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Molecules and Cells

Selection and Characterization of Tenascin C Targeting Peptide

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Since tenascin C is a factor expressed highly in the tumor-associated matrix, it would be a desirable first step for targeting the tumor-specific microenvironment. In fact, a high level of tenascin C expression has been reported in most solid tumors, including lung cancer, colon cancer and glioblastoma. Therefore, the targeted binding of tenascin C in tumor stroma would inhibit tumor metastasis by modulating cancer cell growth and migration. We isolated a peptide that bound to tenascin C by phage display peptide library selection, and the selected peptide specifically recognized tenascin C protein in xenograft mouse tissue. We also observed exclusive staining of tenascin C by the selected peptide in tumor patient tissues. Moreover, the peptide reduced tenascin C-induced cell rounding and migration. We propose that the tenascin C targeting peptide may be useful as a specific anti-cancer diagnostic and therapeutic tool for most human solid tumors.

INTRODUCTION

Tumor tissue is unique in that the cells and extracellular matrix (ECM) are distinct from those of normal tissues (Fidler et al., 2010). The tumor-specific microenvironment may play a role in tumorigenesis by accompanying changes in surrounding stroma and ECM on top of genetic mutations in cancer cells themselves. Therefore, protein components expressed only in tumor tissue might be biomarkers for tumor sites *in vivo*, consequently making them valuable target proteins for therapeutics.

The tenascin proteins (C, X, R, and W) are large ECM glycoproteins that form multimeric complexes and may contribute to pathological states by remodeling tissues. Tenascin C (TNC) is expressed during development but not in normal adult tissues (Chiquet-Ehrismann, 2004; Chiquet-Ehrismann and Tucker, 2004). TNC is secreted from the cells and localized to the extracellular matrix in tumor microenvironments or to stromal cells surrounding tumor cells, so its exuberant expression appears to play a role in tumor progression by regulating cell adhesion and migration (Orend, 2005; Orend and Chiquet-Ehrismann, 2006). Even though the exact roles of TNC in tumorigenesis remain to be determined, the prominent expression of the protein in tumor tissues may potentially provide an excellent opportunity for targeting tumor sites for diagnosis and treatment of diverse solid tumors *in vivo* (Hsia and Schwarzbauer, 2005).

A large isoform of TNC is generated by alternative splicing of mRNA and its protein expression has been shown to be associated with tumor progression (Adams et al., 2002; Berndt et al., 2006; Juuti et al., 2004). To further enhance the targeting potential, the large isoform of TNC can be used as a target for tumor-specific microenvironments (Takeda et al., 2007). Thus, anti-TNC antibodies represent an effective means of tumor targeting and a radiolabeled monoclonal antibody is currently undergoing clinical evaluation (Akabani et al., 2005; Brack et al., 2006; Silacci et al., 2006). In addition, RNA aptamers have been developed for use as targeting and imaging tools (Hicke et al., 2001; 2006). However, to expand the potential utility of TNC as a tumor microenvironment target, a versatile alternative molecule such as a small peptide should also be developed (Aggarwal et al., 2006; Jäger et al., 2007; Lee et al., 2007; Rasmussen et al., 2002).

To develop TNC binding small peptides, we performed two independent selections against TNC proteins. We showed that the peptides recognized TNC in xenograft mouse models. Furthermore, it specifically stained TNC rich stroma in the tissues of patients with lung cancer that may represent the metastatic invasion front. Moreover, inhibition of tumor migration was achieved by the peptide. We propose here that the TNC binding peptide can be used as a targeting tool for tumor tissues and for therapeutic agents.

MATERIALS AND METHODS

Ethics statement

The lung tissue array was composed of 36 adenocarcinomas,

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15 squamous cell carcinomas, one bronchioloalveolar cell carcinoma, and one normal lung, and was constructed from archived paraffin blocks at Bundang Hospital, Seoul National University. Using patient tissues for arrays has been approved by the Institutional Review Board (IRB). Informed consent has been exempted, because the tissue array was made from resected tissues after a surgery and was used only for investigation purposes.

