

# Technical Advance

## Identification of a Glioblastoma-Associated Tenascin-C Isoform by a High Affinity Recombinant Antibody

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**Tenascin-C exists in several polymorphic isoforms due to alternative splicing of nine fibronectin-like type III repeats. Large Tenascin-C isoforms are present in almost all normal adult tissues but are upregulated in fetal, regenerating, and neoplastic tissues. Here, we report a human antibody fragment, TN11, derived from a phage library with high affinity for the spliced repeat C and demonstrate that this repeat is undetectable in normal adult tissues, barely detectable or undetectable in breast, lung and gastric carcinomas, meningioma, and low grade astrocytoma, but extremely abundant in high grade astrocytoma (grade III and glioblastoma), especially around vascular structures and proliferating cells. The antibody appears to have potential for development of a therapeutic agent for patients with high grade astrocytoma. (Am J Pathol 1999, 154:1345–1352)**

During tumor progression, the extracellular matrix (EM) of the tissues in which a tumor grows is remodeled through proteolytic degradation and through neosynthesis of new EM components by both neoplastic cells and stromal cells. The EM generated by these processes differs from that found in normal tissues and seems to provide an environment that is more conducive for tumor progression (inductive and/or instructive), of which angiogenesis is a crucial step.<sup>1–4</sup> The tumoral EM contains several tumor-associated antigens that are generally more abundant and possibly more stable than those of the cell

surface.<sup>5–7</sup> Consequently, these antigens represent valuable targets for tumor imaging and therapy.<sup>8–11</sup> Some of these tumor-associated EM molecules are isoforms of proteins with a wide distribution in normal adult tissues, such as fibronectin and tenascin, which are generated by deregulation of the mechanisms of alternative splicing of their primary transcripts.

Tenascin-C (TN-C) is a glycoprotein composed of six similar subunits joined at their NH<sub>2</sub> terminus by disulphide bonds. Each human TN-C subunit includes three types of structural modules: 14.5 epidermal growth factor-like repeats, 17 type III homology repeats, and a COOH-terminal knob made up of a sequence with homology to the globular domain of the  $\beta$  and  $\gamma$  chains of human fibrinogen.<sup>12–15</sup> TN-C is coded for by a single gene and its expression is regulated by a single promoter.<sup>16</sup> Structurally and functionally different human TN-C isoforms are generated by the alternative splicing of the TN-C transcript, nine type III repeats being included or omitted in the mRNA.<sup>17–20</sup> We have previously demonstrated that in neoplastic tissues the alternative splicing of the TN-C pre-mRNA is deregulated and is cell cycle-dependent.<sup>21–23</sup> In order to obtain highly specific human antibodies to tumor-associated TN-C isoform we have attempted to use phage antibody libraries.<sup>24,25</sup>

### Materials and Methods

#### Cell lines, TN-C Purification, Monoclonal Antibodies, and TN-C Recombinant Fragments

SK-MEL-28 human melanoma and GM6114 normal human cell lines were purchased from American Type Cul-

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Supported in part by funds of the Associazione Italiana per la Ricerca sul Cancro (AIRC), by European Union BIOTEC-2 project "Novel Markers of Angiogenesis," by the ETH (Zurich, Switzerland), and by the Stiftung zur Krebsbekämpfung.

Accepted for publication February 3, 1999.

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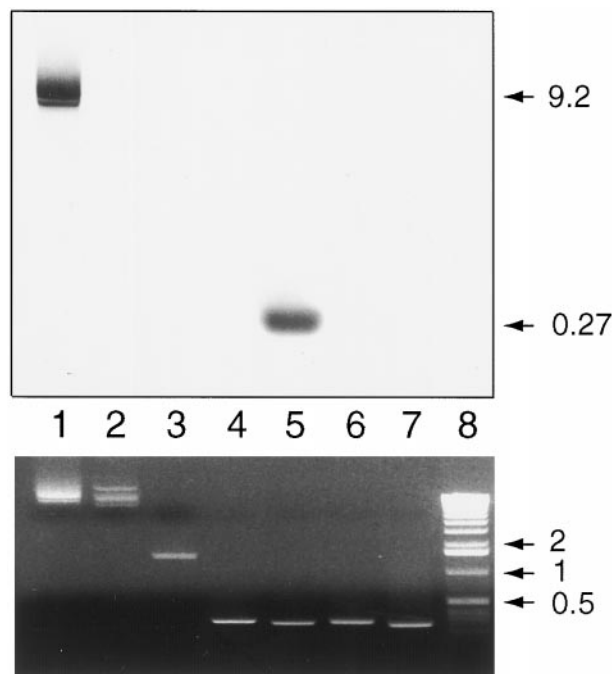
ture Collection (ATCC, Manassas, VA). BHK cells transfected with two cDNA constructs in pNUT expression vector and producing the large and the small TN-C splice variants (TN Large and TN Small) were a gift of Dr. H. P. Erickson.<sup>26</sup> TN-C was purified from the various conditioned media as previously reported.<sup>27</sup> The mAb specific for proliferating cells, KI-67, was purchased from Dako (Carpinteria, CA). The recombinant TN A-D, B-D, C and B fragments, and fusion proteins  $\lambda$ TN27 and  $\lambda$ TNBC were prepared as reported by Balza et al.<sup>27</sup> SDS-PAGE and immunoblotting were carried out as previously reported.<sup>28</sup>

