Tenascin-C Regulates Recruitment of Myofibroblasts during Tissue Repair after Myocardial Injury

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Tenascin-C (TN-C) is an extracellular matrix molecule that is expressed during wound healing in various tissues. Although not detectable in the normal adult heart, it is expressed under pathological conditions. Previously, using a rat model, we found that TN-C was expressed during the acute stage after myocardial infarction and that α -smooth muscle actin (α -SMA)-positive myofibroblasts appeared in TN-C-positive areas. In the present study, we examined whether TN-C controls the dynamics of myofibroblast recruitment and wound healing after electrical injury to the myocardium of TN-C knockout (TNKO) mice compared with wild-type (WT) mice. In TNKO mice, myocardial repair seemed to proceed normally, but the appearance of myofibroblasts was delayed. With cultured cardiac fibroblasts, TN-C significantly accelerated cell migration, α -SMA expression, and collagen gel contraction but did not affect proliferation. Using recombinant fragments of murine TN-C, the functional domain responsible for promoting migration of cardiac fibroblasts was mapped to the conserved fibronectin type III (FNIII)-like repeats and the fibrinogen (Fbg)-like domain. Furthermore, alternatively spliced FNIII and Fbg-like domains proved responsible for the up-regulation of α -SMA expression. These results indicate that TN-C promotes recruitment of myofibroblasts in the early stages of myocardial repair by stimulating cell migration and differentiation. (*Am J Pathol* 2005, 167:71–80)

Tenascin-C (TN-C), an extracellular matrix molecule expressed at high levels during embryonic development and cancer invasive fronts, as well as in response to

injury, is known to influence various cell activities.^{1–4} Each subunit of a hexameric glycoprotein consists of TA (tenascin assembly domain), epidermal growth factor (EGF)-like repeats, fibronectin type III (FN III)-like repeats, and a C-terminal fibrinogen (Fbg)-related domain. Alternative splicing results in several different forms of TN-C, containing variable numbers of FN III repeats. Accumulating results of *in vitro* studies point to each domain having specific functions, for example in the regulation of cell adhesion, migration, or growth.^{1–4}

In the heart, TN-C is expressed at very early stages of embryonic development,⁵ is not detected in normal adult myocardium, but is re-expressed in various pathological conditions.^{6–13} After myocardial infarction, TN-C appears during the acute stages, at the interface between infarcts and intact myocardium.^{7,8} We previously reported that TN-C may loosen the linkage between cardiomyocytes and connective tissue and thus helps with tissue remodeling at the edges of residual myocardium.⁸ Furthermore, we found α -smooth muscle actin (α -SMA)-positive myofibroblasts in TN-C-positive areas and that deposition of TN-C precedes their recruitment.⁸

Myofibroblasts are specialized fibroblasts that share characteristics with smooth muscle cells expressing α -SMA. They play an important role in wound healing by synthesizing collagens and exerting strong contraction forces to minimize wound areas.^{14–17} It is thought that residential interstitial fibroblasts at the edges of injured tissue differentiate into myofibroblasts and migrate into damaged areas. In the present study, we investigated whether TN-C contributed to myocardial tissue repair, with particular attention to recruitment of myofibroblasts. For this purpose TN-C knockout (TNKO) and wild-type (WT) mice were compared with regard to the healing processes after electrical injury to the myocardium. Fur-

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thermore, the effect of TN-C on cell proliferation, migration, and differentiation of cardiac fibroblasts into myofibroblasts was examined *in vitro*. Functional domains were also determined using recombinant fragments of murine TN-C.

Materials and Methods

TN-C Knockout Mice

The originally produced TN-C knockout mouse¹⁸ was backcrossed with BALB/c inbred mice.

Myocardial Injury Model

TNKO and WT mice, 8 weeks old, were handled according to guidelines approved by the Mie University Animal Experiment and Care Committee. Animals were anesthetized by an intraperitoneal injection of 50 mg/kg of sodium pentobarbital and the left diaphragm was exposed through a midline incision in the abdomen. The ventricular wall was injured through the diaphragm by an electric pulse current (160 μ A, 0.5 second) using an electric coagulator (OPERER II-F; J. Morita, Tokyo, Japan) that was delivered with a probe of 2 mm in diameter. This procedure caused a transmural injury at the postinferior wall of the left ventricle of ~2 to 3 mm in diameter. Mice were sacrificed 1, 2, 3, and 5 days after injury. The hearts were removed and fixed in a 4% paraformaldehyde solution at 4°C for 16 hours, embedded in paraffin, and cut into 4- μ m-thick sections. To examine cell proliferation, some mice received an intraperitoneal administration of 20 mg/kg of 8-bromodeoxyuridine (BrdU) 30 minutes before sacrifice.