Cell lines

Colorectal cancer cell lines (HT-29, HCT116, and SW620) and glioblastoma cell lines (U118MG, U251, and T98G) were purchased from the American Type Culture Collection (ATCC; USA). All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS).

RT-PCR

Total cellular RNA was isolated with TRIzol (Invitrogen, USA), reverse transcribed with M-MuLV reverse transcriptase (Stratagene, USA), and used in polymerase chain reaction (PCR) analysis. The following PCR primers were used: TNC, 5′-GGTACAGTGGGACAGCAGGTG-3′ (forward) and 5′-AACTG GATTGAGTGTTCGTGG-3′ (reverse); GAPDH 5′-TGACATC AAGAAGGTGGTGA-3′ (forward) and 5′-TCCACCACCCTGT TGCTGTA-3′ (reverse). The complimentary DNA was subjected to standard PCR and the products were analyzed on a 2% agarose gel followed by ethidium bromide staining.

Recombinant proteins

Full-length TNC protein was purchased from Chemicon (USA). Fibronectin (FN) was purchased from Sigma-Aldrich (USA). Recombinant $(His)_6$-tagged TNC protein was generated from the TNCfnA-D plasmid containing TNC alternative splice domain (kindly provided by Dr. Harold P. Erickson, Duke University Medical Center, USA). TNCfnA-D plasmid was amplified with TNCfnA-D sense (5′-ATAGGATCCGAACAAGCCCCT-3′) and the TNCfnA-D antisense (5′-GCCGGATCCCTATGTTGT TGC-3′) primers. The amplified fragment was inserted into the *Bam*HI site of the pET28a+ vector (Novagen, USA) to generate $(His)_6$-tagged-TNCfnA-D. The ligated DNA was used to transform *Escherichia coli* BL21 (DE3) cells and the recombinant DNA was confirmed by DNA sequence analysis. To prepare $(His)_6$-tagged-TNCfnA-D fusion proteins, transformed bacteria were cultured in LB medium with kanamycin to an optical density of 0.6 at 600 nm. Next, 0.4 mM isopropyl-1-thio-D-galactopyranoside (IPTG) was added and the cultures were incubated for another 4 h at 30°C. The cells were collected by centrifugation, resuspended in 15 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0) with 1 mM phenylmethanesulfonyl fluoride and 1 mg/ml lysozyme, and sonicated for 7 min with a 30% pulse. After the lysate was cleared by centrifugation at 13,000 rpm for 30 min at 4°C, the $(His)_6$-tagged-TNCfnA-D fusion protein was purified with Ni-NTA agarose (Qiagen, USA) according to the manufacturer's instructions.

Biopanning of TNC-binding phages

The phage-display peptide library Ph.D.-12 (New England Biolabs, USA) was panned in 96-well plates coated with 0.15 ml $(His)_6$-tagged-TNCfnA-D (10 μg/ml) or full-length TNC (20 μg/ml) in 0.1 M $NaHCO_3$ (pH 8.6) overnight at 4°C. The wells were blocked a with buffer comprised of 0.1 M $NaHCO_3$ (pH 8.6), 0.5% bovine serum albumin (BSA) and 0.02 % NaN_3 for 1 h at 4°C, and 2×10^{11} plaque forming units/ml were added to the target protein-coated plates in blocking buffer. After incubation for 30 min at 25°C, unbound or weakly bound phages were removed by rinsing ten times with Tris buffered saline-Tween 20, and bound phages were eluted by incubation for 8 min in 0.1 ml of an elution buffer comprised of 0.2 M glycine-HCl (pH 2.2) and 0.1% BSA. The recovered phages were used to infect *E. coli* ER2738 (New England Biolabs), amplified and were purified by precipitation with 1/6 volume of a mixture containing 20% (w/w) polyethylene glycol 8000 and 2.5 M NaCl (PEG/NaCl). The purified phages were used in the next round of panning. After three rounds, independent clones were isolated on LB/IPTG/X-gal plates, and phage titers were calculated from the number of plaques formed.

