Invasion of Melanoma in Double Knockout Mice Lacking Tenascin-X and Tenascin-C

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The roles of extracellular matrix glycoproteins belonging to the tenascin family in the regulation of tumor cell proliferation, invasion, and metastasis are not known. To address this issue, we generated tenascin-X (TNX) and tenascin-C (TNC) double knockout mice and compared findings in these mice with those in single knockout (TNX+/+TNC−/− and TNX−/−TNC+/+) mice. We investigated the proliferation and invasion of B16-BL6 melanoma cells after these cells had been injected into the footpads of mice in each group. The primary tumor size and invasion to the ankle adjacent to the primary tumor site were examined at 35 days after injection of the melanoma cells. The primary tumor size in TNX−/−TNC+/+ mice was significantly larger than that in wild-type mice, but those of TNX+/+TNC−/− and double knockout mice were comparable to that in the wild-type mice. On the other hand, invasion to the ankle was obviously promoted in TNX−/− TNC+/+ and double knockout mice compared with that in the wild-type mice, but invasion to the ankle in TNX+/+TNC−/− mice was only slightly promoted. Gelatin zymography confirmed increased matrix metalloproteinase (MMP)-9 activity in the dorsal skin of TNX−/−TNC+/+, TNX+/+TNC−/− and double knockout mice. However, the amounts of MMP-9 mRNA in the dorsal skins of all mice were almost the same, indicating that the increased activity of MMP-9 in the single and double knockout mice is regulated at the MMP-9 processing level. These results indicate that MMP-9 is activated in all TN-deficient mice, but that TNX exerts a greater effect on tumor invasion than does TNC.

Key words: Double knockout mice — Matrix metalloproteinase (MMP) — Tenascin-X — Tenascin-C

The extracellular matrix consists of a complex network of molecules that interact with cells to effect a wide range of cellular functions. During tumor proliferation and metastasis, extracellular matrix proteins act as substrates for tumor cell attachment and motility.[1] The tenascin (TN) family of extracellular matrix proteins is known to play an important role in progression of cancer.[2] So far, five members of the tenascin family have been identified in vertebrates: tenascin/cytotactin (tenascin-C, TNC), restrictin/J1-160/180 (tenascin-R, TNR), tenascin-X (TNX), tenascin-Y (TNY), and tenascin-W (TNW).[3–6] Members of the family have similar domain structures, i.e., an amino-terminal cysteine-rich region involved in oligomerization, an epidermal growth factor-like (EGF) domain, a series of fibronectin type III-like (FNIII) domains, and a fibrinogen-like domain at the carboxyl terminus. TNC and TNX have been reported to be involved in tumor malignancy.[7, 8]

TNC is a large extracellular matrix protein that is expressed in the developing brain, cartilage, and mesenchyme and is re-expressed in tumors, wound healing, and inflammation.[9] The expression level of TNC in the stroma of a brain tumor is up to 4 times higher than that in normal tissues.[10, 11] In general, TNC expression becomes stronger as the cancer malignancy advances.[12] Recently, Huang et al.[13] showed that interference by TNC with syndecan-4 binding to fibronectin blocks glioblastoma and breast carcinoma adhesion, resulting in the stimulation of tumor cell proliferation. However, in TNC-deficient (−/−) mice crossed with a mouse strain in which mice develop mammary tumors followed by metastasis, the occurrence of tumors and metastasis to the lungs was not different from that in TNC−/− or wild-type mice.[14] This finding implies that the expression of TNC with the advance of malignancy is a consequence of tumor progression and that TNC does not induce the progression of malignancy. Alternatively, the lack of a phenotype with regard to tumor progression in TNC−/− mice may be due to compensation by functionally homologous molecule(s).

