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Original Article

Tumor necrosis factor-α inhibits epithelial differentiation and morphogenesis in the mouse metanephric kidney *in vitro*

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ABSTRACT Tumor necrosis factor-α (TNF-α), an inflammatory cytokine, has diverse actions both within and outside the immune system and has been implicated in the etiology of a wide range of pathological conditions. Evidence is accumulating that it may also have important roles in the normal development of the embryo. In this study we demonstrated that the addition of recombinant TNF-α to metanephric organ culture induced a dose dependent and reversible decrease in growth and development, with inhibition of ureteric bud branching and nephron formation beyond the condensate stage and despite appropriate expression of the transcription factor pax-2. TNF-α also increased the point prevalence of apoptosis after only 1 day of culture. We also noted that macrophages were present in renal rudiments at the inception of nephrogenesis and their numbers significantly increased during the culture period. This effect was enhanced by TNF-α. We have also demonstrated expression of mRNAs for TNF-α and its receptors in whole mouse metanephroi from the inception of renal development. TNF-α protein was also detected, predominantly at mesenchymal/epithelial interfaces. In addition, TNF-α mRNA and protein were expressed by clonal renal mesenchymal cells *in vitro*, suggesting that these cells are a source of TNF-α *in vivo*.

KEY WORDS: *TNF-α, metanephros, mesenchyme, Pax 2, macrophage*

Introduction

The cytokine TNF-α was originally described as a potent mediator of tumor necrosis and septic shock (Vassalli, 1992). More recently it has been shown to have wide ranging roles both within and outside the immune system (Vassalli, 1992), mediated by the widespread presence of TNF receptors (TNFRs) on adult cells (Vandenabeele *et al.*, 1995).

Three prominent actions of TNF-α on various adult cells, namely enhancement of cell proliferation and programmed cell death and modulation of the extracellular matrix (Vassalli, 1992), are also key processes in normal embryonic development (Bard, 1990). Several lines of evidence suggest that the inflammatory cytokines, and in particular TNF-α, may have important actions during embryonic growth and organogenesis. Firstly, mRNA for TNF-α is expressed in human pre-implantation embryos (Sharkey *et al.*, 1995) as well as in mid to late gestation mouse (Kohchi *et al.*, 1994) and human (Jarvis *et al.*, 1995) embryos. Secondly, TNF-α protein has been localized to areas of apoptosis in chick embryos (Wride and Sanders, 1993) and finally, this factor has been functionally implicated in the proliferation of germ cells (Kawase *et al.*, 1994) and also in the development of the lung (Jaskoll *et al.*, 1994) and thymus (de Kossodo *et al.*, 1992). However, the expression and possible roles of TNF-α have not yet been examined in the mammalian embryonic metanephros, the direct precursor of the adult kidney.

In the developing kidney, mesenchymal to epithelial conversion occurs during the formation of nephrons (glomeruli and

Abbreviations used in this paper: BrdU, bromodeoxyuridine; DNA, deoxyribonucleic acid; E11 (18), embryonic day 11 (18); ELISA, enzyme linked immunosorbant assay; FACS, flow cytometry; FCS, fetal calf serum; GDNF, glial cell line-derived neurotrophic factor; HGF, hepatocyte growth factor; HRP, horse radish peroxidase; IFNγ, interferon gamma; LIF, leukaemia inhibitory factor; MDCK, Madin-Darby canine kidney; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; PBS, phosphate buffered saline; PFA, paraformaldehyde; PI, propidium iodide; RT-PCR, reverse transcriptase polymerase chain reaction; SD, standard deviation; TGF-α, transforming growth factor alpha; TGF-β, transforming growth factor beta; TNF-α (R), Tumor necrosis factor-alpha (receptor); TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; WT-1, Wilms tumor.

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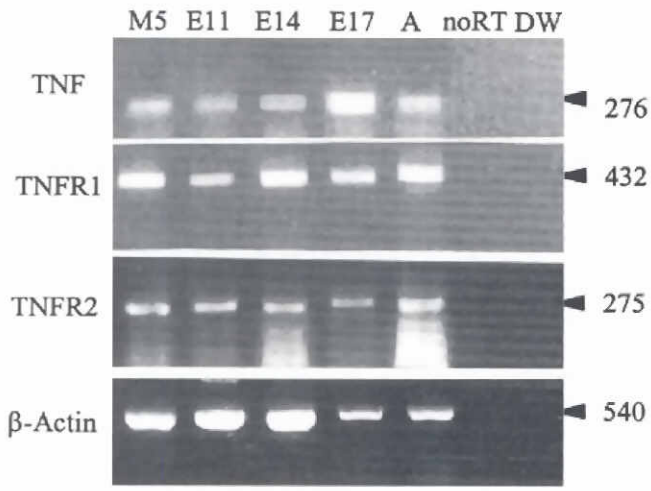

Fig. 1. TNF-α mRNA in nephrogenesis. *RT-PCR of TNF-α, TNFR1, TNFR2 and β-actin in the M5 renal mesenchymal cell line (M5), E11 to E17 metanephroi (E11-E17) and adult kidney (A). Negative controls, comprising no RT or distilled water only with no RNA (DW), were negative on all occasions (representative samples shown). RT-PCR products at 276, 432, 275 and 540bp are indicated by arrowheads.*

proximal tubules) via condensate, vesicle, and comma- and S-shape stages (see Fig. 1 in Woolf *et al.*, 1995). At the same time, collecting ducts form by branching of the ureteric bud epithelium. Stromal cells develop alongside nephrons and both may have common precursors in the renal mesenchyme (Herzlinger *et al.*, 1992). This process of normal organogenesis includes mechanisms with either positive or negative effects on overall growth. The former include cell proliferation and the acquisition of complex three-dimensional forms, such as branching morphogenesis, while negative aspects include programmed cell death and as yet poorly defined mechanisms which terminate morphogenesis. Dysregulation of these tightly controlled processes is associated with disease. For example, in humans and in animals uncontrolled proliferation of metanephric cells is associated with tumor and epithelial cyst formation (Dressler *et al.*, 1993; Winyard *et al.*, 1996b) while an excess of programmed cell death contributes to the pathogenesis of kidney malformations (Veis *et al.*, 1994; Winyard *et al.*, 1996a).

The molecules mediating the complex interactions occurring during normal nephrogenesis are under intense investigation, but have not yet been fully elucidated. To date, several classes of molecules have been implicated in these processes. These include: cytokines and growth factors (Woolf and Cale, 1997), transcription factors (Rothenpieler, 1996) and cell adhesion molecules (Ekblom, 1996). In the current study, we have demonstrated that TNF-α mRNA and protein are expressed within the murine metanephros from the inception of nephrogenesis. Addition of exogenous TNF-α to metanephric culture decreases both ureteric bud branching and nephron formation. Our data also suggests that these effects may be mediated either by apoptosis of cells destined to form nephrons early in the culture period or by actions of TNF-α on downstream targets of pax-2 which mediate epithelial differentiation and morphogenesis in the embryonic kidney.

Results

TNF-α is expressed by the metanephros

mRNAs for TNF-α and both its receptors were expressed in freshly dissected whole organs from the inception of nephrogenesis i.e. embryonic day 11 (E11), through periods of active nephron formation and ureteric bud branching (E14-17) and in adulthood, as assessed by RT-PCR (Fig. 1).

Western blotting (Fig. 2) demonstrated the presence of multimeric forms of TNF-α protein in E11 and E13 metanephroi. Pre-incubation of TNF-α antibody with murine recombinant TNF-α considerably diminished staining for the major species (arrowed in Fig. 2). Both membrane bound and circulating TNF-α are active as trimers (Vandenabeele *et al.*, 1995) and the major species detected here, at approximately 70 kDa, may represent a trimer. Similar polymeric forms have been detected in murine lung development (Jaskoll *et al.*, 1994). Some of these polymeric forms are due to differential glycosylation patterns, as we found that they were considerably diminished by prior deglycosylation of the protein (asterisks in Fig. 2). TNF-α was also detected by ELISA in whole organ homogenates and, although no TNF-α was detected in unconditioned, serum-supplemented media, low levels (0.09 ng/ml) were detected in conditioned media after 36 h of culture.

At E11 a low level of diffuse TNF-α immunoreactivity was observed in both the renal mesenchyme and ureteric bud (Fig. 3A). At E13 more intense immunostaining for TNF-α localized to areas of mesenchymal-epithelial interaction, particularly areas surrounding the ureteric bud branches and in mesenchyme around primitive nephrons (Fig. 3D). In E18 kidneys, immunoreactivity was confined to papillary collecting ducts, which are large branches of the ureteric bud (Fig. 3F and G), whilst glomeruli and

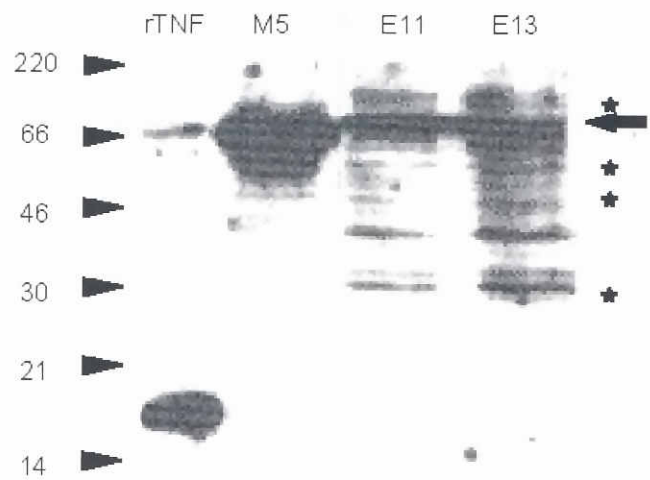

Fig. 2. Western blot for TNF-α. *Protein extracted from the M5 cell line (M5), E11 kidneys (E11) and E13 kidneys (E13) all demonstrated immunoreactivity for polymeric forms of TNF-α. The arrowed band, at approximately 70 kDa, was considerably diminished when the antibody was pre-absorbed with recombinant TNF-α and no bands were detectable with omission of the primary antibody or its replacement with non-immune serum. Asterisks denote bands diminished after in vitro deglycosylation of the protein. Recombinant, 17 kDa murine TNF-α used as a positive control (rTNF) demonstrated both single and multimeric forms.*

proximal tubules were negative (data not shown). Controls, comprising a non-immune rabbit serum, TNF-α antibody preabsorbed with murine recombinant TNF-α and omission of the primary antibody, all confirmed the specificity of TNF-α labeling (Fig. 3B and data not shown).