### Antibody Fragment Isolation and scFv Purification

A human scFv phage library<sup>25</sup> and TN Large, as antigen, were used for the selection of recombinant antibodies. The selection was performed as previously reported.<sup>8</sup> Enzyme-linked immunosorbent assay (ELISA) screening of bacterial supernatants using TN large and TN small as antigens allowed the identification of the TN large-specific clone TN11, which was then selected for further characterization. Single bacterial colonies were grown as reported by Carnemolla et al<sup>8</sup> and supernatants containing scFv TN11 or TN12 were purified using the recombinant TN A-D fragment or TN-C conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden), respectively. Real-time interaction analysis with surface plasmon resonance detection of the affinity and kinetic constants of the scFv was carried out as previously described.<sup>10</sup>

### RNA Extraction, Northern Blot Analysis, RT-PCR, and Immunohistochemical Procedure

Total RNA was isolated from human glioblastoma tissues or from human fibroblast cell line as reported.<sup>5</sup> RNAs from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon (no mucosa), and peripheral blood leukocyte and fetal brain, lung, liver, and kidney blotted on a nylon membrane (Hybridization-ready Human Multiple Tissues Blots) were purchased from Clontech Laboratories Inc. (Palo Alto, CA) and the hybridization was carried out as reported.<sup>5</sup> For the identification of TN-C mRNA containing the type C repeat, we used a <sup>32</sup>P-labeled DNA probe of 1078 bp containing 270 bp of human TN-C (4630–4899 bp of the sequence) of Siri et al<sup>18</sup> plus 801 bp of  $\lambda$ gt11 vector. For the identification of all the different TN-C mRNAs we used the HT11 cDNA probe<sup>18</sup>, and to normalize Northern blots, the human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech). Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed using 100 ng of total RNA, oligonucleotides BC-482 (5'GCTACCCCCTAGTACTGATTTTATTGTCTA, position: bases 4542-4571 of the TN-C sequence (Siri et al<sup>18</sup>) and BC-485 (5'TTTCCAGTGGCTCAGACTGC, complementary sequence, position: bases 5028-5047) or BC-482 and BC-484 (5'CTGGTCTGAGTCTTGGTTCCGTC, complementary sequence, position: bases 5322-5345) and Titan

