hypertrophied podocytes and a protein-losing nephropathy. This nephropathy is not present at birth, but develops during early childhood, consistent with a role for WT1 in podocyte function beyond the stage of differentiation.

During nephrogenesis, the growing ureteric bud induces the mesenchyme to convert into epithelium. This differentiation is accompanied by drastic changes in the composition of the extracellular matrix. Once induced, cells of the metanephric blastema switch from the expression of interstitial collagens (collagen types I and III) to the production of basement membrane components such as collagen type IV, laminin and proteoglycans (Ekblom, 1981; Mounier et al., 1986). This change in expression precedes any morphological events and is thus one of the first detectable responses after tubule induction. The expression pattern of WT1 shows a high degree of similarity in temporal and spatial distribution. In addition, similar basement membrane

components are produced by Sertoli cells (Skinner et al., 1985), another major site of WT1 expression. Given similar temporal and spatial patterns of expression, as well as the observed effect on kidney function of some germ-line *WT1* mutations, it is tempting to speculate that this putative transcription factor modulates the expression of genes that are involved in basement membrane production.

Wilms' tumor appears to develop from persistent blastema, and, in addition to containing areas of primitive blastema, may differentiate along stromal and epithelial lines (Beckwith, 1983). In rare cases, these tumors include immature glomeruli. Such glomeruli contain basement membrane material (laminin, collagen type IV), podocytes and primitive mesangial cells but no patent capillaries (Payton et al., 1988). As in the described cases, the tumor investigated in this study showed a relatively large number of glomeruloid structures. Within these structures, nuclear staining was evident in selective cells. Judging from the expression in the kidney, these cells had most likely developed along the podocyte pathway. Expression of WT1 mRNA in blastema and not stroma confirmed the original observation by Pritchard-Jones et al. (1990). Our results are the first to demonstrate directly that WT1 protein is synthesized in Wilms' tumors.

The expression of WT1 in tissues other than the kidney should focus research on the possible involvement of WT1 in other tumors besides Wilms' tumor. Extrarenal sites of tumors histologically identified as Wilms' tumors have been reported (Sarode et al., 1992; Broecker et al., 1989). Such tumors could possibly arise from extrarenal sites of expression such as the visceral epithelium, which expresses WT1 protein. In one patient with Denys-Drash syndrome, reduction to homozygosity for the mutated *WT1* allele was demonstrated in a juvenile granulosa cell tumor that had developed in one case (Pelletier et al., 1991c). Thus, WT1 may contribute to tumorigenesis in other organs that express the protein. The use of the herein described anti-WT1 antibodies will greatly facilitate the search for WT1 expression in other tumors.

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