Extracellular matrix protein Tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration

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Abstract

The bone marrow (BM) microenvironment is required for the maintenance, proliferation, and mobilization of hematopoietic stem and progenitor cells (HSPCs), both during steady-state conditions and hematopoietic recovery after myeloablation. The extracellular matrix (ECM) meshwork has long been recognized as a major anatomical component of the BM microenvironment; however, the molecular signatures and functions of the ECM to support HSPCs are poorly understood. Of the many ECM proteins, the expression of Tenascin-C was found to be dramatically upregulated during hematopoietic recovery after myeloablation. The TN-C gene was predominantly expressed in stromal cells and endothelial cells, known as BM niche cells supporting the function of HSPCs. Mice lacking Tenascin-C (TN-C/-) showed normal steady-state hematopoiesis; however, TN-C/- mice failed to reconstitute hematopoiesis after BM ablation and showed high lethality. The capacity to support transplanted wild-type hematopoietic cells to regenerate hematopoiesis was reduced in TN-C/- recipient mice. 

In vitro culture on a Tenascin-C substratum promoted the proliferation of HSPCs in an integrin α9-dependent manner, and upregulated the expression of the cyclins (cyclinD1 and E1) and downregulated the expression of the CDK inhibitors (p57Kip2, p21Cip1, p16Ink4a). Taken together, these results identify Tenascin-C as a critical component of bone marrow microenvironment required for hematopoietic regeneration.
Introduction

The bone marrow (BM) is the main hematopoietic organ in the adult. It provides an efficient microenvironment for hematopoiesis, which contributes to the maintenance, proliferation, and differentiation of hematopoietic stem cells and progenitor cells (HSPCs). A well-accepted concept regarding the hematopoietic microenvironment is that of the hematopoietic stem cell (HSC) niche.\textsuperscript{1-3} The HSC niche is subdivided into the osteoblastic niche\textsuperscript{4-7} and the vascular niche.\textsuperscript{8,9} The BM vasculature is surrounded by perivascular niche cells such as macrophages\textsuperscript{10,11} and stromal cells (i.e., reticular cells) of mesenchymal lineage,\textsuperscript{12,13} which cooperatively regulate HSC activity.

By contrast to the well investigated cellular niches, the functions of extracellular matrix (ECM) proteins as a niche are poorly understood. The ECM of the BM comprises fibrous proteins such as types I and IV collagen and fibronectin (FN)\textsuperscript{14} and non-fibrous proteins such as tenascin-C (TN-C).\textsuperscript{15} Previously, we showed that long-term BrdU-label-retaining cells reside in the hypoxic areas distant from the endothelial tubes, closely attached to non-endothelial ECM structures.\textsuperscript{16} In vitro culture systems also suggest the importance of the ECM in the maintenance of HSPCs.\textsuperscript{17} Therefore, a role for the ECM as a BM niche has been suggested, yet little is known about how the ECM affects HSPCs \textit{in vivo}. 
TN-C is a highly conserved ECM glycoprotein expressed mainly during embryogenesis.\(^\text{18}\) TN-C-deficient mice show normal development, with no defects in gross organization.\(^\text{18}\) In adult tissues, TN-C expression is restricted to sites of active tissue remodeling (e.g., inflammation\(^\text{19,20}\) and wound healing\(^\text{21}\)) and plays a significant function in these pathologies.\(^\text{19-21}\) Expression of TN-C in the BM is limited to the endosteal regions.\(^\text{15,22}\) TN-C acts by binding with high affinity to specific integrins, such as integrin \(\alpha_9\), or to other matrix proteins such as FN.\(^\text{23}\) Colony-forming capacity of BM cells is lower in TN-C-deficient mice, although their mononuclear cell count and BM architecture show no detectable abnormalities, suggesting a significant function of TN-C in stressed conditions.\(^\text{24}\) Neutralizing integrin \(\alpha_9\) inhibits HSPC proliferation and adhesion to primary cultured osteoblasts.\(^\text{25}\) TN-C displays cytoadhesive properties toward hematopoietic cells in \textit{in vitro} adhesion assays,\(^\text{15}\) however, the role played by TN-C during hematopoiesis \textit{in vivo} is still unclear.

In the present study, we identify a prominent role for TN-C in BM reconstitution after myeloablation. TN-C expression in the BM was highly upregulated, becoming much more widely distributed after 5-FU administration. TN-C-deficient mice (\(TN-C^{-/-}\)) showed impaired BM recovery and high lethality after myeloablation. Also, TN-C promoted the proliferation of HSPCs both \textit{in vivo} and \textit{in vitro}. Taken together, the results of this study demonstrate a requirement for TN-C in the BM microenvironment.
primed for hematopoietic regeneration.