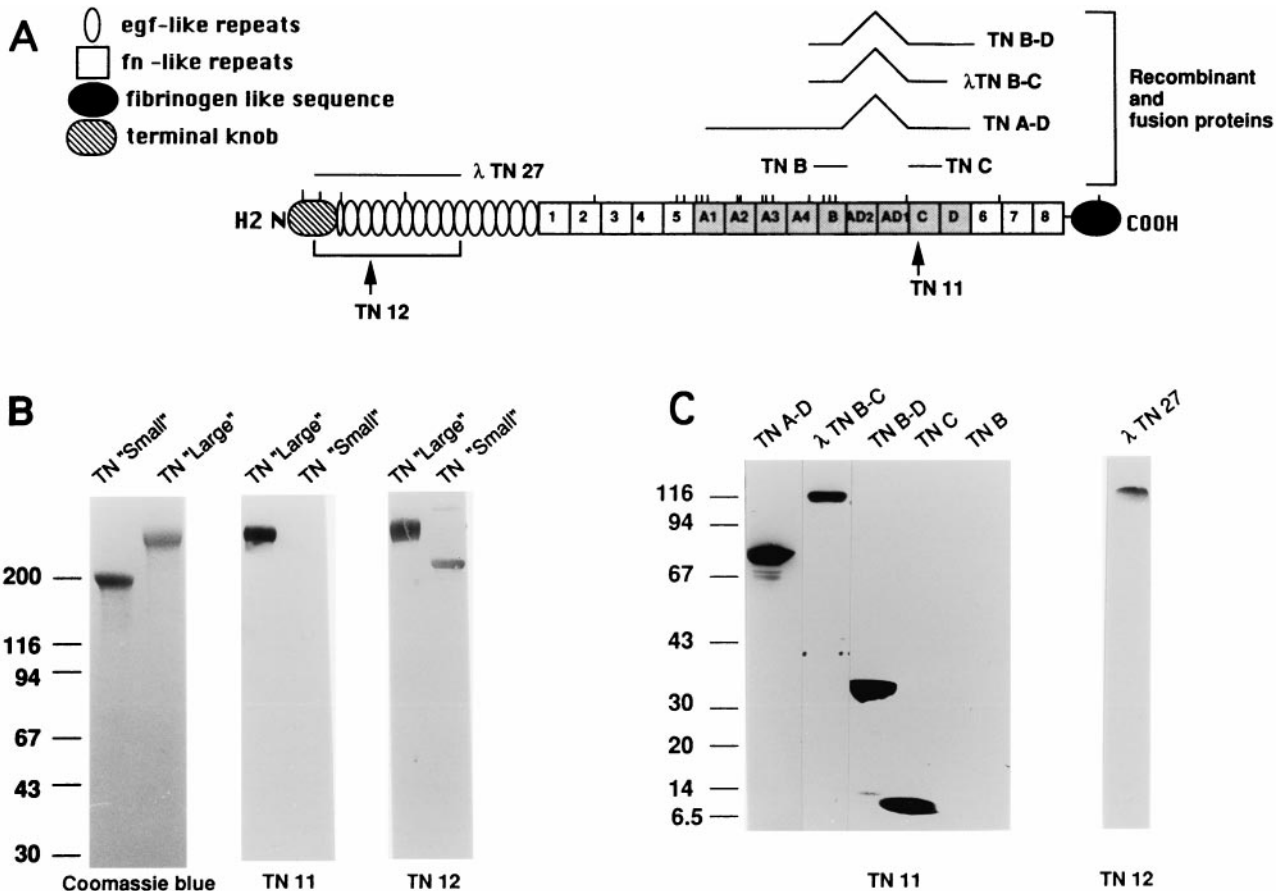


**Figure 1.** Confirmation by Southern blot analysis of the specificity of the cRNA probe used for *in situ* hybridization experiments. **Bottom:** ethidium bromide staining of the agarose gel. **Lane 1:** TNfnALL (including human tenascin DNA from type III repeat 2 to type III repeat 7, including all the alternatively spliced type III repeats). **Lane 2:** TNfn 1-8 (same sequence as TNfnALL but lacking all the alternatively spliced repeats). **Lane 3:** All TNegf like repeats. **Lane 4:** Alternatively spliced type III repeat D. **Lane 5:** Alternatively spliced type III repeat C. **Lane 6:** type III repeat 1. **Lane 7:** TNegf like repeats from 8 to 10. **Lane 8:** 1-Kb standard. **Above:** Southern blot showing DIG-hybrids detection. The numbers on the right are expressed in kilobases.

One Tube RT-PCR system (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's manual. Normal and neoplastic tissues were obtained from specimens taken during the course of therapeutic surgical procedures. Immunohistochemical studies were carried out as previously described.<sup>29</sup>

### In Situ Hybridization

For the *in situ* hybridization we used a modification of the Schaeren-Wiemers and Gerfin-Moser method<sup>30</sup> as previously described by Ponassi et al.<sup>31</sup> Briefly, paraformaldehyde-fixed cryostat sections were hybridized for 16–20 hours at 68°C with digoxigenin-labeled cRNA probes generated from templates obtained by PCR. The templates carried the T3 or T7 RNA polymerase promoters included before their transcription start sites. The visualization of the signal was accomplished by a color reaction with 4-nitrobluetetrazolium chloride (NBT) (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim) via an anti-DIG antibody conjugated to alkaline phosphatase (AP) (Boehringer Mannheim). Both sense and antisense probes entirely covered the TN repeat C, but only the last gave hybridization signal. The specificity of the probe was established by Southern blot using different DNA fragments, some including and others not including the repeat C, of TN-C (Figure 1) using the same stringency conditions used in the *in situ* hybridization experiment.



