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Original Article

Characterization of monoclonal antibodies against tenascin-C: no apparent effect on kidney development *in vitro*

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ABSTRACT Tenascin-C is an extracellular matrix glycoprotein found in embryonic mesenchyme. The precise biological function of tenascin-C is unknown, but different parts of the molecule have effects on cell adhesion and other cellular activities. We studied the expression and role of tenascin-C in the embryonic mouse kidney. By Northern blots, no tenascin-C was detectable in uninduced mesenchyme from day 11 embryonic kidneys, but after 24 hours of *in vitro* culture both major splice variants of tenascin-C were detected. The larger variant was the predominant form. By *in situ* hybridization tenascin-C mRNA in 13-day old embryonic kidneys was detected in the mesenchyme surrounding newly formed epithelial structures. In 17-day old embryonic kidneys, tenascin-C mRNA was detected in mesenchyme around the forming epithelial structures in the cortex, and expression was also seen in mesenchyme surrounding the capsular epithelium of glomeruli. In newborn kidneys, expression had shifted to the medulla but was still confined to mesenchymal areas. We have characterized 6 new monoclonal antibodies against mouse tenascin-C, which all stain embryonic kidneys from different stages in a pattern consistent with earlier reports and with the mRNA data. The binding sites of the monoclonal antibodies on the tenascin-C molecule were mapped to discrete regions of tenascin-C. These six and five previously described antibodies against tenascin-C were tested in antibody perturbation experiments. Three of these have been shown by *in vitro* assays to perturb function of other cell types. Despite this, none of them inhibited development of mouse kidneys in organ culture, although they were tested at 1 mg/ml. It raises the possibility that tenascin-C is not crucial for kidney development. Alternatively, tenascin-C has more subtle functions which could not be identified with the assays used here.

KEY WORDS: *kidney, tenascin-C, development*

Introduction

The formation of organized epithelial sheets with polarized cells is an important event during organogenesis. There is good evidence that cell-cell adhesion mediated by cadherins (Gumbiner, 1992; Larue *et al.*, 1994) and adhesion of cells to basement membranes (Klein *et al.*, 1988) are crucial for the initiation of epithelial cell polarity. The embryonic kidney is a good system to study development of epithelial cells. Formation of basement membranes and development of the kidney can be perturbed by antibodies against the basement membrane protein laminin-1 (Klein *et al.*, 1988) and its receptors integrin $\alpha_6\beta_1$ and dystroglycan (Sorokin *et al.*, 1990; Durbeej *et al.*, 1995). All these molecules are produced by the epithelium. Interestingly, development can also be perturbed with antibodies which block binding of nidogen to laminin-1, although nidogen is a mesenchymal extracellular matrix protein (Ekblom *et al.*, 1994). Less is known about the physiological role of other mesenchymally produced glycoproteins. One protein expressed at high levels in embryos close to developing epithelial cells is tenascin-C (Chiquet-Ehrismann *et al.*, 1986; Aufderheide *et al.*, 1987).

The tenascin-C molecule forms a hexamer of six peptide chains. Each tenascin-C polypeptide chain is composed of an N-terminal domain where it can bind the other chains, a serial arrangement of many EGF-like repeats, varying numbers of fibronectin type III-repeats and a C-terminal fibrinogen-like knob (Spring *et al.*, 1989; Weller *et al.*, 1991). The precise biological function of tenascin-C is not known, but different parts of the molecule have been shown

Abbreviations used in this paper: mAbs, monoclonal antibodies; WT1, Wilms tumor gene; TNfn #, tenascin-fibronectin repeat type III domain #; PBS, phosphate buffered saline.

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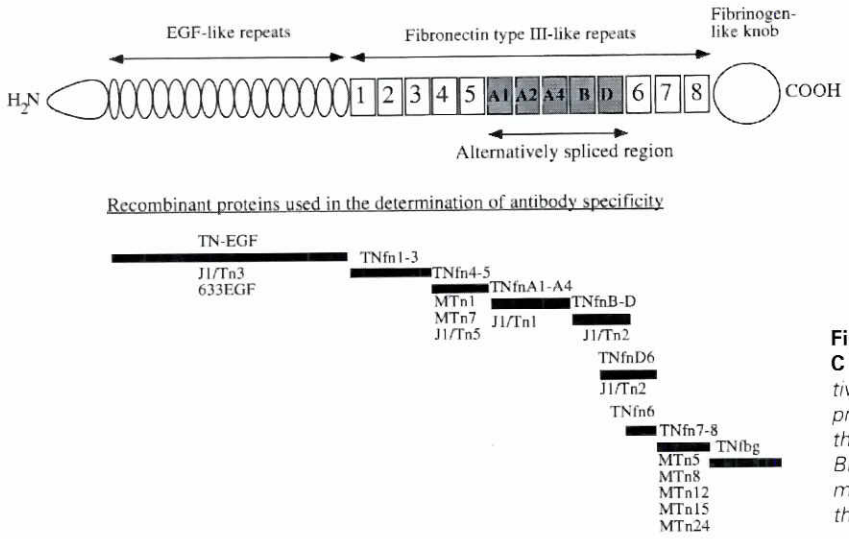

Fig. 1. Model of the domain structure of a mouse tenascin-C subunit. *The fibronectin-like repeats that undergo alternative splicing are indicated by gray shading. Recombinant proteins used in this study are indicated by black bars below the part of the tenascin-C molecule they correspond to. Binding regions defined by the ELISA assays for the used monoclonal antibodies against tenascin-C are indicated by their names below the recombinant proteins.*

to affect cell adhesion and other activities *in vitro* (Chiquet-Ehrismann *et al.*, 1988; Faissner and Kruse, 1990; Lochter *et al.*, 1991; Murphy-Ullrich *et al.*, 1991; Julian *et al.*, 1994). Although it contains adhesive sites the whole molecule appears to have both de-adhesive and anti-spreading effects on cells *in vitro*. Moreover, the alternatively spliced fibronectin type III-repeats and the last three fibronectin type III-repeats respectively have been shown to inhibit epithelial branching of embryonic lung in organ culture (Young *et al.*, 1994). Thus the possibility of multiple activities is supported by *in vitro* and cell culture experiments. Despite this, concerns about the functional role of tenascin-C have been raised by two reports showing that knock-out mice lacking tenascin-C develop normally (Saga *et al.*, 1992; Forsberg *et al.*, 1996). Since tenascin-C appears to have effects *in vitro*, whereas knock-out mice are phenotypically normal, it is possible that functionally related genes compensate for the loss of tenascin-C. In fact, at least two other tenascins have been reported (Matsumoto *et al.*, 1992; Norenberg *et al.*, 1992; Bristow *et al.*, 1993).