DNA sequence analysis

Thirty-five clones from two independent selections were sequenced. Individual phage clones were purified by precipitation with PEG/NaCl. The phage pellets were suspended in iodide buffer comprised of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 4 M NaI, and single-stranded phage DNA was precipitated with ethanol. The nucleotide sequences of the isolated DNAs were determined with an ABI Prism® automatic sequencer (Applied Biosystems, USA) and primer 5′-CCCTCATAGTTAGCGTAA CG-3′.

Synthesis of peptides

The selected peptides and a scrambled peptide were synthesized by Peptron Inc. (Korea). The peptide sequences were FHKHKSPALSPVGGG (peptide #1) and VSPKSHLKAHPFGGG (scramble peptide). All peptides were synthesized with amino-terminal conjugated biotin residues.

Biosensor assay

BIAcore3000 was used for Surface Plasmon Resonance (SPR) experiments. To attach the protein to the CM5 sensor chip, the surface of the chip was pre-equilibrated with HEPES and activated with 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N′-(dimethylaminopropyl) carbodiimide (EDC) to modify the carboxymethyl groups of dextran. After pre-activation, FN was injected into two of the flow cells and the surface of the chip was deactivated with 1 M ethanolamine hydrochloride, pH 8.5. The TNC was injected into one of the cells whereas binding buffer was injected into the other. To overcome the limitation of measuring small size peptide binding on large size target TNC protein, we conjugated peptide #1 to quantum dots (QD; Invitrogen, USA). When the baselines were stabilized, various concentrations of the QD conjugated peptide #1 were injected. Following each experiment, the sensor chip was regenerated with 10 mM NaOH. Kinetics parameters were obtained with BIA software.

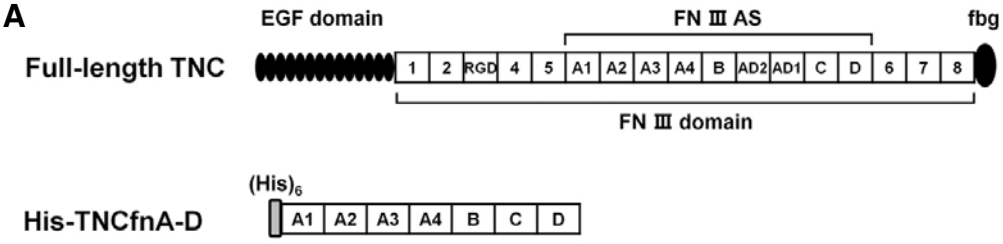
Immunocytochemistry

Cells were grown on coverslips, fixed with 3.7% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate buffered saline (PBS). They were washed and blocked in 10% normal calf serum and 0.5% gelatin in PBS for 30 min at room temperature. Staining was carried out with biotin-labeled peptide #1 for 1 h at room temperature. A 1:200 dilution of Texas Red-conjugated streptavidin (Calbiochem, USA) was applied at room temperature for 50 min in the dark. The cells were counterstained with Hoechst 33258 and visualized by microscopy (Carl Zeiss Axioplan 2; Carl Zeiss, Germany).

Immunofluorescence analysis

The tumor xenograft was established by subcutaneous injec-

A



B

Group	Sequences	Frequency
Ⅰ	F H K H K S P A L S P V (peptide #1)	19
	F H K H – – P – – S P – (consensus)	
Ⅱ	F H K P F F P K G S A R (peptide #2)	13
	F H K P F – P K – S A – (consensus)	
	F H K H – – P – – S P – P A	
Ⅲ	Non consensus	3
	Total clones	35