**Figure 2.** A: Model of the domain structure of a human TN-C subunit. The ovals and the squares represent the EGF-like and FN-like repeats, respectively. The globular N-terminal knob and the fibrinogen-like C-terminal domain are also depicted. The FN-like repeats A1 to D, whose expression is regulated by the alternative splicing of the pre-mRNA, are shaded. The upper part of the figure also shows the TN-C- $\beta$ -galactosidase fusion proteins or recombinant proteins used. The arrows show the sequence in which each epitope of recombinant or monoclonal antibodies was localised.  $\wedge$  indicates contiguity. B: Sodium dodecyl sulfate polyacrylamide gel electro-phoresis (4-18%) of recombinant Large TN-C (containing repeats from A1 to D) and Small TN-C (without repeats from A1 to D) stained with Coomassie blue and immunoblots stained with scFv TN11 and TN12. C: Immunoblots of different fusion and recombinant proteins (A) using the scFv TN11 and TN12. Values on the left indicate molecular masses (in kilodaltons) of the standards.

## Results

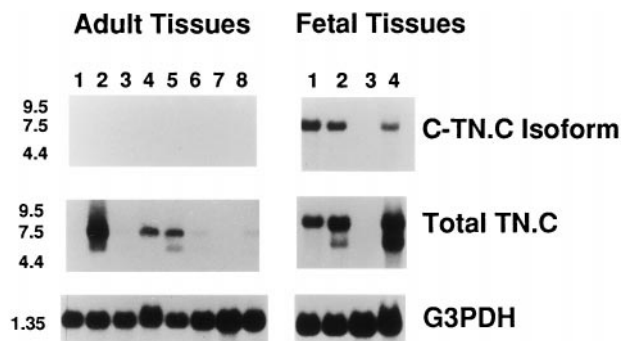
### Isolation of Two Human Antibody Fragments against the Large and Small Human TN-C Isoforms

The phage antibody library<sup>25</sup> was selected using recombinant human large TN-C. Several clones (including TN12) exhibited a strong reactivity with large and small TN-C isoforms in ELISA assays. One clone (TN11) gave a strong ELISA signal only with the large TN-C isoform. Antibodies TN11 and TN12 were therefore selected for further characterization. The binding affinity of TN11 and TN12 to the TN Large recombinant protein was determined by real-time interaction analysis with surface plasmon resonance detection (see Materials and Methods) and the dissociation constants of TN11 and TN12 were  $1.3 \times 10^{-10}$  and  $2.7 \times 10^{-8}$  mmol/L respectively. Sequencing of the V-gene of TN11 and comparison of the sequences with VBASE (the complete collection of human V-gene segments at <http://www.mrc-cpe.cam.ac.uk/imt-doc>) identified human V<sub>H</sub> segment DP10 and

V<sub>L</sub> segment DPL11/10 with V<sub>H</sub> CDR3 sequence of SR-RITIFGGGAFDI and V<sub>L</sub> CDR3 sequence of SSSY-TRSTRV, and sequencing of the V-gene of TN12 identified human V<sub>H</sub> segment DP38 and V<sub>L</sub> segment DPL12 with V<sub>H</sub> CDR3 sequence of ALPYYYYGMDV and V<sub>L</sub> CDR3 sequence of AAWDDSLSEFL.

### TN11 Reacts with the Spliced Repeat C whereas TN12 Reacts with the EGF-Like Repeats

The binding of TN11 and TN12 to human TN-C recombinant fragments was analysed by immunoblotting (Figure 2). TN12 reacted with both the large and small recombinant isoforms, as well as with a TN-C fusion protein containing only the EGF-like repeats in the NH<sub>2</sub> terminus part of the molecule. TN12 did not react with any other TN-C fusion proteins or recombinant fragments tested or with purified human FN (data not shown). Thus, the epitope recognized by TN12 is located within the EGF-like repeats.



**Figure 3.** Northern blots of poly(A)-rich RNA from human adult heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), and pancreas (8) tissues and fetal brain (1), lung (2), liver (3), and kidney (4) tissues using the cDNA probe (see Materials and Methods) specific for c-TN-C isoform, the HT11 probe that recognizes all TN-C isoforms, and the human G3PDH cDNA to normalize the blots (see Materials and Methods). Numbers on the left are the size, in kb, of the standards.

TN11 reacted with the large recombinant TN-C but not with the small isoform, and also reacted only with the recombinant fragments TNA-D, TNB-D, TNC, and fusion protein  $\lambda$ TN BC (Figure 2). These findings demonstrated that the epitope recognized by TN11 is localized within the TN-C repeat C (cTN-C). Using this scFv in Western blot experiments, we observed that the repeat C was undetectable in the large isoform of purified TN-C from cultured normal human fibroblasts and a melanoma cell line, SKMEL 28 (data not shown). These results were confirmed by RT-PCR experiments using RNA from the same cell lines (data not shown). The RT-PCR experiments also revealed that the repeat C was present in almost all the mRNA samples from glioblastoma, whereas it was undetectable in RNA samples from meningioma specimens. These data were confirmed by Western blotting using the recombinant antibodies TN11 and TN12 and TN-C from human glioblastoma and meningioma specimens. While TN12 reacted with both the TN-C preparations, TN11 reacted only with the TN-C preparation from glioblastoma (data not shown).