Immunohistochemistry

Production and characterization of an anti-TN-C polyclonal rabbit antibody and mouse monoclonal antibody clones 4F10TT (IBL, Gunma, Japan) and 4C8MS (IBL), as well as the immunostaining procedures with tissue sections, were previously described.^{8,9,19} 4C8MS specifically recognizes the alternative splicing sites, whereas 4F10TT reacts with constitutive sites of TN-C molecules. Sections on slides were incubated with either rabbit polyclonal antibodies (1 μ g/ml), 4F10TT (1 μ g/ml), or 4C8MS (5 μ g/ml), overnight at 4°C, and subsequently with peroxidase-conjugated anti-mouse or anti-rabbit IgG Fab' (1:500; MBL, Nagoya, Japan) for 1 hour. After washing, diaminobenzidine/H₂O₂ solution was used to demonstrate antibody binding. The sections were then lightly counterstained with methyl-green or hematoxylin to facilitate orientation. Incorporated BrdU in tissue sections was immunostained according to Yoshimura and colleagues.²⁰ The labeled cells excluding inflammatory cells in the injured areas near the border zone were counted in three different fields of $\times 40$ objective, and the average of each sample ($n = 5$ for each) was calculated. Myofibroblasts were labeled by a direct immunoperoxidase method

Figure 1. Diagram of mouse TN-C and its recombinant fragments. FL: FNIII repeats including both conserved (1 to 5, 6 to 9) and alternatively spliced repeats (A1, A2, A4, B, D). SV: Alternatively spliced FNIII repeats. SO: Conserved FNIII repeats. EGF: the EGF-like domain. Fbg: fibrinogen-like domain.

with anti- α -SMA antibody (EPOS; Dako Japan, Kyoto, Japan) and the α -SMA-positive cells in the injured areas were also counted. Double immunohistochemistry for TN-C and α -SMA was performed as previously described.⁸

Purification of TN-C and Its Recombinant Fragments

TN-C was purified from conditioned medium of the U-251MG human glioma cell line.²¹ Recombinant fragments of TN-C (Figure 1): FNIII repeats including the alternative splicing site (FL), FNIII repeats of the alternative splicing site (SV), FNIII repeats without the site (SO), the EGF-like domain, and the fibrinogen (Fbg)-like domain, were obtained from conditioned media of CHO K-1 cells permanently transfected with cDNAs encoding the respective domains, and purified.¹⁹

Cell Cultures

Primary cultures of cardiac fibroblasts were obtained from cardiac ventricles of either TNKO or WT mice. Five mice were used for each preparation. The mice were sacrificed by cervical dislocation and the hearts were quickly removed under sterile conditions. Ventricular tissue was excised, thoroughly minced, and digested with 0.1% collagenase (class II; Worthington, Freehold, NJ) in Dulbecco's phosphate-buffered saline. The isolated cells were collected by centrifugation, resuspended in Iscov's modified Dulbecco's medium (IMDM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY), and plated on a 75-cm² Primaria culture flask (Becton Dickinson Labware, Franklin Lakes, NJ). After the cardiac fibroblasts had attached to the bottom surface of the flask, nonadherent cells (primarily myocytes, leukocytes, and endothelial cells) could be washed away. This approach enabled us to obtain virtually pure cultures of fibroblasts. The cells were grown in IMDM with 10% FBS at 37°C in a humidified incubator gassed with 5% CO₂. Experiments were performed using cells at the second passage. The effects of intact TN-C and recombinant fragments of TN-C on fibroblasts were examined using the cells from TN-C-null mice to exclude the influence of intrinsically synthesized TN-C.