Although immunofluorescence studies have previously shown that tenascin-C is expressed selectively in some parts of the mesenchyme in embryonic kidneys (Aufderheide *et al.*, 1987), more detailed expression studies with other assays have not been performed. Moreover, the possible function of tenascin-C in the embryonic kidney has not been studied. Since it has been reported that antibodies against tenascin-C affect lung epithelial morphogenesis (Young *et al.*, 1994) we performed a more comprehensive study of the expression and role of tenascin-C during development of the mouse kidney. We have characterized the binding sites of several rat monoclonal antibodies (mAbs) against mouse tenascin-C, and have studied whether they can perturb kidney development in organ culture.

Results

Determination of immunoglobulin subtype and mapping of antibody binding sites of tenascin-C monoclonal antibodies

Ouchterlony immunodiffusion assays showed that MTn 1, MTn 7, MTn 8, MTn 12 and MTn 24 were of the IgG_1 subtype, MTn 15 was of the IgG_{2A} subtype, whereas MTn 5 was an IgM.

By ELISA assays using recombinant domains of tenascin-C (Fig. 1), the binding sites of all mAbs used in this study were mapped to discrete regions of tenascin-C (Fig. 2). MTn 1 bound to tenascin-C fibronectin repeats 4-5 (TNfn 4-5), a region to which mAb J1/Tn5 had previously been mapped. Monoclonal antibodies MTn 5, MTn 8, MTn 12, MTn 15 and MTn 24 bound to TNfn 7-8. For each antibody binding to other fragments of tenascin-C was low and comparable to binding to laminin-1, showing that the mAbs were specific. The mAb MTn 7 did not react strongly in ELISA, but a weak reaction was seen with TNfn 4-5. In western blotting against the different recombinant proteins MTn 7 was also found to react with TNfn 4-5 (not shown). Additional previously described antibodies (Götz *et al.*, 1996) also used in the perturbation studies were mAb J1/Tn2 which binds to TNfnD, mAb J1/Tn1 which binds to TNfn A1-A4 and mAbs J1/Tn3 and 633EGF which bind to TN-EGF (Fig. 1). The J1/Tn1 antibody is thus specific for the larger splice variant.

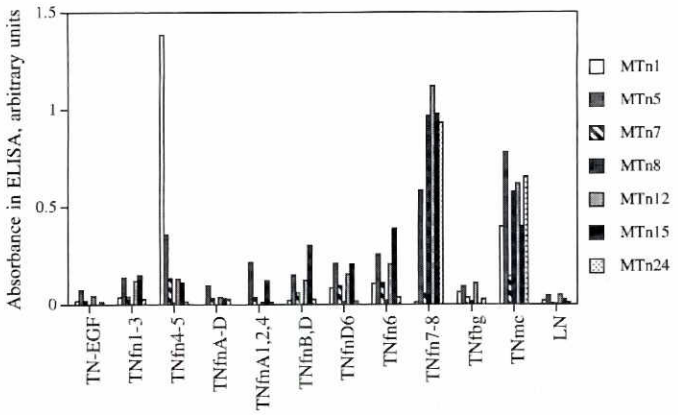

Fig. 2. Identification of binding sites of monoclonal antibodies against tenascin-C by ELISA. *The diagram shows the reactivity of the mAbs to different short recombinant proteins of the different parts of the tenascin-C molecule (see Fig. 1), to the complete tenascin-C polypeptide (TNmc) and to laminin-1.*

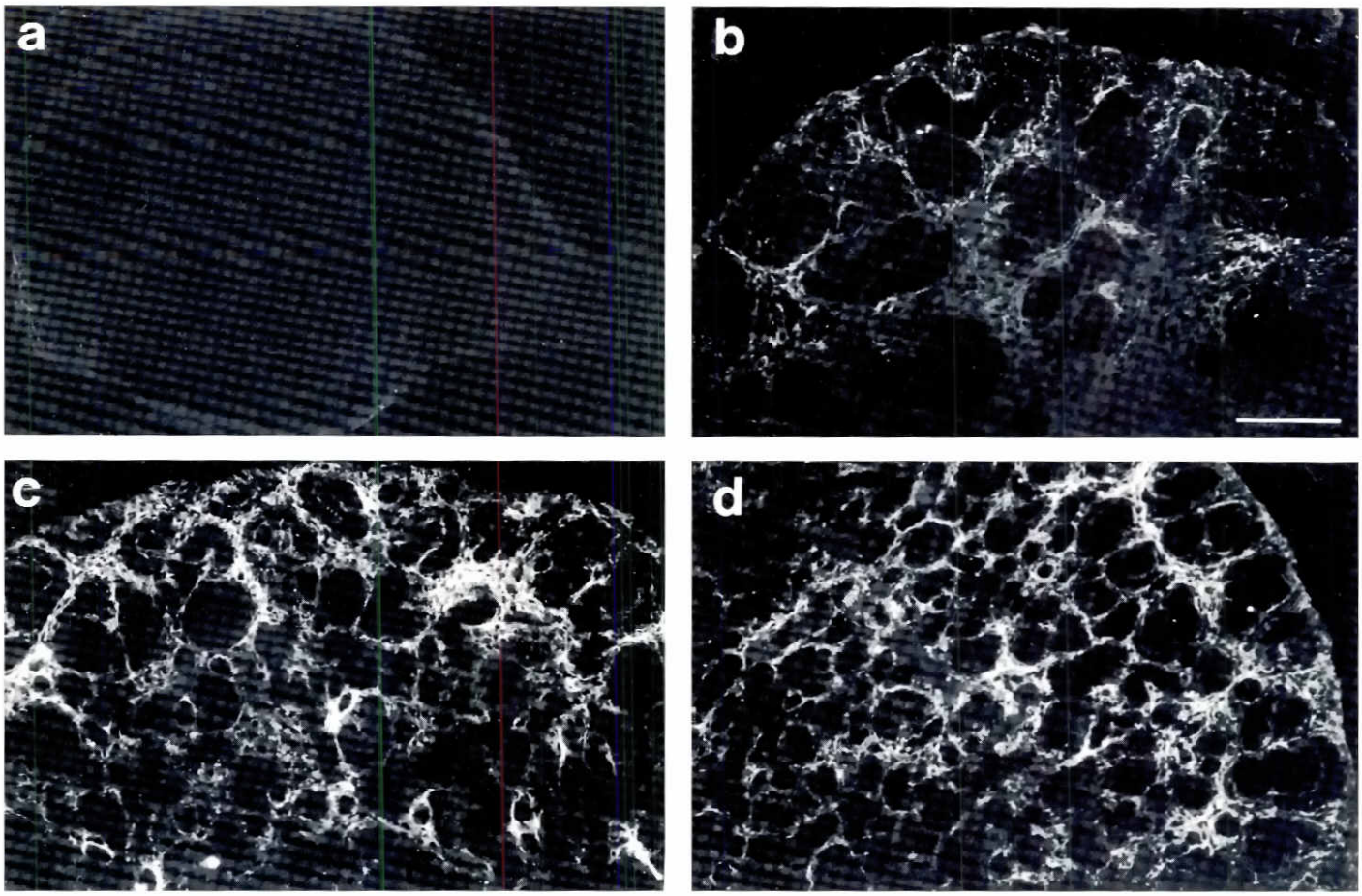

Fig. 3. Immunofluorescence staining of embryonic kidneys. *Kidneys from mouse embryonic day 12* **(a)**, *day 14* **(b)**, *and cortical parts of day 18* **(c)** *and newborn* **(d)** *kidneys stained with monoclonal antibody MTn 15. Note that only the cortical part of the kidneys of embryonic day 18 and newborn kidneys are shown. Bar, 100 μm.*