The M5 cell line derived from E11 renal mesenchyme expressed mRNA for TNF-α and its receptors by RT-PCR (Fig. 1) and TNF-α protein, as assessed by ELISA, western blotting (Fig. 2) and immunohistochemistry (Fig. 3C). Secreted TNF-α was also detected by ELISA (0.23 ng/ml) in the conditioned media from the cell line. Although we detected scattered macrophages (potential sources of TNF-α) in the mesenchymal compartment of freshly dissected whole metanephroi (Fig. 3E), it should be emphasized that the isolated renal mesenchymal cell line was negative for the F4/80 macrophage marker (data not shown).

Exogenous TNF-α decreases growth and differentiation in metanephric organ culture

The majority of experiments assessed the effects of TNF-α on metanephric culture over 4 days from E11. A second series of experiments investigated effects after 7 days of culture.

Control metanephric cultures grow and differentiate over 4 days

E11 metanephroi were cultured from the stage at which the ureteric bud had branched only once, resulting in two branch tips (Figs. 4A and 7A). In control cultures we observed an increase in the area of organs with time. This was detectable after 2 days of culture and continued to day 4 (mean 174% increase in area over 4 days) (Figs. 4B and 7A,G). Explant area is a crude means of assessing growth and morphogenesis as the organs are not of a uniform depth and the degree of differentiation cannot be easily seen. We therefore used a variety of other techniques to assess these parameters in more detail. Growth in culture was accompanied by branching of the ureteric bud, demonstrated by anti-laminin staining after 2 days of culture (Fig. 4C) with a mean +SD number of branch tips per organ of 8.4+2.9 (n= 28) (Table 1). By day 4 control cultures had an active nephrogenic zone containing multiple nephron forming units (Fig. 4D). Some of these nephrons were mature enough to express galactosidase (mean per metanephros +SD of 3.2+2.5) (Table 1). Flow cytometric analysis of single cell suspensions of metanephroi demonstrated that the mean number of cells significantly increased by 30% from day 0 to day 4 in these control cultures (Fig. 5H).

TABLE 1

NUMBERS OF URETERIC BUD BRANCHES AND NEPHRONS IN CULTURED METANEPHROI

	Ureteric bud branch tips Day 2		Mature nephrons Day 4	
	n	mean (SD)	n	mean (SD)
Control	28	8.4 (2.9)	15	3.2 (2.5)
TNF-α	27	5.3 (2.3)	18	0.7 (1.2)

Numbers of ureteric bud branch tips were counted after 2 days of culture in laminin stained control and TNF-α (100 ng/ml) treated metanephric cultures. This demonstrated a significant ($p < 0.05$) increase in branching in control cultures compared to TNF-α treated. In addition, after 4 days, control cultures contained significantly more ($p < 0.05$) nephrons staining positive for galactosidase than TNF-α treated cultures.

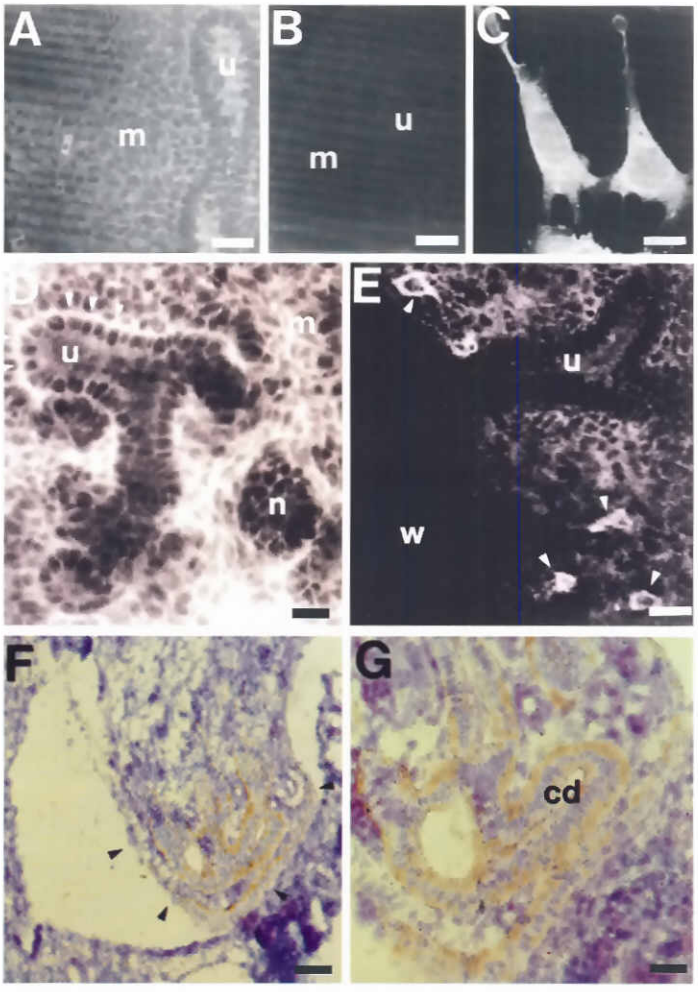

Fig. 3. TNF-α immunoreactivity in the metanephros. *Freshly dissected E11* **(A and B)**, *E13* **(D)** *and E18* **(F and G)** *metanephroi were stained with an antibody to TNF-α (A,D,F and G) or the secondary antibody only (B). (A-E) are confocal laser scanning images and conventional microscopy was used in (F) and (G). At E11 (A) TNF-α immunoreactivity was diffusely spread throughout the organ with an absent signal in the control (B). M5 cells isolated from renal mesenchyme at E11 also showed immunoreactivity for TNF-α (C). At E13 (D) TNF-α immunoreactivity was intense at mesenchymal/epithelial interfaces (arrowheads) and at E18 (F and G) immunoreactivity, depicted by peroxidase (brown) staining, was confined to the branched collecting ducts in the renal papilla [arrowheads in (F)]. (E) shows a confocal section of an E12 metanephros stained with F4/80 macrophage antibody with the ureteric bud branching off the Wolffian duct. Macrophages (arrowheads) are seen within the mesenchyme. Key: u, ureteric bud; m, metanephric mesenchyme; n, primitive nephron; cd, collecting duct and w, Wolffian duct. Bars, 50 μm (C and G), 100 μm (A, B and F) and 200μm (D and E).*

TNF-α inhibits growth and differentiation of metanephric cultures over 4 days

Addition of TNF-α to metanephric organ culture from E11 disrupted all of these processes (Figs. 4,5). Addition of TNF-α resulted in a decrease in the final planar area of organ cultures (mean increase in area at 4 days of 135% with 10 ng/ml, 115%

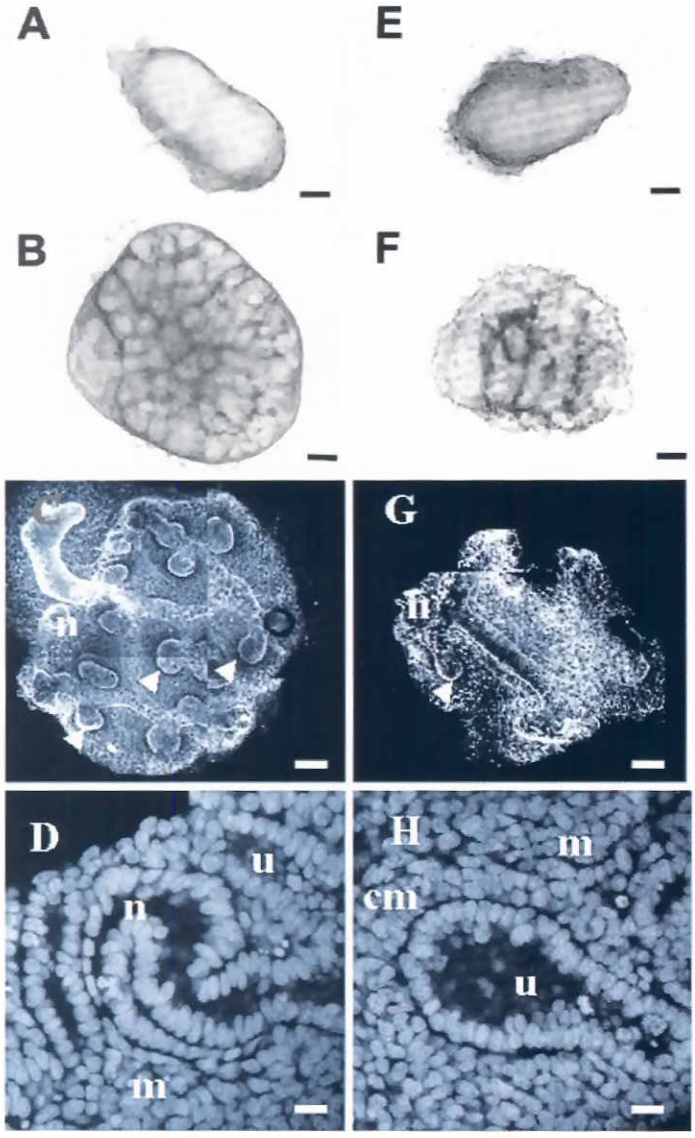

Fig. 4. TNF-α inhibits ureteric bud branching and nephron formation. **(A)**, **(B)**, **(E)** *and* **(F)** *are photomicrographs of whole organ cultures.* **(C)** *and* **(G)** *are composite confocal microscope images of laminin-stained cultures.* **(D)** *and* **(H)** *are higher power confocal images of PI stained organ cultures. E11 metanephroi (at least 10 per group) were cultured from a stage where the ureteric bud had branched once (A and E) for 4 days in serum containing media alone (B-D) or with the addition of 100 ng/ml of TNF-α (F-H). Control cultures increased in area (B), complexity of ureteric bud branching (branch tips indicated by arrowheads) (C), and the generation of nephron forming units (n in C,D and G). In contrast, the addition of TNF-α resulted in a smaller increase in area of the organ (F) and decreased arborization of the ureteric bud (G). Comparison of the confocal microscope pictures from control (D) and TNF-α treated cultures (H), shows that the latter contain poorly branched ureteric bud (u), with some condensed mesenchyme (cm) and large areas of loose, undifferentiated mesenchyme (m). Bars, 750 μm in (A), (B), (E) and (F); 85 μm in (C) and (G); and 35 μm in (D) and (H).*