#### Northern Blot of TN-C mRNA in Normal and Fetal Tissues

Northern blot was performed to study the levels of the mRNAs of total TN-C and of the cTN-C using the probes described in Materials and Methods and the RNA from various normal adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon (no mucosa), and peripheral blood leukocytes) and from four different fetal tissues (brain, lung, liver, and kidney). The results demonstrated the presence of large amounts of the cTN-C in fetal brain, lung, and kidney, whereas this mRNA was undetectable or barely detectable in all the adult tissues tested (Figure 3). By contrast, total TN-C mRNA was present in almost all the tissues tested, as previously reported (Figure 3).<sup>5</sup>

**Table 1.** Reactivity of the scFv TN11 and TN12 with Primary Tumors of Various Histotypes

Tumor type	Number positive/number tested	
	TN11	TN12
Brain glioblastoma and anaplastic astrocytomas	15/16	16/16
Astrocytomas II	0/5	5/5
Pilocytic astrocytoma	0/2	2/2
Neurinoma	1/1	1/1
Ependimoma	0/1	1/1
Oligodendroglioma	1/2*	2/2
Meningioma	1/23 <sup>†</sup>	23/23
Brain metastasis	7/15 <sup>‡</sup>	15/15
Breast adenocarcinoma	3/27 <sup>§</sup>	27/27
Stomach adenocarcinoma	0/2	2/2
Lung carcinoma	0/2 <sup>¶</sup>	2/2

All tumors tested strongly positive with scFv TN12, which recognizes all different TN-C isoforms. The scFv TN11 recognizes only the TN-C isoform containing the C domain.

\*The positive case showed staining only in some vascular structures.

<sup>†</sup>The positive case was a transitory meningioma and showed staining only in some vascular structures.

<sup>‡</sup>Among the 7 positive cases, 3 showed positivity both in connective tissue and some vascular structures, and 3 cases showed positivity only occasionally in some vascular structures.

<sup>§</sup>In the 3 positive cases the staining was barely detectable.

<sup>¶</sup>Rare focal positivity in both cases.

#### Distribution of the cTN-C in Normal and Neoplastic Tissues

Immunohistochemical analyses of a variety of normal adult tissues (brain (2 specimens), lung (4), breast (4), stomach (1), endometrium (2), prostate (1), skin (2), thyroid (1), fallopian tubes (1), vein (1), kidney (1), spleen (1), didymous (1), liver (1), adrenal cortex (1), thymus (1), striate muscle (1), colon (1), prostate (1), and peripheral nerve (1)) using the scFv TN11, which is specific for the type III repeat C, and the scFv TN12, which recognizes all different TN isoforms, showed that in normal adult tissues, although total TN-C had a widespread distribution, the presence of the repeat C was undetectable by immunohistochemistry in all the tissues tested with exception of lymph node and thymus, in which very rare focal staining was observed, mainly in vascular structures.

Furthermore, we analyzed the distribution of total TN-C and of the cTN-C in 92 human tumors of different histotypes using the scFv TN12 and TN11, respectively. Glioblastoma expressed the highest levels of the repeat C, with 14 out of 15 specimens showing strong positivity (Table 1 and Figure 4). Presence of this TN-C isoform was detected mainly around vascular structures, surrounding areas with high proliferative activity, in the stroma of tumor nests (Figure 4, A, B, C, E, and G), and within proliferating cells (Figure 4F), as demonstrated by double staining using the mAb KI67 and TN11. By contrast, no positive staining was seen in other brain tumors, with the exception of two meningiomas out of 23 that were weakly positive around vascular structures (Table 1 and Figure 5). Furthermore, some rare focal positivity was found in 7 of 15 brain metastases from lung and breast carcinomas