Methods

Mice

All mice were of a C57BL/6 background. *TN-C-deficient* mice were obtained from RIKEN Bio resource center. All animal experiments were approved by Keio University and performed in accordance with the Guidelines of Keio University for Animal and Recombinant DNA experiments.

5-FU administration and sub-lethal irradiation

Myeloablation was induced by either 5-FU (Sigma-Aldrich; intraperitoneal (i.p.) injection at 250 mg/kg body weight) or sub-lethal irradiation (6.5 Gy). Peripheral blood counts were measured at 3 day intervals. Briefly, peripheral blood was collected from the tail vein in a heparinized microtube (Drummond Scientific) and analyzed using CellTac (NIHON KOHDEN). Mice were sacrificed at the indicated times after 5-FU administration.
Antibodies

The primary antibodies used for immunohistochemistry (IHC) were hamster anti-CD31 (2H8; Millipore), rat anti-CD31 (MEC13.3; Becton Dickinson (BD)), and anti-tenascin-C (Abcam). The primary polyclonal antibodies were fibronectin (DAKO), collagen IV (Cosmo Bio), laminin (Sigma-Aldrich) and c-Kit (R&D). Secondary antibodies were Alexa 488 fluorescence-conjugated IgGs (Molecular Probes) or Cy3/Cy5/DyLight549/DyLight649-conjugated IgGs (Jackson ImmunoResearch). Specimens were treated with DAPI (Molecular Probes) for nuclear staining. For blocking the function of integrin $\alpha_9$ in vitro, hamster monoclonal antibodies against murine integrin $\alpha_9$ (clone: 55A2C) \textsuperscript{27} were used. As a negative control study, we used anti-integrin $\alpha_9$ antibodies (clone: 18R18D) \textsuperscript{27} that does not have such an inhibitory effect.

Immunostaining of bone marrow

Isolated femurs were fixed in 4% PFA in PBS overnight at 4°C and then immersed in 0.5 M EDTA solution for at least 7 days for decalcification. Femurs were embedded in OCT compound (Tissue Tek) and cryosections (12 μm) were made. For IHC, samples were stained with primary antibodies overnight at 4°C. Staining with secondary antibodies was done for 1 hour at room temperature. Thereafter, samples were
post-fixed in 4% PFA and mounted using a Prolong Antifade Kit (Molecular Probes). For the BrdU incorporation assay, 100 μg of BrdU (BD Pharmingen) per gram of body weight was dissolved in sterile PBS and injected i.p. 2 h before sacrifice. Prepared sections were stained using a BrdU immunohistochemistry system (Calbiochem).

**Confocal microscopy**

Fluorescence images were obtained using a confocal laser-scanning microscope (FV1000; Olympus). Scanning was performed in sequential laser emission mode to avoid scanning at other wavelengths.

**Quantitative-PCR assay**

Total RNA was prepared from bone marrow at the indicated times after 5-FU administration and reverse transcribed using Superscript II (Invitrogen). Quantitative PCR assays were performed using an ABI 7500 Fast Real-Time PCR System, TaqMan Fast Universal PCR master mixture (Applied Biosystems, Foster City, CA), and a TaqMan® Gene Expression Assay Mix comprising *cyclinD1* (Mm03053889_s1), *cyclinD2* (Mm00438070_m1), *cyclinD3* (Mm01612362_m1), *cyclinEl* (Mm01266311_m1), *cyclinE2* (Mm00432367_m1), *cyclinG1* (Mm00438084_m1),
cyclinG2 (Mm01354285_m1), p21\(^{Cip1}\) (Mm00432448\_m1), p57\(^{Kip2}\) (Mm01272135\_g1), p16\(^{Ink4a}\) (Mm00494449\_m1), p16\(^{Indc}\) (Mm00483243\_m1), fn1 (Mm01256744\_m1), itgna4 (Mm00439770\_m1), itgna5 (Mm00439797\_m1), itgna7 (Mm00434400\_m1), itgna9 (Mm00519293\_m1), itgnav (Mm00434506\_m1), itgnb1 (Mm01253227\_m1), itgnb3 (Mm00443980\_m1), itgnb6 (Mm01269869\_m1), c-myc (Mm00487803\_m1) and tn-c (Mm00495662\_m1). A mouse β-actin (Mm00607939\_sl) assay mix served as an endogenous control. Data was analyzed using 7500 Fast System SDS Software 1.3.1. Each experiment was performed with four replicates from each sample and the results were averaged.