BrdU Incorporation

Cells (1×10^5) of cardiac fibroblasts from TNKO mice were plated on each well of Falcon four-chamber culture slides (Becton Dickinson Labware), grown in IMDM/10% FBS for 12 hours, washed with serum-free medium, and then incubated in medium with 0.1% FBS for 24 hours. Thereafter, TN-C was added to a final concentration of 1 to 10 $\mu\text{g/ml}$. After incubation for 12 hours, the cells were labeled with BrdU (10 $\mu\text{g/ml}$) for 2 hours, fixed with 100% ethanol at -20°C for 30 minutes, and treated with 1 N HCl solution at room temperature for 20 minutes. Labeled nuclei were detected with monoclonal anti-BrdU antibody (Dako Japan) and peroxidase-conjugated goat anti-mouse IgG (MBL). The BrdU-positive nuclei and total number of nuclei (more than 500) were counted and percentage values were determined.

Migration Assays

We first compared the migration of cardiac fibroblasts from TNKO and WT mice, by a transwell migration assay using cell culture inserts (8- μm pore size, Becton Dickinson Labware). Cells (5×10^4 of either type) in 0.5% bovine serum albumin/serum-free IMDM were plated into the inner chamber. The medium, containing 5% FBS as a chemoattractant, was poured into the outer chamber (Falcon 24-well plate, Becton Dickinson Labware). To examine the effect of TN-C on cell migration, intact TN-C (0 to 10 $\mu\text{g/ml}$) or one of the recombinant fragments (10 $\mu\text{g/ml}$) was added to medium of the upper chamber of TN-C-null cells. The cells were allowed to migrate to the lower membrane surface for 8 hours. The cells remaining on the upper surface were then wiped off and the inserts were fixed with 100% ethanol and stained with 0.1% crystal violet (Sigma) in 10% ethanol. Stained cells on 1 mm^2 of the lower membrane surface were counted under a $\times 10$ objective lens.

Expression of α -SMA

Cardiac fibroblasts from either TNKO or WT mice were plated on Falcon four-chamber culture slides (5×10^4 for each well) in IMDM/10% FBS for 3 hours, washed with serum-free medium, then incubated in medium with 0.1% FBS for 24 hours. Thereafter, intact TN-C (5 to 20 $\mu\text{g/ml}$) or one of the recombinant fragments (10 $\mu\text{g/ml}$) was added to TN-C-null fibroblasts. After incubation for 24 hours, the cells were fixed with 4% paraformaldehyde and labeled with antibodies against α -SMA (Sigma) for 1.5 hours, then exposed to secondary antibodies (fluorescein isothiocyanate-conjugated anti-mouse IgG, MBL) and rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 1 hour. Cells were examined with a $\times 20$ objective lens by epifluorescence microscopy (BX-50; Olympus, Tokyo, Japan). Polyclonal monospecific antibodies against either TN-C or calsequestrin were affinity-purified with antigen-conjugated columns. Calsequestrin is protein of the sarcoplasmic reticulum, and anti-calsequestrin antibody was used as a control IgG. Purified TN-C (10

$\mu\text{g/ml}$) was incubated with either antibodies (10 $\mu\text{g/ml}$) at room temperature for 30 minutes, and then added to the culture medium.

Collagen Gel Contraction Assay

Cellular collagen gel contraction assays were performed in Falcon 24-well plates. A collagen solution in IMDM with 0.1% FBS was prepared from porcine collagen I according to the manufacturer's instructions (Nitta Gelatin, Osaka, Japan) and combined with cardiac fibroblasts of either TNKO or WT mice at $2 \times 10^5/\text{ml}$. Five hundred μl of the collagen/cell mixture (final collagen concentration, 1.0 mg/ml) was plated into each well and allowed to polymerize at 37°C . After incubation for 24 hours, 0.5 ml of IMDM containing 0.1% FBS with or without TN-C (10 $\mu\text{g/ml}$) was added to each well, and the gels were carefully detached from the dishes. The mean size of the gel ($n = 3$ for each sample) was taken at each time point with the aid of a flatbed scanner (Epson, Tokyo, Japan). To estimate the contractility of the cells, the size of the gel was subtracted from the starting area and expressed as a percentage. This experiment was repeated three times.

Results

Wound Healing and Expression of TN-C in Injured Mouse Myocardium

In WT mice, coagulation necrosis of cardiomyocytes and edema around lesions were apparent 1 day after injury (Figure 2A), becoming more pronounced on day 2 (Figure 2B). On day 3, granulation tissues began to be formed in the border zone and eventually replaced necrotic masses (Figure 2C). TN-C began to be expressed within 1 day after injury at the border between intact myocardial tissues and necrotic areas (Figure 2D) and was gradually deposited in developing granulation tissue (Figure 2, E and F). Using the monoclonal antibody 4C8MS, it was confirmed that the deposited molecules contained the large splice variants (Figure 2G). In TNKO mice, myocardial healing appeared to proceed normally and there was no obvious difference from WT mice on routine histological analyses (Figure 3).