TNX is the largest member in the tenascin family.[15–18] It is ubiquitously expressed, especially in skeletal muscle and the heart. In skin and tissues of the digestive tract, the distribution of TNX is often reciprocal to that of TNC.[19]

As for tumor malignancy, the expression of TNX is down-regulated in high-grade astrocytomas as the grade of malignancy progresses.[8] The same finding has been reported in swine malignant melanoma.[20] Strong TNX staining at the dermo-epidermal junction and in the dermis of the skin was detected, but this signal had almost completely disappeared in the tumor. Interestingly, this relationship between tumor malignancy and TNX expression is the opposite to that between tumor malignancy and TNC expression. To determine whether the deduced expression of TNX is a cause of the tumor progression or a consequence of tumor malignancy, we have generated TNX-deficient (−/−) mice by homologous recombination using embryonic stem cells and have injected highly invasive and metastatic melanoma B16-BL6 cells into the footpads of the mice to compare the findings with those in wild-type mice.[21] After 30 days, TNX−/− mice showed a significant promotion of tumor invasion and metastasis due to the increased activities of matrix metalloproteinase (MMP)-2 and MMP-9 compared to those in wild-type mice. This finding suggests that a lack of TNX is sufficient for tumor invasion and metastasis and that reduced expression of TNX is a cause of tumor progression.

In the present study, TNC−/−TNX−/− double knockout mice were generated, and experiments were carried out using these mice to determine the functional role of tenascin family proteins in tumor progression and to determine whether the lack of an obvious phenotype in TNC−/− mice with regard to tumor progression is due to compensation by TNX. It was found that TNX deficiency-induced tumor cell proliferation in the primary tumor site was suppressed by TNC deficiency. On the other hand, TNX deficiency-induced invasion of neighboring tissues was not accelerated by TNC deficiency. These findings suggest that TNC is not involved in tumor invasion, but that it suppresses TNX deficiency-induced tumor proliferation in the primary tumor site.

MATERIALS AND METHODS

TNX−/− and TNC−/− mice TNX−/− mice were created by *TNX* gene targeting in murine embryonic stem (ES) cells as described previously.[21] TNC−/− mice were originally generated using homologous recombination in ES cells to disrupt the *TNC* gene.[22] TNC−/− mice were further established by backcrossing original TNC−/− mice into a congenic line, C57BL/6.[23] Mice were housed at the Department of Animal Experimentation, Hokkaido University Graduate School of Pharmaceutical Sciences. This experiment was performed in accordance with the Hokkaido University Guide for the Care and Use of Laboratory Animals.

Generation of TNX−/−TNC−/− double knockout mice Since the *TNX* and *TNC* genes are localized on different chromosomes in the mouse genome,[15, 24] TNX−/−TNC−/− double knockout mice were generated by intercrossing of single knockouts. The breeding of F2 generation double heterozygous mice yielded TNX+/+TNC+/+, TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/− mice. For genotype analysis, genomic DNA was extracted from a tail biopsy sample, and then polymerase chain reaction (PCR) analysis was performed as described in a previous paper.[25] The primer sets used were as follows. For the detection of TNX wild-type allele, forward GT-KpnI primer 5′-AAGCACATGCAGCAGAGTGGG-GTC-3′ and reverse rGT-AflII primer 5′-GTGCCAGCT-TGGGCCGCAGTGGGAC-3′ were used. Following PCR analysis, an approximately 850-bp band was expected. For detection of TNX-targeted alleles, forward rSP2 primer 5′-AAGCTTCAAGTGCTTCACTACAGAGAGTT-3′ and reverse rPGK1 primer 5′-TAAAGCGCATGCTCCA-GACTGCCTTGGGAA-3′ were used. An approximately 1.7-kb band was expected. On the other hand, for detection of TNC wild-type alleles, forward G1-1 primer 5′-GGTACCTGATTCCGAAGTGCATTGTCACGT-3′ and reverse 3′TN primer 5′-AAGATGCCTGGCAGTAGC-CAGGTCAC-3′ were used. An approximately 0.9-kb band was expected. For detection of TNC-targeted alleles, forward G1-1 primer 5′-GGTACCTGATTCCGAAGTGCAT-TGTCACGT-3′ and reverse LacZ primer 5′-CTCCAT-GCTTGGAACAACGAGCGCAGC-3′ were used. An approximately 1.0-kb band was expected. All primers used for detection of TNC alleles were constructed according to the method described in a previous paper.[22] PCR products were then separated on 1.5% agarose gel.

Tumor lines A highly invasive and metastatic cell line, B16-BL6 melanoma cells, was initially established by Hart.[26] It was kindly provided by Dr. J. Hamada (Hokkaido University) and was maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), penicillin (10 units/ml) and streptomycin (10 μg/ml).

