MATRIX PATHOBIOLOGY

Tenascin-C Induces Phenotypic Changes in Fibroblasts to Myofibroblasts with High Contractility through the Integrin $\alpha v\beta 1$ /Transforming Growth Factor β /SMAD Signaling Axis in Human Breast Cancer

Daisuke Katoh,^{*} Yuji Kozuka,[†] Aya Noro,[‡] Tomoko Ogawa,[‡] Kyoko Imanaka-Yoshida,^{*§} and Toshimichi Yoshida^{*§}

From the Departments of Pathology and Matrix Biology,^{*} Pathologic Oncology,[†] and Breast Surgery,[‡] Graduate School of Medicine, Mie University, Tsu; and the Research Center for Matrix Biology,[§] Mie University, Tsu, Japan

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Address correspondence to
Toshimichi Yoshida, M.D.,
Ph.D., Department of Pathology
and Matrix Biology, Graduate
School of Medicine, Mie Uni-
versity, 2-174 Edobashi, Tsu,
Mie 514-8507, Japan. E-mail: t-yoshida@doc.medic.mie-u.ac.jp.

Tenascin-C (TNC) is strongly expressed by fibroblasts and cancer cells in breast cancer. To assess the effects of TNC on stromal formation, we examined phenotypic changes in human mammary fibroblasts treated with TNC. The addition of TNC significantly up-regulated α -smooth muscle actin (α -SMA) and calponin. TNC increased the number of α -SMA- and/or calponin-positive cells with well-developed stress fibers in immunofluorescence, which enhanced contractile ability in collagen gel contraction. The treatment with TNC also significantly up-regulated its own synthesis. Double immunofluorescence of human breast cancer tissues showed α -SMA- and/or calponin-positive myofibroblasts in the TNC-deposited stroma. Among several receptors for TNC, the protein levels of the αv and $\beta 1$ integrin subunits were significantly increased after the treatment. Immunofluorescence showed the augmented colocalization of αv and $\beta 1$ at focal adhesions. Immunoprecipitation using an anti- αv antibody revealed a significant increase in coprecipitated $\beta 1$ with TNC in lysates. The knockdown of αv and $\beta 1$ suppressed the up-regulation of α -SMA and calponin. The addition of TNC induced the phosphorylation of SMAD2/3, whereas SB-505124 and SIS3 blocked myofibroblast differentiation. Therefore, TNC enhances its own synthesis by forming a positive feedback loop and increases integrin $\alpha v\beta 1$ heterodimer levels to activate transforming growth factor- β signaling, which is followed by a change to highly contractile myofibroblasts. TNC may essentially contribute to the stiffer stromal formation characteristic of breast cancer tissues. (*Am J Pathol* 2020; 190: 2123–2135; <https://doi.org/10.1016/j.ajpath.2020.06.008>)

Tenascin-C (TNC) is an extracellular matrix (ECM) glycoprotein of the matricellular family and is formed by hexamers of 250- to 400-kDa subunits. TNC is rarely expressed in normal adult tissues, but is strongly up-regulated in pathologic tissues undergoing remodeling, such as inflammation, wound healing, and cancer.¹ Invasive ductal carcinoma of the breast is a tumor that shows the dense deposition of TNC,^{2,3} which has been associated with the unfavorable prognosis of patients with cancer.⁴ The immunohistochemical expression of TNC in cancer cells and the cancer stroma predicts a significantly poorer outcome than its deposition in the stroma alone. Furthermore, the expression of TNC at the site of invasion

correlated with a higher proliferation rate of breast cancer as well as a higher incidence of local and distal recurrence.^{5,6} The findings of these clinicopathologic studies have been reinforced by many recent studies on the roles of TNC in the regulation of the malignant behaviors of breast cancer cells. The exogenous application of TNC to breast cancer cells has

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been shown to enhance the proliferation, migration, and invasion of breast cancer cells *in vitro* and up-regulate matrix metalloproteinase 9.^{3,7} The overexpression of alternative splice variants of TNC in breast cancer cells up-regulates matrix metalloproteinase 13 expression and promotes cancer cell invasion.⁸ Furthermore, a co-culture with transfected fibroblasts enhanced cancer cell invasion. TNC is one of six genes regulated by miR-335 in metastatic breast cancer. The loss of miRNA expression in breast cancer cells is associated with poor distant metastasis-free survival.⁹ Moreover, cancer cell-derived TNC facilitates the survival and outgrowth of breast cancer cells at the initiating steps of lung metastases by promoting Notch and Wnt signaling.¹⁰ TNC also induced an epithelial-mesenchymal transition-like change in human breast cancer cells *in vitro*.^{11,12} These findings revealed the involvement of TNC in cancer progression mainly from the aspect of the malignant properties of cancer cells.

TNC is produced by tumor and stromal cells in breast cancer tissues. Fibroblasts of the cancer stroma are a major cellular source of TNC in fully invasive scirrhous cancers, whereas cancer cells mostly produce TNC when large cancer nests, such as intraductal components, are formed.² Fibroblasts in the stroma associated with cancer have been termed cancer-associated fibroblasts (CAFs) and are activated by tumor microenvironments.^{13–16} Although CAFs are a heterogeneous population, they classically show myofibroblast properties and express α -smooth muscle actin (α -SMA). Double labeling by *in situ* hybridization for TNC mRNA and α -SMA immunohistochemistry of human colon cancer tissues revealed the synthesis of TNC by α -SMA-positive myofibroblasts.¹⁷ TNC production by CAFs is required for the stimulation of cancer cell invasion.¹⁸ Although myofibroblasts are found in the majority of solid tumors, breast cancer contains a tumor stroma that is intensely rich in myofibroblasts, similar to prostatic, colon, and pancreatic cancers.^{19,20} These cancers generally have dense deposits of TNC in the stroma.^{2,21–23}