Recruitment of Myofibroblasts in Granulation Tissue of the Heart

Immunostaining of α -SMA of WT mice showed that on day 1, only vascular walls were positive and few myofibroblasts were present, although TN-C deposition was clearly detectable (Figure 2D). On day 2, myofibroblasts emerged in TN-C-positive areas of the interstitium of the border zone (Figure 2E), and increased in number on day 3, moving into necrotic areas. The number of myofibroblasts per optic field in WT mice on days 1, 2, and 3 were 2.2 ± 0.3 , 16.6 ± 6.2 , and 30.6 ± 0.3 , respectively (Figure 4). In TNKO mice, they were 0.7 ± 0.6 , 4.9 ± 3.3 , and 33.2 ± 3.5 . On days 1 and 2, myofibroblasts in WT

Figure 2. Tissue healing and TN-C expression after electrical injury of the myocardium at day 1 (**A**, **D**), day 2 (**B**, **E**), and day 3 (**C**, **F**, **G**). **A–C:** H&E staining; **D**, **E:** double immunolabeling for α -SMA (blue) and TN-C (brown). **F** and **G:** Immunolabeling with the antibody clone 4F10TT reacting with constitutive sites of TN-C (**F**), and 4C8MS that specifically recognizes an alternative spliced repeat of TN-C (**G**). On day 1, TN-C deposition is clearly detectable in the interstitial spaces of border zone myocardium, but only vascular walls are positive for α -SMA (**D**). **E:** On day 2, myofibroblasts are apparent in TN-C-positive areas. **G:** Note staining for large splice variants of TN-C. int, intact area; inj, injured area. Scale bar, 50 μ m.

mice were significantly more numerous than in TNKO mice ($P < 0.01$), but the difference disappeared on day 3 (Figure 4).

Cell Proliferation in Granulation Tissue of the Heart

Since it is well known that TN-C enhances proliferation of some cell types, we examined DNA synthesis in granulation tissue by BrdU incorporation assay. The percent-

ages of BrdU-labeled nuclei in WT mice on days 1, 2, and 3 were $5.7 \pm 3.4\%$, $17.5 \pm 2.7\%$, and $24.2 \pm 5.9\%$, respectively, whereas in TNKO mice they were $6.1 \pm 2.9\%$, $17.7 \pm 2.8\%$, and $26.6 \pm 7.0\%$, with no significant differences between the two animal groups (Figure 5A).

Effect of TN-C on Proliferation, Migration, and Differentiation of Cultured Cardiac Fibroblasts in Vitro

To confirm the *in vivo* results, we first investigated the effect of TN-C on cell proliferation of cultured cardiac fibroblasts from TNKO mice by BrdU incorporation assay. Addition of TN-C in various concentrations to the fibroblasts was without significant effect (Figure 5B). In transwell migration assays, the number of TN-C-null fibroblasts that migrated through insert membranes was significantly lower than those from the WT mice (28.8 ± 3.7 cells/mm² versus 42.0 ± 3.6 cells/mm², $P < 0.01$). Addition of TN-C to TN-C-null cells at the concentration of 5 and 10 μ g/ml, significantly increased the number of migrated cells to 50.8 ± 12.3 cells/mm² and 53.1 ± 5.2 cells/mm², respectively ($P < 0.01$ and $P < 0.001$, Figure 6).

Differentiation to myofibroblasts was assessed by expression of α -SMA (Figure 7) and collagen gel contraction assay (Figure 8). Cardiac fibroblasts in culture from TNKO mice showed well developed stress fibers and some cells expressed α -SMA (Figure 7A). However, the percentage of α -SMA-positive cells was significantly lower than that of WT cardiac fibroblasts ($35.4 \pm 2.03\%$

Figure 3. Comparison of the histopathology of myocardial repair in a WT (**A**) and TN-C knockout mouse (**B**) 5 days after electric injury. H&E staining. Scale bar, 50 μ m.