**Flow cytometry**

Dissected tibias and femurs were flushed with 2% fetal calf serum in PBS using a 23G needle. The dissociated bone marrow cells were collected and bone marrow mononuclear cells (BMMNCs) isolated by density centrifugation on Lymphoprep (Axis-Shield). BM MNCs were pre-incubated with Fc block (BD, Franklin Lakes, NJ) to avoid nonspecific binding of antibodies, and then incubated with the intended antibodies. The primary antibodies (purchased from BD) were anti-c-Kit (2B8), -Sca-1 (E13-161.7), -CD4 (L3T4), -CD8 (53-6.72), -B220 (RA3-6B2), -TER-119, Gr-1 (RB6-8C5), -CD11b (M1/70), -CD3 (500A2), -Flt3 (AF10.1), -CD31 (MEC13.3), -CD41 (MWRReg30),
CD45.2 (104) and CD45.1 (A20). Primary antibodies from other manufactures included anti-integrin α9 (R&D systems AF3827), -integrin β1 (BD, Ha2/5), -CD34 (eBioscience KAM34), -PDGFRα (eBioscience APA5), -CD48 (Biolegend B120132), and -CD150 (Biolegend TC15-12F12.2). A mixture of CD4, CD8, B220, TER-119, Mac-1, and Gr-1 was used as the lineage mix (Lin). Propidium iodide was used to identify and exclude dead cells. Stained cells were analyzed and sorted using a SORP FACSaria (BD) and the data analyzed with FlowJo software (TreeStar, Ashland, OR).

For cell cycle analyses of HSCs, BrdU (1 mg) was injected i.p. four times at 12 hour intervals prior to sacrifice. HSC fractions were sorted, fixed on MAS-coated slides (Matsunami Japan) and stained using a BrdU IHC system (Calbiochem).

**BM transplantation (BMT)**

BMMNCs were obtained from either *TN-C*+/+ or *TN-C*−/− mice as described above. *TN-C*+/+ or *TN-C*−/− mice-derived BMMNCs (2 × 10⁵ cells or 1 × 10⁴ cells) were transplanted into lethally-irradiated Ly5.1 mice. For reverse BMT, BMMNCs from C57Bl/6-Ly5.1 mice (2 × 10⁵) were transplanted into lethally-irradiated *TN-C*+/+ or *TN-C*−/− mice (Ly5.2). Peripheral blood chimerisms in recipient mice were analyzed at 1 month intervals. Recipient mice were sacrificed for analyses 4 months after BMT.
LSK cell culture on TN-C substrata

Recombinant full-length human TN-C (R&D systems) or recombinant proteins of a fibronectin type III repeat domain of human TN-C lacking the arginine-glycine-aspartic acid (RGD) sequence (TN-C\textsuperscript{FNIII})\textsuperscript{27} at a concentration of 2 μg/cm\textsuperscript{2} was coated onto FN-coated culture slides (BD Bioscience) for 1 hour at 37\textdegree{}C. FACS sorted LSK cells were obtained and cultured on FN with or without TN-C/TN-C\textsuperscript{FNIII} in SF-O3 medium (Sanko Junyaku Co., Ltd.) containing 100 ng/ml SCF and 100 ng/ml thrombopoietin for 48 hours. Prior to staining, cultured cells were incubated with BrdU (3 μg/ml medium) for 2 hrs and then fixed with methanol and stained using a BrdU IHC system (Calbiochem). For neutralizing \(\alpha_9\) integrin, culture medium was supplemented with hamster monoclonal antibodies against murine integrin \(\alpha_9\) (55A2C or 18R18D) at a concentration of 10μg/ml as described previously.\textsuperscript{27}

Western blotting

Western blot analysis was performed as previously described \textsuperscript{28} using anti-TN-C (Abcam) as the primary antibody. The amount of total protein was examined by re-blotting with anti-β-actin (Sigma).
Statistical analysis

All results are expressed as the mean ± SD. The two-tailed Student's t test and log rank test were used for comparisons between two-group experiments. The Wilcoxon signed-rank test was performed on complete blood counts (CBCs) after 5-FU administration.

RESULTS

TN-C is upregulated and widely distributed in the bone marrow during hematopoietic recovery after myeloablation