with 100 ng/ml and 91% with 1000 ng/ml of TNF-α). These effects were dose dependent (Fig. 5) with maximal and statistically significant inhibition at 100 and 1000 ng/l. As significant perturbation of growth was seen at 100 ng/ml, this concentration of the factor was used for subsequent analyses. These inhibitory effects on growth were reversible as removal of TNF-α from the culture medium after 2 days resulted in further differentiation and growth of the organs (data not shown). Moreover, detailed histological analysis of explants failed to demonstrate non-specific toxic effects such as extensive necrosis (see below).

Addition of 100 ng/ml of TNF-α significantly ($p < 0.05$) decreased branching of the ureteric bud, with the mean +SD number of branch tips being 5.3+2.3 (n= 27) after 2 days of culture (compare Fig. 4G with 4C) (Table 1). These effects were abolished by pre-incubation of TNF-α with a TNF-α specific antibody (data not shown). In the TNF-α treated cultures, although areas of condensed mesenchyme were seen around the majority of existing ureteric bud branches, further differentiation failed to occur and these metanephroi consisted mainly of poorly branched ureteric bud and large areas of loose, undifferentiated mesenchyme (compare Fig. 4H with 4D). Furthermore, staining for galactosidase demonstrated only a mean +SD of 0.7+1.2 nephron forming units per culture on day 4 (Table 1). Hence, addition of TNF-α to metanephric organ culture disrupts both ureteric bud branching and mesenchymal/epithelial conversion. Finally, the number of cells in TNF-α treated day 4 cultures was less than that in both day 4 control and day 0 cultures ($p < 0.05$) (Fig. 5H). Mean cell diameter was not statistically different in control and TNF-α treated cultures (data not shown).

TABLE 2

A. APOPTOTIC INDICES IN CULTURED METANEPHROI

	Day 1 Apoptotic index (PI) mean (SD)		Day 4 Apoptotic index (PI) mean (SD)		Day 4 Apoptotic index (TUNEL) mean (SD)	
	Control	TNF-α	Control	TNF-α	Control	TNF-α
All cells	1.6 (0.6)	2.7 (1.3)*	1.0 (0.6)	0.7 (0.4)	7.3 (3.4)	6.2 (2.3)
Mesenchyme cells	1.8 (0.7)	3.1 (1.5)	1.5 (0.9)	0.9 (0.4)	11.4 (5.0)	7.3 (2.2)
Ureteric bud cells	0	0	0.4 (0.5)	0.5 (0.4)	1.9 (1.2)	3.5 (3.2)
Nephron forming unit cells	0	0	1.0 (0.2)	0 (0)	1.4 (1.2)	0.3 (0.9)

B. PROLIFERATIVE INDICES IN CULTURED METANEPHROI

	Day 1 Proliferative index (BrdU) mean (SD)		Day 4 Proliferative index (BrdU) mean (SD)	
	Control	TNF-α	Control	TNF-α
All cells	14.9 (8.0)	10.8 (3.3)	12.6 (3.2)	15.4 (3.0)
Mesenchyme cells	14.4 (4.6)	11.5 (3.7)	17.6 (4.6)	21.9 (3.0)**
Ureteric bud cells	8.3 (7.0)	2.1 (0.7)	4.8 (1.9)	4.3 (1.6)
Nephron forming unit cells	0	0	10.3 (5.8)	3.9 (3.5)***

* $p = 0.04$, ** $p=0.02$ and *** $p= 0.008$ for TNF-α v control

Total proliferative (assessed by BRdU incorporation) and apoptotic (assessed by PI staining or TUNEL) indices (i.e. the % of the total cells examined) were estimated in control and TNF-α treated metanephroi after 1 or 4 days of culture, as discussed in the Methods. Significantly more apoptosis was seen on day 1, but not day 4, in cultures treated with TNF-α. The observed differences in proliferation in nephron forming units in TNF-α treated versus control cultures is difficult to interpret as so few nephrons were formed in the presence of TNF-α and those formed were very primitive. n = 10 for all groups.

TNF-α alters the point prevalence of programmed cell death on day 1 but not day 4 of metanephric culture

The decreased cell number we recorded in TNF-α treated explants may be due to abnormalities of cell death or proliferation. However, the point prevalence of apoptotic nuclei detected by PI or TUNEL staining in TNF-α treated and control cultures was not statistically different after 4 days of culture (Table 2A). However, analysis of apoptosis using PI staining on day 1 demonstrated a significant (p= 0.04) increase in apoptosis in TNF-α treated cultures compared to controls (Table 2A).

TNF-α does not alter the point prevalence of proliferation in metanephroi on days 1 or 4 of culture

The point prevalence of cells undergoing DNA synthesis and hence proliferation, as assessed by BrdU incorporation, was the same overall in TNF-α treated and control cultures on days 1 and 4 of culture (Table 2B). In fact, analysis of individual cell compartments demonstrated a small, but statistically significant (p= 0.02) increase in proliferation in the mesenchymal compartment of TNF-α treated cultures compared to controls on day 4 (Table 2B). Finally, there was a significant (p= 0.008) decrease in the percentage of proliferating cells in the few early nephrons that did form in the TNF-α treated cultures. The importance of this observation must remain uncertain as the number of nephrons in TNF-α treated cultures were small, and any nephrons which did start to form were much less well differentiated than those in control cultures.

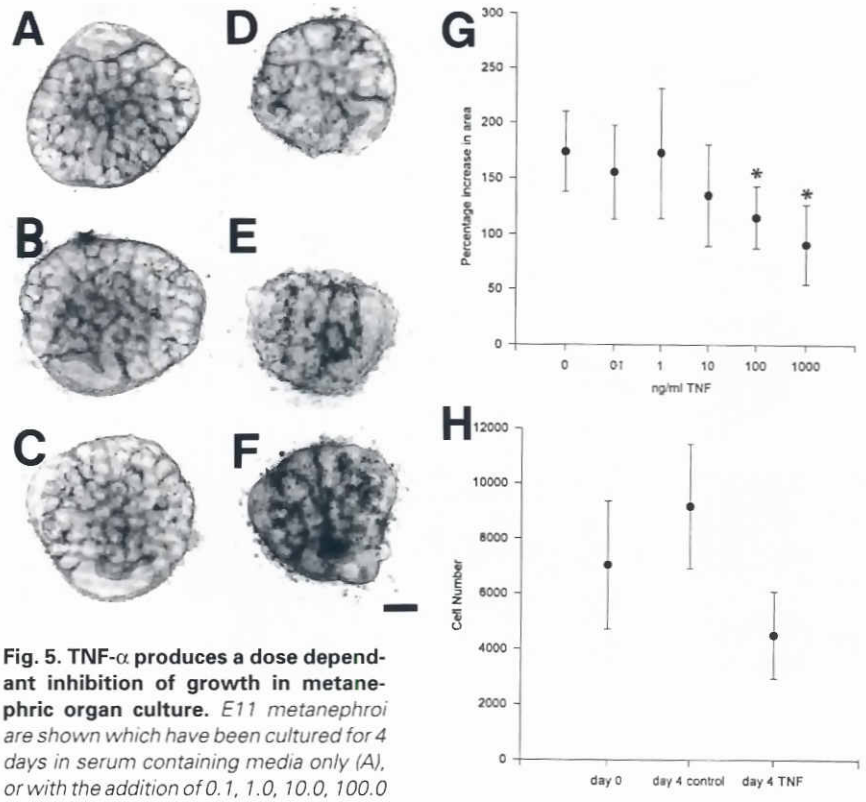

Fig. 5. TNF-α produces a dose dependant inhibition of growth in metanephric organ culture. *E11 metanephroi are shown which have been cultured for 4 days in serum containing media only (A), or with the addition of 0.1, 1.0, 10.0, 100.0 or 1000.0 ng/ml (B-F) of TNF-α. This produces a dose dependent decrease in final area. The results of one representative experiment are depicted graphically in* ***(G)*** *as mean + SD (n= 7-10 per concentration of TNF-α). Although a decrease in mean area is seen at 10 ng/ml, this does not reach statistical significance until 100 ng / ml (*p < 0.05 compared to control). (H) shows a cell cytometric analysis of cell number. Single cell suspensions of day 0 (n = 8) and day 4 cultures without (day 4 control, n= 9) or with the addition of 100 ng/ml of TNF-α (day 4 TNF, n= 9), were analyzed flow cytometrically and the number of cells counted after 40 sec at a constant flow rate. These are plotted as mean ± SD. The number of cells in TNF-α treated day 4 cultures is significantly less than day 4 control cultures (p < 0.05). Although a significant increase in cell number is clearly seen in day 4 control cultures compared to day 0 organs (p < 0.05), the number of cells in day 4 TNF-α treated cultures is significantly less than at day 0 (p < 0.05).*

Macrophage numbers increase during metanephric culture

Macrophages are present in metanephroi at E11, the start of the culture period (Fig. 3E). The numbers of macrophages (assessed by expression of F4/80) increased during the culture period in control cultures (Fig. 6A and Table 3). These effects were enhanced by the addition of TNF-α to the culture medium (Fig. 6B and Table 3). Mitotic figures were seen within macrophages (Fig. 6D), demonstrating that active proliferation was occurring. In addition, macrophages could be seen to contain nuclear debris (Fig. 6C), suggesting that they are actively scavenging dying cells.