To clarify the relationship between TNC expression and phenotypic changes in CAFs, the present study investigated the involvement of TNC in the phenotypic changes of cultured human mammary fibroblasts (HMFs) to CAFs. To detect the differentiation to CAFs, changes in the protein expression of CAF markers^{13–16} by the exogenous application of purified TNC into the medium were examined by immunoblotting. α -SMA, calponin, and caldesmon were examined as specific markers of myofibroblast differentiation.^{24–26} S100A4 (alias fibroblast-specific protein 1) was used as a marker for the fibroblastic phenotype of CAFs.^{27,28} Podoplanin,²⁹ fibroblast-activation protein α ,³⁰ neural/glial antigen 2, prolyl-4-hydroxylase, and platelet-derived growth factor receptor- β are also common markers that define CAFs in breast cancer tissues.³¹ Caveolin-1 expression was analyzed because the loss or up-regulation of caveolin-1 in the CAFs of human breast cancer tissues has been reported.^{32,33} TNC-induced phenotypic changes in HMFs in *in vitro* experiments were confirmed by immunohistochemistry of human breast cancer tissues. The altered gene expression of ECM proteins was also examined.

Furthermore, the contribution of TNC receptors was investigated by examining expression levels and alterations during CAF differentiation using a quantitative RT-PCR (RT-qPCR) and Western blotting. The heterodimer formation of subunits was examined by immunofluorescence and immunoprecipitation. The involvement of the transforming growth factor- β (TGF- β) signaling pathway in phenotypic changes was also investigated. In addition, differences in responses to the TNC treatment between fibroblasts derived from mammary glands and the dermis were assessed and discussed.

Materials and Methods

Antibodies

The antibodies used in the present study are summarized in Table 1.

Cell Culture

HMFs were purchased from ScienCell Research Laboratories (lot numbers 4066 and 15990; Carlsbad, CA). Human dermal fibroblasts (HDFs) derived from the abdominal skin of a 50-year-old female were obtained from Lifeline Cell Technology (lot number 02081; Oceanside, CA). Cells were grown in FibroLife Serum Free Medium (Complete Kit; Lifeline Cell Technology). Sixteen hours after seeding at 1.5×10^4 cells/cm² in multiwell plates (BD Falcon, Franklin Lakes, NJ), 10 μ g/mL of TNC purified from the conditioned medium of U-251MG³⁴ was added to the medium. After a 4-day culture, cells were analyzed by immunoblotting and immunofluorescence staining. Heparin (grade I-A; Sigma-Aldrich, St. Louis, MO), SB-505124 (TGF- β receptor I kinase inhibitor; Calbiochem, La Jolla, CA), or SIS3 (SMAD3 inhibitor; Cayman Chemical, Ann Arbor, MI) was added 30 minutes before the TNC treatment.

Immunoblotting

Cells were lysed using sample buffer (1% SDS, 5% 2-mercaptoethanol, and 50 mmol/L Tris-HCl buffer, pH 6.8). Lysates were heated at 98°C for 5 minutes and electrophoresed on 5% to 20% polyacrylamide gradient gels. Proteins were electrically transferred to Immobilon membranes (Merck Millipore, Carrigtwohill, Ireland), labeled with the primary antibodies (400- to 20,000-fold diluted) shown in Table 1, and then exposed to peroxidase-labeled anti-mouse IgG (3000- to 30,000-fold diluted; Bio-Rad, Hercules, CA) or anti-rabbit IgG (Bio-Rad). Bands were visualized with ECL Prime Western Blotting Detection Reagents (GE Healthcare, Waukesha, WI) using a cooled charge-coupled device camera. Band intensities were quantified using ImageJ software, Fiji version 1.52p (NIH, Bethesda, MD; <https://imagej.net/welcome>), and the values obtained were generally normalized to the intensities of α -tubulin bands. The phosphorylation of SMADs was evaluated after normalization by total SMADs. All

Table 1 Antibodies

Target	Clone	Method	Origin	Supplier
α -SMA	1A4	WB, IF, IHC	M	Abcam, Cambridge, UK
	EPR5368	WB, IF, IHC	R	Abcam
Caldesmon	EP789Y	WB	R	Abcam
Calponin	EP798Y	WB, IF, IHC	R	Abcam
Caveolin-1	D46G3	WB	R	Cell Signaling Technology, Danvers, MA
FAP	Polyclonal	WB	R	EMD Millipore, Temecula, CA
NG2	EPR9195	WB	R	Abcam
P4H	Polyclonal	WB	R	Abcam
PDGFR β	Y92	WB	R	Abcam
Podoplanin	18H5	WB	M	Abcam
S100A4	Polyclonal	WB	R	EMD Millipore
α -Tubulin	DM1A	WB	M	Cedarlane, Burlington, ON, Canada
Collagen I	EPR7785	WB	R	Abcam
Collagen III	EPR17673	WB	R	Abcam
Fibronectin	Polyclonal	WB	R	Sigma-Aldrich, St. Louis, MO
LOX	EPR4025	WB	M	Abcam
Syndecan 4	Polyclonal	WB	R	IBL, Takasaki, Japan
Tenascin-C	4F10TT	WB, IF, IHC	M	IBL
Integrin α 2	H-293	WB	R	Santa Cruz, Santa Cruz, CA
Integrin α v	EPR16800	WB	R	Abcam
	Polyclonal	IF	G	R&D Systems, Minneapolis, MN
	P2W7	IP	M	R&D Systems
Integrin β 1	EP1041Y	WB, IHC	R	Abcam
	P5D2	IF	M	R&D Systems
Integrin β 3	EPR17507	WB	R	Abcam
SMAD2	86F7	WB, IF	R	Cell Signaling Technology
pSMAD2 (Ser465/467)	Polyclonal	WB	R	Cell Signaling Technology
SMAD3	C67H9	WB, IF	R	Cell Signaling Technology
pSMAD3 (Ser423/425)	C25A9	WB	R	Cell Signaling Technology
SMAD2/3	Polyclonal	IF	R	GeneTex, Irvine, CA

α -SMA, α -smooth muscle actin; FAP, fibroblast activation protein α ; G, goat; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; LOX, lysyl oxidase; M, mouse; NG2, neural/glial antigen 2; P4H, prolyl 4-hydroxylase; PDGFR β , platelet-derived growth factor receptor- β ; pSMAD, phosphorylated SMAD; R, rabbit; S100A4, S100 calcium binding protein A4; WB, Western blot.

quantitative analyses were performed at least in triplicate. Precision plus protein dual-color standards (Bio-Rad) were used as molecular weight markers. Dots at the left margins of clipped gel images indicate the positions of the nearest marker bands at 15, 20, 25, 37, 50, 75, 100, 150, or 250 kDa.