C

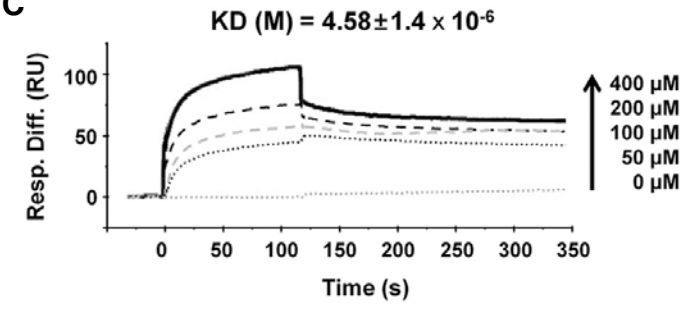


Fig. 1. Development of TNC binding peptides. (A) Schematic diagram of the recombinant TNC proteins used for selecting peptides. FNIII; fibronectin type III, fbg; fibrinogen glob, AS; alternative spliced domain. (B) Consensus motifs in the selected peptides. Frequency denotes the number of identical phage clones isolated. (C) Binding affinity of peptide #1 was measured by Surface Plasmon Resonance (SPR). Dissociation constants (Kd) are presented as means of three independent SPR measurements.

tion of 2×10^6 U118MG or HT29 cells into one flank of nude mice. When the tumor diameter reached approximately 9-10 mm, the mice were killed and the tumor tissue was obtained and frozen for storage at -80°C. Frozen sections 8-10 μm in thickness were cut, fixed with 3.7% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS. The tissues were washed and blocked with 10% FBS, 10% non fat dry milk, and 3% BSA in PBS for 2 h at room temperature. They were stained with biotin-labeled peptide #1 overnight at 4°C. Secondary Texas Red-conjugated streptavidin at 1:1000 dilutions were used for 1 h at room temperature in the dark. Cells were counterstained and visualized as described above.

Immunofluorescence analysis of tissue array

Sections were dewaxed in xylene and rehydrated in graded concentrations of ethanol and distilled water. For antigen retrieval, sections were heated for 20 min using a pressure cooker in Target Retrieval Solution consisting of Tris/EDTA (pH 9) (Dako, Denmark). Staining was carried out with 1:1000 dilutions of anti-TNC antibody (BC24; Sigma-Aldrich) or biotin-labeled peptide #1 overnight at 4°C. Secondary fluorescein isothiocynate (FITC)-conjugated anti-mouse IgG antibody (Sigma-Aldrich) or Texas Red-conjugated streptavidin was used for 1 h at room temperature in the dark. Cells were counterstained and visualized as described above.

Adhesion assay

Microtiter plates (6-well; Nunc, Denmark) were coated with 1 μg/cm^2 of FN or FN+TNC overnight at 4°C. The non-coated plastic surface was blocked with 1% heat-inactivated BSA in PBS. Before plating, cells were serum starved for 18 h in DMEM and treated with trypsin. After treatment with soybean trypsin inhibitor, cells were resuspended in serum-free medium and counted using a hematocytometer. Approximately 1×10^5 cells were plated in each well and the selected peptide #1 or scrambled peptide was treated for 18 h. Cells were observed by optical microscope and counted.