Pax-2 and tenascin are expressed normally in TNF-α treated cultures

Pax-2 has been implicated in both ureteric bud branching and mesenchymal/epithelial conversion (Rothenpieler and Dressler, 1993; Torres *et al.*, 1995). We therefore assessed the effects of TNF-α on pax-2 protein expression in metanephric organ culture. *Pax-2* was expressed in ureteric bud, Wolffian duct and mesenchyme at the start of the culture period (Fig. 7D), as previously reported by Rothenpieler and Dressler (1993). After 4 days in culture, control rudiments continued to express pax-2 strongly in the branches of the ureteric bud, condensed mesenchyme and early nephrons (Fig. 7E). Expression was absent in undifferentiated mesenchyme and mature nephron elements, notably the podocyte layer (arrowed in Fig. 7E). In TNF-α (100 ng /ml) treated cultures, pax-2 expression was restricted to the ureteric bud and mesenchymal condensates, with a lack of expression in the loose, undifferentiated mesenchyme (Fig. 7F). Hence, pax-2 expression was appropriate for the level of morphological differentiation of TNF-α treated cultures. Finally, we reasoned that the lack of mesenchymal to epithelial conversion in the presence of exogenous TNF-α might also have been caused by diversion of mesenchyme into a stromal lineage. However, patterns of stromal differentiation, as assessed by tenascin immunostaining, were identical in TNF-α treated and untreated organ cultures, with minimal expression in the cortical mesenchyme (Fig. 8).

Prolonged exposure exacerbates the effects of TNF-α on metanephric culture

If cultures were grown for 1 week in the presence of 0, 0.1, 1.0, 10 or 100 ng/ml TNF-α, a potentiation of its effects were seen compared to day 4 cultures. Not only was a significant decrease

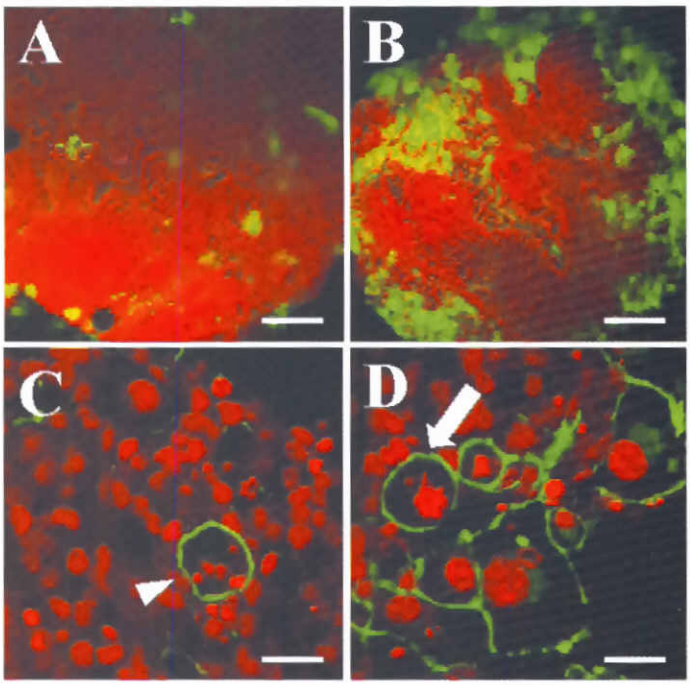

Fig. 6. Macrophages in day 4 metanephric culture. *Nuclei have been stained red with PI, and macrophages are green (F4/80 antibody). In* **A**, *macrophages are scattered throughout a control culture.* **B** *demonstrates the increase numbers seen in a TNF-α treated culture. In* **C**, *a macrophage (arrow head) is seen to contain nuclear debris and a mitotic figure within a macrophage is seen in* **D** *(arrow).*

in planar area detectable at 10 ng/ml TNF-α, but the number of mature, galactosidase expressing, nephrons was also significantly less at this concentration (mean 7.0 in controls, 4.7 at 10 ng/ml and 0.6 at 100 ng/ml) (Table 4).

Neutralizing endogenous TNF-α has no effects in metanephric organ culture over 4 days

As we had demonstrated the presence of endogenous TNF-α at the onset of the period of organ culture, we investigated the effects that neutralizing this activity had on growth. Addition of a neutralizing antibody to organ culture had no effects on overall growth of the organs by inspection, branching of the ureteric bud or the formation of nephron forming units (data not shown). The amount of antibody used (1 mg/ml), calculated from data provided by the manufacturers, neutralized in excess of 100 ng/ml of TNF-α, the concentration at which marked inhibition of growth was seen. In addition, immunohistochemistry using a fluorescent secondary antibody, confirmed that the anti-TNF-α antibody had penetrated the organ culture (data not shown). Specificity of the antibody was demonstrated by abolition of the effects of TNF-α on metanephric growth *in vitro* by its preabsorption with the antibody.

TABLE 3

MACROPHAGE PROLIFERATION IN METANEPHRIC CULTURE

	Control				TNF-α		
	Day 0	Day 1	Day 2	Day 4	Day 1	Day 2	Day 4
n	10	15	15	12	15	16	11
mean (SD)	0.7 (0.2)**	4.4 (1.3)	4.6 (1.4)*	4.7 (1.5)*	4.6 (1.4)	8.4 (3.3)	6.6 (1.7)

* p<0.001 control v same day TNF

** p < 0.001 day 0 v days 1, 2 and 4 of control and TNF cultures

Metanephroi were cultured for 0,1,2 or 4 days with the addition of 0 (control) or 100 ng/ml TNF-α. The number of macrophages per field was counted using confocal microscopy. This demonstrated a significant increase in macrophages in control cultures over time, and was significantly enhanced on days 2 and 4 by the addition of TNF-α.

Discussion

Normal nephrogenesis involves complex and reciprocal interactions between mesenchyme and ureteric bud which result in sequential branching of the ureteric bud and the formation of nephrons by initial mesenchymal condensation and subsequent mesenchymal to epithelial conversion. The data presented in this paper leads us to suggest that enhanced levels of TNF-α inhibit metanephric epithelial differentiation and morphogenesis .

Expression of TNF-α during nephrogenesis

We demonstrated that TNF-α and its receptors were present in metanephroi throughout the nephrogenic period. As nephrons started to form, TNF-α protein localized to regions of maximal mesenchymal-epithelial interaction, where the factor would be well placed to affect mesenchymal-epithelial inductive processes. In more mature kidneys (E18), expression was confined to medullary collecting ducts, which are ureteric bud derived structures. We also found that a cell line derived from E11 renal mesenchyme expressed both TNF-α and its receptors. This observation suggests that the factor may have an autocrine effect on renal mesenchyme. Further studies are necessary to determine whether ureteric bud expresses TNFR's, but it is of note that MDCK cells, a collecting duct cell line, bind TNF-α, with consequent increased MHC class II antigen expression (Zucker *et al.*, 1994) and inhibition of viral replication (Van Campen, 1994).

Exogenous TNF-α inhibits epithelial growth in the metanephros

Addition of increasing concentrations of TNF-α to metanephric organ culture resulted in a dose dependent inhibition of growth. Close examination, using a variety of histological techniques, demonstrated a block of mesenchymal to epithelial conversion after the stage of mesenchymal condensation, as well as decreased arborization of the ureteric bud. Previous experiments using mice with null mutations and studies with metanephric organ culture have suggested that metanephric cytokines and growth factors can have positive or negative effects on growth (Woolf and Cale, 1997). Factors with positive effects on growth include: transforming growth factor-α (Rogers *et al.*, 1992), insulin-like growth factors I and II (Rogers *et al.*, 1991), HGF (Woolf *et al.*, 1995), basic fibroblast growth factor (Karavanova *et al.*, 1996), bone morphogenetic protein-7 (Luo *et al.*, 1995), wnt-4 (Stark *et al.*, 1994) and GDNF (Sanchez *et al.*, 1996, Towers *et al.*, 1998).

Before the current study, only three growth factors had been described with negative effects on metanephric growth in organ culture: transforming growth factor-β1 (TGF-β1) (Rogers *et al.*, 1993), activin (Ritvos *et al.*, 1995) and leukaemia inhibitory factor (LIF) (Bard and Ross, 1991). Of these, only TGF-β1 (Rogers *et al.*, 1993) and activin (Ritvos *et al.*, 1995) are expressed in metanephroi. However, organ cultures grown in the presence of LIF show an arrest of nephron formation at the condensate stage similar to

that seen with TNF-α (Bard and Ross, 1991). If endogenous levels of these molecules are important in the normal process of nephrogenesis *in vivo*, one might expect that null mutations of these genes would result in kidney overgrowth. Null mutation mice for these molecules have been generated but appear to have grossly normal kidneys (Stewart *et al.*, 1992; Shull *et al.*, 1992; Matzuk *et al.*, 1995; Eugster *et al.*, 1996). It is feasible, however, that more subtle abnormalities, such as increased nephron number, might be present in these mutants. Using our *in vitro* organ culture system, we failed to demonstrate an acceleration of growth when endogenous TNF-α was neutralized with an antibody known to block its bioactivity. It is possible that multiple negative regulators may need to be blocked to cause significant overgrowth *in vitro* or *in vivo*. Such a mechanism has been proved for certain molecules which are positive regulators of nephrogenesis, namely *hoxa-11* and *hoxd-11* transcription factors (Davis *et al.*, 1995) as well as retinoic acid receptors αγ and αβ2 (Mendelsohn *et al.*, 1994).