Injection of the melanoma cells into footpads A total of 2.5×10^5 B16-BL6 melanoma cells were suspended in 100 μl of phosphate-buffered saline (PBS) and subcutaneously injected into the footpad of each mouse. At 35 days after the injection, the degree of swelling of the primary tumor site and that of the neighboring ankle were measured with a slide caliper.

RNA purification and reverse transcription-PCR (RT-PCR) Dorsal skin from adult mice (3−4 months old) with each genotype was dissected, frozen, and powdered in liquid nitrogen. Total RNA was isolated, and poly(A)-rich RNA was selected using an mRNA purification kit (Amersham Biosciences, Tokyo). RT-PCR conditions were the same as those reported previously.[25] For the detection of TNX, TNC, and MMP-9 mRNAs, the following PCR

primers were used. For the detection of mouse TNX mRNA, forward fRT/X primer 5′-ATGGCAGCTCAGTG-CACCCCGTCTA-3′ and reverse rRT/X primer 5′-AAGA-CACCGTGGAGGCTGCAGAGGC-3′ were used. An approximately 410-bp band was expected. For the detection of mouse TNC mRNA, forward fRT/C primer 5′-ACCTGGCTACTGCCAGGCATCTTTC-3′ and reverse rRT/C primer 5′-CAGGTTGGAGGCAACAGCCTGTAC-3′ were used. An approximately 440-bp band was expected. For the detection of mouse MMP-9 mRNA, forward fRT/MMP9 primer 5′-CAGCCCCTGCTCCTG-GCTCTCCTG-3′ and reverse rRT/MMP9 primer 5′-ACTCGTCGTCGTCGAAATGGGCAT-3′ were used. An approximately 610-bp band was expected. To get quantitative information on MMP-9 mRNA expression in each group, we examined the PCR conditions which showed linear kinetics of amplification. The optimum conditions were as follows. Initial denaturation at 94°C for 1 min was followed by 25 PCR cycles at 98°C for 20 s and at 68°C for 5 min. After a final extension at 72°C for 10 min, PCR products were separated on 2% agarose gel.

Gelatin zymography The preparation of soluble and membrane fractions from the dorsal skin of mice of each genotype and gelatin zymography were carried out as described in a previous paper.[21] Briefly, the dorsal skin of each mouse was dissected and mixed with 200 μl of a solution containing 20 mM Hepes (pH 7.4), 250 mM sucrose, 10 mM EDTA, 0.01% (w/v) Triton X-100, and "Complete" protease inhibitor set (Roche Molecular Biochemicals, Indianapolis, IN), and then this solution was homogenized with a Polytron homogenizer (Central Scientific Commerce, Sapporo). The homogenate was centrifuged at 700g for 15 min at 4°C. The supernatant was then collected and centrifuged at 105 000g for 30 min at 4°C (soluble fraction). The pellet fraction was solubilized for 1 h on ice in 100 μl of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, "Complete" protease inhibitor set] and then centrifuged at 105 000g for 30 min at 4°C (membrane fraction). The protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL).

Samples were mixed with SDS sample buffer without a reducing agent, incubated for 30 min at 25°C, and separated on 10% SDS-PAGE gel containing 0.1% gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1.5 h and then incubated in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl$_2$, 0.02 mg/ml NaN$_3$, 1% Triton X-100, and 1 μM Zn(CHCOO)$_2$ at 37°C for 24 h. The gel was stained with 0.1% Coomassie Brilliant Blue R250.

Statistical analysis Statistical significance for the difference of survival rates after injection of melanoma between different genotypes was calculated according to the Kaplan-Meier method and differences were tested with the log-rank test by StatView software. Statistical evaluation of the differences of tumor cell proliferation and invasion in different genotypes was carried out using the Mann-Whitney U test. Differences were considered to be statistically significant when the P value was less than 0.05.

RESULTS

Generation of TNX−/−TNC−/− double knockout mice To understand the functional similarities and differences between the two family members, TNX−/−TNC−/− double knockout mice were generated by intercrossing of TNX−/− and TNC−/− single knockout mice. For identification of the genotype, PCR analysis was performed using each allele-specific primer. As shown in Fig. 1A, mice of each of the four genotypes (TNX+/+TNC+/+, TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/−) appeared in approximately Mendelian proportions. RT-PCR analysis using mRNA extracted from the dorsal skin confirmed the presence and absence of TNX and TNC mRNA transcripts consistent with the genotype (Fig. 1B).