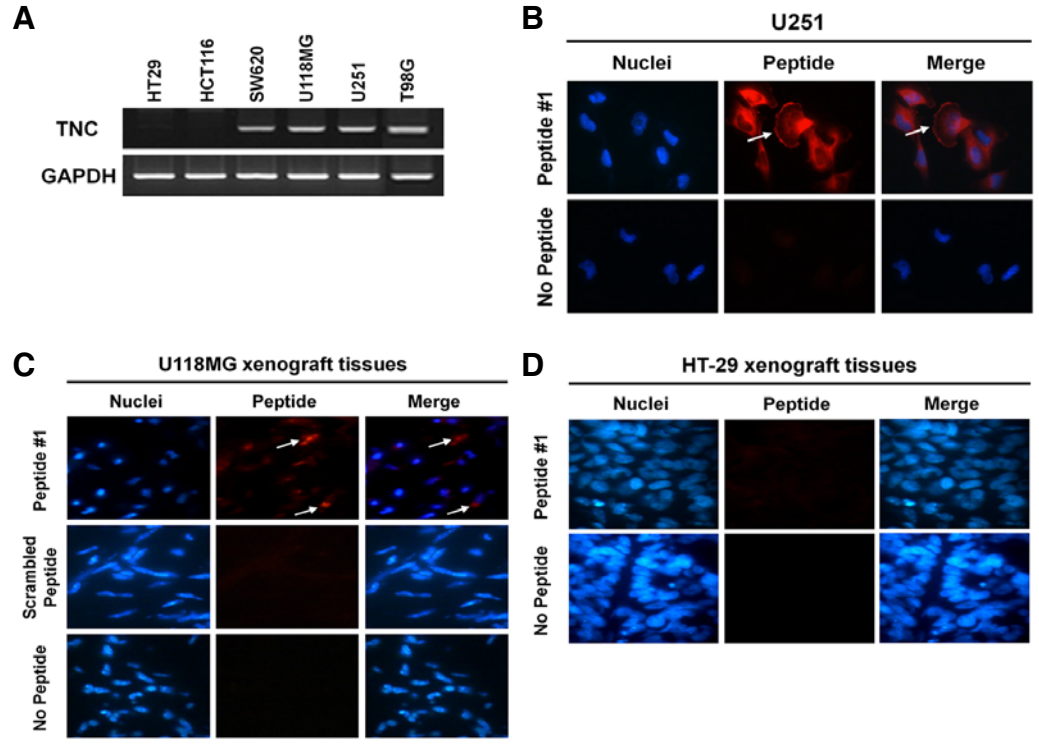


Fig. 2. Recognition of TNC in xenograft mouse tissues. (A) Total RNA from cultured human cells was analyzed by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. (B) Staining of the biotin-labeled peptide #1 to U251 cells. Nuclei were counterstained with Hoechst33258 and visualized by microscope with × 630 magnification. Arrows indicate examples of positively stained cells. (C) Immunofluorescence detection of TNC protein in U118MG-derived tumor xenograft mouse by peptide #1. (D) Immunofluorescence detection in HT29-derived tumor xenograft mouse. Nuclei were counterstained with Hoechst33258 and visualized by microscope with ×630 magnification. The scrambled peptide was used as a control. Arrows indicate examples of positively stained cells.