Figure 4. Myofibroblasts in the border zones of necrotic cardiac tissue. **A:** Myofibroblasts labeled with anti- α -SMA antibody. On day 2, α -SMA-positive cells are evident (arrows) in the myocardial interstitium near the necrotic area in a WT mouse. Fewer cells are apparent in a TN-C knockout (TNKO) mouse. **B:** α -SMA-positive cells were counted in three fields of view under a $\times 40$ objective and the average of each sample was calculated. On days 1 and 2, the myofibroblasts in WT mice were more frequent than in TNKO mice ($P < 0.01$), but there was no difference on day 3. The data are averages and SDs of results from five animals. Scale bar, 50 μ m.

versus $50.8 \pm 3.7\%$, $P < 0.01$). Addition of TN-C significantly increased the α -SMA-positive ratio of the TN-C-null fibroblasts in a dose-dependent manner (Figure 7B). To confirm this result, we tested whether addition of an antibody against TN-C blocked this effect and found that treatment with the TN-C antibody ($95 \pm 5\%$) restored the

Figure 5. Effect of TN-C on proliferation of cardiac fibroblasts *in vivo* and *in vitro*. **A:** BrdU-labeled cells in the injured areas of WT and TNKO mice. BrdU-positive nuclei were counted in three fields of view of a $\times 40$ objective and the average of each sample was calculated. The data are expressed as averages and SDs of results from five animals. Note the lack of significant differences between WT and TNKO. **B:** Cells isolated from TNKO mice were plated and grown on culture glass slides. After serum starvation for 24 hours, TN-C was added. Cells labeled with BrdU were visualized by immunocytochemistry and BrdU-positive and total number of nuclei (more than 500) were counted and percentage values generated. The data are averages and SDs from three independent experiments. Note the lack of any increase with TN-C treatment.

α -SMA-positive cells to the levels in fibroblasts that were cultured without TN-C ($100 \pm 5\%$), while addition of control IgG had no effect ($117 \pm 8\%$). Both TN-C-null and WT cells contracted collagen gels, but contraction by TN-C-null cells was significantly weaker than those by WT cells. Addition of TN-C significantly increased the gel contraction of TN-C-null fibroblasts (Figure 8).

Functional Domains of TN-C on Migration and α -SMA Expression of Cardiac Fibroblasts

Having shown that TN-C promoted migration and α -SMA expression, we tried to determine the responsible functional domains of TN-C, using the following recombinant fragments: FNIII repeats including alternative splicing sites (FL), conserved FNIII repeats (SO), alternative splice sites (SV), the EGF-like domain, and the fibrinogen (Fbg)-like domain (Figure 1). On addition to fibroblast cultures, FL and SO increased the numbers of migrating cells to $151 \pm 28\%$ and $151 \pm 18\%$, respectively, of the control value. The Fbg-like domain also caused a $165 \pm 32\%$ elevation, while addition of neither EGF nor SV fragments had significant effects (Figure 9). Therefore, the conserved FNIII repeats and the Fbg-like domain may have the ability to promote migration of fibroblasts.

FL also augmented the proportion of α -SMA-positive fibroblasts ($123 \pm 15\%$) as compared with the controls (Figure 10). Although SO did not exert any apparent influence, SV also caused a significant increase, along with the Fbg-like domain ($118 \pm 16\%$). Addition of EGF was without effect. Therefore, alternatively spliced FNIII repeats and the Fbg-like domain may thus induce differentiation from fibroblasts to myofibroblasts.

Discussion

TN-C has been proposed to play significant roles in wound healing in various tissues because of its dramatic

increase after injury.²² Although TN-C knockout mice were initially reported to be phenotypically normal,¹⁸ several reports have documented abnormal tissue restoration after injury to the cornea,²³ skin,²⁴ or after habu venom-induced glomerulonephritis.^{25,26} Using different models, others have reported no significant differences in the wound healing on morphological analysis,^{27,28} but deposition of fibronectin was found to be reduced in the granulation tissue of skin wounds in TN-C-null mice.²⁷ *In vitro*, TN-C promotes epithelial cell migration and proliferation,¹⁹ which could be partly responsible for abnormal repair of epithelial tissue in TNKO mice.

Because of the limited ability for regeneration of cardiomyocytes, myocardial wound healing mostly depends on interstitial fibroblasts, which form and contract granulation and scar tissue via multiple cell activities.²⁹ Especially, myofibroblasts, specialized fibroblasts, are key players in the myocardial repair.^{30–32} In the present study, we found that their appearance in injured myocardium was clearly delayed in TN-C knockout mice, indicating that TN-C promotes recruitment of myofibroblasts.

