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

Ken-ichi Matsumoto,1 Kazuhisa Takahashi,1 Atsushi Yoshiki,2 Moriaki Kusakabe3,4 and Hiroyoshi Ariga1

1Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6, Kita-ku, Sapporo, Hokkaido 060-0812, 2Experimental Animal Division, Bio Resource Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, 3ANB Tsukuba Institute, ALOKA Co., Ltd., 1103 Fukaya, Kawanagaura-machi, Niihari-gun, Ibaraki 300-0134 and 4Department of Anatomy, Jikei University School of Medicine, 3-25-8 Nishi-Shinbashii, Minato-ku, Tokyo 105-8461

The roles of extracellular matrix glycoproteins belonging to the tenasin family in the regulation of tumor cell proliferation, invasion, and metastasis are not known. To address this issue, we generated tenasin-X (TNX) and tenasin-C (TNC) double knockout mice and compared findings in these mice with those of single knockout (TNX+/− or TNX−−/− and TNX−−/− or 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−−/− or TNC+/+ mice was significantly larger than that in wild-type mice, but those of TNX−−/− or 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−−/− or TNC+/+ and double knockout mice compared with that in the wild-type mice, but invasion to the ankle in TNX−−/− or 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) — Tenasin-X — Tenasin-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.11 The tenasin (TN) family of extracellular matrix proteins is known to play an important role in progression of cancer.2) So far, five members of the tenasin family have been identified in vertebrates: tenasin/cytotactin (tenasin-C, TNC), restrictin/J1-160/180 (tenasin-R, TNR), tenasin-X (TNX), tenasin-Y (TNY), and tenasin-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 tenasin 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

E-mail: kematsum@pharm.hokudai.ac.jp
As for tumor malignancy, the expression of TNX is down-regulated in high-grade astrocytomas as the grade of malignancy progresses. The same finding has been reported in swine malignant melanoma. 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. 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 TNX deficiency. On the other hand, TNX deficiency-induced invasion of neighboring tissues was not accelerated by TNX 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. TNC−/− mice were originally generated using homologous recombination in ES cells to disrupt the TNC gene. TNC−/− mice were further established by backcrossing original TNC−/− mice into a congenic line, C57BL/6. 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, 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. The primer sets used were as follows. For the detection of TNX wild-type allele, forward GT-Kpnl primer 5′-AAGCACATGCAGCAGGTTGGG-GTC-3′ and reverse rGT-AflII primer 5′-GTCGACGT-TGGCCCGAGTGGGAC-3′ were used. Following PCR analysis, an approximately 850-bp band was expected. For detection of TNX-targeted alleles, forward rSP2 primer 5′-AACGTTCAAGTCATCAGAGAGTT-3′ and reverse rPGK1 primer 5′-TAAAGGCATGCTCAGACTGCGT-TGGGAG-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′-GGTACCTGATTCGAAGTGCATTGTCACGT-3′ and reverse rGT-AflII primer 5′-TAAAGGCATGCTCAGACTGCGT-TGGGAG-3′ and reverse 3′TN primer 5′-AAGATGCTCTGGCAGTACGAGTGCAC-3′ were used. An approximately 0.9-kb band was expected. For detection of TNC-targeted alleles, forward G1-1 primer 5′-GGTACCTGATTCGAAGTGCATTGTCACGT-3′ and reverse LacZ primer 5′-CTCCATGCTGGAAACCGAGGCCAGC-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. 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. 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 2.5×10⁵ 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 was 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 (Amerham Biosciences, Tokyo). RT-PCR conditions were the same as those reported previously. 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/T primer 5′-ATGGCGAGCTCATGTGACCCCGTGCTA-3′ and reverse rRT/T primer 5′-AAGACACCGTGAGGCTGCAAGGCG-3′ were used. An approximately 410-bp band was expected. For the detection of mouse TNC mRNA, forward fRT/C primer 5′-ACCTGGCTACTGCGACCATCTTTTC-3′ and reverse rRT/C primer 5′-CAGGTTGGAGGCAACAGCCTGTAC-3′ were used. An approximately 440-bp band was expected. For the 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. 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. 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’T (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.
Increased MMP-9 activities in 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−/− 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−/− (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).
than the activity in wild-type mice despite the fact that the activity in the present study. However, in our previous study21) we demonstrated that were derived from different tissues or to the difference between the genetic backgrounds of the previous generation21) and 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 study21) 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 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 increase 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.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Dr. J. Hamada (Hokkaido Univ.) for providing B16-BL6 melanoma cells. We also thank Kiyomi Takaya and Yoko Misawa for their technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