First, we examined the BM expression of various ECM proteins during steady-state hematopoiesis, immediately after myeloablation, and during hematopoietic recovery. As previously reported, injection of 5-FU resulted in a marked reduction of BM cellularity on Day 2 post-5-FU administration, and recovery was evident by Day 10 (Supplementary Figure 1A-C). A reduction in (Day 2) and recovery of (Day 10) BM c-Kit⁺ HSPC numbers was also noted (data not shown). Only a moderate distortion of ECM components (fibronectin, laminin and type IV collagen) was noted at Day 10 (Supplementary Figure 1D-L). TN-C expression showed a far more drastic change during hematopoietic recovery compared with that of the other ECM molecules. Before 5-FU administration, and as previously reported, TN-C expression was limited to the
periosteal regions (Figure 1A, D & G) with abundant expression in the trabecular bone-rich metaphyseal regions compared with the diaphyseal regions. TN-C protein was detected both on the bone surface (Figure 1G, arrows) and in the stromal regions near to the bone (Figure 1G, arrowheads). TN-C expression did not change on Day 2 (Figure 1B, E) but was markedly upregulated on Day 10 after 5-FU administration. TN-C expression increased and the protein was widely distributed throughout both the metaphyseal (Figure 1C) and diaphyseal (Figure 1F & H) regions. TN-C proteins were detected in the central stromal (Figure 1H, arrowheads) and endosteal regions (Figure 1H, arrows). HSPCs labeled by c-Kit antibodies were observed in close contact with TN-C proteins (Figure 1I). Laminin was stained to highlight the endothelial cell basement membrane (Supplementary Figure 1G–I) to discriminate perivascular TN-C from TN-C expressed further from the vasculature (Figure 1I). HSPCs resided in close contact with TN-C expressed in both the locations, suggesting a functional association between HSPCs and TN-C rather than simply reflecting the well-known association between HSPCs and endothelial cells.8,9 The increase in TN-C expression after 5-FU administration was confirmed by Western blotting (Figure 1J, K). TN-C expressions returned to steady state levels by Day 21 (Figure 1J, K).

TN-C is predominantly expressed in stromal and endothelial cells, and its ligand,
integron α9, is expressed on HSPCs

Next, we performed a detailed analysis of cellular TN-C mRNA expressions. TN-C mRNA expression in the entire BM was markedly upregulated after 5-FU administration (Figure 2A). The immunofluorescence data (Figure 1I) indicated that TN-C was produced by endothelial cells, along with some other lineage cells. PDGFRα is expressed in BM stromal cells.29 A prominent upregulation in TN-C gene expression was noted in CD45+CD31+ endothelial cell and CD45+PDGFRα+ stromal cell populations after 5-FU administration. The CD45+, CD11b+ or Lin-Sca-1+c-Kit+ (LSK) cell populations showed far lower expression than stromal and endothelial cells although these cell populations also exhibited significant upregulation of TN-C after 5-FU administration (Figure 2B, Supplementary figure 2). CXCL-12-abundant reticular cells (CAR cells), a subpopulation of stromal cells, are required for the proliferation and maintenance of HSCs.12,30 IHC of BM samples from Cxcl12+/EGFP knock-in mice showed that a portion of CAR cells were positive for TN-C (Figure 2C-E, arrowheads). TN-C mRNA was much more abundant in CAR cells than in Cxcl12-negative cells and endothelial cells (Figure 2F), suggesting that CAR cells are a major cellular source of TN-C.

TN-C exerts its functions by binding to multiple integrins and ECM components.31 Of these, integrin α9 binds to the FN-III domain of TN-C and promotes
the proliferation of neurons. Whole-BM mRNA transcript levels for \( \text{itgn} \alpha 9, \alpha V, \alpha 5, \alpha 7, \beta 3, \beta 6 \) were significantly upregulated during hematopoietic recovery (Figure 2G, J, K-N). In particular \( \text{itgn} \alpha 9 \) showed prompt and explicit upregulation as early as 2 days after 5-FU treatment, whereas \( \text{itgn} \alpha V, \alpha 5, \alpha 7, \beta 6 \) exhibited more gradual and moderate upregulation. The transcription levels of other integrins, such as \( \text{itgn} \beta 1 \) (Figure 2H) and \( \text{itgn} \alpha 4 \) (Figure 2I), were not significantly upregulated. In accordance with findings of Nilsson and colleagues, flow cytometry analysis revealed that LSK cells expressed high levels of integrin \( \alpha 9 \) (Figure 2O). Integrin \( \beta 1 \), a binding partner of integrin \( \alpha 9 \), was also highly expressed in HSC fractions (Figure 2O). In quantification of mean fluorescence, integrin \( \beta 1 \) was strongly expressed in primitive HSCs (Flt3\( ^+ \)CD34\( ^+ \) or Flt3\( ^- \)CD34\( ^- \) LSKs) compared to the Flt3\( ^+ \)CD34\( ^+ \) cell fraction, although integrin \( \alpha 9 \) was evenly expressed in those subpopulations (Figure 2P). Taken together, these results suggest that the expression of TN-C is predominant in stromal and endothelial cells, and its ligand, integrin \( \alpha 9 \beta 1 \), is highly expressed on HSPCs.