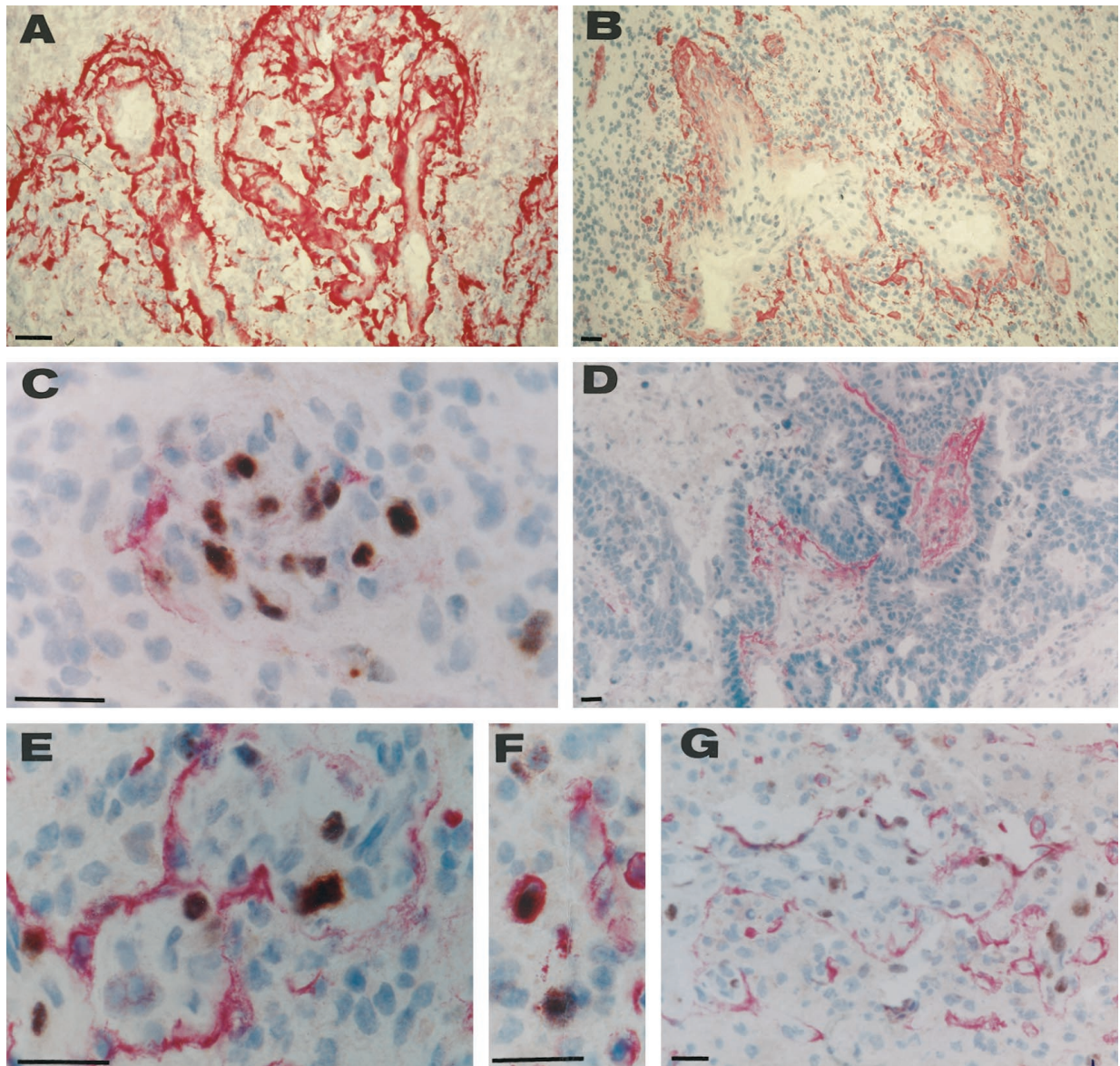


Figure 4. Immunohistochemical experiments on sections of glioblastoma stained using scFv TN11 (A and B) and double-stained using scFv TN11 (red) and mAbKI67 (brown) (C, E, F, and G); a section of a brain metastasis from lung carcinoma stained using scFv TN11 (D). Scale bar, 10  $\mu$ m.

(see Figure 4D). Twenty-seven specimens from patients with invasive breast carcinoma were examined, and some very weak positivity was seen in 3 cases (Table 1 and Figure 5).