Immunofluorescence

Fibroblasts were grown on cover glasses and fixed in 2% paraformaldehyde/phosphate-buffered saline with 0.2% Triton X-100 for 10 minutes. After a treatment with 10% normal goat serum, they were exposed to primary antibodies. Cells were subsequently treated with appropriate secondary antibodies, Alexa Fluor 488-labeled goat anti-mouse IgG or anti-rabbit IgG (200-fold diluted; Life Technology, Eugene, OR), and rhodamine-phalloidin (Life Technology). Alexa Fluor 555-labeled goat anti-mouse IgG Fab2 (Cell Signaling Technology, Danvers, MA) was generally used for double immunofluorescence. Alexa Fluor 488-labeled donkey anti-goat IgG and Alexa Fluor 555-labeled donkey anti-mouse IgG (Abcam, Cambridge, UK) were used for double immunofluorescence for α v and β 1. Rhodamine-phalloidin was purchased from

Molecular Probes (Eugene, OR). Observations were performed under an epifluorescence microscope with appropriate filter sets and a 10 $\times$ to 60 $\times$ objective lens (Olympus, Tokyo, Japan), and images were taken using a cooled charge-coupled device camera (Olympus). To evaluate the protein expression of TNC, cells were treated with 2 μ mol/L monensin (Biolegend, San Diego, CA), an intracellular vesicle transport inhibitor, for 30 minutes before fixation.

Immunohistochemistry

An immunohistochemical analysis was performed on 10 cases of fully invasive ductal carcinoma of the breast, including 7 cases with skin invasion, using archival samples for pathologic diagnoses that had been fixed in formalin and routinely processed for embedding in paraffin. The use of samples was approved by written informed consent from patients under a protocol authorized by the Institutional Review Board of our university. All sections were cut at a thickness of 4 μ m and placed on silane-coated glass slides. Antigen retrieval was performed by Target Retrieval Solution (pH 6.0; S1699; Dako Japan, Tokyo, Japan) using an

autoclave (121°C for 1 minute). Sections were treated with 10% goat or donkey serum before an incubation with primary antibodies overnight. After being washed, sections were treated with the secondary antibodies of Alexa Fluor

488-labeled goat anti-rabbit IgG and Alexa Fluor 555-labeled goat anti-mouse IgG, followed by nuclear staining with DAPI. When the double staining of the integrin α_v and β_1 subunits was performed, Alexa Fluor 488-labeled donkey anti-goat IgG and Alexa Fluor 555-labeled donkey anti-rabbit IgG (Abcam) were used. Observations were performed under an epifluorescence microscope.

Cell Proliferation Assay

HMFs were plated at 5×10^3 cells in 96-well plates (BD Falcon) and, after 16 hours, treated with TNC at 10 $\mu\text{g/mL}$. Seventy-two hours after the addition of TNC, 20 μL /well of MTS Reagent (Abcam) was added to each well and incubated at 37°C for 2 hours. The absorbance of TNC-treated and untreated cells (10 wells each) was measured using a plate reader at 490 nm.

Collagen Gel Contraction Assay

A collagen gel contraction assay was performed using a collagen gel culturing kit (Nitta Gelatin, Osaka, Japan). Type I collagen solution (3 mg/mL), 10 $\times$ minimal essential medium, reconstitution buffer, and the cell suspension were mixed carefully on ice at a ratio of 8:1:1:1. The final cell concentration was 5×10^5 cells/mL. The mixture at a volume of 500 μL was placed in each well of a 24-well plate and gelated in an incubator at 37°C for 30 minutes. After gelation, 500 μL of FibroLife medium was added to the gel in each well, and the medium was replaced by fresh medium three times every 30 minutes. Cells in the gel were cultured for 16 hours and, then, TNC at a concentration of 10 $\mu\text{g/mL}$ was added. Twenty-four hours after the addition of TNC, the gel was carefully detached from the well wall using a spatula. Collagen gel contraction was observed for 48 hours after detachment. Images of gels were scanned from the bottom of plates using a flatbed scanner (Epson Scan

Figure 1 Morphologic changes and the altered expression of cancer-associated fibroblast (CAF) markers in human mammary fibroblasts (HMFs) after the tenascin-C (TNC) treatment. **A:** HMFs are stellate in shape and form clusters, particularly on glass, similar to the hill-and-valley appearance of smooth muscle cells, 4 days after the TNC treatment. **B:** Representative images of the immunoblotting of CAF-related proteins in HMFs treated with/without TNC. **C:** The protein expression of α -smooth muscle actin (α -SMA) and calponin is significantly up-regulated in HMFs by the TNC treatment. Platelet-derived growth factor receptor- β (PDGFR β) is also up-regulated. Other CAF markers are not affected. Values were normalized to the intensities of α -tubulin bands. The graph is an average of three independent experiments and expressed as fold increases relative to non-treated cells. Differences between non-treated and TNC-treated groups were analyzed using the *t*-test with Welch correction. **D:** Immunofluorescence of cultured HMFs reveal well-developed stress fibers showing positive α -SMA and/or calponin staining after the TNC treatment, whereas non-treated cells show thin fibers with faint positive staining. Fibroblasts positive for α -SMA are more dominant. Data are shown as means $\pm$ SD (**C**). * $P < 0.05$, *** $P < 0.001$. Scale bars: 100 μm (**A**); 20 μm (**D**). FAP, fibroblast-activation protein α ; NG2, neural/glial antigen 2; P4H, prolyl 4-hydroxylase; S100A4, S100 calcium binding protein A4.