Staining characteristics of the tenascin-C monoclonal antibodies

Several cell types differentiate simultaneously in the developing kidney. Initially, metanephric kidney rudiments are composed of a small epithelial ureter bud surrounded by undifferentiated stem cells. Interactions between the epithelial ureter bud and mesenchyme initiate the development of the metanephric kidney. The mesenchyme stimulates the ureter to grow and branch and to differentiate into new epithelium. The ureter, in turn, induces a part of the mesenchyme to convert into new epithelium. The first sign of this conversion is the formation of mesenchymal condensates around the tips of the ureter. The condensates subsequently form comma-shaped bodies and then S-shaped bodies. The S-shaped epithelia elongate and join the ureter to form the mature nephron. The newly induced areas are in the most cortical parts of the developing kidney, while the more differentiated areas are found in the medullary part (Ekblom *et al.*, 1990).

In 12-day-old embryonic kidneys no tenascin-C staining could be detected with the mAbs (Fig. 3a), whereas strong expression was found in mesenchymal cells in kidneys of day 14 embryos (Fig. 3b). The staining intensity was even stronger in embryonic day 18 kidneys (Fig. 3c) or newborn stage (Fig. 3d). A gradient of staining intensity was noted in the older embryonic kidneys, with particularly strong staining in the cortical regions where the youngest stages of epithelial development are seen. Staining of embryonic kidneys and kidneys from newborn mice showed the same staining pattern with all mAbs described in this study, consistent with an earlier report which used cross-reacting polyclonal antibodies against chicken tenascin-C (Aufderheide *et al.*, 1987).

Tenascin-C during in vitro conversion of metanephric mesenchyme to epithelium

Tenascin-C mRNA expression during conversion of mesenchyme to epithelium was analyzed and compared to expression of fibronectin mRNA (Fig. 4). More than 200 transfilter-cultures from kidney mesenchyme were used to extract RNA at each time point of culture. In uninduced mesenchyme from 11-day-old embryonic kidneys no tenascin-C mRNA was detectable. At 24 h of culture mRNA for both splice variants of tenascin-C were detected, although the larger variant was more abundant (Fig. 4). Expression of tenascin-C mRNA gradually increased with time and the highest expression level was noted at the latest stage analyzed, at 120 h (Fig. 4).

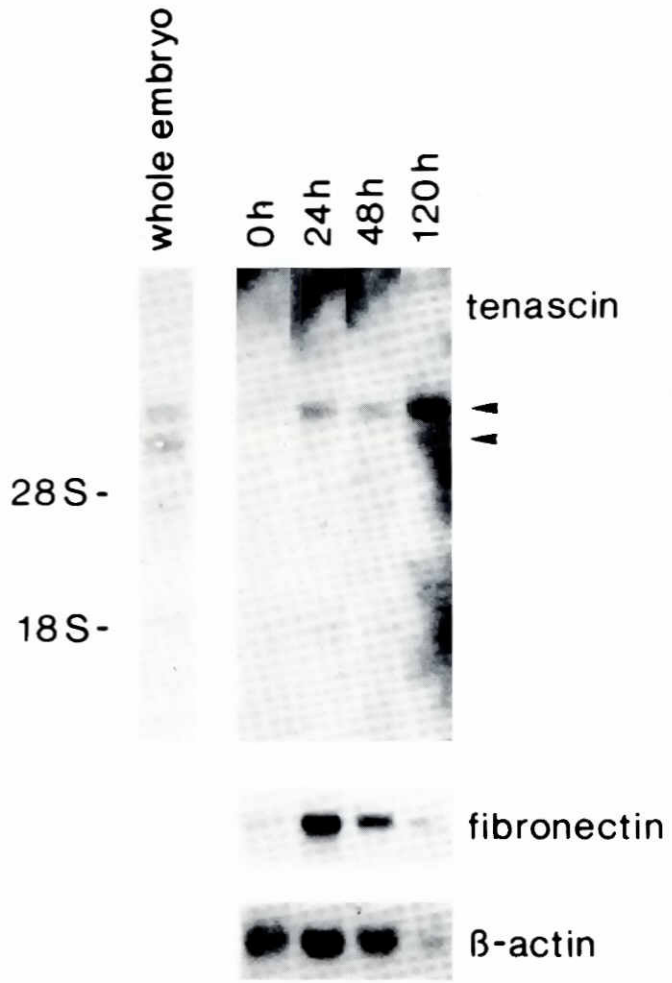

Fig. 4. Tenascin-C and fibronectin mRNA expression in kidney mesenchyme cultured transfilter with inducer tissue. *Ten μg of RNA was loaded into each lane and fractionated by agarose gel electrophoresis. RNA was transferred to Zeta-Probe GT membranes (Bio-Rad) and hybridized to ^{32}P-oligolabeled cDNA clones of tenascin-C, fibronectin and β-actin. The fibronectin signal was approximately 8.5 kb and the β-actin signal was 2.1 kb. For tenascin-C the position of 18s and 28s ribosomal RNA are marked. In the four lanes are the different times the mesenchyme was cultured in vitro.*

Localization of tenascin-C mRNA during in vivo development of kidney

To identify the cells producing tenascin-C mRNA and to elucidate the temporal and spatial distribution of the two splice variants expressed in kidney, we performed *in situ* hybridization. In 13-day-old embryonic kidneys both probes reacted with mRNA in the mesenchyme surrounding newly formed epithelial structures (Fig. 5a,b,c). In the light of the Northern blot results (Fig. 4), it seems likely that the signals were due to expression of the larger isoform. In the 17-day-old embryonic kidney cortical mesenchymal expression of tenascin-C mRNA was detected around the forming epithelial structures, and expression was also seen in mesenchyme surrounding the capsular epithelium of glomeruli. This expression pattern was detected with both probes (Fig. 5d,e,f). The newly induced areas are in the most cortical parts of the developing kidney, while the more differentiated areas are found in the medullary part (Ekblom *et al.*, 1990). In the newborn kidney, expression had shifted to medullary regions but was still confined to mesenchymal areas, showing strong reaction in areas surrounding the elongating straight tubules and also in mesenchyme surrounding the capsular epithelium of glomeruli (Fig. 5g,h,i). At this stage, a signal was still seen with both probes, but the probe for the larger splice variant showed a slightly more restricted pattern of expression (Fig. 5g) than the probe detecting both splice variants (Fig. 5h).