The relatively high concentrations of TNF-α used in our experiments (10-100 ng/ml) reflect the fact that the factor has to diffuse from the medium, through the Millipore membrane and throughout a bulk of tissue to access responsive cells. It has been observed in other organ culture studies that higher concentrations of growth factors (e.g. LIF, TGFβ and GDNF) are needed to mediate actions on organs rather than cells (Bard and Ross, 1991; Alvarez *et al.*, 1992; Serra *et al.*, 1994; Jing *et al.*, 1996; Vega *et al.*, 1996). We found that a longer culture period of 1 week results in significant decreases on growth and differentiation at 10 ng/ml rather than the 100 ng/ml TNF-α required after 4 days (Table 4). Hence, we hypothesize that effects would be seen at yet lower concentrations of TNF-α if the period of exposure was extended. We have observed, however, that culture beyond one week is problematic because of the large size of the explant. In addition, it is of note that the concentration of circulating TNF-α can reach levels of 30 ng/ml in meningococcal septic shock (Waage *et al.*, 1989). Thus, concentrations of TNF-α in the range used in our experiments can be obtained *in vivo*, at least in pathophysiological circumstances.

TABLE 4

GROWTH AND DIFFERENTIATION AFTER ONE WEEK OF CULTURE

Concentration of TNF-α	Percentage increase in area		Mature nephrons	
	n	mean (sd)	n	mean (sd)
100 ng/ml	10	118 (40)**	12	0.6 (0.7)**
10 ng/ml	11	240 (45)*	10	4.7 (2.2)*
1 ng/ml	12	297 (79)	12	7.0 (3.0)
0.1 ng/ml	10	309 (52)	11	6.9 (3.0)
0 ng/ml	10	315 (95)	12	7.0 (1.8)

** $p < 0.001$ v control; * $p < 0.01$ v control

The planar area of metanephroi was measured on day 0 and 7 of culture and the percentage increase in area calculated. This demonstrated a significant decrease in growth in cultures with 100 or 10 ng/ml TNF-α compared to controls. Similarly, control cultures contained significantly more nephrons staining positive for galactosidase than cultures treated with 10 or 100 ng/ml of TNF-α.

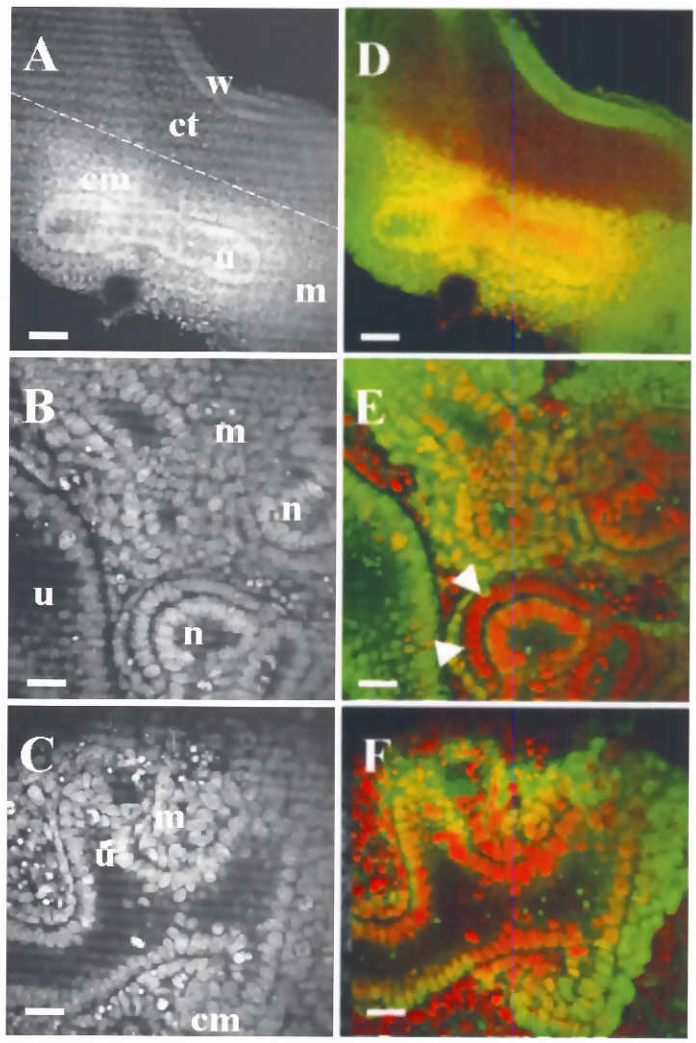

Fig. 7. *Pax 2* is normally expressed in metanephroi cultured with TNF-α. (A-C) *show confocal microscope images stained with a nuclear marker, PI;* **(D-F)** *are combined images showing PI (in red) and Pax-2 antibody (in green). (A) and (D) are E11 metanephroi with the adjacent Wolffian duct (w), surrounding loose mesenchyme (m) and connective tissue (ct). The dotted line in (A) represents the plane of dissection before organ culture, which ensures that the Wolffian duct and connective tissues are not cultured. At E11, pax-2 protein was expressed in the renal mesenchyme, first ureteric bud branches and Wolffian duct but not in the surrounding connective tissue. (B) and (E) are representative images from control organs after 4 days in culture. Pax-2 continued to be expressed in the ureteric bud (u) and early nephrons, but was not detectable in loose mesenchyme or the more differentiated, podocyte layer (arrowheads) of the nephrons. In TNF-α treated cultures, (C) and (F), pax-2 was expressed in the poorly branched ureteric bud and adjacent condensed mesenchyme but not in loose, undifferentiated mesenchyme. Bars, 50 μm in (A) and (D) and 20 μm in (B-D) and (F).*

Mechanism of action of TNF-α

We performed a variety of experiments to attempt to elucidate the mechanism(s) of action of TNF-α in our experimental model. A fine balance between proliferation and cell death is necessary for normal nephrogenesis. We therefore measured the total number of cells in explants and also quantified incorporation of BrdU and pyknotic PI stained nuclei. We found that TNF-α treated

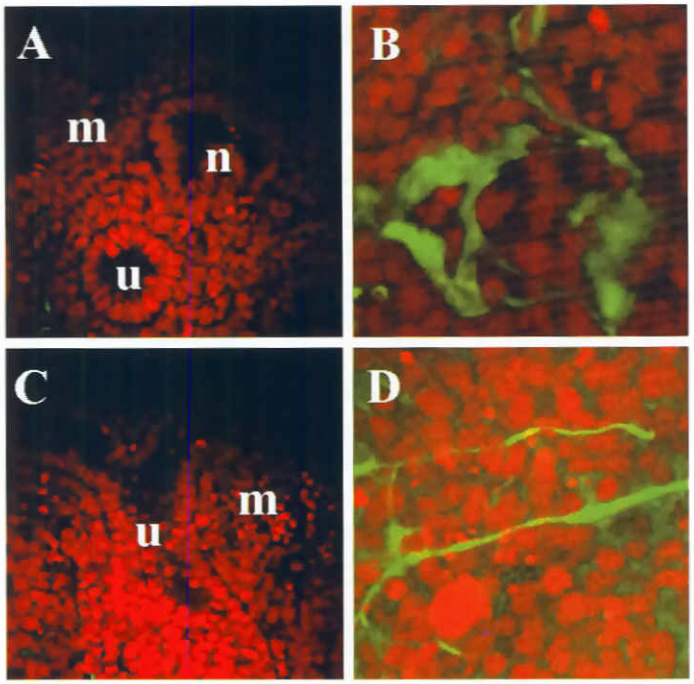

Fig. 8. Tenascin immunostaining in TNF-α treated cultures. *Organs grown in culture for 4 days without* **(A and B)** *and with* **(C and D)** *the addition of 100 ng/ml of TNF-α were stained for tenascin (green) and nuclei counterstained with PI (red). In both conditions tenascin staining was minimal in superficial mesenchyme, (A) and (C), but was detected in stromal cells deep in the explants, (B) and (D). Key: u, ureteric bud; m, mesenchyme. Bars, 50 μm (A and C), 25 μm (B and D).*

explants contained fewer cells at 4 days than time matched control cultures. Furthermore, we found that there were fewer cells in TNF-α treated 4 day explants compared to explants analyzed at the start of the culture period. However, quantification of the point prevalence of cell turnover in defined cellular compartments failed to show any increase in apoptosis or decrease in proliferation in TNF-α treated organ cultures compared to controls at day 4. Theoretically, if TNF-α induced large amounts of necrosis, final cell numbers could be less despite normal apoptotic and proliferative indices. However, no areas of necrosis were seen in any of the TNF-α treated cultures examined and the inhibitory effects of TNF-α were reversible. We therefore also determined apoptotic indices after 1 day of culture and were able to show a significant increase in the point prevalence of apoptosis in TNF-α treated cultures compared to controls. This raises the possibility that the group of cells most sensitive to apoptosis, and therefore being eliminated on day 1 of culture, are those that ultimately form nephrons. It is of note, however, that the rate of apoptosis or clearance of apoptotic nuclei cannot be calculated using these techniques.