**TN-C-deficient mice show defects in hematopoietic recovery after 5-FU treatment and sub-lethal irradiation**

The expression analysis described in the previous section suggested that TN-C functions during BM reconstitution. Before analyzing the function of TN-C during hematopoietic
recovery, we checked the steady-state status of \( TN-C^{+/+} \) mice. \( TN-C^{-/-} \) mice did not show apparent defects in steady state hematopoietic parameters, including peripheral blood counts, total BM cellularity, HSC frequency, and BM lineage composition (Figure 3A-K). For assessing the proliferation rate in HSPCs, we stained LSK cells from \( TN-C^{+/+} \) and \( TN-C^{-/-} \) mice with Ki67 and Hoechst 33342 and found no significant difference in cell cycle frequencies (Figure 3L, M).

We next challenged TN-C-deficient mice with 5-FU-induced myeloablation. TN-C immunoreactivity, which was detected in wild-type control (\( TN-C^{+/+} \)) mice, was not detected in \( TN-C^{-/-} \) mice (Figure 4A, B). This confirmed the specificity of the TN-C antibody used. \( TN-C^{-/-} \) mice showed a significantly high level of lethality (Figure 4C) compared with \( TN-C^{+/+} \) mice after 5-FU administration, and peripheral blood counts showed a significant delay in the recovery of white blood cell, hemoglobin and platelet counts (Figure 4D-F). No differences in BM lineage frequencies were noted during hematopoietic recovery after 5-FU (Figure 4G). Within the BM of the \( TN-C^{-/-} \) mice, c-Kit+ HSPCs were dramatically reduced in number compared with those in \( TN-C^{+/+} \) mice (Figure 4H, I, L). Furthermore, BrdU incorporation into c-Kit+ HSPCs was significantly lower in the \( TN-C^{-/-} \) BM (Figure 4J, K, M). Flow cytometry revealed a significantly lower LSK cell frequency in \( TN-C^{-/-} \) mice on day 10 after 5-FU (Figure 4N, O). Similarly, increased lethality and impairment of hematopoietic recovery was
observed in \(TN-C^{-/-}\) mice when myeloablation was induced by sub-lethal irradiation (6.5 Gy) \(\text{(Figure 4P)}\).

**TN-C\(^{-/-}\) mice are vulnerable recipients to BMT**

Regarding the susceptibility to 5-FU and irradiation of \(TN-C^{-/-}\) mice \(\text{(Figure 4)}\), the expression pattern of TN-C \(\text{(Figure 2)}\) indicated that TN-C is particularly important in the non-hematopoietic BM stroma. To test this hypothesis, a series of BMT were conducted. First, donor cells \((2 \times 10^5 \text{ BMMNCs})\) isolated from \(TN-C^{+/+}\) or \(TN-C^{-/-}\) mice (Ly5.2) were transplanted into lethally-irradiated Ly5.1 mice \((n = 10 \text{ for each group})\). There was no difference in the survival rates between the groups \(\text{(data not shown)}\). Examination of peripheral blood chimerism at one month intervals also showed no difference between the two groups \(\text{(Figure 5A)}\). BM cellularity, LSK cell frequency and chimerism were also equivalent between the two groups 4 months after transplantation \(\text{(Figure 5B-D)}\). We next asked whether TN-C deficiency in the recipient mice affected BM reconstitution during transplantation. BMMNCs \((2 \times 10^5 \text{ or } 1 \times 10^4 \text{ cells})\) from Ly5.1 mice were transplanted into lethally-irradiated \(TN-C^{-/-}\) or \(TN-C^{+/+}\) mice (Ly5.2). When \(2 \times 10^5 \text{ BMMNCs}\) were transplanted, the survival rates were the same for both groups \(\text{(Figure 5E)}\). Flow cytometry analysis of the BM of recipient mice at 4 months post-BMT revealed normal frequency \(\text{(Figure 5F)}\) and chimerism \(\text{(Figure 5G)}\) of
Flt3-CD34-LSK cells and CD48-CD41-CD150+LSK cells; however, when the recipients were challenged with $1 \times 10^4$ BMMNCs, none of the $TN-C^{-/-}$ recipient mice survived beyond Day 15 post-BMT compared with a proportion (40%) of $TN-C^{+/+}$ recipients (Figure 5E). Four months after BMT with $2 \times 10^5$ BMMNCs, recipient $TN-C^{+/+}$ or $TN-C^{-/-}$ mice were subjected to 5-FU-induced myeloablation. The $TN-C^{-/-}$ recipients showed higher levels of lethality (Figure 5J) and a delay in hematopoietic recovery in the peripheral blood (Figure 5K-M). This confirmed that the impairment of hematopoietic recovery in $TN-C^{-/-}$ mice was attributable to TN-C-deficiency in the hematopoietic microenvironment, rather than the hematopoietic cells.