Antibody perturbation experiments of kidney development in vitro

Eleven mAbs against tenascin-C were tested in antibody perturbation experiments (Table 1). Initial experiments were carried out at 0.15 mg/ml but none of the antibodies seemed to perturb kidney development. All of the mAbs were therefore tested at 1 mg/ml in at least two independent experiments, each consisting of 5 to 10 individual embryonic kidneys. The concentration used is a fairly high concentration since mAbs against some basement membrane proteins and their receptors were found to inhibit kidney development at 0.1-0.2 mg/ml (Klein *et al.*, 1988; Sorokin *et al.*, 1990, 1992). However, none of the mAbs against tenascin-C reduced the number of tubules formed during *in vitro* development at 1 mg/ml (Table 1).

By stereo microscopy analysis, explants cultured in the presence of the anti-tenascin antibodies did not differ from control kidneys cultured only in medium or in the presence of control Ig of the same subtype (Fig. 6a,b), as shown here for mAb J1/tn 2 (Fig. 6c). Histological analysis of explants grown for three days showed an apparent normal development in both controls (Fig. 6b) and in explants treated with mAbs against tenascin-C (Fig. 6d).

During *in vitro* development of the kidney, the different segments can be detected by the appearance of specific molecular markers. Glomerular differentiation occurs fairly late and indicates advanced development (Bernstein *et al.*, 1981; Ekblom, 1981; Lehtonen *et al.*, 1983). The Wilms tumor gene (WT1) is at later developmental stages expressed only in glomerular podocytes (Pritchard-Jones *et al.*, 1990; Mundlos *et al.*, 1993; Larsson *et al.*, 1995) and we therefore analyzed the presence of podocytes by immunostaining for the WT1 gene product. Both in control explants and in explants treated with mAb MTn 24 against tenascin-C, well developed tubules expressing laminin α1 chain could be detected, and tubules were connected to laminin α1 chain-negative glomerular-like bodies with cells expressing the WT1 gene product in their nuclei (Fig. 7). Similar confocal microscopy images with apparently completely normal glomeruli and tubuli were obtained in cultures treated with 1 mg/ml of each of the 11 different antibodies against tenascin-C.

Discussion

Tenascin-C is expressed in several locations in the embryo, during wound healing, and in the stroma of many malignant tumors. The precise biological function of tenascin-C is not known, but different parts of the molecule have effects on cell adhesion and other cellular activities *in vitro* (Erickson, 1993). Expression of tenascin-C is prominent in embryonic mesenchyme close to actively growing epithelium. With the use of a number of monoclonal

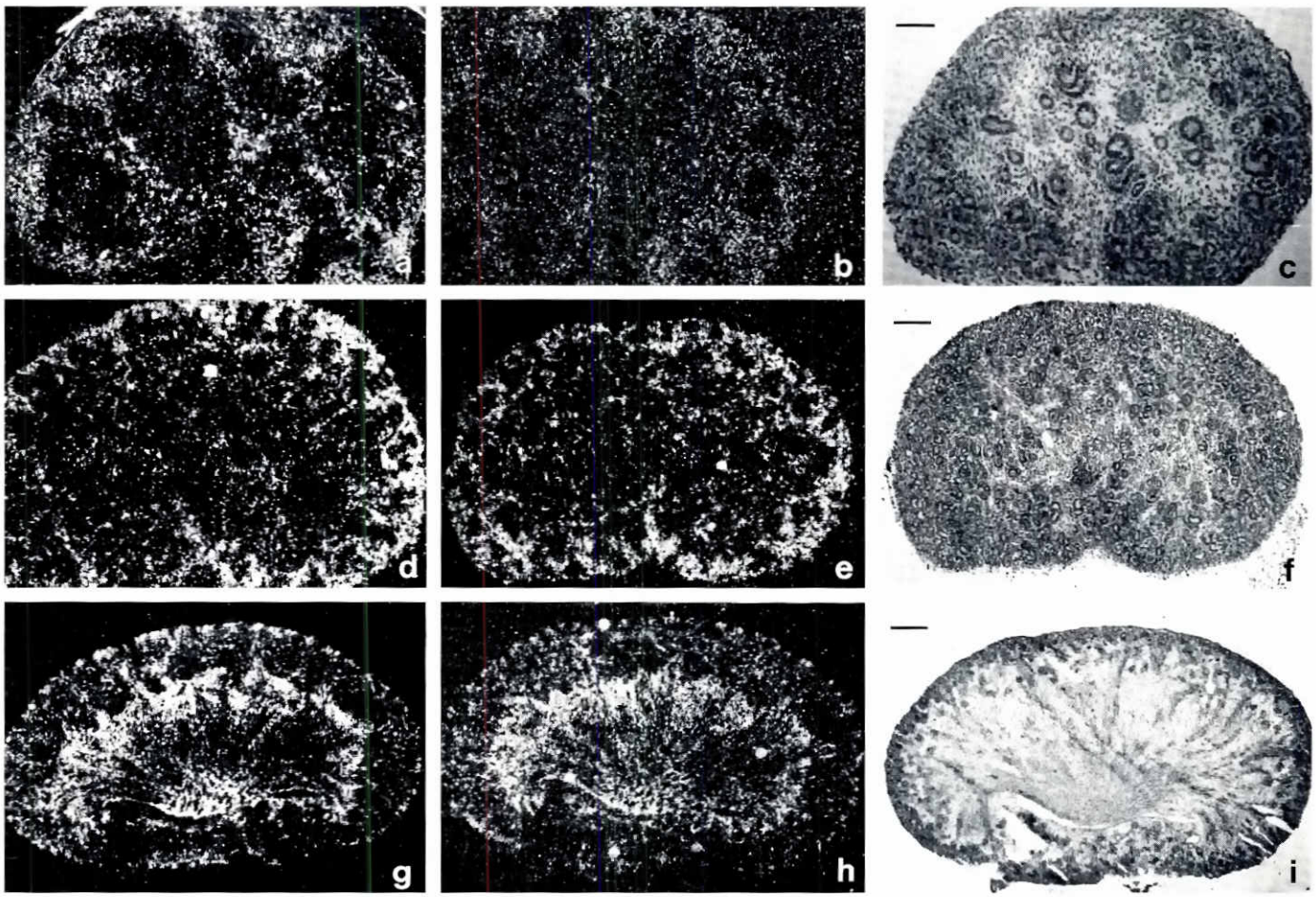

Fig. 5. *In situ* hybridization demonstrating tenascin-C mRNA expression in three developmental stages of mouse kidneys. *Serial sections of 13-day-old embryonic kidney* **(a,b and c)**, *17-day-old embryonic kidney* **(d,e and f)** *and newborn kidney* **(g,h and i)** *were used. Sections depicted in* **(a,d and g)** *are hybridized with a probe reacting with TNfn A2 and thus only with the large splice variant. Sections depicted in* **(b,e and h)** *are hybridized with a probe reacting with TNfn8 and thus reacting with both splice variants. Sections depicted in* **(c,f and i)** *are the same as those in* **(b,e and h)** *shown in bright field illumination. The sections are stained with Ehrlich-Hematoxylin. Bar: 125 μm in 13-day sections, 250 μm in 17-day sections, and 500 μm in sections from newborn kidneys.*