(Received May 20, 2002/Revised June 28, 2002/Accepted July 10, 2002)
REFERENCES

1) Fidler, J. L. and Nicholson, G. L. The process of cancer invasion and metastasis. Cancer Bull., 39, 126–131 (1987).
2) Sakakura, T. and Kusakabe, M. Can tenascin be redundant in cancer development? Perspect. Dev. Neurobiol., 2, 111–116 (1994).
3) Erickson, H. P. Tenascin-C, tenasin-R and tenasin-X: a family of talented proteins in search of functions. Curr. Opin. Cell Biol., 5, 869–876 (1993).
4) Chiquet-Ehrismann, R., Hagios, C. and Matsumoto, K. The tenasin gene family. Perspect. Dev. Neurobiol., 2, 3–7 (1994).
5) Hagios, C., Koch, M., Spring, J., Chiquet, M. and Chiquet-Ehrismann, R. Tenasin-Y: a protein of novel domain structure is secreted by differentiated fibroblasts of muscle connective tissue. J. Cell Biol., 134, 1499–1512 (1996).
6) Weber, P., Montag, D., Schachner, M. and Bernhardt, R. R. Zebrafish tenasin-W, a new member of the tenasin family. J. Neurobiol., 35, 1–16 (1998).
7) Mackie, E. J., Chiquet-Ehrismann, R., Pearson, C. A., Inaguma, Y., Taya, K., Kawarada, Y. and Sakakura, T. Tenasin is a stromal marker for epithelial malignancy in the mammary gland. Proc. Natl. Acad. Sci. USA, 84, 4621–4625 (1987).
8) Hasegawa, K., Yoshida, T., Matsumoto, K., Katsuta, K., Waga, S. and Sakakura, T. Differential expression of tenasin-C and tenasin-X in human astrocytomas. Acta Neuro-pathol., 93, 431–437 (1997).
9) Erickson, H. P. and Bourdon, M. A. Tenasin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. Annu. Rev. Cell Biol., 5, 71–92 (1989).
10) Higuchi, M., Ohnishi, T., Arita, N., Hiraga, S. and Hayakawa, T. Expression of tenasin in human gliomas: its relation to histological malignancy, tumor differentiation and angiogenesis. Acta Neuro-pathol., 85, 481–487 (1993).
11) Zagzag, D., Friedlander, D. R., Miller, D. C., Dosik, J., Cangiarella, J., Kostianovsky, M., Cohen, H., Grumet, M. and Greco, M. A. Tenasin expression in astrocytomas correlates with angiogenesis. Cancer Res., 55, 907–914 (1995).
12) Sakakura, T., Ishihara, A. and Yatani, R. Tenasin in mammary gland development: from embryogenesis to carcinogenesis. In “Regulatory Mechanisms in Breast Cancer,” ed. M. Lippmann and R. Dickson, pp. 383–400 (1991). Kluwer Academic Publ., Boston.
13) Huang, W., Chiquet-Ehrismann, R., Moyano, J. V., Garcia-Pardo, A. and Orend, G. Interference of tenasin-C with syndecan-4 binding to fibronectin blocks cell adhesion and stimulates tumor cell proliferation. Cancer Res., 61, 8586–8594 (2001).
14) Talts, J. F., Wirl, G., Dictor, M., Muller, W. J. and Fassler, R. Tenasin-C modulates tumor stroma and monocyte/macrophage recruitment but not tumor growth or metastasis in a mouse strain with spontaneous mammary cancer. J. Cell Sci., 112, 1855–1864 (1999).
15) Matsumoto, K., Arai, M., Ishihara, N., Ando, A., Inoko, H. and Ikemura, T. Cluster of fibronectin type III repeats found in the human major histocompatibility complex class III region shows the highest homology with the repeats in an extracellular matrix protein, tenasin. Genomics, 12, 485–491 (1992).