Figure 2 Cell proliferation, collagen gel contraction, and the expression of extracellular matrix (ECM) proteins and lysyl oxidase (LOX) after the tenascin-C (TNC) treatment. **A:** MTS assay. TNC treatment significantly promotes human mammary fibroblast (HMF) proliferation. Data are shown from 10 wells. **B:** Representative images of HMFs embedded in collagen gels with/without TNC. Images were taken 4 hours (left panel) and 36 hours (right panel) after gel detachment. **C:** Time course for decreases in gel areas. The graph is plotted by the average area of three gels at each point of time when images were taken and expressed as a percentage of the initial size. TNC treatment enhances the collagen gel contraction of HMFs. Differences between the non-treated and TNC-treated groups were analyzed using the *t*-test. **D:** Expression changes in ECM proteins analyzed by quantitative RT-PCR after the TNC treatment. The exogenous TNC treatment up-regulates the gene expression of *TNC* itself. Results were normalized to *GAPDH* expression. The graph represents an average of four independent experiments and shows fold increases relative to non-treated cells. Differences between non-treated and TNC-treated groups were analyzed using the *t*-test with Welch correction. **E:** Immunocytochemistry of TNC after the treatment with 2 μ mol/L monensin, to block intracellular vesicular traffic, for 30 minutes. TNC-treated cells with thick stress fibers show the accumulation of large amounts of TNC-loaded transport vesicles, indicating up-regulated synthesis of TNC protein. **F** and **G:** Immunoblot analysis of cell lysates for collagen I (COL1) and III (COL3) and fibronectin (FN, also known as FN1). Their protein deposits are significantly increased by the exogenous application of TNC. **H** and **I:** Expression of the LOX protein analyzed by immunoblotting after the TNC treatment. LOX expression did not change by the TNC treatment. Values were normalized to the intensities of α -tubulin bands. The graph is an average of three independent experiments. Data are shown as the means $\pm$ SD (**A**, **C**, **D**, **G**, and **I**). **P* < 0.05, ****P* < 0.001 TNC- versus TNC+. Scale bars = 20 μ m (**E**). α -SMA, α -smooth muscle actin.

GT-9800F; Seiko Epson, Suwa, Japan) and analyzed using ImageJ to measure the gel area.

RT-qPCR

The expression of mRNAs for the integrin subunits α V (ITGAV), α 2 (ITGA2), α 7 (ITGA7), α 8 (ITGA8), α 9 (ITGA9), β 1 (ITGB1), β 3 (ITGB3), and β 6 (ITGB6), and for COL1A2, COL3A1, SDC4, FN1, TNC, and GAPDH was quantified by qPCR. QuantiTect primers for genes were purchased from Qiagen (Valencia, CA). Total RNA (1 μ g) extracted using the ISOGEN reagent (Nippon Gene, Tokyo, Japan) was reverse transcribed using a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) with anchored-oligo (dT)₁₈ or random hexamer primers. qPCR was performed using a LightCycler 96 real-time PCR instrument and FastStart essential DNA green master kit (Roche Diagnostics). Reactions were performed

at 95°C for 10 minutes to activate the polymerase, and this was followed by 55 cycles at 95°C/10 seconds, 60°C/10 seconds, and 72°C/10 seconds. The specificity of the PCR was confirmed with a heat dissociation protocol (between 65°C and 95°C). Quantification cycle values were obtained using the second derivative maximum method with LightCycler 96 Software version 1.1.0.1320. Quantitative analyses of mRNA expression changes after the treatment were performed at least in triplicate.

Immunoprecipitation

Cells were lysed in ice-cold lysis buffer, 1% Nonidet P-40, 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L CaCl₂, and 1 mmol/L MgCl₂ with protease inhibitors (complete mini EDTA free; Roche Diagnostics). Lysates were centrifuged at 40,000 $\times$ *g* for 20 minutes, and the supernatants obtained were then precleared with protein G

Figure 3 Immunohistochemical analyses of human breast cancer tissues. **A and B:** Double immunofluorescence of human breast cancer tissues shows the colocalization of α -smooth muscle actin (α -SMA)—positive (**A**) or calponin-positive (**B**) fibroblasts within tenascin-C (TNC)—positive stroma. **C and D:** In the double immunohistochemical staining of α -SMA and calponin, double-positive fibroblasts are sparsely distributed at the invasive front, whereas cells co-expressing α -SMA and calponin are dense near the center of the tumor. Cells positive for α -SMA are more dominant. **E and F:** Narrow areas adjacent to the basement membrane of ductules are positive for TNC. Only a few fibroblasts in these areas are positive for α -SMA or calponin. Myoepithelia and capillary vessel walls are positive. Scale bars = 100 μ m (**A–F**). d, ductules with myoepithelia; v, vessel walls with smooth muscle cells as intrinsic positive controls.

Mag Sepharose (GE Healthcare) for 30 minutes and incubated with antibodies against α v or mouse IgG (Sigma-Aldrich) as a negative control at 4°C overnight. Complexes were precipitated with protein G beads for 2 hours. Beads were then washed three times with lysis buffer and boiled with sample buffer. Samples were subjected to immunoblotting using primary antibodies with a rabbit origin. Representative data are shown from at least three independent experiments that yielded similar results.

Silencing of *ITGAV* and *ITGB1*

siRNAs against *ITGAV* (Hs_ITGAV_7 FlexiTube siRNA) and *ITGB1* (Hs_ITGB1_4 FlexiTube siRNA) and Allstars negative control siRNA were purchased from Qiagen.

HMFs were grown in FibroLife Serum Free Medium for 12 hours, and the transfection of siRNAs was performed using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Carlsbad, CA), according to the manufacturer's protocol. TNC was added 8 hours later, and cells were incubated for 72 hours. Cell lysates were analyzed using immunoblots.