antibodies and mRNA analyses we confirm and extend previous reports showing a restricted expression of tenascin-C in selected parts of the mesenchyme and stroma of embryonic kidney and absence from the stem cells (Aufderheide *et al.*, 1987; Prieto *et al.*, 1990). We also tested whether different rat mAbs against mouse tenascin-C can perturb kidney development in organ culture. Of the eleven mAbs tested that recognize tenascin-C, three have been shown to have effects *in vitro* in neurite extension- and granule cell migration-assays (Husmann *et al.*, 1992) and one of the new mAbs described here (MTn 15) also affects hematopoietic cells grown *in vitro* (Talts *et al.*, 1997). Despite this, we did not detect any effects of these antibodies on mouse embryonic kidney development in the assays used here.

Several regions of the tenascin-C molecule have been implicated in binding both extracellular matrix molecules as well as cell surface proteins. A tenascin-fibronectin domain (TNfn 5) and the fibrinogen-like knob have been shown to bind heparin (Aukhil *et al.*, 1993; Weber *et al.*, 1995) and potentially could bind cell-surface proteoglycans such as syndecan (Salmivirta *et al.*, 1991). TNfn 3 has been shown to bind $\alpha_9\beta_1$ integrin (Yokasaki *et al.*, 1994). TNfn

TABLE 1

DEVELOPMENT OF CONDENSATES IN ANTIBODY-TREATED KIDNEY EXPLANTS

Antibody (1 mg/ml)	Number of explants	Number of condensates (% of control)
–	58	100
Anti-Rat IgG	59	102
MTn1	19	82
MTn5	26	104
MTn7	22	84
MTn8	35	101
MTn15	16	100
MTn24	25	104
J1/Tn1	14	86
J1/Tn2	21	77
J1/Tn3	20	87
J1/Tn5	20	84
633EGF	20	97

No differences in the number of condensates formed in the different antibody treatments could be found. Standard deviations varied between 24 and 35%.

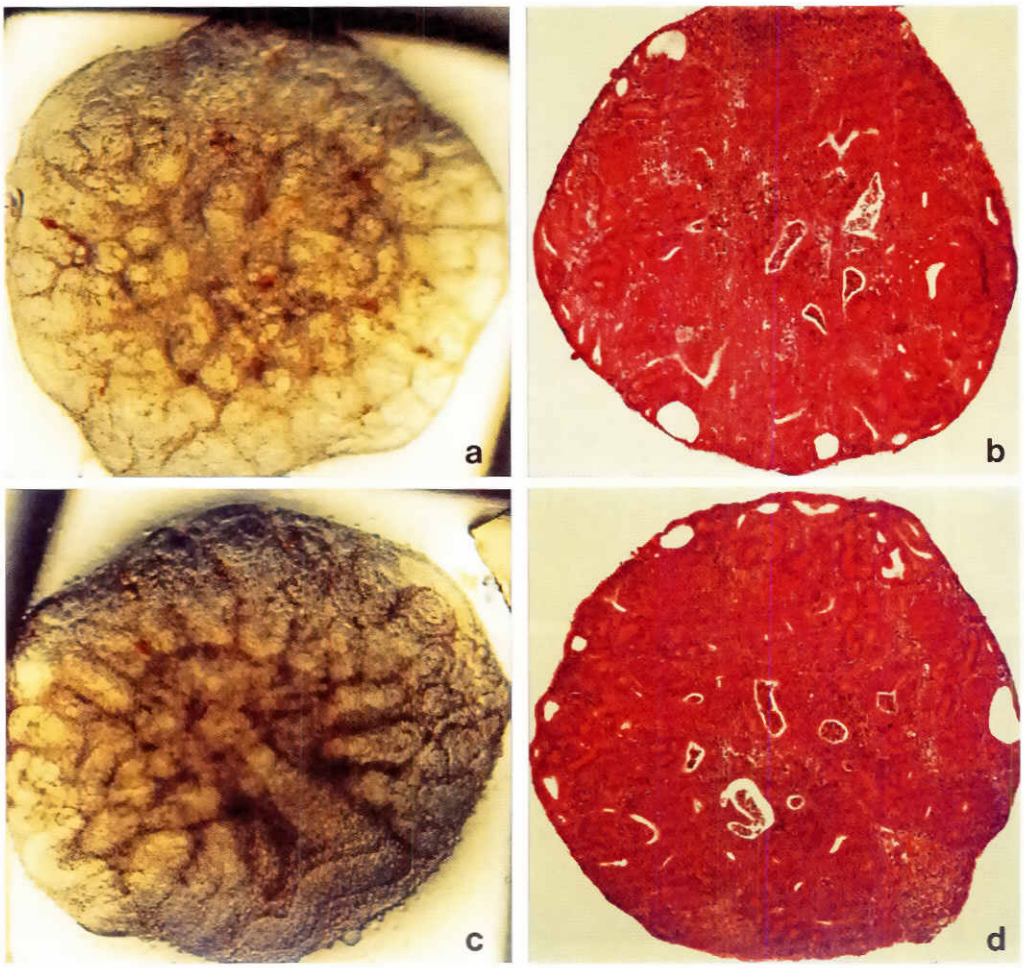

Fig. 6. Antibody perturbation experiment of kidney development. *Shown here are kidneys cultured in the presence of 1 mg/ml rat anti-IgG* **(a and b)** *and 1 mg/ml monoclonal antibody J1/Tn2* **(c and d)**, *an antibody known to affect neuronal cells in culture. The kidneys in* **(a and c)** *were photographed live by stereo microscopy on day 3 of culture. They were then fixed in Bouin's solution, processed for histology and stained with Ehrlich-Hematoxylin* **(b and d)**. *Note well developed tubules both in control cultures and in cultures treated with the antibody J1/Tn2.*

8 has been proposed to contain a cell binding site (Chiquet-Ehrismann *et al.*, 1988; Spring *et al.*, 1989) which interferes with cell binding to fibronectin.