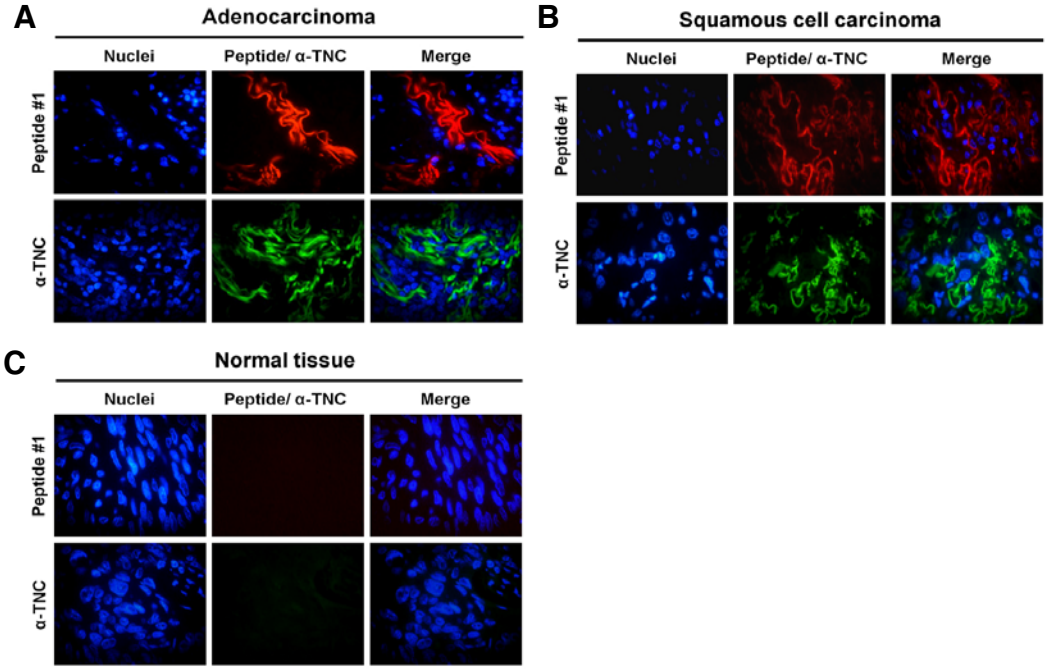


Fig. 3. Localized streak staining of TNC by the peptide in lung tissues of human patients. Paraffin-embedded lung tissue was incubated with anti-TNC antibody or with biotin-labeled peptide #1. Nuclei were counterstained with Hoechst33258 and visualized by microscope with × 630 magnification. (A) A lung tissue from an adenocarcinoma lung cancer patient. (B) A lung tissue from a squamous cell carcinoma lung cancer patient. (C) A lung tissue from a cancer-free individual.

Wound scratch assay and statistical analysis

The U118MG cells were grown on a 35 mm dish until they be came confluent and were serum starved for 18 h in DMEM. A straight line was drawn with a marker on the outer surface of the dish bottom and an artificial wound was made in the cell lawn using a pipet tip. The dish was rinsed and then incubated with the serum free medium in the presence of the selected peptide #1 or scrambled peptide. After 18 h incubation, cells were counted. Student's *t*-test was performed for most data using Microsoft Excel (Microsoft, USA).

RESULTS

Identification of TNC binding peptide sequences

Most solid tumors express the large isoform of TNC which contains EGF domain, fibrinogen glob (fbg) and fibronectin III (FN III) domains with the inclusion of alternatively spliced exons in the middle (Fig. 1A). Due to the complexity and sizes of the tumor specific TNC protein, we utilized two different versions of TNC proteins and performed two independent selections. One is the tumor-specific large isoform of TNC (Full-length TNC) expressed in eukaryotic cells and the other is the alternatively spliced domain (A1-D) of TNC expressed in bacteria (His-TNCfnA-D, Fig. 1A). Of the 35 clones derived from two independent selections, 19 had the same sequences (designated peptide #1), and 13 others were also identical (designated peptide #2), while the remainder had similar sequences (Fig. 1B). Most sequences contained amino terminal FHKH and SP or $PX_{2\text{-}4}P$ motifs. Since one peptide sequence was representatives of the selected sequences, we synthesized peptide #1 (FHKHKSPALSPV), with flanking sequences (-GGG) from the M13 coat protein. As a control, we also synthesized the scrambled form of peptide #1 (VSPKSHLKAHPFGGG).

We used Surface Plasmon Resonance (SPR) analysis to estimate the binding affinity of the peptide to TNC protein. We made FN (fibronectin)/TNC substratum in one flow cell of the sensor chip to mimic TNC-rich ECM within tumor tissue and analyzed the binding profiles of the peptide. In another flow cell, only the FN was injected and used as a mimic for TNC-free ECM in normal tissue. By injecting various concentrations of the peptide under kinetic conditions, we obtained their dissociation constants (Fig. 1C). The binding affinity of peptide #1 to TNC was 4.58 ± 1.4 μM, which was extremely high for such a small size.

Recognition of TNC by the peptide in xenograft mouse models

As a first step for testing specific binding of the peptide to TNC protein, we examined a number of cancer cell lines to assess the relationship between tumor type and TNC expression. Colorectal cancer cell lines (HT-29, HCT116, and SW620) and glioblastoma cell lines (U118MG, U251, and T98G) were used as models for TNC expression analyses (Fig. 2A). All three glioblastoma cell lines expressed TNC mRNA. However, in the case of three colorectal cancer cell lines, only the single cell line (SW620) expressed TNC mRNA (Fig. 2A). Based on the expression pattern of TNC in cancer cell lines, we chose U118MG and U251 cells as TNC positive cell lines, whereas HT29 and HCT116 cells represented TNC negative cell lines.

To test whether the selected peptide could recognize TNC protein, we stained the cancer cell lines using biotin-labeled peptide. Interestingly, peptide #1 yielded a characteristic staining pattern of long streaks or spots in a polarized region of the plasma membranes of U251 cell lines (Fig. 2B). The signal seemed to be specific because no staining was observed when the peptide was omitted. When we used peptide #1 for staining HCT116 that did not express TNC, no signal was detected (data not shown).