16) Matsumoto, K., Ishihara, N., Ando, A., Inoko, H. and Ikemura, T. Extracellular matrix protein tenasin-like gene found in human MHC class III region. Immunogenetics, 36, 400–403 (1992).
17) Bristow, J., Tee, M. K., Gitelman, S. E., Mellon, S. H. and Miller, W. L. Tenasin-X: a novel extracellular matrix protein encoded by the human XB gene overlapping P450c21B. J. Cell Biol., 122, 265–278 (1993).
18) Lethias, C., Descollonges, Y., Boutillon, M. M. and Garrone, R. Flexilin: a new extracellular matrix glycoprotein localized on collagen fibrils. Matrix Biol., 15, 11–19 (1996).
19) Matsumoto, K., Saga, Y., Ikemura, T., Sakakura, T. and Chiquet-Ehrismann, R. The distribution of tenasin-X is distinct and often reciprocal to that of tenasin-C. J. Cell Biol., 125, 483–493 (1994).
20) Geffroin, C., Horak, V., Crèchet, F., Traicud, Y., Lethias, C., Vincent-Naultreau, S. and Vielh, P. Opposite regulation of tenasin-C and tenasin-X in MeliM swine heritable cutaneous malignant melanoma. Biochim. Biophys. Acta, 1524, 196–202 (2000).
21) Matsumoto, K., Takayama, N., Ohnishi, J., Ohnishi, E., Shirayoshi, Y., Nakatsuji, N. and Ariga, H. Tumour invasion and metastasis are promoted in mice deficient in tenasin-C. Genes Cells, 6, 1101–1111 (2001).
22) Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. Mice develop normally without tenasin. Genes Dev., 6, 1821–1831 (1992).
23) Nakao, N., Hiraiwa, N., Yoshiki, A., Ike, F. and Kusakabe, M. Tenasin-C promotes healing of Habu-snake venom-induced glomerulonephritis: studies in knockout congenic mice and in culture. Am. J. Pathol., 152, 1237–1245 (1998).
24) Pilz, A., Moseley, H., Peters, J. and Abbott, C. Comparative mapping of mouse chromosome 4 and human chromosome 9: Lv, Orm, and Hxb are closely linked on mouse chromosome 4. Mamm. Genome, 3, 247–249 (1992).
25) Ikuta, T., Sagowa, N., Ariga, H., Ikemura, T. and Matsumoto, K. Structural analysis of mouse tenasin-X: evolutionary aspects of reduplication of FNIII repeats in the tenasin gene family. Gene, 217, 1–13 (1998).
26) Hart, I. R. The selection and characterization of an invasive variant of the B16 melanoma. Am. J. Pathol., 97, 587–600 (1979).
27) Mao, J. R., Taylor, G., Dean, W. B., Wagner, D. R., Afzal, V., Lotz, J. C., Rubin, E. M. and Bristow, J. Tenasin-X deficiency mimics Ehlers-Danlos syndrome in mice through
alteration of collagen deposition. *Nat. Genet.*, **30**, 421–425 (2002).

28) Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D. L. and Quigley, J. P. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J. Biol. Chem.*, **274**, 13066–13076 (1999).

29) Yang, Z., Kyriakides, T. R. and Bornstein, P. Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. *Mol. Biol. Cell.*, **11**, 3353–3364 (2000).

30) Ruegg, C. R., Chiquet-Ehrismann, R. and Alkan, S. S. Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. *Proc. Natl. Acad. Sci. USA*, **86**, 7437–7441 (1989).

31) Koyama, Y., Kusubata, M., Yoshiki, A., Hiraiwa, N., Ohashi, T., Irie, S. and Kusakabe, M. Effect of tenascin-C deficiency on chemically induced dermatitis in the mouse. *J. Invest. Dermatol.*, **111**, 930–935 (1998).