TNX−/−TNC−/− mice did not exhibit any gross anatomical abnormalities, grew to normal size, were fertile, and had a normal life span. However, they showed hyperextensibility of their skin, as was found in TNX−/− mice.[27] More detailed analysis is being carried out.

Promotion of tumor invasion in TNX−/− single and TNX−/−TNC−/− double knockout mice We previously demonstrated that the degrees of invasion and metastasis of B16-BL6 melanoma cells subcutaneously injected into TNX−/− mice are greater than those in wild-type mice as a result of the increased activity of MMPs.[21] To determine the specific roles of TNX and TNC in tumor cell proliferation and invasion, highly invasive B16-BL6 melanoma cells were subcutaneously injected into footpads of TNX+/+TNC+/+, TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/− mice in the present study. We first examined the survival rates of mice of each genotype. The long-term survival curves for the four groups of mice are shown in Fig. 2. The TNX−/−TNC+/+, TNX−/−TNC−/−, and TNX+/+TNC−/− mice died at 39.8 days ($n=17$), 41.5 days ($n=17$), and 44.2 days ($n=18$) on average after the injection, respectively. In contrast, TNX+/+TNC+/+ mice survived significantly longer, for an average of 59.0 days, $n=21$. Log-rank test confirmed the different survival rates between wild-type (TNX+/+TNC+/+) and TNX−/−TNC+/+ ($P=0.0027$), TNX+/+TNC−/− ($P=0.0388$), TNX−/−TNC−/− ($P=0.0116$) mice, but no significant difference was detected among TNX−/−TNC+/+, TNX+/+TNC−/−, and TNX−/−TNC−/− mice ($P>0.33$).

Next, the degrees of swelling of the footpads at the primary tumor site in which B16-BL6 melanoma cells had been injected were measured at the 35 days after the injec-

tion (Fig. 3A). The TNX−/−TNC+/+ mice exhibited large degrees of swelling at the primary tumor site (mean±SE, 12.30±0.71 mm, n=6). On the other hand, the TNX+/+TNC−/− mice (9.75±1.30 mm, n=5) and

TNX−/−TNC−/− mice (9.96±1.00 mm, n=6) showed almost the same extent of swelling as that in the wild-type mice (10.13±0.76 mm, n=10). The difference between the degrees of swelling at the primary tumor site was statisti-
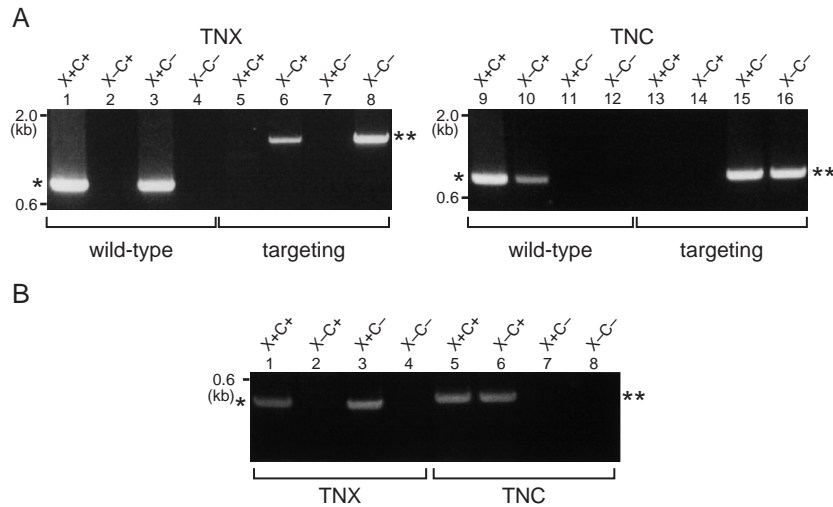