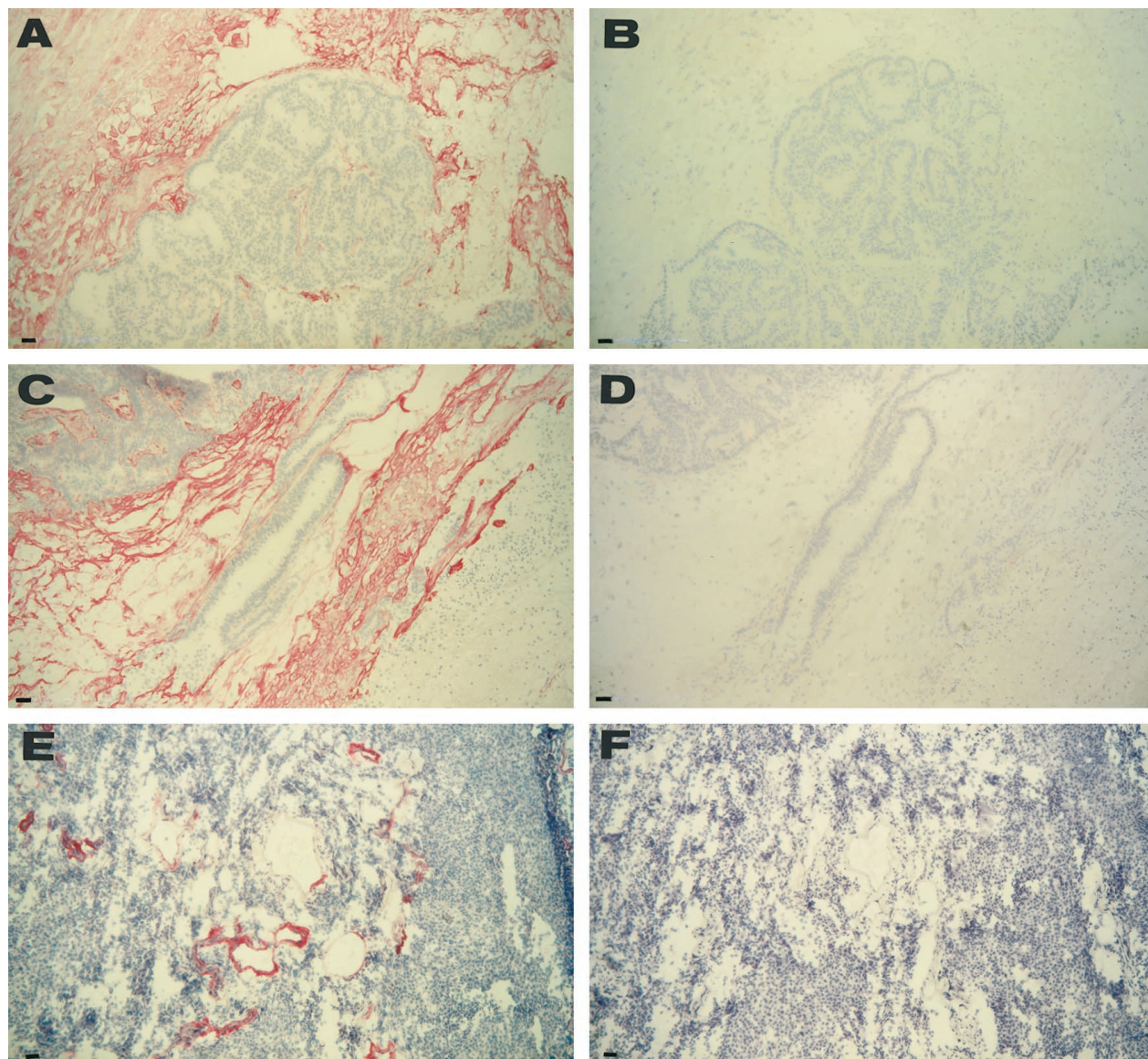
To establish which kind of cells were responsible for the production of the cTN-C isoform, we prepared a DIG-labeled cRNA probe specific for the cTN-C (see Figure 1 and the Materials and Methods section) and performed *in situ* hybridization on glioblastoma cryostat sections (Figure 6, A and B). The results demonstrate that the cTN-C isoform was produced by tumoral cells, even though not all tumoral cells produce the cTN-C isoform.

### Discussion

The large TN-C isoform is expressed in many normal adult tissues but it is expressed at higher levels in neo-

plastic tissues, particularly glioblastoma. Glioblastomas are usually highly invasive but well compartmentalised, and in general do not metastasize. Nevertheless, due to the lack of specific therapeutic agents, the prognosis of patients with glioblastoma is very poor. With current treatment, which includes palliative surgical resection together with radiotherapy and steroids, the mean length of survival after diagnosis is only 8–10 months, with fewer than 10% of patients alive after 2 years. Glioblastomas have already responded to clinical approaches with TN-C monoclonals (mAbs) and two mAbs, BC-2 and 81C6, both specific for the large TN-C isoform, have found clinical application.<sup>9,11,32–35</sup> In fact, the expression of the large TN-C isoform is the most constant feature of glioblastoma. Using a monoclonal antibody specific for the large TN-C isoform (BC-2), staining of the extracellular stroma and around the walls of hyperplastic blood