On tissue injury, primarily in response to mechanical stress, interstitial fibroblasts evolve into proto-myofibroblasts with a contractile microfilamentous apparatus, then, with various stimuli, into differentiated myofibroblasts characterized by expression of α -SMA. These then migrate into the injured areas, generate contraction forces, synthesize, organize, and degrade collagen and other ECMs, and finally results in a shortening of collagen matrix with corresponding wound closure.^{14,15,33,34}

A close relationship between TN-C and myofibroblasts in cancer stroma or normal tissue has been reported.^{35–37} In cancer stroma, myofibroblasts have often been identified as the source of TN-C deposits³⁸ and it is supposed that they may provide a proinvasive signal to cancer cells mediated with TN-C.³⁹ By careful sequential observation in a rat model after myocardial infarction, we have found that, initially α -SMA-negative interstitial cells in the border zones express TN-C, and then myofibroblasts appear in the TN-C-positive areas.⁸ Similar results were observed in mouse myocardial wound healing in this study. It seems likely that interstitial cells synthesize TN-C, which, in an autocrine and paracrine manner, then causes interstitial cells to change their phenotype to differentiated myofibroblasts and promotes migration into damaged areas.

Our *in vitro* findings support this possibility. Cardiac fibroblasts from TNKO mice showed lower cell migration and α -SMA expression than WT cells that synthesize TN-C in culture. Addition of TN-C to TN-C-null cells recovered both cell migration and α -SMA expression. Expression of α -SMA is a critical step for myofibroblast

Figure 6. Effects of TN-C on migration of cardiac fibroblasts in a transmigration assay. Cells isolated from cardiac ventricles of either TNKO or WT mice were plated on the culture inserts, treated with TN-C (5 or 10 μ g/ml) or without TN-C, and allowed to migrate for 8 hours. **A:** Cells migrating through the membrane were stained with 0.1% crystal violet. **B:** Migration of TN-C-null cells was significantly lower than that of WT-cells, and addition of TN-C significantly enhanced cell migration in a dose-dependent manner. Cells were counted in three fields of view of 1 mm² in each insert, and the data are averages and SDs of results from three independent experiments.

A

B

Figure 7. Effects of TN-C on α -SMA expression in cardiac fibroblasts. Cells isolated from cardiac ventricles of either TNKO or WT mice were plated and grown on the culture glass slides. After 24-hour serum starvation, TN-C (0 or 20 μ g/ml) was added to TN-C-null cells and incubated for another 24 hours. **A:** α -SMA expression was detected by indirect immunofluorescent staining with fluorescein isothiocyanate-conjugated secondary antibody, and all cells were counterstained with rhodamine-phalloidin. The α -SMA-positive ratio of TN-C-null cells was significantly lower than that of WT cells. **B:** Addition of TN-C significantly up-regulated α -SMA expression in a dose-dependent manner. The α -SMA-positive and total cells (more than 200) were counted to allow generation of percentage values. The data are averages and SDs of results from three independent experiments.

Figure 8. Effects of TN-C on collagen gel contraction by cardiac fibroblasts. Cardiac fibroblasts of either TNKO or WT mice were combined with collagen gel mixture and allowed to polymerize. After 24 hours medium with or without TN-C (10 μ g/ml) was added, then the gels were detached from the dish. Percentages of the contraction were measured at 4, 8, 12, and 24 hours. Contraction by WT cells (closed circle) was significantly stronger than that by TN-C-null cells (open triangle) at each time point. Addition of TN-C (closed triangle) significantly increased the gel contraction of TNKO fibroblasts at 8, 12, and 24 hours. The data are expressed as averages and SDs of triplicate samples.

differentiation and functionally important for force generation.^{40,41} Although gel contraction could be regulated by various factors including differentiation of myofibroblasts, cell-matrix adhesion, and maturation of cytoskeletal contractile apparatus,^{34,42–44} the increment of contraction by addition of TN-C in our experiment should partly reflect the up-regulation of α -SMA expression. Although TN-C is well known to stimulate migration of various types of cells, it has also been suggested to modulate differentiation of cells.^{45–47}

Functional domains responsible for differentiation into myofibroblasts were mapped to alternative spliced FNIII repeats, but not the conserved repeats, and the Fbg-like domain of TN-C. In contrast, the responsible domain for

Figure 9. Determination of functional domains promoting cell migration using recombinant fragments of TN-C. TN-C-null cardiac fibroblasts were plated on culture inserts and treated with TN-C (10 μ g/ml) or TN-C fragments (10 μ g/ml), and allowed to migrate for 8 hours. The cells were counted in three fields of view of 1 mm² in each insert, and the data are averages and SDs of results from six independent experiments, relative to the control without TN-C. *P < 0.01.