Fig. 1.   Genomic PCR and RT-PCR analysis of the four groups of mice. (A) Confirmation of the genotypes by genomic PCR analysis with allele-specific primers. Total genomic DNA was prepared from tail samples obtained from the four groups of mice. Then PCR analysis was performed using allele-specific primers, GT-KpnI and rGT-AflII (lanes 1−4), rSP2 and rPGK1 (lanes 5−8), G1-1 and 3′TN (lanes 9−12), and G1-1 and LacZ (lanes 13−16) for the TNX wild-type alleles, TNX targeted alleles, TNC wild-type alleles, and TNC targeted alleles, respectively. Following PCR analysis, an 850-bp band indicated by an asterisk, an 1.7-kb band indicated by two asterisks, an 0.9-kb band indicated by an asterisk, and a 1.0-kb band indicated by two asterisks were detected on 1.5% agarose gel for the TNX wild-type allele, TNX targeted allele, TNC wild-type allele, and TNC targeted allele, respectively. (B) Lack of TNX and TNC mRNA transcripts in TNX−/−TNC−/− double knockout mice. To confirm the presence and absence of TNX and TNC transcripts consistent with the genotype, RT-PCR analysis was performed using dorsal skin RNA. Following RT-PCR, a 410-bp band indicated by an asterisk and a 440-bp band indicated by two asterisks were detected on 2% agarose gels for TNX (lanes 1 and 3) and TNC (lanes 5 and 6) transcripts, respectively.
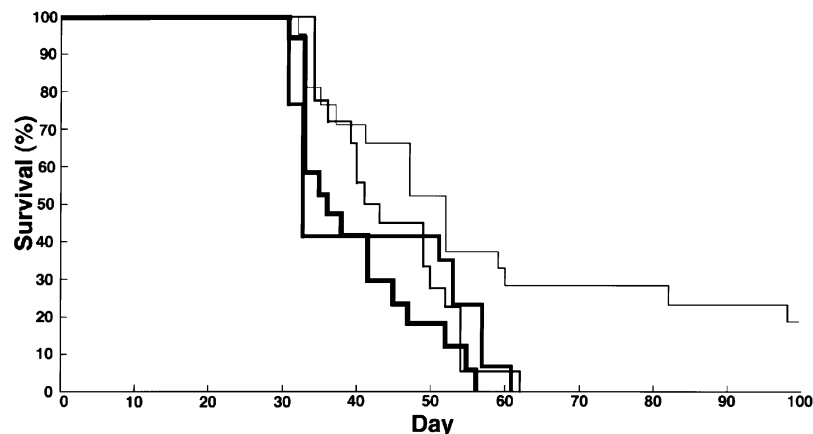

Fig. 2.   Long-term survival in the four groups of mice after injection of B16-BL6 melanoma cells into footpads. TNX+/+TNC+/+ mice, very thin line; TNX−/−TNC+/+ mice, very thick line; TNX+/+TNC−/− mice, thin line; TNX−/−TNC−/− mice, thick line.
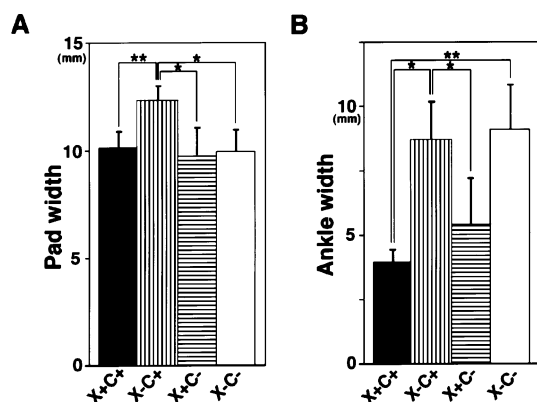
Fig. 3. Growth of B16-BL6 melanoma at the primary tumor site (A) and its invasion to the ankle (B) in the four groups of mice. (A) Swelling of the primary tumor site. B16-BL6 melanoma cells were subcutaneously injected into the footpads of mice in each group. At 35 days after injection, the degree of swelling of the primary tumor site was measured. (B) Invasion of melanoma cells to the ankle adjacent to the primary tumor site. At 35 days after the injection of B16-BL6 melanoma cells into the footpad, the degree of swelling of the ankle was measured. ∗∗ $P<0.01$ and ∗ $P<0.05$, Mann-Whitney U test.
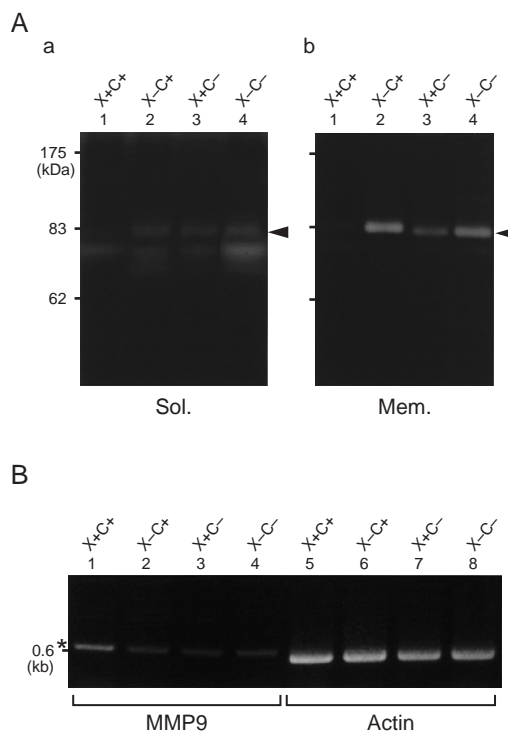