Since TNC protein is expected to secrete from the cell and localize to the ECM in the tumor microenvironment, we established a xenograft mouse model using U118MG cells. Tumor tissue sections were prepared and stained using biotin-labeled peptide #1. Most remarkably, biotin-labeled peptide #1 stained as the prominent spot or blob-like staining patterns (Fig. 2C). This peptide staining pattern was specific because no staining was observed when the peptide was omitted or when the scrambled peptide was used. We also developed a xenograft mouse model with HT-29 cells, which do not express TNC and no peptide signal was detected (Fig. 2D).

Peptide-mediated staining of TNC in lung cancer patient tissues

To see whether the peptide was capable of detecting TNC in the tissues of human cancer patients, we used a tissue array prepared with tissues from 52 lung cancer patients (36 adenocarcinomas, 15 squamous cell carcinomas, and one bronchioalveolar carcinoma) and one normal lung. The use of anti-TNC antibody revealed the prominent expression of the protein, which was exclusively located outside of the cells, possibly in the ECM (Fig. 3). A streaky pattern of protein expression was observed in every tumor tissue sample, and was especially strong in the adenocarcinomas (Fig. 3A) and squamous cell carcinomas (Fig. 3B), with some spots evident in the bronchioalveolar carcinoma (data not shown). Such stromal bands may represent delineated packets of invasive metastatic tumor cells. Remarkably, peptide #1 also stained stromal bands with striking similarity to the staining patterns obtained with the antibody. No peptide staining was detected in normal tissue (Fig. 3C). These observations are entirely consistent with the suggestion that the selected peptide can specifically detect TNC protein in human tumor tissues, especially in stromal bands near tumor cells, which are the possible invasive front of the metastasis.

Suppression of cell migration by the peptide

TNC has anti-adhesive properties by causing cell rounding and migration promotion, which is consistent with a role as a critical regulator for tumor progression and metastasis. Appropriately, we tested whether peptide #1 could inhibit TNC-induced cell rounding and migration in U118MG cells. The cells were plated on either the FN or the FN+TNC substratum and cell morphology was microscopically assessed. While most of the FN plated cells had a spreading morphology, most of the TNC plated cells adopted a round morphology (Figs. 4A and 4B). Notably, we observed a significant reversion of TNC-induced rounded morphology by peptide #1 (Figs. 4A and 4B). We also tested the migration potential of the glioblastoma cells and found that TNC-induced cell migration was significantly reduced by the incubation of peptide #1. No significant reduction in cell migration was observed when the scrambled peptide was used (Fig. 4C). Taken together, these results suggest that the peptide may be useful as a therapeutic tool for TNC expressing tumors.

DISCUSSION

Since the TNC protein is exuberantly produced in most solid tumors, development of specific ligands would be a first step for TNC-targeted diagnosis and therapeutics. We have shown here that the phage selected peptide can specifically recognize the TNC in xenograft mouse models. Furthermore, specific