Both we and other authors (Camp and Martin, 1995) have noted macrophages within the murine metanephros at the inception of nephrogenesis, and these macrophages contain nuclear debris. In the present series of experiments we noted that macrophage numbers increase over the culture period in control cultures, an effect which is significantly enhanced by TNF-α. Hence, macrophages actively scavenging apoptotic nuclei may mask any differences in apoptotic rates on day 4 of culture. Furthermore, macrophages are potent producers of cytokines and other mediators (Gordon, 1995), including known modulators of normal nephrogenesis such as TGF-β. These inflammatory cells and mediators have been shown to have important roles in the pathogenesis of the inflammatory glomerulonephritides (Cattell 1994). We suggest that macrophages may contribute directly to the altered morphology seen with TNF-α. Macrophage depletion of metanephroi prior to culture may resolve these issues. Our recent unpublished data (Cale *et al.* manuscript in preparation) demonstrated that macrophages are also present in the developing human metanephros, and, moreover, that their distribution is abnormal in the undifferentiated tissue found in renal dysplasia.

The transcription factor *pax-2* is expressed in the Wolffian duct and upregulated in the mesenchyme at the inception of nephrogenesis. It continues to be expressed in branches of the ureteric bud and mesenchymal condensates, but is down regulated as nephrons mature and is not expressed in loose, undifferentiated mesenchyme (Dressler *et al.*, 1990). Mice homozygous for a null-mutation in *pax 2* lack kidneys secondary to a failure of outgrowth of the ureteric bud (Torres *et al.*, 1995). Furthermore, addition of antisense oligonucleotides to organ cultures from E11 in mice produces a complete block in nephron formation at or even before the condensate stage (Rothenpieler and Dressler, 1993). In TNF-α treated cultures, we found that *pax-2* was expressed in mesenchymal condensates and ureteric bud branches i.e., the expression of this transcription factor is consistent with the level of morphological differentiation of the explant. In our cultures, condensates were seen around the poorly arborized branches of the ureteric bud. Thus, the actions of pax-2 in inducing condensation do not appear to be inhibited by TNF-α. The failure of further differentiation in TNF-α treated cultures suggests that the expression of pax-2 alone is insufficient for mesenchymal to epithelial conversion beyond the condensate stage. It is possible that TNF-α may be acting on downstream targets of pax-2. One established target for pax-2 expression is the Wilms tumor gene, *WT-1* (Dehbi

TABLE 5

PRIMER SEQUENCES FOR RT-PCR

cDNA	Sense Primer 5' to 3' (nucleotide numbers)	Antisense Primer 5' to 3' (nucleotide numbers)	Product size (bp)
β-Actin (CLONTECH)	GTGGGCCGCTCTAGGCACCAA (25-45)	CTCTTTGATGTCACGCACGATTTC (564 -541)	540
TNF-α (Macica, *et al.*, 1994)	ATGAGCACAGAAAGCATGATC (157-177)	TACAGGCTTGTCACTCGAATT (432-412)	276
TNFR1 (Lewis *et al.*, 1991)	CTTTACGGCTTCCCAGAATTACCTCAGGCA (458-487)	CTGATGAAGATAAAGGATAGAAGGCAAAGA (889-860)	432
TNFR2 (Lewis *et al.*, 1991)	AGGATGCAGTAGGCCTTGAGCAGCAGCACC (969-998)	GGAAGAGCACTGAGAACTGTGGTCAGAGCT (1243 - 1214)	275

et al., 1996). Future work will assess the expression of this gene in TNF-α treated cultures.

An alternative explanation for the failure of differentiation of metanephric mesenchyme in the presence of TNF-α is that it is inducing differentiation of mesenchymal cells along a non-epithelial route, such as tenascin expressing stromal cells (embryonic interstitial fibroblasts) (Ekblom and Weller, 1991). This does not appear to be the case as expression patterns of tenascin were unchanged in TNF-α treated cultures compared to controls. It is possible that other, non-tenascin expressing lineages are transdifferentiating. One example would be expression of α-smooth muscle actin (Daikha-Dahmane *et al.*, 1997), which has been demonstrated in dysplastic kidneys. The fate of these mesenchymal cells will be investigated further in a separate study.

Finally, TNF-α is capable of influencing the production of multiple cytokines from a variety of cells (Vassalli, 1992), for example TNF-α induces TGF-β production in adult renal proximal tubular cells (Phillips *et al.*, 1996). It is therefore conceivable that TNF-α may act in our *in vitro* system by either increasing negative growth factors or decreasing positive growth factors.

Complex patterns of cytokine production and interactions with immune and non-immune cells are documented in many disease processes. Our data suggests that the role of TNF-α in human renal maldevelopment merits further investigation.

Materials and Methods

All reagents were obtained from Sigma unless otherwise stated. All tissues were obtained from the CD1 strain of mouse, apart from the transgenic cell line which was derived from the H-2Kb-tsA58 mouse (Woolf *et al.*, 1995). All tissue culture reagents used were endotoxin free and the fetal calf serum (FCS) batches used did not contain TNF-α, as assessed by ELISA (see below).

Metanephric organ culture

E11 metanephroi were isolated by microdissection and maintained in organ culture on transparent permeable supports, essentially as previously described (Woolf *et al.*, 1995), for 1, 2, 4 or 7 days in DMEM/Ham's F12 medium (Gibco, BRL, Paisley, UK) supplemented with 5% vol./vol. FCS (Gibco, BRL). In some experiments we added: i) recombinant murine TNF-α (0.1-1000 ng/ml) (Sigma) ii) 1 mg/ml TNF-α neutralizing antibody (Pharmingen, San Diego, USA) or iii) 1 mg/ml rat IgG1 (Pharmingen). Each experimental condition was repeated at least twice and media and additives were changed on days 3 and 5 *in vitro*. The recombinant TNF-α used was made in E. Coli and was demonstrated by the manufacturers, using an SDS-PAGE technique, to be more than 97% pure and to contain less than 1 ng endotoxin per μg TNF. Their data also demonstrated, using a cell inhibition assay, an EC50 of 0.433 ng/ml. Organs were photographed on days 0, 2, 4 and 7 using an inverted microscope. As the explants are disc shaped in culture, the cross-sectional area was used as a measure of overall growth and the areas of individual organs were calculated from photographs by computer scanning and analysis using NIH Image (Version 1.59). Percentage increase in area was calculated for each organ. Detailed histological analysis of organs was performed after fixation in 4% paraformaldehyde (PFA) and nuclear staining with propidium iodide (PI), (4 mg/l with RNase A 100 mg/l) using confocal microscopy (Confocal Laser Scanning Microscope; Aristoplan-Leica, Heidelberg, Germany) (Woolf *et al.*, 1995).

Quantification of branching in cultured metanephroi

Metanephroi were fixed in 4% PFA after 2 days of culture and stained with an anti-laminin antibody as described below (Sariola *et al.*, 1988). This method clearly outlined the branches and terminal buds of the developing collecting duct system. Using confocal microscopy, the numbers of branch tips at all levels of the culture could be visualized and counted. Nephron forming units, which acquire a laminin-containing basement membrane, were easily distinguished by counter-staining with PI. Cultures were analyzed after 2 days *in vitro* rather than 4 as the structure of the control cultures is too complex by 4 and 7 days to allow accurate counting of branches.

Quantification of nephron forming units in cultured metanephroi

Differentiated kidney epithelia, especially proximal tubules, contain endogenous galactosidase and can therefore be detected using X-gal staining (Bard and Ross, 1991; Woolf *et al.*, 1995). Although ureteric bud structures also stain blue, the color intensity is much less and nephrons can easily be distinguished. Immature nephrons are not detected by this method. After 4 or 7 days of culture, metanephroi were fixed in 4% PFA for 15 min and analyzed for galactosidase expressing nephrons as described elsewhere (Bard and Ross, 1991; Woolf *et al.*, 1995).

Measurement of metanephric cell number and size by flow cytometry

Single metanephroi were too small to analyze separately. Metanephroi were cultured for 4 days, removed from the filter in groups of 3 and dissociated to a single cell suspension by incubation in 1 mg/ml collagenase for 30 min. Cells were pelleted and resuspended in 200 μl of phosphate buffered saline (PBS)/ 1% FCS. These single cell suspensions (verified microscopically) were analyzed flow cytometrically (FACSCalibur, Becton Dickinson, Oxford, UK). One clearly defined population of cells was detected, hence allowing an estimation of cell diameter. The number of cells detected after 40 sec at a constant flow rate for all analyses was recorded for at least 8 groups for each condition.

Quantification of apoptosis and proliferation in cultured metanephroi

Staining with PI and viewing by confocal microscopy allowed morphological assignment of individual cells to one of three compartments: mesenchyme (loose and condensed taken together), ureteric bud structures, or nephron forming units (vesicle, comma- and S-shaped bodies as well as early glomeruli). In addition, apoptotic nuclei are brightly staining and condensed with PI (Woolf *et al.*, 1995; Winyard *et al.*, 1996a). Apoptotic nuclei were also detected on day 4 by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL), using methodology detailed by the manufacturers (Boehringer Mannheim, Mannheim, Germany). The total number of cells and the number of apoptotic nuclei in mesenchyme, nephron forming units and ureteric bud were counted for 5 fields per organ and the percentage of apoptotic nuclei per organ calculated (= apoptotic index for each organ). This represents the point prevalence of apoptosis (number of events at a particular time (total number in population at that time). Cell proliferation was analyzed using a fluorescent bromodeoxyuridine (BrdU) incorporation kit as recommended by the manufacturers (Boehringer Mannheim). Briefly, for whole organ cultures, the medium was replaced with BrdU incorporation medium for the last 2 h of organ culture, organs were washed with PBS and fixed with 70% ethanol in 50 mM glycine buffer at -20°C for 20 min. Cells were washed with PBS and incorporated BrdU detected using a fluorescin conjugated secondary antibody. Proliferative indices were calculated as for apoptotic indices.

Quantification of macrophages in metanephric culture

Metanephroi were cultured for 0, 1, 2 or 4 days with or without 100 ng/ml TNF-α and fixed and stained for macrophages as described below. Organs were then analyzed using confocal microscopy. The number of macrophages per field was counted for 10 fields per organ and a mean number of macrophages per field derived from this.