Statistical Analysis

Results are shown as means $\pm$ SD. Statistical analyses were performed using the *t*-test with Welch correction or a one-way analysis of variance with Tukey multiple comparison test. $P < 0.05$ was considered to be significant.

Results

Morphologic Changes and Alterations in the Expression of CAF Markers after the TNC Treatment

HMFs had a stellate shape 4 days after the addition of TNC (10 μ g/mL), but generally had a spindle shape aligned in a certain direction on a plastic substratum. More aggregated cluster formation was observed on glass surfaces, similar to the hill-and-valley appearance of smooth muscle cells (Figure 1A). The protein expression of α -SMA and calponin was significantly up-regulated in HMFs after the TNC treatment, indicating differentiation to myofibroblasts. No significant increases were observed in the expression of high-molecular-weight caldesmon, which is more characteristic of smooth muscle cells (Figure 1, B and C). The expression of platelet-derived growth factor receptor- β was significantly up-regulated, possibly indicating smooth muscle cell differentiation. No significant changes were observed in the expression of fibroblast-activation protein α , neural/glia antigen 2, prolyl-4-hydroxylase, podoplanin, or S100A4. TNC treatment may not induce a CAF subset other than myofibroblasts. Immunofluorescence of cultured HMFs revealed that stress fibers associated with positive α -SMA and/or calponin staining were well developed after the treatment, whereas nontreated cells showed scant and thin fibers with faint positive staining (Figure 1D). Fibroblasts showing α -SMA staining were more dominant. Thus, the TNC treatment induced the myofibroblastic differentiation of HMFs.

Cell Proliferation Assay, Collagen Gel Contraction Assay, and Alterations in the Expression of ECM Proteins

The TNC treatment significantly increased the proliferation of HMFs (Figure 2A). Because HMFs after the addition of TNC differentiated to myofibroblasts, contractility was assessed using a collagen gel contraction assay. An incubation with TNC for 24 hours enhanced the contractility of cells (Figure 2B), suggesting that the myofibroblastic

Figure 4 Gene and protein expression of tenascin-C (TNC) receptors of human mammary fibroblasts (HMFs) after the TNC treatment. **A:** mRNA was extracted from cells stimulated with TNC (10 μ g/mL) for the synthesis of cDNA, which was then used in real-time PCR to obtain the quantification cycle (Cq) values for each subunit. Differences in the Cq values of TNC receptor mRNAs from that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in TNC-treated cells are shown using quantitative PCR. The Cq values of $\alpha 7/8$ and $\beta 6$ are high, indicating only limited expression. The expression of $\alpha 9$ is not detected. Calibration curves were obtained using each target gene in samples. **B:** Changes in receptor mRNA expression for $\alpha v/2$, $\beta 1/3$, and syndecan 4 (SDC4) after the treatment with TNC. Results were normalized to GAPDH expression. Integrin $\beta 3$ significantly decreases after the TNC treatment. The graph is an average of four independent experiments and expressed as a fold increase relative to non-treated cells. Differences between groups were analyzed using the *t*-test with Welch correction. **C:** Representative results of the immunoblotting of integrin subunits and SDC4 in HMF lysates. **D:** The quantification of bands of receptor proteins in treated cells reveal significant increases in αv , $\beta 1$, and SDC4 after the TNC treatment from the levels noted with no treatment. Integrin $\beta 3$ bands were separately evaluated as upper ($\beta 3U$) and lower ($\beta 3L$) bands at 85 and 135 kDa, respectively. Results were normalized to the intensities of α -tubulin bands. The graph is an average of three independent experiments. **E** and **F:** To block the binding of the heparan sulfate chains of SDC4 to TNC, 100 μ g/mL heparin was added to the medium, but it did not inhibit the up-regulation of calponin expression. The graph is an average of three independent experiments and expressed as a fold increase relative to non-treated cells. Data are given as means $\pm$ SD (**B**, **D**, and **F**). **P* < 0.05, ***P* < 0.01.

phenotype with the increased expression of smooth muscle cell proteins was more contractile and potentially followed by the formation of a dense collagen matrix in cancer stroma. Differences in contractility appeared to be sustained for >48 hours (Figure 2C). The gene expression of ECM proteins was also evaluated using RT-qPCR. Although the expression of *COL1A2*, *COL3A1*, and *FN1* mRNAs was not significantly affected by the TNC treatment, that of *TNC* mRNA was significantly up-regulated (Figure 2D). To confirm alterations in the expression of TNC protein, immunofluorescence of cells treated with 2 μ mol/L monensin, a protein transport inhibitor, was performed for 30 minutes. Many transport vesicles that were positive for TNC accumulated in treated cells with well-developed stress fibers containing α -SMA, whereas the number of vesicles in non-treated cells was negligible (Figure 2E). An immunoblot analysis of cell lysates showed a significant increase in the protein deposition of collagens and fibronectin after the TNC treatment (Figure 2, F and G). The protein expression of lysyl oxidase cross-linking collagen molecules was also not significantly affected (Figure 2, H and I).

Immunohistochemical Analysis of Human Breast Cancer Tissues

Double immunofluorescence of human breast cancer tissues revealed α -SMA— and/or calponin-positive fibroblasts in the TNC-rich stroma (Figure 3, A and B). Fibroblasts showing α -SMA staining were more dominant than calponin-positive cells as well as *in vitro*. Positive cells were preferentially adjacent to cancer nests. Although positively stained fibroblasts were sparsely distributed at the invasive front, a dense population of cells co-expressing α -SMA and calponin was observed near the center of the tumor (Figure 3, C and D). Positive fibroblasts were rarely visible in normal breast tissues conserved around cancer (Figure 3, E and F).