**TN-C-coating enhances in vitro proliferation and expression of cell cycle-promoting genes in HSPCs in an integrin α9-dependent manner.**

The *in vivo* data indicated that HSPC adhesion to TN-C proteins increases HSPC proliferation. Therefore, we performed *in vitro* experiments using cultured HSPCs to confirm this. The TN-C substratum works cooperatively with the FN substratum *in vitro*, and the TN-C substratum alone is not effective.\(^{37}\) Indeed, TN-C and FN share a common receptor, integrin $\alpha 9$.\(^{38,39}\) Culture on slides coated with TN-C plus FN yielded significantly more BrdU-positive proliferating LSK cells than culture on slides coated with FN alone (Figure 6A, B, F). Next, we went on to inquire whether the binding
action of TN-C to integrin α9 is indeed important for the HSPC-expanding effects of TN-C. The effects of TN-C proteins was abrogated by neutralizing antibodies for integrin α9 (55A2C), whereas non-functional anti-integrin α9 antibodies (18R18D) did not show such inhibitory effects (Figure 6B, D, F). Furthermore, we utilized synthesized proteins of a fibronectin type III repeat domain of TN-C lacking the RGD sequence (TN-C^FNIII), which bind integrin α9 but not other integrins such as integrin αVβ3 and α5β1. TN-C^FNIII enhanced the proliferation of LSK cells when combined with FN (Figure 6A, C, F), which effect was abrogated by 55A2C but not 18R18D (Figure 6C, E, F).

We next examined changes in cell cycle related genes. TN-C coating enhanced the expression of the cell cycle-promoting genes c-myc, cyclinD1, cyclin E1 and cyclinG2, and suppressed cell cycle-inhibitory genes p57^Kip2, p18^Ink4c, p21^Cipl, and p16^Ink4a (Figure 6G). To test the correlation between in vitro and in vivo settings regarding these cell cycle regulators, we quantified the expression of those genes in LSK cells in TN-C^−/− mice on day 10 after 5-FU administration (Supplementary Figure 4). As expected, the expression of some cell cycle-promoting genes (cyclin D3, cyclin E1 and cyclin G2) was downregulated in the cells derived from TN-C^−/− mice. However, some cell cycle-inhibitory genes (p57^Kip2 and p18^Ink4c) were not significantly changed and others (p21^Cipl and p16^Ink4a) were rather downregulated in TN-C^−/− mice, suggesting
downregulated cyclins dominate the changes in cell cycle-inhibitory genes in \( TN-C^{-/-} \) mice.

**Discussion**

The results of the present study show that changes in the amount and distribution of TN-C are most prominent for various ECM proteins during hematopoietic recovery after myeloablation. The TN-C gene was predominantly expressed in endothelial cells and stromal cells, including CAR cells. \( TN-C^{-/-} \) mice were vulnerable to myeloablation due to meager levels of HSPC supportive environment. Taken together, the results of this study identify TN-C as a critical component of the bone marrow microenvironment required for hematopoietic regeneration.

\( TN-C^{-/-} \) mice showed high levels of lethality and defective hematopoietic recovery after myeloablation induced by 5-FU, sub-lethal irradiation, or BMT using low numbers of donor cells. However, \( TN-C^{-/-} \) recipients of BM transplants were rescued by higher donor cell doses. Moreover, \( TN-C^{-/-} \) mice did not show any prominent defects in steady-state hematopoiesis, indicating that other factors may compensate for TN-C deficiency. TN-C associates with fibronectin, and these two proteins work cooperatively; indeed TN-C and fibronectin share a common receptor, integrin \( \alpha 9 \).\(^{38,39} \)
neutralization of which inhibits HSPC proliferation and adhesion to primary cultured osteoblasts.\textsuperscript{25} Since TN-C expression co-localized with fibronectin (data not shown), it is plausible to assume that TN-C functionally interacts with fibronectin. A significant induction of TN-C expression in response to 5-FU was also detected in hematopoietic cells (Fig. 2B). Moreover, it was previously shown that colony-forming capacity of BM cells is lower in TN-C-deficient mice.\textsuperscript{24} These results suggest that hematopoietic cells also contribute to the deposition of TN-C proteins in the BM microenvironment, and that TN-C in those cells plays a role although not so drastic as in the stromal side.