Fig. 4. Analysis of the activity of MMP-9 and the level of MMP-9 mRNA in the four groups of mice. (A) Increased activity of MMP-9 in the dorsal skin of TNX−/−TNC+/+ (lane 2), TNX+/+TNC−/− (lane 3), and TNX−/−TNC−/− (lane 4) mice compared with that in the dorsal skin of the wild-type (lane 1) mice. A soluble fraction (a) and membrane fraction (b) were prepared from homogenates of dorsal skins obtained from mice in the four groups, and they were analyzed for MMP activity by gelatin zymography on a 10% SDS-PAGE gel. Active MMP-9 is indicated with an arrowhead. (B) Levels of MMP-9 mRNA transcript in the four groups of mice. RT-PCR analysis was performed using dorsal skin RNA with MMP-9-specific primers. Following RT-PCR, we detected a 610-bp band, indicated by an asterisk, due to MMP-9 transcripts on 2% agarose gels (lanes 1–4). To confirm that equal RNA amounts were used for RT reactions, the level of actin mRNA transcript was examined by RT-PCR with actin-specific primers (lanes 5–8). Note that the levels of MMP-9 in mice of the four groups are almost the same (lanes 1–4).

cally significant ($P<0.05$ by the Mann-Whitney U test, TNX−/−TNC+/+ vs. other groups). This result indicates that TNC does not regulate tumor growth at the primary tumor site but that TNX deficiency-induced tumor cell proliferation at the primary tumor site is suppressed by TNC deficiency.

We next carried out experiments to determine whether invasion of melanoma cells to the ankle contiguous to the primary tumor-injected site in the footpad is dependent on the presence or absence of the TN family members. At 35 days after injection of melanoma cells into the footpad, the degree of swelling of the ankle was measured (Fig. 3B). Large degrees of swelling of the ankle were found in TNX−/−TNC+/+ mice (8.67±1.48 mm, $n=6$) and TNX−/−TNC−/− mice (9.09±1.74 mm, $n=6$) compared to those in TNX+/+TNC+/+ mice (3.95±0.52 mm, $n=11$). However, the degree of swelling of the ankle in TNX+/+TNC−/− mice (5.42±1.80 mm, $n=5$) was almost the same as that in TNX+/+TNC+/+ mice. This result indicates that TNC, in contrast to TNX, is not involved in tumor invasion.

Increased MMP-9 activities in TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/− mice To determine the molecular mechanisms by which tumor proliferation at the primary tumor-injected site in TNX−/−TNC+/+ mice and invasion in TNX−/−TNC+/+ and TNX−/−TNC−/− mice are promoted, we examined the activities of MMPs using gelatin zymography. Soluble

fractions containing MMP-2 (gelatinase A) and MMP-9 (gelatinase B) activities, as well as membrane fractions containing membrane-bound MMP activities, were prepared from dorsal skin homogenates of mice of each genotype. As shown in Fig. 4A, the gelatinolytic activities of active MMP-9 (82 kDa) in both fractions of TNX−/−TNC+/+, TNX+/+TNC−/−, and TNX−/−TNC−/− mice were significantly higher than those in control wild-type mice. However, activity of active MMP-2 form (64 kDa) was not detected in the dorsal skin homogenates

from mice of any genotype. Next, in order to determine whether the increased activity of MMP-9 detected in the skin homogenates of both single and double knockout mice is dependent on the amount of its RNA or arises through an activation process, total RNA from the dorsal skin of mice of each genotype was isolated, and RT-PCR analysis was performed using mouse MMP-9-specific primers. As shown in Fig. 4B, the amounts of MMP-9 RNA in the dorsal skin of the mice were almost the same, indicating that the increased activity of MMP-9 detected in the single and double knockout mice is due to promotion of the activity of MMP-9 during the activation process, not to an increase in the amount of MMP-9 mRNA at the transcriptional level. It is interesting that invasion to the ankle was not promoted, despite the increased activity of MMP-9 in TNX+/+TNC−/− mice compared with that in wild-type mice (Figs. 3B and 4B).