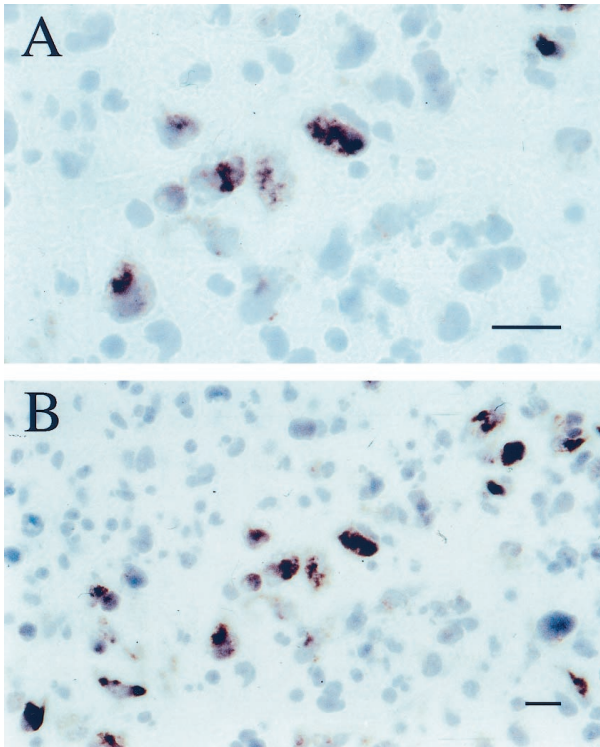


**Figure 5.** Immunohistochemical experiments on serial sections of invasive ductal breast carcinoma stained using scFv TN12 (A and C) and scFv TN11 (B and D) and on serial sections of meningioma stained using scFv TN12 (E) and scFv TN11 (F). Scale bar, 10  $\mu$ m.

vessels was reported.<sup>36</sup> On the contrary, TN-C is barely or not present in the white matter and meninges of different areas of normal adult cerebrum, cerebellum, and spinal cord, whereas only focal and weak staining is reported in the cerebral cortical matrix. Furthermore, the large TN-C isoform is not detectable in normal brain tissues. However, these mouse mAbs used in the radioimmunotherapy (RIT) of glioblastoma are of very limited specificity because they react with a number of normal adult tissues.

Using phage display technology we isolated human antibody fragments binding to human TN-C and identified a fragment directed against the type III repeat C of the large TN-C isoform (TN11). This revealed that the repeat C appeared to be absent from all the normal adult human tissues tested. Likewise, the repeat C was undetectable in the mRNA of normal adult tissues but present in fetal lung, kidney and brain; this finding is consistent

with earlier reports showing the presence of the repeat C in fetal tissues but not in adult tissues.<sup>37,38</sup> However, the antibody revealed the presence of the repeat C (confirmed by mRNA studies) in anaplastic astrocytoma and glioblastoma, mostly associated with vascular structures and around proliferating cells. This suggests that this TN-C isoform could be produced mainly by proliferating cells. Seven cases out of 15 brain metastases from lung and breast carcinoma showed positive staining for cTN-C, although this isoform was barely detectable in the primary tumors. The mechanisms responsible for the expression of the cTN-C isoform in these metastases are, at present, only a matter of speculation. However, one explanation could be the different environment in which tumoral cells are located and that could induce the expression of the cTN-C isoform. In fact, we have previously demonstrated that environmental conditions, such as the extracellular pH,<sup>23</sup> play an important role in controlling



**Figure 6.** Two different magnifications of an *in situ* hybridization experiment using human glioblastoma cryostat sections with the DIG-labeled cRNA repeat C probe (see Material and Methods section). Positive signal was visible only in some tumoral cells with large nuclei.

the alternative splicing of the TN-C pre mRNA. We are presently investigating the mechanism regulating the expression of the cTN-C in brain metastasis.

The type III repeat C of TN-C appears to be a highly specific target for therapy of glioblastoma, and the human antibody fragment TN11 an attractive candidate for scintigraphic and therapeutic applications. In fact, small human antibody fragments are rapidly cleared from circulation, do not accumulate in the liver, are not immunogenic, and exhibit improved tissue penetration compared to conventional immunoglobulins.<sup>39,40</sup> Further studies on the biological activities of the c-TN-C may help to identify new potential targets for therapeutic intervention.

### Acknowledgments

We thank Tristan Vaughan at Cambridge Antibody Technology for supplying the human scFv phage library and Mr. Thomas Wiley for manuscript revision.

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