Some *in vitro* functional assays have demonstrated that tenascin-C affects cells. For example, the differentially spliced segment (TNfn A1, A2, A4, B, D) has been demonstrated to down regulate focal adhesion sites (Murphy-Ullrich *et al.*, 1991) and to have a negative effect on uterine epithelial cell adhesion (Julian *et al.*, 1994). Epitopes on TNfn 3-5 and TNfn A1-A4 have been shown to have a positive effect on granule cell migration (Husmann *et al.*, 1992). An epitope on TNfn D has been shown to have a positive effect on neurite outgrowth (Husmann *et al.*, 1992). More recently neurite outgrowth promotion has been shown for TNfn D,6 and TNfn D (Götz *et al.*, 1996). Exogenously added tenascin-C affects mammary epithelial cell development (Wirl *et al.*, 1995) and was shown to down regulate β-casein synthesis. This effect could be ascribed to TNfn 1-2 and the differentially spliced segment (Jones *et al.*, 1995). Tenascin-C promoted sprouting of endothelial cells (Canfield and Schor, 1995) and this could be inhibited with antibodies against tenascin-C. Several other *in vitro* assays have shown that cells can respond to tenascin-C. Nonetheless, homozygous mice with a null mutation for *tenascin-C* gene develop normally and do not display any obvious phenotype (Saga *et al.*, 1992). It has been suggested that the normal phenotype in the knock-out mice described by Saga *et al.* (1992) is due to the presence of a shorter form of tenascin-C (Mitrovic and Schachner, 1995) but this seems unlikely, since independently produced *tenascin-C -/-* knock-out mice with no tenascin-C protein also develop normally and do not display any obvious phenotype (Forsberg *et al.*, 1996).

Of the eleven antibodies tested in functional assays, six rat mAbs against mouse tenascin-C were characterized in the current study. By immunofluorescence microscopy all of them stain embryonic kidneys from different stages in a pattern consistent with an earlier report using polyclonal antibodies (Aufderheide *et al.*, 1987). In 12-day-old embryonic kidneys, no tenascin-C staining could be detected, whereas in day 14 embryonic kidneys strong expression was found in mesenchymal cells which had differentiated into stromal cells. The staining intensity was higher in embryonic day 18 kidneys. At all stages, staining remained restricted to cortical regions where the youngest stages of epithelial development are seen. In the newborn kidney only some medullary staining could be observed. These results support our previous findings of lack or low amounts of tenascin-C in non-induced mesenchymal stem cells (Aufderheide *et al.*, 1987), although tenascin-C was reported to be present in the blastemal stem cells in human embryonic kidneys (Natali *et al.*, 1991; Klingel *et al.*, 1993; Daïkha-Dahmane *et al.*, 1995). In the current study the lack of tenascin-C in mouse stem cells could be verified at the mRNA

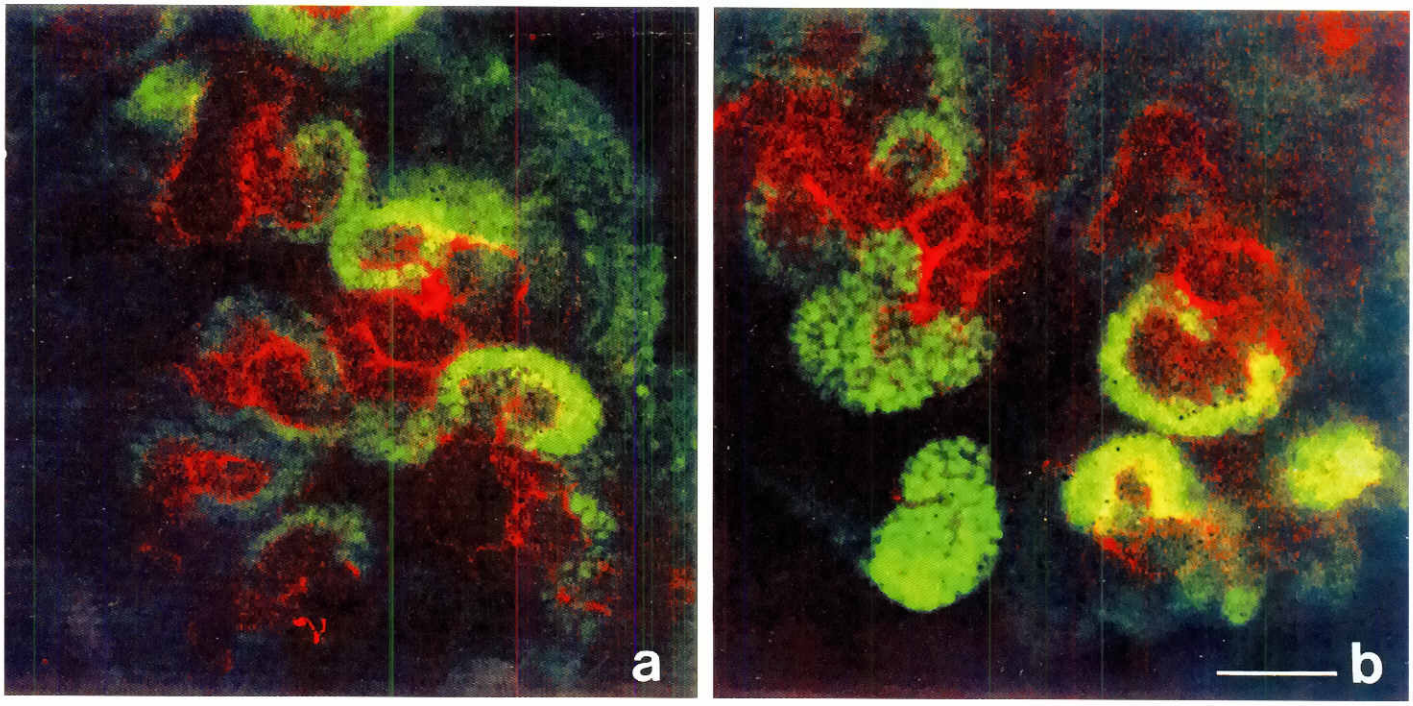

Fig. 7. Expression of segmentation markers demonstrated by confocal microscopy. *Confocal images were obtained of 12-day-old embryonic kidneys cultured for 4 days in the presence of 1 mg/ml rat anti-IgG* **(a)** *or 1 mg/ml monoclonal antibody MTn 24* **(b)**. *Whole kidneys from organ cultures were stained with monoclonal antibody 200 against laminin α1 chain (red) and antibodies against WT1 gene product (green) and photographed with a confocal microscope. Laminin α1 staining (in red) shows basement membranes in forming tubuli, and WT-1 staining (in green) shows podocyte nuclei in more mature glomeruli. Bar, 50 μm.*

level. In Northern blots of isolated stem cells we could detect very little, if any, tenascin-C message. The apparent discrepancy between the findings in mouse and human embryonic kidneys might be due to the different stages analyzed. We also find that tenascin-C mRNA and protein are expressed in many parts of the uppermost cortex at later developmental stages. At this stage, it is difficult to distinguish between stem cells and stromal cells immediately adjacent to them. The location of stem cells at the later stages is in fact unclear and much debated. In the studies of human embryonic kidneys the blastemal stem cells were not specifically isolated from the earliest embryonic stages (Daïkha-Dahmane *et al.*, 1995) as was done in the current study, probably because the earliest stages of human kidney development can be difficult to detect. The data in mouse and human embryonic kidneys are thus not necessarily contradictory.