Renal mesenchymal cells in culture

In order to study gene expression in purified populations of cells, we used a conditionally-immortalized cell line derived from mouse E11 renal mesenchyme. This line, called M5 in Woolf *et al.* (1995) expresses

classical markers of mesenchymal cells [e.g. vimentin and hepatocyte growth factor (HGF) (Woolf *et al.*, 1995)] but does not stain for epithelial (Woolf *et al.*, 1995) or macrophage markers (see Results). Proliferation is driven by a temperature sensitive Simian Virus 40 T antigen which can be induced by low levels of interferon-γ (IFN-γ) (40 U/ml) (Woolf *et al.*, 1995). For analyses in this paper, the cell line was studied after culture in non-permissive conditions for 48 h; this includes the withdrawal of IFN-γ.

Reverse transcriptase-polymerase chain reactions (RT-PCR)

All analyses were performed at least twice. The sequences of PCR primers are shown in Table 5. All tissues were freshly harvested and immediately frozen in liquid nitrogen prior to analysis. RNA was extracted from metanephroi, adult kidneys and cell lines using Tri-reagent (Molecular Research Center, Inc., Cincinnati, USA) and 300 ng was subjected to reverse transcription and PCR amplification as previously described (Woolf *et al.*, 1995) with the following modifications. Tubes were heated to 94°C for 5 min and 2.5 U Taq DNA polymerase added. Each PCR cycle was: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Twenty eight cycles were performed for β-actin and TNF-α, and 30 for TNF receptors (TNFR) 1 and 2. Finally, the reaction was held at 72°C for 10 min and cooled to 30°C for 20 min (Hybaid Omnigene PCR machine, Teddington, UK). β-actin primers were used as a positive control to confirm the integrity of RNA in all samples. Negative controls contained no RNA or no reverse transcriptase. No attempt was made to quantitate RT-PCR assays. Specificity of products obtained was confirmed by restriction digestion. For each reaction, 5 μl of product was digested for 1 h at 37°C in a 20 μl reaction mix. Enzymes used (Promega) were: Acc I (digested products 146 and 130) and Dde I (digested products 38, 96 and 141) for TNF-α, Hinf I (digested products 216, 142 and 74) and Pst I (digested products 172 and 260) for TNFR1 and Alu I (digested products 60, 107 and 108) and Dde I (digested products 73 and 202) for TNFR2.

Western Analysis

Tissues and cells were dissociated by passage through needles in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) plus freshly added protease inhibitors (1 μM sodium orthovanadate, 100 μg/ml of phenylmethylsulphonyl fluoride and 30 μl/ml aprotinin). Samples were electrophoresed using 5% stacking and 15% resolving Tris-glycine SDS-polyacrylamide gels (Protogel, National Diagnostics, Atlanta, Georgia). Proteins were transferred electrophoretically onto a membrane (Hybond-C extra, Amersham, UK) by semi-dry blotting. Filters were blocked in 5% marvel and 0.05% Tween in PBS and incubated with a rabbit anti-mouse polyclonal TNF-α (antibody (Genzyme, West Malling, UK) at a dilution of 1:500. Primary antibodies were detected by horseradish peroxidase conjugated secondary antibodies (HRP goat anti-rabbit, Dako, High Wycombe, UK) and enhanced chemiluminesence reagent (Amersham Life Science, Amersham, UK). Recombinant TNF-α was used as a positive control and the specificity of the antibody was assessed by pre-incubation of the antibody with TNF-α. In a further experiment, protein from E13 kidneys was prepared in buffer (20 mM Tris HCl, 1 mM EDTA, 50 mM NaCl, 0.02% sodium azide, 0.5% SDS and 5% β-mercaptoethanol) and deglycosylated by incubation for 0, 6 or 24 h with peptide-N-glycosidase F (Oxford Glycosciences, Oxford, UK). Products were analyzed by western blotting as above.

Enzyme linked immunosorbant assay (ELISA) for TNF-α

Ten E11 metanephroi were cultured in 200 μl of serum-supplemented medium for 36 h. Cell lines were analyzed at confluence. Conditioned media from cells and organ cultures were spun prior to analysis to remove cellular debris and protein extracts prepared as for Western analysis. TNF-α rat anti-mouse monoclonal antibody (Pharmingen) was diluted to a concentration of 4 μg/ml in 0.1 M sodium hydrogen carbonate and 50 μl added to wells of an Immulon 4 (Dynatech) plate. This was incubated overnight at 4°C and then washed with phosphate buffered saline/0.1% vol./vol. Tween-20 (PBST). Non-specific binding was blocked by incubation of the plate for 2 h at room temperature with 200 μl per well of PBS/10% FCS (Gibco, BRL). Standard curves were generated using serial dilutions of recombinant murine TNF-α. Fifty μl of standards and samples were incubated for 4 h at room temperature and the plate washed with PBST. Fifty μl of the detection antibody (biotinylated rabbit anti-mouse TNF-α polyclonal antibody [Pharmingen]) diluted to 2 μg/ml in PBS/10% FCS, was added to the wells and the plate incubated for 45 min at room temperature. The plates were again washed and incubated for 30 min with 100 μl/well of a 1:1000 solution of streptavidin-alkaline phosphatase (Amersham Life Science). The reaction was detected, after final washing of the plate with PBST, with a solution of 0.1 M NaHCO3, 0.6x10-6M magnesium chloride and p-nitrophenylphosphate (1 g/l). The plate was left for 2-3 h for a colour reaction to develop and optical densities read at 410 nm (Dynatech MRX Elisa plate reader). The lower limit of detection of the assay was 0.07 ng/ml.

Immunohistochemistry

All tissues were fixed in 4% PFA at room temperature.

E11-13 metanephroi, organ cultures and cell lines

Tissues were permeabalized in methanol at -20°C and non-specific staining blocked by incubation for 1 h at room temperature in 10% vol./vol. FCS, 1% w/vol. bovine serum albumin (BSA) in PBS. They were incubated overnight at 4°C with the primary antibody in serum/PBS, washed (3x10 min) in PBS and then incubated at 4°C overnight with the appropriate secondary flourescin-conjugated antibody at a concentration of 1:50. After washing in PBS (3x10 min) organs were counterstained with PI and visualised using confocal laser scanning microscopy (Woolf *et al.*, 1995). Primary antibodies used were: rabbit anti-mouse TNF-α (Genzyme) diluted to 1:100; FITC-conjugated rat anti-mouse macrophage F4/80 (1:50) (Serotec, Oxford, UK); rabbit anti-mouse Pax-2 (1:50) (Winyard *et al.*, 1996b); rabbit anti-mouse laminin for detection of basement membrane (1:50); FITC-conjugated monoclonal anti-mouse tenascin, a marker of stromal fibroblasts (Ekblom and Weller, 1991) (1:100). These methods were also used to assess the penetration of TNF-α neutralizing antibody through organ cultures.

E18 metanephroi

E18 organs are too large to image as whole-mounts and were therefore paraffin embedded, sectioned at 5 (m and processed for immunohistochemistry as previously described (Winyard *et al.*, 1996b) but using the Genzyme TNF-α antibody described above.

Statistical analysis

Normally distributed data was expressed as mean +SD and analyzed using a one way ANOVA or t-test for comparison of multiple or two groups respectively. Differences were considered significant when $p < 0.05$.

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References

ALVAREZ, R.J., SUN, M.J., HAVERTY, T.P., IOZZO, R.V., MEYERS, J.C. and NEILSON, E.G. (1992). Biosynthetic and proliferative characteristics of tubulointerstitial fibroblasts probed with paracrine cytokines. *Kidney Int. 41:* 14-23.

BARD, J.B.L (1990). The cellular and molecular processes of developmental anatomy. In *Morphogenesis* (Eds P.W. Barlow, D.B. Bray, P.B. Green, and J.M.W. Slack). Cambridge University Press, Cambridge. pp 65-173.

BARD, J.B.L. and ROSS, A.S.A. (1991). LIF, the ES-cell inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. *Development 113:* 193-198.

CAMP, V. and MARTIN, P. (1995). Programmed cell death and its clearance in the developing mammalian kidney. *Exp Nephrol 4:* 105-111.

CATTELL, V. (1994). Macrophages in acute glomecular inflammation. *Kidney Int. 45:* 945-952.

DAIKHA DAHMANE, F., DOMMERGUES, M., MULLER, F., NARCY, F., LACOSTE, M., BEZIAU, A. and DUMEZ, Y. (1997). Development of human fetal kidney in obstructive uropathy: correlations with ultrasonography and urine biochemistry. *Kidney Int. 52:* 21-32.

DAVIS, A.P., WITTE, D.P., HSIEH-LI, H.M., POTTER, S.S. and CAPECCHI, M.R. (1995). Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. *Nature 375:* 791-795.

DE KOSSODO, S., GRAU, G.E., DANEVA, T., POINTAIRE, P., FOSSATI, L., ODY, C., ZAPF, J., PIGUET, P., GAILLARD, R.C. and VASSALLI, P. (1992). Tumor Necrosis Factor α is involved in mouse growth and lymphoid development. *J Exp. Med. 176:* 1259-1264.

DEHBI, M., GHAHREMANI, M., LECHNER, M., DRESSLER, G. and PELLETIER, J. (1996). The paired-box transcription factor, PAX2, positively modulates expression of the Wilms' tumor suppressor gene (WT1). *Oncogene 13:* 447-453.

DRESSLER, G.R., DEUTSCH, U., CHOWDHURY, K., NORNES, H.O. and GRUSS. P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development 109:* 787-795.

DRESSLER, G.R., WILKINSON, J.E., ROTHENPIELER, U.W., PATTERSON, L.T., WILIAMS-SIMONS, L. and WESTPHAL, H. (1993). Deregulation of pax-2 expression in transgenic mice generates severe kidney abnormalities. *Nature 362:* 65-67.