Figure 10. Determination of functional domains up-regulating α -SMA expression using recombinant fragments of TN-C. TN-C-null cardiac fibroblasts were plated and grown on the culture glass slides. After 24-hour serum starvation, they were treated with TN-C (10 μ g/ml) or TN-C fragments (10 μ g/ml), and incubated for 24 hours. α -SMA expression was detected by immunofluorescence. α -SMA-positive cells and total number of cells (more than 200) were counted and the percentage values were generated. The data are averages and SDs of results from six independent experiments, relative to the control without TN-C. * $P < 0.01$, ** $P < 0.05$.

promoting migration of cardiac fibroblasts was mapped to the conserved FNIII repeats and the Fbg-like domain. Several receptors might bind to the respective domains of TN-C and transmit multiple signals that could trigger various cellular functions.

Interestingly, using the same recombinant fragments and the same assays, our previous study demonstrated that the alternative spliced FNIII domains promoted migration of breast cancer cells, but the conserved repeats and Fbg domain did not.¹⁹ The conserved repeats of FNIII 3 and 6 have similarly been demonstrated to be involved in glioma migration.⁴⁸ It has also been reported that the Fbg-like domain, but not the entire FNIII repeats, can mediate migration of aortic smooth muscle cells on a TN-C substrate.⁴⁹ Conversely, a recombinant fragment of alternatively spliced FNIII A to D, but not the conserved FN III repeats or the Fbg domain, was found to enhance migration of endothelial cells.⁵⁰ Therefore, cell-type specificity is apparent. A number of molecules, such as members of the integrin family, annexin II, EGF receptor, cell adhesion molecules (CAMs), syndecan, and phosphacan/receptor-type protein tyrosine phosphatase ζ/β , have been reported as receptors for TN-C.³ Moreover, TN-C may also regulate cell behavior by direct or indirect modulation of other ECM proteins. For example, it has been reported that TN-C may inhibit fibronectin initiating signaling by interfering fibronectin-syndecan 4 binding.^{44,51,52} Therefore, functional activities provided by TN-C could occur through different mechanisms, possibly using separate receptors depending on the cell.

In this study, we demonstrated that TN-C plays critical roles in stimulating myofibroblasts in response to injury. However, after 3 days there was no longer any difference in myofibroblasts in TN-C-null and control mice, and myocardial healing did not show distinct morphological differences so that a compensatory mechanism must exist. Indeed, TN-C is not the only factor that controls behavior of myofibroblasts. For example, the transition between proto-myofibroblasts and differentiated myofibroblasts is

known to be stimulated by many factors, such as various cytokines, growth factors, endothelin 1, angiotensin II, and newly synthesized extracellular matrix proteins.¹⁶ It is well established that the combined action of the splice variant of cellular fibronectin containing ED-A and transforming growth factor- β is important in this regard.^{53,54} In cancer tissues, TN-C is often co-expressed with fibronectin containing ED-A,^{37,55} and also can enhance the functions of transforming growth factor- β in cancer cells.⁵⁶ Therefore, TN-C might control behavior of myofibroblasts in a complex way, collaborating and interfering with many other factors.

Nevertheless, it is clear that TN-C is a major factor for recruitment of myofibroblasts in early stages of myocardial tissue repair. Although this would increase contraction forces to prevent ventricular dilatation, TN-C also has been reported to inhibit cell contraction by suppressing focal adhesion kinase and Rho A activity in fibrinogen-fibronectin matrix.^{43,44} Furthermore, TN-C may loosen adhesion of cardiomyocytes⁸ and stimulate MMP expression in some types of cells.⁵⁶ Although these effects, as well as integrin shedding, should be beneficial for cells to modify cell-ECM interaction during tissue remodeling,⁵⁷ they also have potential to cause slippage of myocytes resulting in ventricular dilatation. Therefore, TN-C could be a key molecule in controlling the balance of beneficial and undesirable cellular responses in cardiac remodeling.

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