The isotypes of the new mAbs were determined and their binding sites were mapped to discrete regions of tenascin-C. Together with other already characterized mAbs against tenascin-C also used in this study (Aufderheide and Ekblom, 1988; Götz *et al.*, 1996) they cover most regions of tenascin-C. Eleven mAbs against tenascin-C were tested in antibody perturbation experiments. We did not observe any perturbation of kidney development with any of the mAbs. The number of tubules appeared to be normal, and proper segmentation into tubules and glomeruli occurred during *in vitro* development in the presence of high concentrations of antibodies against tenascin-C. Normal segmentation into the different parts of the nephron could be reliably demonstrated by confocal microscopy and double immunofluorescence for a marker of tubular basement membranes (Ekblom *et al.*, 1990; Sorokin *et al.*, 1992) and for a nuclear protein expressed selectively in glomerular podocytes (Pritchard-Jones *et al.*, 1990; Mundlos *et al.*, 1993; Larsson *et al.*, 1995).

Our apparent failure to perturb kidney development with fairly high concentrations of antibodies against tenascin-C can be contrasted with previous studies using antibodies to different domains of laminin-1 and its receptors. Certain antibodies against laminin-1 or its receptors profoundly affected kidney development *in vitro* (Sorokin *et al.*, 1990, 1992; Ekblom *et al.*, 1994; Durbeej *et al.*, 1995). Taken together these studies indicate a role for laminin-1 in kidney development, but the role of tenascin-C in kidney development remains enigmatic. One possibility is that tenascin-C is not important for kidney development, in agreement with the knock-out results (Saga *et al.*, 1992; Forsberg *et al.*, 1996). Another possibility is that our antibodies do not bind to domains of tenascin-C important for kidney development although some of the antibodies used here clearly affect other cell types. It has been shown that a polyclonal antiserum against tenascin-C could perturb lung epithelial morphogenesis (Young *et al.*, 1994), and this serum might have reacted with many important epitopes. It is also possible that several epitopes together are important for tenascin-C function and therefore a polyclonal serum may be more effective in perturbing function. This serum was not tested here. Finally, it is possible that the assays we used here were too crude to detect more subtle effects of the antibodies.

Although we could not provide any evidence for a role of tenascin-C it could also be argued that tenascin-C dependent developmental processes occur *in vivo* but not in our organ cultures. Tenascin-C has been implicated in endothelial sprouting (Canfield and Schor, 1995)

and in angiogenesis in astrocytomas (Zagzag *et al.*, 1995). A remote possibility is thus that tenascin-C may be involved in the vascularization of kidneys rather than in epithelial morphogenesis. Kidney glomeruli which form in the *in vitro* kidney cultures are lacking proper endothelial cells (Bernstein *et al.*, 1981; Ekblom, 1981) and vascularization is not essential for the *in vitro* kidney cultures. Nutrients in the medium pass freely into all parts of the explants. Further studies are thus necessary before we can completely rule out that tenascin-C has a role in the developing kidney or in other epithelial-mesenchymal interactions.

Materials and Methods

Hybridomas

Generation of the hybridomas producing rat mAbs against mouse tenascin-C (MTn 12, MTn 5) has been previously described (Aufderheide and Ekblom, 1988). The hybridomas used in the current study were produced earlier but the obtained mAbs (MTn 1, MTn 5, MTn 7, MTn 8, MTn 15, MTn 24) were not characterized in detail (Aufderheide, 1988).

Purification of monoclonal antibodies

Propagation of cells was in DMEM containing 10% FBS and production of supernatants for mAb production was achieved either in DMEM (Gibco Life Technologies AB, Stockholm, Sweden) containing 1% MCA-1 (Costar, Cambridge, MA, USA) or in Serum Free Media (Gibco). Culture supernatant was diluted 1:4 with 25 mM 2(N-Morpholino)etansulfonic acid, pH 4.0 and applied to a Bakerbond ABx column (J.T. Baker Inc., Phillipsburg, NJ, USA) connected to a Fast Protein Liquid Chromatography system (Pharmacia, Uppsala, Sweden) as previously described (Aufderheide and Ekblom, 1988). The column was then washed with 8 volumes of 10 mM 2(N-Morpholino)etansulfonic acid, pH 5.6, and eluted with step gradients at varying percentages of 1 M Na-acetate, pH 7.0 (buffer B), over 500 ml. Antibodies eluted between 20 and 40% buffer B. They were then dialyzed against IMEM, concentrated by ultrafiltration (Centricon, Millipore Co., Bedford, MA, USA) and sterile filtered. Their purity was checked by SDS-PAGE using Phastgels 10-15% (Pharmacia) and the Pharmacia Phast system. Immunoglobulin subtypes were identified using a rat monoclonal typing kit (ICN Biomedical, Eschwege, Germany).

Recombinant proteins and mapping of antibody binding sites on tenascin-C

The mAbs J1/tn1, J1/tn2, J1/tn3, J1/tn5 and 633EGF have been shown to bind to specific regions on tenascin-C (Fig. 1), but nothing was known regarding the binding sites for the other mAbs used in this study. Therefore, recombinant tenascin-C segments were produced in a bacterial expression system. Tenascin-C segments were expressed as fusion proteins from cDNA clones and were purified on nickel-chelate columns (Weller *et al.*, 1991; Götz *et al.*, 1996). Ten mouse tenascin-C segments were used for the present study (Fig. 1).

ELISA

Microplates were coated overnight with 50 µl/well of solutions containing 20 µg/ml of the different tenascin-C recombinant proteins in phosphate buffered saline (PBS), intact tenascin-C purified from mouse brain (TnMc) (Faissner and Kruse, 1990), or laminin-1 (Boehringer Mannheim, gmbH, Mannheim, Germany) at 20 µg/ml in PBS. After coating, plates were washed five times with PBS and blocked for 1 h at room temperature with 2 mg/ml bovine serum albumin, washed twice with PBS, and incubated for 1 h at 37°C with the following antibodies: MTn 1, MTn 5, MTn 7, MTn 8, MTn 12, MTn 15 and MTn 24. After three washes with PBS, 0.05% Tween 20 (PBS-Tween), plates were incubated for 1 h with anti-rat immunoglobulin coupled to peroxidase, washed three times in PBS-Tween and developed using 2,2´-azino-di-(3-ethylbenzthiazoline sulfonate). The reaction was stopped by addition of SDS to a final concentration of 0.2%, and the absorbance read at 405 nm using an ELISA reader (Titertek Multiskan, Flow laboratories, Meckenheim, Germany).