DISCUSSION

This paper represents the roles of two TN family members, TNX and TNC, in tumor proliferation and invasion. In TNX single knockout mice, promotion of both tumor proliferation at the primary tumor site and tumor invasion to the adjacent ankle was found, but no difference in tumor proliferation or invasion in TNC single knockout mice compared with those in wild-type mice was found. On the other hand, in TNX and TNC double knockout mice, TNX deficiency-induced tumor cell proliferation was suppressed. These results suggest that TNX is profoundly involved in tumor cell proliferation and invasion, whereas TNC does not participate in these processes, but it does play a role in the TNX deficiency-induced tumor cell proliferation.

In this study, we used homogenates from dorsal skins to examine the MMP activities. As shown in Fig. 4A, the amounts of active MMP-9 form in homogenates from dorsal skins of TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/− mice were significantly greater than in homogenates from dorsal skins of wild-type mice. However, in our previous study[21] we demonstrated that proMMP-9, proMMP-2 and active MMP-2 forms were increased in homogenates from footpads of TNX−/− mice compared with those of wild-type mice. The results, showing that different kinds of MMPs were activated in homogenates from dorsal skins and footpad skins of TNX−/− mice, might be due to the fact that homogenates were derived from different tissues or to the difference between the genetic backgrounds of the previous generated TNX−/− mice and the TNX−/−TNC+/+ mice used in the present study.

The activities of MMP-9 in TNX−/−TNC+/+, TNX+/+TNC−/−, and TNX−/−TNC−/− mice were greater than the activity in wild-type mice despite the fact that the levels of MMP-9 mRNA in all of the mice were the same. MMP-3 (also known as stromelysin-1) is the most efficient activator of MMP-9 identified to date, and it has been proposed as a candidate for a physiological activator of MMP-9 *in vivo*.[28] It is possible that either the level of MMP-3 mRNA is increased in single and double knockout mice or the efficiency of the conversion of proMMP-9 form to active MMP-9 form by MMP-3 is increased in both single and double knockout mice. Yang *et al.*[29] reported that thrombospondin-2 (TSP2)-null fibroblasts produced a twofold increase in MMP-2 activity and protein compared to those in wild-type cells and that this increase contributes to the adhesive defect in TSP2-null fibroblasts. TN family members (at least TNC) and TSP-2 are known to possess anti-adhesive activities. Thus, we speculate that the MMP activity is increased when an anti-adhesive component in extracellular matrices and on the cell surface is lacking.

In this study, it was shown that the tumor growth at the primary tumor site was enhanced only in TNX−/−TNC+/+ mice, whereas the survival rates were shortened in TNX+/+TNC −/− and TNX−/−TNC−/− mice as well as TNX−/−TNC+/+ mice bearing B16-BL6 melanoma cells as compared with that in the wild-type mice. At present, the reason why survival rates were decreased in TNX+/+TNC−/− and TNX−/−TNC−/− mice in spite of no promotion of tumor growth in the primary tumor site of these mice as compared with that in the wild-type mice is not clear. Although we do not know the role of TNX in the immune system, it is known that TNC inhibits *in vitro* T cell activation induced by antigens.[30] The role of TNC in inhibiting T cell activation *in vitro* is supported by the observation that TNC−/− mice develop severe dermatitis in response to hapten sensitization.[31] Thus, the differences in the host immuno-response among TNX−/−TNC+/+, TNX+/+TNC−/− and TNX−/−TNC−/− mice may explain the similar survival rates, despite the different tumor growth in the primary tumor site in these mice.

In summary, the results of our study have demonstrated that TNX is more significantly involved in tumor proliferation and invasion than is TNC, suggesting that a lack of TNX can not be functionally compensated for by TNC, and that TNX and TNC have distinct roles *in vivo*.

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