EKBLOM, P. (1996). Extracellular matrix and cell adhesion molecules in nephrogenesis. *Exp Nephrol 4:* 92-96.

EKBLOM, P. and WELLER, A. (1991). Ontogeny of tubulointerstitial cells. *Kidney Int. 39:* 394-500.

EUGSTER, H., MULLER, M., KARRER, U., CAR, B.D., SCHNYDER, B., ENG, V.M., WOERLY, G., LE HIR, M., DI PADOVA, F., AGUET, M., ZINKERNAGEL, R., BLUETHMANN, H. and RYFFEL, B. (1996). Multiple immune abnormalities in tumor necrosis factor and lymphotoxin-a double-deficient mice. *Int. Immunol. 8:* 23-36.

GORDON, S. (1995). The Macrophage. *BioEssays 17:* 977-986.

HERZLINGER, D., KOSEKI, C., MIKAWA, T. and AL-AWQATI, Q. (1992). Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development 114:* 565-572.

JARVIS, J.N., DENG, L., BERRY, S.M., ROMERO, R., and MOORE, H. (1995). Fetal cytokine expression in utero detected by reverse transcription polymerase chain reaction. *Pediatr. Res. 37:* 450-454.

JASKOLL, T., BOYER, P.D. and MELNICK, M. (1994). TNF-a and embryonic lung morphogenesis. *Dev. Dynamics 201:* 137-150.

JING, S., WEN, D., YU, Y., HOLST, P.L., LUO, Y., FANG, M., TAMIR, R., ANTONIO, L., HU, R., LOIUS, J., HU, S., ALTROCK, B.W. and FOX, G.M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. *Cell 85:* 1113-1124.

KARAVANOVA, I., DOVE, L.F., RESAU, J.H. and PERANTONI, A.O. (1996). Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme. *Development 122:* 4159-4167.

KAWASE, E., YAMAMOTO, H., HASHIMOTO, K. and NAKATSUJI, N. (1994). Tumor Necrosis Factor-α (TNF-α) stimulates proliferation of mouse primordial germ cells in culture. *Dev. Biol. 161:* 91-95.

KOHCHI, C., NOGUCHI, K., TANABE, Y., MIZUNO, D., and SOMA, G. (1994). Constitutive expression of TNF-α and -β genes in mouse embryo: roles of cytokines as regulator and effector on development. *Int. J. Biochem. 26:* 111-119.

LEWIS, M., TARTAGLIA, L.A., LEE, A., BENNETT, G.A., RICE, G.C., WONG, G.H.W., CHEN, E.Y. and GOEDDEL, D.V. (1991). Cloning and expression of cDNA's for two distinct murine tumour necrosis factor receptors demonstrates one receptor is species specific. *Proc. Natl. Acad. Sci. USA 88:* 2830-2834.

LUO, G., HOFMANN, C., BRONCKERS, A.L.J.J., SOHOCKI, M., BRADLEY, A. and KARSENTY, G. (1995).BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev. 9:* 2808-2820.

MACICA, C.M., ESCALANTE, B.A., CONNERS, M.S. and FERRERI, N.R. (1994). TNF production by the medullary thick ascending limb of Henle's loop. *Kidney Int. 46:* 113-121.

MATZUK, M.M., LU, N., VOGEL, H., SELLHEYER, K., ROOP, D.R. and BRADLEY, A. (1995). Multiple defects and perinatal death in mice deficient in follistatin. *Nature 374:* 360-366.

MENDELSOHN, C., LOHES, D., DECIMO, S., LUFKIN, T., LEMEUR, M., CHAMBON, P. and MARK, M. (1994). Function of the retinoic acid receptors (RARs) during development (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development 120:* 2749-2771.

PHILLIPS, A.O., TOPLEY, N., STEADMAN, R., MORRISEY, K. and WILLIAMS, J.D. (1996). Induction of TGF-β1 synthesis in D-glucose primed human proximal tubular cells by IL-1β and TNFα. *Kidney Int. 50:* 1546-1554.

RITVOS, O., TUURI, T., ERAMAA, M., SAINIO, K., HILDEN, K., SAXEN, L. and GILBERT, S.F. (1995). Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mech. Dev. 50:* 229-245.

ROGERS, S.A., RYAN, G. and HAMMERMAN, M.R. (1991). Insulin-like Growth Factors I and II are produced in the metanephros and are required for growth and development *in vitro. J. Cell Biol. 113:* 1447-1453.

ROGERS, S.A., RYAN, G. and HAMMERMAN, M.R. (1992). Metanephric transforming growth factor-α is required for renal organogenesis *in vitro. Am. J Physiol. 262:* F533-F539.

ROGERS, S.A., RYAN, G., PURCHIO, A.F. and HAMMERMAN, M.R. (1993). Metanephric transforming growth factor-β1 regulates nephrogenesis *in vitro. Am. J Physiol. 264:* F996-F1002.

ROTHENPIELER, U.W. (1996). Roles of Pax genes in nephrogenesis. *Exp Nephrol 4:* 86-91.

ROTHENPIELER, U.W. and DRESSLER, G.R. (1993). Pax-2 is required for mesenchyme-to-epithelial conversion during kidney development. *Development 119:* 711-720.

SANCHEZ, M.P., SILOS-SANTIAGO, I., FRISEN, J., HE, B., LIRA, S.A. and BARBACID, M. (1996). Renal agenesis and the absence of enteric neurones in mice lacking GDNF. *Nature 382:* 70-73.

SARIOLA, H., HOLM, K. and HENKE-FAHLE, S (1988). Early innervation of the metanephric kidney. *Development 104:* 589-599.

SERRA, R., PELTON, R.W. and MOSES, H.L. (1994). TGFβ1 inhibits branching morphogenesis and N-myc expression in lung bud organ cultures. *Development 120:* 2153-2161.

SHARKEY, A.M., DELLOW, K., BLAYNEY, M., MACNAMEE, M., CHARNOCK-JONES, S. and SMITH, S.K. (1995). Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. *Biol. Reprod. 53:* 974-981.

SHULL, M.M., ORMSBY, I., KIER, A.B., PAWLOWSKI, S., DIEBOLD, R.J., YIN, M., ALLEN, R., SIDMAN, C., PROETZEL, G., CALVIN, D., ANNUNZIATA, N. and DOETSCHMAN, T. (1992). Targeted disruption of the mouse transforming growth factor-(1 gene results in multifocal inflammatory disease. *Nature 359:* 693-699.

STARK, K., VAINIO, S., VASSILEVA, G. and MCMAHON, A.P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature 372:* 679-683.

STEWART, C.L., KASPAR, P., BRUNET, L., BHATT, H., GADI, I., KONTGEN, F. and ABBONDANZO, S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature 359:* 76-78.

TORRES, M., GOMEZ PARDO, E., DRESSLER, G.R. and GRUSS, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development 121:* 4057-4065.

TOWERS, P.R., WOOLF, A.S. and HARDMAN P. (1998). GDNF stimulates ureteric bud outgrowth and enhances survival of ureteric bud cells *in vitro. Exp Nephrol* (In press).

VAN CAMPEN, H. (1994). Influenza A virus replication is inhibited by tumor necrosis factor-alpha *in vitro. Arch. Virol. 136:* 439-446.

VANDENABEELE, P., DECLERCQ, W., BEYAERT, R. and FIERS, W. (1995). Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol. 5:* 392-399.

VASSALLI, P. (1992). The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol. 10:* 411-452.

VEGA, Q.C., WORBY, C.A., LECHNER, M.S., DIXON, J.E. and DRESSLER, G.R. (1996). Glial cell line-derived neurotrophic factor actives the receptor tyrosine kinase RET and promotes kidney morphogenesis. *Proc. Natl. Acad. Sci. USA 93:* 10657-10661.

VEIS, D.J., SORENSON, C.M., SHUTTER, J.R. and KORSMEYER, S.J. (1994). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. *Cell 75:* 229-240.

WAAGE, A., BRANDTZAEG, P., HALSTENSEN, A., KIERULF, P. and ESPEVIK, T. (1989). The complex pattern of cytokines in serum from patients with meningococcal septic shock. *J. Exp. Med. 169:* 333-338.

WINYARD, P.J.D., NAUTA, J., LIRENMAN, D.S., HARDMAN, P., SAMS, V.R., RISDON, R.A. and WOOLF, A.S. (1996a). Deregulation of cell survival in cystic and dysplastic renal development. *Kidney Int. 49:* 135-146.

WINYARD, P.J.D., RISDON, R.A., SAMS, V.R., DRESSLER, G.R. and WOOLF, A.S. (1996b). The PAX2 transcription factor is expressed cystic and hyerproliferative dysplastic epithelia in human kidney malformations. *J. Clin. Invest. 98:* 451-459.

WOOLF, A.S. and CALE, C.M. (1997). Roles of growth factors in renal development. *Curr. Opin. Nephrol. Hypertens. 6:* 10-14.

WOOLF, A.S., KOLATSI-JOANNOU, M., HARDMAN, P., ANDERMARCHER, E., MOORBY, C., FINE, L., JAT, P.S., NOBLE, M.D. and GHERADI, E. (1995). Roles of hepatocyte growth factor/scatter factor and the Met receptor in the early development of the metanephros. *J. Cell Biol. 128:* 171-184.

WRIDE, M.A. and SANDERS, E.J. (1993). Expression of tumour necrosis factor-α (TNFα)-cross-reactive proteins during early chick embryo development. *Dev. Dynamics 198:* 225-239.

ZUCKER, K., LU, P., FULLER, L., ASTHANA, D., ESQUENAZI, V. and MILLER, J. (1994). Cloning and expression of the cDNA for canine tumor necrosis factor alpha in E. coli. *Lymphokine Cytokine. Res 13:* 191-196.

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