Tissues and organ culture

NMRI mouse embryos were used. Twelve-day-old embryonic kidneys were isolated for whole organ culture and cultured in I-MEM (Gibco) (Grobstein, 1955) supplemented with 1% L-glutamine and 10% fetal bovine serum.

Transfilter cultures were prepared using 11-day-old metanephric mesenchyme which was microsurgically separated from the ureter bud and cocultured with a heterologous inducer, the spinal cord (Grobstein, 1956; Saxén *et al.*, 1968). We used I-MEM medium (Gibco) supplemented with transferrin and 10% fetal bovine serum (Klein *et al.*, 1988). For Northern blots, freshly isolated mesenchymes and mesenchyme induced for 24 h, 48 h and 120 h *in vitro* were analyzed. The inducer spinal cord was removed from the filter and discarded.

Whole organ cultures were incubated in the presence of varying amounts of concentrated and purified antibodies. Control incubations were performed with a rat IgG antibody at the same concentrations as the experiments or no antibody at all. In control cultures both the ureter epithelium and the epithelium derived from mesenchyme will form within a two-day period (Grobstein, 1956). At 72 h of culture the explants were examined under stereomicroscope, photographed, and the number of condensates were counted by two persons independently without prior knowledge of the experiment code. Samples were collected and frozen for immunocytochemistry or processed for histological examination. Tissues for histological examination were fixed in Bouin solution, embedded in paraffin and 5 µm sections were stained with Ehrlich-Hematoxylin.

The purified mAbs against tenascin-C used in the antibody perturbation assays include MTn 1, MTn 5, MTn 7, MTn 8, MTn 15, MTn 24 characterized in this study, and the previously described antibodies J1/tn1, J1/tn2, J1/tn3, J1/tn5, and 633EGF (Götz *et al.*, 1996). Antibody concentration was measured after the final dialysis against I-MEM.

Immunofluorescence

For immunofluorescence staining, frozen sections (5 µm) were fixed in -20°C methanol and blocked in 1% bovine serum albumin in PBS. Anti-mouse tenascin-C mAbs used in antibody perturbation assays were used in immunofluorescence diluted 1:250. Secondary antibody used in immunofluorescence staining procedures was fluorescein (DTAF)-conjugated affinipure goat anti-rat diluted 1:50 (Jackson Immunoresearch Laboratories, Inc, West Grove, PA, USA). For confocal microscopy immunofluorescence, the primary antibodies were rabbit anti-Wilms' tumor (WT) antibody C-19 (Larsson *et al.*, 1995) (Santa Cruz Biotech., Santa Cruz, CA, USA) diluted 1:100, and undiluted supernatant of monoclonal antibody 200 against laminin α1 chain (Sorokin *et al.*, 1992). Goat anti-rabbit antibodies conjugated to FITC (diluted 1:500) were used to detect the anti-WT antibodies. As the kidneys had been incubated with rat antibodies to perturb development, the rat anti-laminin antibody was initially incubated overnight at 4°C with Fab fragments of goat anti-rat conjugated to lissamine rhodamine (Jackson ImmunoResearch). Excess Fab fragments were separated from the rat IgG/anti-rat Fab fragment complex by passing over a Sephadex G-100 column (Pharmacia). Kidneys were fixed in methanol (-20°C) for 20 min, rinsed in PBS and incubated in blocking buffer (2% bovine serum albumin/PBS) overnight. The anti-WT antibody was added to the kidneys and incubated overnight at 4°C. Kidneys were then extensively washed with 0.05% Tween 20/PBS for 6 h at room temperature before a further overnight incubation at 4°C with a mixture of the goat anti-rabbit and the coupled anti-laminin antibodies. After extensive washing as above, the kidneys were sandwiched between slides containing a spacer of 4 layers of parafilm (to preserve the morphology) and mounted in Vectashield (Vector Lab., Burlingame, CA, USA).

Confocal microscopy

Kidneys were analyzed in a confocal microscope. Images from the confocal laser microscope were captured by epifluorescence microscopy

using the MultiProbe 2001 (Molecular Dynamics, Sunnyvale, CA, USA) consisting of an inverted Nikon microscope, a Nikon 60X 1.40 oil immersion lens, and an Argon/Krypton laser. The excitation and barrier filters used for fluorescein visualization had wavelengths of 488 nm and 500-560 nm, respectively. For visualizing lissamine rhodamine, the filter wavelengths were 510 nm for excitation and 590 nm and up for the barrier filter. Image reconstruction was done using the Molecular Dynamics software.

Northern blotting

Tissues were frozen in liquid nitrogen and total RNA was isolated (Chirgwin *et al.*, 1979). Ten µg total RNA was denatured with glyoxal and analyzed by electrophoresis on a 1% agarose gel. After transfer to Zeta-Probe GT membranes (Bio-Rad, Solna, Sweden), RNA was fixed by UV cross-linking with a stratalinker (Stratagene, AH Diagnostics AB, Skarholmen, Sweden) under conditions recommended by the manufacturer. RNA was hybridized to ^{32}P-labeled cDNA clones M20/1 (Weller, 1990; Weller *et al.*, 1991), M20/2 (Talts *et al.*, 1995), β-actin (Minty *et al.*, 1983) in a 0.25 M sodium-phosphate solution, pH=7.2, containing 7% SDS at 65°C. The filters were subsequently washed in 0.02 M sodium-phosphate solutions containing 5% and 1% SDS for 2 + 2x1 h before auto radiography.

In situ hybridization

In situ hybridization was performed with synthetic oligonucleotides as described (Durbeej *et al.*, 1993). To detect tenascin-C mRNA for both splice variants we used a synthetic 48-mer oligonucleotide probe 5´- GGA ATC GAG GTC TGT GGT AAA CTT GGT GGC GAT GGT AGA GCT CTT CTG -3´, complementary to nucleotides 5200-5247 of mouse tenascin-C (Weller *et al.*, 1991) corresponding to TNfn8. To detect tenascin-C mRNA for the larger splice variant we used a synthetic 45-mer oligonucleotide probe 5´- GGA GTT GTC CCT GTG GAG GTC TCG GCA GAG AGC ACT GGT GTT CTA -3´, complementary to nucleotides 3579-3623 of mouse tenascin-C (Weller *et al.*, 1991) corresponding to TNfnA2. The oligonucleotides were selected with the oligo™4.0 software (National Biosciences Inc., Plymouth, MN, USA). Control sections hybridized with the same amount of labeled probe plus unlabeled probe in excess did not show any specific binding.

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