\textit{In vitro} culture of HSPCs on a TN-C substratum enhanced their proliferation. Also, we showed that integrin \(\alpha_9\), for which TN-C shows the highest affinity of all the integrins, was expressed abundantly on LSK cells. TN-C acts as either a positive or negative regulator of cell functions such as proliferation,\textsuperscript{41} migration,\textsuperscript{42} and cell spreading.\textsuperscript{37} The binding of TN-C to integrin \(\alpha_9\) results in the phosphorylation of FAK and Erk2 in colon carcinoma cells and thereby facilitates cell proliferation.\textsuperscript{31} The high frequency of integrin \(\alpha_9\) on HSPCs suggests that these cells bind to TN-C and subsequently proliferate. We identified that TN-C is expressed beyond the endosteal regions during BM reconstitution. This expansion may enable HSPCs to migrate to a more spacious location for proliferation and differentiation, rather than being stationed in the endosteal zone. Osteopontin, another ligand for integrin \(\alpha_9\), is mainly expressed
in the endosteal regions of the BM and thereby controls HSC homing to this area. Upregulation of TN-C expression within the central BM may functionally antagonize integrin α9 binding to osteopontin and enable it to promote HSC proliferation during hematopoietic recovery. Another known function for ECM molecules is the sequestration of growth factors and cytokines. We attempted to co-immunoprecipitate TN-C with the hematopoiesis-promoting cytokines SDF-1, SCF and M-CSF, but failed to obtain positive results (data not shown). The hematopoiesis-enhancing effect of TN-C seems to be restricted to its binding to integrin-expressing cells.

In conclusion, the results of the present study clarify the in vivo role of TN-C in supporting HSPC proliferation during BM reconstitution after myeloablation. Regulating the ECM should provide essential cues for the treatment of BM pathologies and for controlling normal hematopoiesis.

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Author contributions

A. N. performed the experiments, analyzed the data, and wrote the paper. Y. OK., K. O. and Y. OM. performed the experiments and analyzed the data. J. M. and T. U provided TN-C^{FNIII} proteins and the neutralizing antibodies against integrin α9. T. N. analyzed the data and assisted in preparing the manuscript. T. S. designed the experiments, interpreted the results, and assisted with manuscript preparation. Y. K. designed the experiments, interpreted the results, and wrote the paper.

Conflicts of interest disclosure

The authors declare no competing financial interests.
References


Figure legends

Figure 1. TN-C is markedly upregulated in the BM during myeloablation and hematopoietic recovery.

BM sections from Days 0, 2 and 10 stained for TN-C (red) in the metaphyseal (A-C) and diaphyseal regions (D-F) of the femoral bone. The bone surfaces are outlined by white dotted lines (A-F). The asterisks in (C) and (F) show the upregulation of TN-C beyond the endosteal areas in both the metaphyseal and diaphyseal regions. (G, H) Enlargement of the dotted squares in (D) and (F). Arrows indicate the bone surface; arrowheads indicate stromal TN-C expression. (I) High magnification of Day 10 BM samples stained for TN-C (red), c-Kit (green), laminin (blue), and DAPI (grey). c-Kit-positive cells adhered to TN-C expressed peri-vascularly (co-stained with laminin; open arrowheads) or away from the vasculature (arrowheads). Western blotting for TN-C in total BM proteins. (J). TN-C proteins were detected in two bands: 280 kDa and 220 kDa. Quantification of Western blot expression (K). The mean fluorescence ratio was calculated by dividing the mean fluorescence of the TN-C band by that of the corresponding β-actin band. *P < 0.05. Scale bars = 200 μm in A-F; 40 μm in G, H; and 10 μm in I.

Figure 2. TN-C is predominantly expressed in stromal and endothelial cells, and its
ligand, integrin α9, is expressed on HSPCs

(A, B) Relative RT-PCR for TN-C mRNA expression on whole-BM (A), CD45+, CD11b+, CD31+CD45+, PDGFRα+CD45+ and LSK cells (B) (n=5). (C-E) IHC of BM obtained from Cxcl12+/EGFP mice on Day 10. Note that TN-C proteins (red) are deposited around CD31+ (blue) endothelial cells (arrows) or CAR cells (green) (arrowheads). (F) Relative expression of TN-C mRNA in isolated CD45-Ter119‘CD31+Scal1+ (ECs), Cxcl12neg and Cxcl12high cells on Day 10 (n=5). (G-N) Relative expressions of integrin α9 (G), integrin β1 (H), integrin α4 (I) integrin αV (J), integrin α5 (K), integrin α7 (L), integrin β3 (M), integrin β6 (N) in whole BM cells (n=6). (O) Flow cytometric analysis of integrin α9 and integrin β1 expression by LSK cells. (P) Ratio (Lin+ =1) of mean fluorescence for integrin α9 and integrin β1 in the Lin+ and LSK fractions (n=7). *P < 0.05; Scale bar = 50 μm.

Figure 3. Normal steady-state hematopoiesis in TN-C-/- mice.