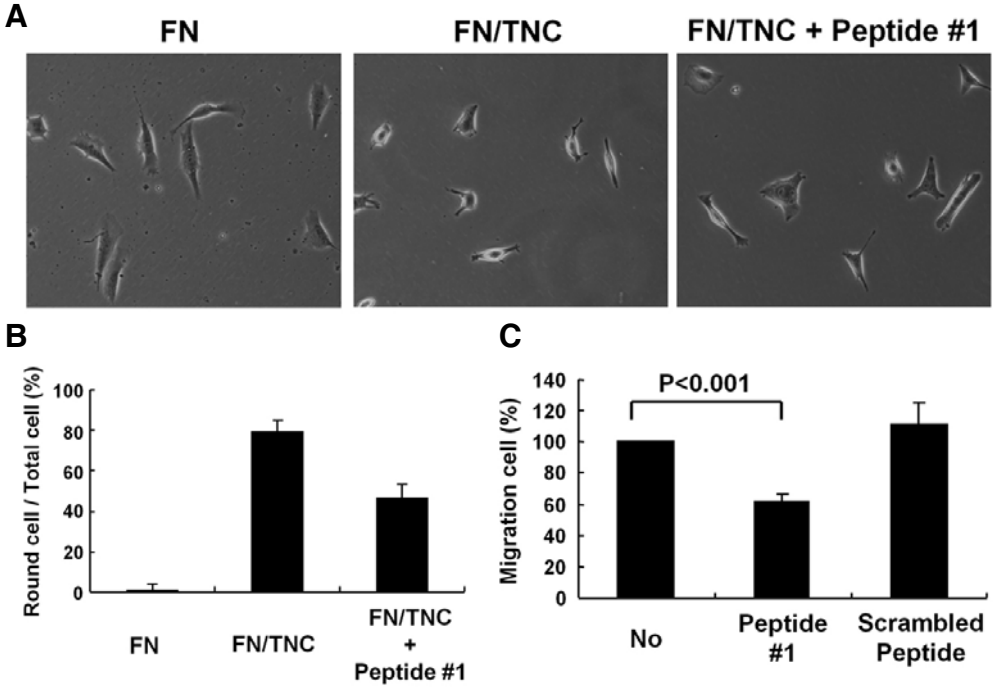


Fig. 4. Inhibition of TNC-induced cell migration by the peptide. (A) Reversion of TNC-induced cell rounding by peptide #1. U118MG cells were plated on the FN+TNC substratum with peptide #1 and observed microscopically (magnification, × 100). (B) Numbers of rounding cells were scored and are presented as a percentage of total cells. Error bars represent SD of four independent experiments. (C) Modulation of cell migration by peptide #1. Peptide #1 or scrambled peptide was added. Error bars represent SD of three independent experiments.

staining patterns of human lung cancer patient tissues suggested the critical roles of TNC in the tumor microenvironment, possibly at the invasive front. Most significantly, we observed that the peptide inhibits TNC-induced cell rounding and migration, which are hallmarks of cancer cell metastasis. To understand the mechanism behind the inhibition of cell adhesion, we have tested the effect of the TNC binding peptide on the downstream signaling pathway. TNC was reported to regulate focal adhesion kinase (FAK); however, unfortunately we could not detect any noticeable changes in FAK activation by the peptide incubation. Even though it is not clear at this point how the peptide regulates cell adhesion, it clearly suppress the TNC mediated rounding and migration of the cells in culture. Since it has been reported that shRNA and siRNA for TNC inhibited the migration and metastasis of lung metastatic cells without knowing the downstream pathways, we hope to develop the peptide as an inhibitors for cancer cell migration and metastasis (Tavazoie et al., 2008).

There are many advantages to the use of small peptides including low cost, absence of significant immunogenicity, and ease of coupling to cytotoxic agents. The most important advantage of peptides is their versatility (Aggarwal et al., 2006; Jäger et al., 2007; Lee et al., 2007; Rasmussen et al., 2002). For example, tumor binding peptide sequences can be incorporated into the coat proteins of viruses (Grifman et al., 2001; White et al., 2004), and novel targeting tools, such as liposomes and nanoparticles, can be used together with the peptides (Hajitou et al., 2006). It is also the first test of the utility of the peptide as a therapeutic tool for TNC, such as glioblastoma cancer cells, as well as lung cancer adenocarcinoma and squamous cell carcinoma.

Due to its highly localized expression, TNC could be an extremely valuable target for tumor-specific targeting of therapeutic agents. For that reason, a TNC-specific monoclonal antibody is already in clinical trials (Akabani et al., 2005; Reardon et al., 2002) and the refinement of monoclonal anti-TNC antibodies is under study (Bellofiore et al., 2006). In addition, RNA aptamers have been developed for tumor imaging (Hicke et al., 2006). As an alternative to antibody- and RNA aptamer-based TNC targeting, we propose to use small peptides that bind selectively to TNC-expressing tumors. Considering the vast potential of peptides in cancer therapy (Khandare and Minko, 2006), we hope to develop the peptide as a starting point for tumor microenvironment targeted therapeutics.

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