Detection of TNC Receptors Expressed in Mammary Fibroblasts

Integrins reported as TNC receptors include $\alpha 2/7/8/9/v$ and $\beta 1/3/6$ subunits.³⁵ The strong mRNA expression of the αv , $\alpha 2$, $\beta 1$, and $\beta 3$ subunits was validated by RT-qPCR (Figure 4A), and

Figure 5 Enhancement of integrin $\alpha v\beta 1$ heterodimer formation by the tenascin-C (TNC) treatment. **A** and **B**: In double immunofluorescence, the αv and $\beta 1$ subunits colocalize at focal adhesion sites after the TNC treatment. **C** and **D**: Immunoprecipitation using the anti- αv antibody shows a significant increase in the coprecipitated $\beta 1$ subunit after the TNC treatment and a slight decrease in $\beta 3$ coprecipitation. TNC also coprecipitates with integrin heterodimers. The graph is an average of three independent experiments and expressed as a fold increase relative to non-treated cells. Differences between groups were analyzed using the *t*-test with Welch correction. **E** and **F**: Silencing of the integrin αv and $\beta 1$ subunits by siRNA transfection. The protein levels of the αv and $\beta 1$ subunits are suppressed by 80 and 93%, respectively, following the transfection of siRNA against *ITGAV* (AV) and *ITGB1* (B1). The expression of α -smooth muscle actin (α -SMA) and calponin is significantly weaker than the transfection of negative control siRNA (NC). Differences between groups were analyzed using a one-way analysis of variance. **G** and **H**: In the double immunofluorescence of human breast tissues, spindle-shaped cells co-expressing the αv and $\beta 1$ subunits are dominantly observed in the cancer stroma (**H**), but not around normal ducts (**G**). Data are given as means $\pm$ SD (**D** and **F**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars: 20 μ m (**A** and **B**); 100 μ m (**G** and **H**).

these subunits were readily detected in an immunoblot analysis of HMFs (Figure 4C). Quantification cycle values for the $\alpha 7/8$ and $\beta 6$ subunits were markedly higher than that for glyceraldehyde-3-phosphate dehydrogenase, indicating the lack of significant expression in these cells. A signal for the $\alpha 9$ subunit

was not detected. Syndecan 4 (SDC4), a TNC receptor, was also detected at the mRNA and protein levels. A comparison of expression levels between non-treated and TNC-treated cells revealed that the gene expression of *ITGB3* significantly decreased after the TNC treatment (Figure 4B). The protein levels of αv , $\beta 1$, and SDC4 were significantly increased in TNC-treated cells (Figure 4D). SDC4 is a cell surface proteoglycan with heparan sulfate chains that bind to TNC. Because soluble heparin inhibits the binding of TNC to SDC4, 100 μ g/mL heparin was added to the medium 30 minutes before the TNC treatment. However, the TNC-induced up-regulation of calponin expression was not inhibited by heparin (Figure 4, E and F). SDC4 may not be the receptor responsible for the signal that up-regulates calponin.

Formation of the Integrin $\alpha v\beta 1$ Heterodimer Is Enhanced by the TNC Treatment

Because the integrin αv and $\beta 1$ subunits were increased in immunoblots, their localization was analyzed by immunofluorescence. Double immunofluorescence showed the increased colocalization of αv and $\beta 1$ at focal adhesion sites with the TNC treatment (Figure 5, A and B). Immunoprecipitation using the anti- αv antibody revealed that the TNC treatment significantly increased the coprecipitation of $\beta 1$ subunits (Figure 5, C and D). TNC was also coprecipitated. In addition, a slight decrease was observed in $\beta 3$ coprecipitation. The functions of the integrin αv and $\beta 1$ subunits were investigated using siRNA-mediated knockdown. Although the protein levels of the αv and $\beta 1$ subunits decreased by 80% and 93%, respectively, after silencing, the expression of α -SMA and calponin was significantly down-regulated (Figure 5, E and F). These results indicated that the formation of integrin $\alpha v\beta 1$ heterodimer was responsible for the up-regulation of α -SMA and calponin, followed by myofibroblast differentiation. Double immunofluorescence of human breast tissues showed that fibroblasts dominantly co-expressed the αv and $\beta 1$ subunits in the cancer stroma, particularly around cancer nests (Figure 5H), but not around normal ducts (Figure 5G). Integrin $\alpha v\beta 1$ of fibroblasts in breast cancer tissues may function as a major TNC receptor.

Activation of TGF- β Signaling by the TNC Treatment

TGF- $\beta 1$ is secreted as an inactive homodimer together with its latency-associated peptide³⁶ by cancer cells and fibroblasts. Recent studies reported that integrin $\alpha v\beta 1$ directly binds to an Arg-Gly-Asp (RGD)-binding site in the latency-associated peptide of TGF- $\beta 1$ and induces a putative conformational change that liberates TGF- $\beta 1$.^{36–38} Although the chemically defined medium used in the present study contained 30 pg/mL TGF- $\beta 1$, the phosphorylation of SMAD2/3 was not apparent in non-treated cells. The TNC treatment induced significant increases in the phosphorylation of SMAD2 at Ser465/467 and SMAD3 at Ser423/425, with a peak being observed 2 hours after the treatment (Figure 6, A and B). In

Figure 6 Activation of transforming growth factor- β 1 signaling by the tenascin-C (TNC) treatment. **A:** Representative images of the immunoblotting of phosphorylated SMAD2/3 (pSMAD2/3) and total SMADs. **B:** The TNC treatment induces significant increases in the phosphorylation of SMAD2/3, with a peak being observed 2 hours after the treatment. Phosphorylated SMADs were normalized to the intensities of total SMAD bands. The graph is an average of three independent experiments and expressed as a fold increase relative to non-treated cells. Significant differences are noted in phosphorylation levels from those in non-treated cells at all time points. **C and D:** Immunofluorescence staining shows the nuclear translocation of SMAD2/3. **E:** The administration of 2 μ mol/L SB-505124 and SIS3 completely blocks the up-regulation of α -smooth muscle actin (α -SMA) and calponin by TNC. Differences between groups were analyzed using a one-way analysis of variance. **F:** The double immunofluorescence of α -SMA and calponin show diminished stress fibers after administration of the inhibitors. Data are given as means $\pm$ SD (**B** and **E**). * P < 0.05, ** P < 0.01, and *** P < 0.001 versus no treatment at all time points. Scale bars = 20 μ m (**C**, **D**, and **F**).