(A-C) FACS plots showing BM lineage frequency. (D) Quantification in each lineage (n = 4). (E) FACS plot showing the detection of LSK cells. (F, G) Total MNC count (F) and LSK cell count (G) (n = 4). (H-K) FACS plots (H, I) and quantification (n = 4) (J, K) of Flt3 -CD34-LSK cell and CD48-CD41-CD150+LSK cell frequency. (L, M) Cell cycle status frequencies assessed through Ki-67 and Hoechst staining of LSK cells (n =
6). *P < 0.05.

Figure 4. TN-C<sup>−/−</sup> mice showed impaired hematopoietic recovery after 5-FU administration.

(A, B) IHC for TN-C (red) and DAPI (blue) for Day 7 BMs. TN-C<sup>−/−</sup> mice lack TN-C immunoreactivity. (C) Survival curve for TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> mice after 5-FU administration (n = 12; combination of 3 independent experiments). (D-F) Peripheral blood counts for white blood cells (WBC) (D), hemoglobin (Hb) (E), and platelets (Plt) (F) after 5-FU administration. (G) BM lineage frequencies on day 10 after 5-FU administration (n=4). (H, I) IHC for c-Kit (green) and DAPI (blue) in TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> BM on Day 10 after 5-FU administration. (J, K) IHC for BrdU (red) and c-Kit (green) in TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> BM on day 10 after 5-FU administration. (L) Quantification of the percentage c-Kit<sup>+</sup> cells among DAPI positive BM cells (n = 6). (M) Quantification of the percentage of BrdU<sup>+</sup> cells among c-Kit<sup>+</sup> cells (n = 6). (N, O) FACS plot and quantification showing the detection of LSK cells on day 10 after 5-FU administration (n=4). (P) Survival curves for TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> mice subjected to 6.5 Gy irradiation (n = 5; combination of 3 independent experiments). *P < 0.05. Scale bars = 100 μm in A, B, H, I; 20 μm in J, K.
Figure 5. TN-C<sup>−/−</sup> are vulnerable recipients for BMT.

(A) Peripheral blood chimerism analyses of recipient mice infused with 2 × 10<sup>5</sup> donor cells from TN-C<sup>+/+</sup> or TN-C<sup>−/−</sup> mice (n = 10). (B-D) BMMNC count (B), LSK cell frequency (C) and LSK cell chimerism (D) of recipient mice 4 months after transplantation with 2 × 10<sup>5</sup> donor cells from TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> mice (n = 4). (E) Survival curves for TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> recipient mice infused with 2 × 10<sup>5</sup> cells (white and black boxes) or 1 × 10<sup>4</sup> cells (white and black circles) (n > 10 for each group; combination of 3 independent experiments). (F, G) Flow cytometric analysis of the BM in TN-C<sup>+/+</sup> or TN-C<sup>−/−</sup> recipient mice 4 months post-BMT (2 × 10<sup>5</sup> cells) showing the frequency and chimerism of Flt3 CD34<sup>+</sup>LSK cells and CD48<sup>+</sup>CD41<sup>+</sup>CD150<sup>+</sup>LSK cells (n = 4). (H, I) BM lineage composition (H) and chimerism of cells (I) within each lineage in the BM of TN-C<sup>+/+</sup> or TN-C<sup>−/−</sup> recipient mice 4 months post-BMT (2 × 10<sup>5</sup> cells) (n = 4). (J) Survival curves for TN-C<sup>+/+</sup> and TN-C<sup>−/−</sup> recipient mice challenged with 5-FU 4 months post-transplantation (2 × 10<sup>5</sup> cells) (n = 10; combination of 3 independent experiments). (K-M) Peripheral blood counts of mice in (J) (n = 10). *P < 0.05.

Figure 6. TN-C coating enhanced the in vitro proliferation and expression of cell cycle-promoting genes in HSPCs in an integrin α9-dependent manner.

(A-E) Short-term BrdU incorporation assay for LSK cells cultured for 48 hours on
slides coated with fibronectin alone (FN), fibronectin plus full-length TN-C (FN + TN-C), or fibronectin plus TN-C$^{^{FNIII}}$ (FN+TN-C$^{^{FNIII}}$) with functional integrin $\alpha 9$ antibodies (55A2C) or non-functional integrin $\alpha 9$ antibodies (18R18D). (F) Percentage of BrdU$^{+}$LSK cells in (A-E) (n = 4). (G) Relative mRNA expression for the cell cycle regulators $c$-myc, $cyclinD1$, $cyclinD2$, $cyclinD3$, $cyclinE1$, $cyclinE2$, $cyclinG1$, $cyclinG2$, $p57^{kip2}$, $p18^{ink4c}$, $p21^{cip1}$ and $p16^{ink4a}$ (n = 4). *$P < 0.05$. 


Figure 3

A

B

C

D

E

F

G

H

J

K

L

M

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Extracellular matrix protein Tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration

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