immunofluorescence, the nuclear translocation of SMAD2/3 was observed at 2 hours (Figure 6, C and D). The administration of 2 μ mol/L SB-505124 and SIS3, TGF- β signaling inhibitors, completely blocked the up-regulation of α -SMA and calponin by TNC in immunoblots and immunofluorescence (Figure 6, E and F). The treatment with 5 ng/mL TGF- β 1 alone also up-regulated the expression of α -SMA, calponin, and TNC more strongly than the TNC treatment (Supplemental Figure S1, A and B). The TGF- β 1 treatment induced greater increases in the phosphorylation of SMAD2/3, with a peak being observed within 30 minutes (Supplemental Figure S1, C and D). TGF- β 1 induces the phosphorylation of SMADs more rapidly than TNC. Immunohistochemistry using an anti-SMAD2/3 antibody showed the nuclear translocation of SMADs in the fibroblasts of cancer stroma with dense TNC deposition and cancer cells (Supplemental Figure S1F), but not in the fibroblasts around normal ducts (Supplemental Figure S1E).

Effects of TNC on HDFs

Immunohistochemistry of human breast cancer tissues revealed that the cancer stromal fibroblasts that localized in mammary glands expressed α -SMA and calponin in all 10 cases. However, the expression of calponin by dermal fibroblasts was not observed at the skin invasion sites of breast cancer, whereas the expression of α -SMA and dense deposition of TNC between cancer nests (Figure 7, A and B) were detected in all seven cases. In these cases, the expression of calponin was only observed in primary breast cancer. These results suggest that the response of fibroblasts to TNC may differ between dermal and mammary tissues. Therefore, differences in the effects of TNC between HMFs and HDFs were also investigated. Although the expression of α -SMA and calponin in HDFs was significantly up-regulated by the TNC treatment, this response was weaker than that in HMFs (Figure 7, C and D).

Figure 7 Effects of tenascin-C (TNC) on phenotypic changes in human dermal fibroblasts. **A:** Expression of α -smooth muscle actin (α -SMA), but not calponin, by dermal fibroblasts in immunohistochemistry of the skin invasion lesions of human breast cancers. **B:** Representative images of the immunoblotting of cancer-associated fibroblast (CAF)-related proteins in human dermal fibroblasts (HDFs) treated with/without TNC. **C:** The expression of α -SMA and calponin in HDFs is significantly up-regulated by the TNC treatment as well as human mammary fibroblast (HMF). The expression of neural/glial antigen 2 (NG2) is also significantly increased. Podoplanin is slightly up-regulated. Values were normalized to the intensities of α -tubulin bands. The graph is an average of three independent experiments and expressed as a fold increase relative to non-treated cells. Differences between groups were analyzed using the *t*-test with Welch correction. **D:** A comparison of the expression levels of CAF-related proteins between HMFs and HDFs with the TNC treatment. **E:** The expression of α -SMA, caldesmon, fibroblast-activation protein α (FAP), and calponin are significantly weaker in HDFs. The graph is an average of three independent experiments and expressed as a fold increase relative to HDFs. Differences between groups were analyzed using the *t*-test with Welch correction. Data are given as means $\pm$ SD (**C** and **E**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars = 100 μ m (**A**). P4H, prollyl 4-hydroxylase; PDGFR β , platelet-derived growth factor receptor- β ; s, bundles of smooth muscle cells in the deep dermis as intrinsic positive controls; S100A4, S100 calcium binding protein A4.

In a comparison of the expression of CAF markers after the TNC treatment between HMFs and HDFs, the expression levels of α -SMA, caldesmon, fibroblast-activation protein α , and calponin were significantly lower in HDFs (Figure 7E). The expression levels of S100A4 and neural/glial antigen 2 appeared to be higher than those in HMFs. These characteristic responses to the TNC treatment are consistent with the immunohistochemical findings of breast cancer tissues with skin invasion.

Discussion

The exogenous application of TNC to the medium induced the differentiation of HMFs to myofibroblasts. In immunoblots, the expression of α -SMA and calponin significantly increased in HMFs, indicating differentiation to myofibroblasts. Immunofluorescence revealed well-developed stress fibers that were positive for α -SMA and/or calponin. Observations with phase contrast microscopy revealed that cells formed aggregated clusters that were similar to the hill-and-valley appearance of smooth muscle cells. Immunohistochemistry of human breast cancers revealed a close relationship between myofibroblasts positive for α -SMA and/or calponin and the deposition of TNC. The

colocalization of α -SMA-positive myofibroblasts and the deposition of TNC in the stroma of colon cancers was previously demonstrated.¹⁷ Furthermore, after electrical injury to the myocardium of TNC-null mice, the appearance of myofibroblasts was delayed. In cultured cardiac fibroblasts, TNC significantly accelerated cell migration, α -SMA expression, and collagen gel contraction, but did not affect proliferation.³⁹ The present results also revealed that TNC promoted the differentiation of HMFs to myofibroblasts. Myofibroblasts expressing calponin in breast cancer stroma were observed in a previous study on breast cancers.²⁴ Calponin and high-molecular-weight caldesmon are factors that influence late differentiation in smooth muscle cells during embryonic development.⁴⁰ During the development of the coronary arteries of the embryonic heart, epicardial cells were differentiated into the smooth muscle cells, expressing α -SMA and/or calponin, of coronary arterial walls by TNC.⁴¹ TNC appears to function as a strong inducer of differentiation to myofibroblasts/smooth muscle cells. In the gel contraction assay, myofibroblasts that were differentiated by the TNC treatment appeared to be highly contractile. The contraction of the collagen matrix is one of the primary functions of myofibroblasts *in vivo*, increasing collagen fiber density and tissue stiffness. *In vitro* studies demonstrated that matrix stiffening contributed to

maintaining the myofibroblast phenotype, thereby establishing a self-reinforcing positive feedback loop⁴² and enhancing cancer cell proliferation⁴³ via the *YAP/TAZ* pathway. In a syngeneic implantation model of mammary cancer cells in mice, tumor stiffness estimated by ultrasound elastography positively correlated with collagen and myofibroblast density.⁴⁴ Increased tumor stiffness has also been identified as a poor prognostic marker of various cancers, including human breast cancer.^{45,46} TNC may be responsible for enhancing the malignant behavior of the tumor through the formation of a stiffer cancer stroma.

Although the gene expression of *COL1A2*, *COL3A1*, and *FNI* were investigated for the formation of a stiffer ECM and the protein expression of lysyl oxidase to cross-link collagen fibers, expression changes were not observed after the TNC treatment. However, the protein deposition of collagens and fibronectin was increased by the addition of TNC. TNC may accelerate the accumulation of ECM proteins in breast cancer stroma. In addition, the exogenous application of TNC up-regulated the expression of TNC itself in a positive feedback manner. TNC-producing cells were shown to be the myofibroblastic phenotype with α -SMA—positive thick stress fibers in immunofluorescence. This is consistent with the previous finding of double labeling with the *in situ* hybridization of TNC mRNA and α -SMA immunohistochemistry in colon cancer tissues.¹⁷ In the TNC immunohistochemistry of breast cancer, micro-invasion sites around the duct have often shown the dense deposition of TNC.³ TNC produced by cancer cells may induce the synthesis of TNC by stromal cells, thereby facilitating cancer cell invasion. In scirrhous carcinoma, TNC secreted by activated fibroblasts may induce the synthesis of TNC in an autocrine manner, and this is followed by the promotion of stromal remodeling. In lung metastasis, cancer cell—derived TNC has been reported to promote the survival and outgrowth of pulmonary micrometastases.¹⁰ Cancer cell—derived TNC may also be an inducer of TNC production by stromal cells at metastasis-initiating sites, comprising a colonized metastatic niche. The positive feedback loop may be an important mechanism for breast cancer progression at any site.

It was previously demonstrated that integrin α v β 1 and α v β 6 are receptors for TNC and that binding to TNC is conducive for epithelial-mesenchymal transition—like changes in MCF7 cells.¹² Double immunofluorescence revealed the colocalization of the α v and β 1 subunits after the TNC treatment, and an immunoprecipitation assay using the anti- α v antibody showed a significant increase in coprecipitated β 1. TNC also coprecipitated with these subunits. The exogenous application of TNC appears to enhance the formation of integrin α v β 1 heterodimers. The knockdown of the α v and β 1 subunits down-regulated the expression of α -SMA and calponin. Double immunofluorescence of human breast cancer tissues showed the predominant co-expression of the α v and β 1 subunits in cancer stromal fibroblasts. Integrin α v β 1 of fibroblasts was recently

shown to directly bind to the RGD sequence of the latency-associated peptide of TGF- β 1 and activate the latent TGF- β complex.^{36–38} Augmented TGF- β signaling causes organ fibrosis in the liver³⁷ and kidney.³⁸ These fibrotic diseases also strongly express TNC during disease progression.^{47–49} The present results showing that TNC enriches integrin α v β 1 heterodimers on activated fibroblasts are important for clarifying the contribution of TNC to fibrotic diseases as well as cancer stromal formation.

Among several signaling pathways, TGF- β /SMAD signaling is widely recognized as a main contributor to myofibroblastic differentiation in various tissues.^{50,51} Differentiation to myofibroblasts after the TNC treatment was inhibited by a TGF- β receptor type I kinase inhibitor and SMAD3 inhibitor, indicating that myofibroblast differentiation induced by TNC is driven by TGF- β /SMAD signaling. The present study showed that the TNC treatment also induced the phosphorylation of SMAD2/3. Phosphorylation peaked 2 hours after the TNC treatment and within 30 minutes of the application of TGF- β . Translocation to nuclei was also observed after 2 hours. Delayed phosphorylation indicated that TNC did not directly stimulate TGF- β signaling. These different times may be necessary for another step, such as exchanging the heterodimer partners of integrins. When bovine aortic endothelial cells are treated with TNC, focal adhesion initially decreases, peaks by 45 minutes, and persists for at least 4 hours in the continued presence of TNC. When cells are washed free of TNC, this effect is fully reversible and independent of *de novo* protein synthesis.⁵² When the heterodimers of integrins are exchanged, focal adhesion may be lost. The longer stimulation times of TNC than TGF- β may be necessary for exchanging the heterodimer partners of integrins. TNC may contribute to the formation of α v β 1 heterodimers to activate the latent forms of TGF- β 1 and enhance TGF- β signals.

In the immunohistochemistry of breast cancer tissues, calponin expression by activated fibroblasts at skin invasion sites was negligible, whereas α -SMA was expressed. Global gene expression by fibroblasts derived from various tissues/sites was recently reported, and topological and/or anatomic diversities were elucidated.^{53–55} When the expression of CAF markers between HMFs and HDFs were compared after the TNC treatment, the protein amounts of α -SMA and calponin were markedly lower in HDFs than in HMFs. HMFs may acquire the myofibroblastic phenotype more readily and robustly than HDFs. These results suggest that the diversity of fibroblasts causes the tissue-specific features of the cancer stroma in several cancers.

In conclusion, this study clearly demonstrates that TNC up-regulates the production of TNC itself and promotes myofibroblastic differentiation with high contractility via the integrin α v β 1/TGF- β signaling axis. Myofibroblasts induced by TNC may contribute to remodeling of the stroma and increases in the stiffness of breast cancer. Further studies are needed to examine the effects of TNC on fibroblasts other than mammary and dermal fibroblasts.

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Author Contributions

T.Y. and D.K. designed the research, performed experiments, analyzed the data, and wrote the manuscript; A.N. and T.O. provided patient samples; Y.K. analyzed the histology of tissue samples; K.I.-Y. reviewed the manuscript; T.Y. acquired research funding; all authors of this article have read and approved the final version. T.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for their integrity and the accuracy of the data analysis.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.06.008>.

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