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# Peptide TNIIIA2 Derived from Tenascin-C Contributes to Malignant Progression in Colitis-Associated Colorectal Cancer via $\beta$ 1-Integrin Activation in Fibroblasts

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**Abstract:** Inflammatory bowel diseases increase the risk of colorectal cancer and colitis-associated colorectal cancer (CAC). Tenascin-C, a matricellular protein, is highly expressed in inflammatory bowel diseases, especially colorectal cancer. However, the role of tenascin-C in the development of CAC is not yet fully understood. We previously showed that a peptide derived from tenascin-C, peptide TNIIIA2, induces potent and sustained activation of  $\beta$ 1-integrin. Moreover, we recently reported that peptide TNIIIA2 promotes invasion and metastasis in colon cancer cells. Here, we show the pathological relevance of TNIIIA2-related functional site for the development of CAC. First, expression of the TNIIIA2-containing TNC peptides/fragments was detected in dysplastic lesions of an azoxymethane/dextran sodium sulfate (AOM/DSS) mouse model. In vitro experiments demonstrated that conditioned medium from peptide TNIIIA2-stimulated human WI-38 fibroblasts induced malignant transformation in preneoplastic epithelial HaCaT cells. Indeed, these pro-proliferative effects stimulated by peptide TNIIIA2 were abrogated by peptide FNIII14, which has the ability to inactivate  $\beta$ 1-integrin. Importantly, peptide FNIII14 was capable of suppressing polyp formation in the AOM/DSS model. Therefore, tenascin-C-derived peptide TNIIIA2 may contribute to the formation of CAC via activation of stromal fibroblasts based on  $\beta$ 1-integrin activation. Peptide FNIII14 could represent a potential prophylactic treatment for CAC.

**Keywords:** tenascin-C; colitis-associated colorectal cancer;  $\beta$ 1-integrin; cancer-associated fibroblast; AOM/DSS model

## 1. Introduction

The association of chronic inflammation with the pathogenesis of numerous malignancies has been well documented. In particular, it has been established that patients with inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease, have an increased risk of developing colorectal cancer, or colitis-associated colorectal cancer (CAC) [1–3]. Unlike sporadic colorectal cancer, the development of CAC does not display an adenoma-carcinoma sequence, but rather an inflammation-dysplasia-carcinoma sequence [4,5]. However, the molecular basis for the development of CAC remains unclear.

Tenascin-C (TNC), one of the typical matricellular proteins, is highly expressed in inflammatory lesions and tumor tissues [6,7]. A number of studies have also reported that upregulated expression of TNC occurs in ulcerative colitis, Crohn's disease, and colon cancer [8–11]; however, the extent of the role of TNC in the pathogenesis of these diseases is not fully understood. We previously found that TNC harbors a cryptic functional site composed of the amino acid sequence YTITIRGV, which becomes active following proteolytic cleavage [12]. A 22-mer TNC peptide containing this functional sequence, termed peptide TNIII A2, has a potent and sustained ability to activate  $\beta$ 1-integrins [13]. Since this active sequence resides within the cancer-associated alternative splicing domain—fibronectin type III repeat A2 (FNIII-A2)—of the TNC molecule [14], it is presumed that the  $\beta$ 1-integrin activation induced by TNIII A2-containing TNC peptides/fragments may contribute to the pathogenesis of cancer. Recently, peptide TNIII A2 was shown to act directly on colon cancer cells to promote their *in vitro* ability to invade through enhanced secretion of matrix metalloproteinase [15]. Furthermore, an *in vivo* experiment using a spontaneous metastasis model showed that peptide TNIII A2 is also involved in the metastasis of colon cancer cells to the lung. On the other hand, we also found previously that a peptide derived from fibronectin, termed peptide FNIII14, induces inactivation of  $\beta$ 1-integrins [16]. Interestingly, peptide FNIII14 has the ability to abrogate cell regulation by peptide TNIII A2 [17].

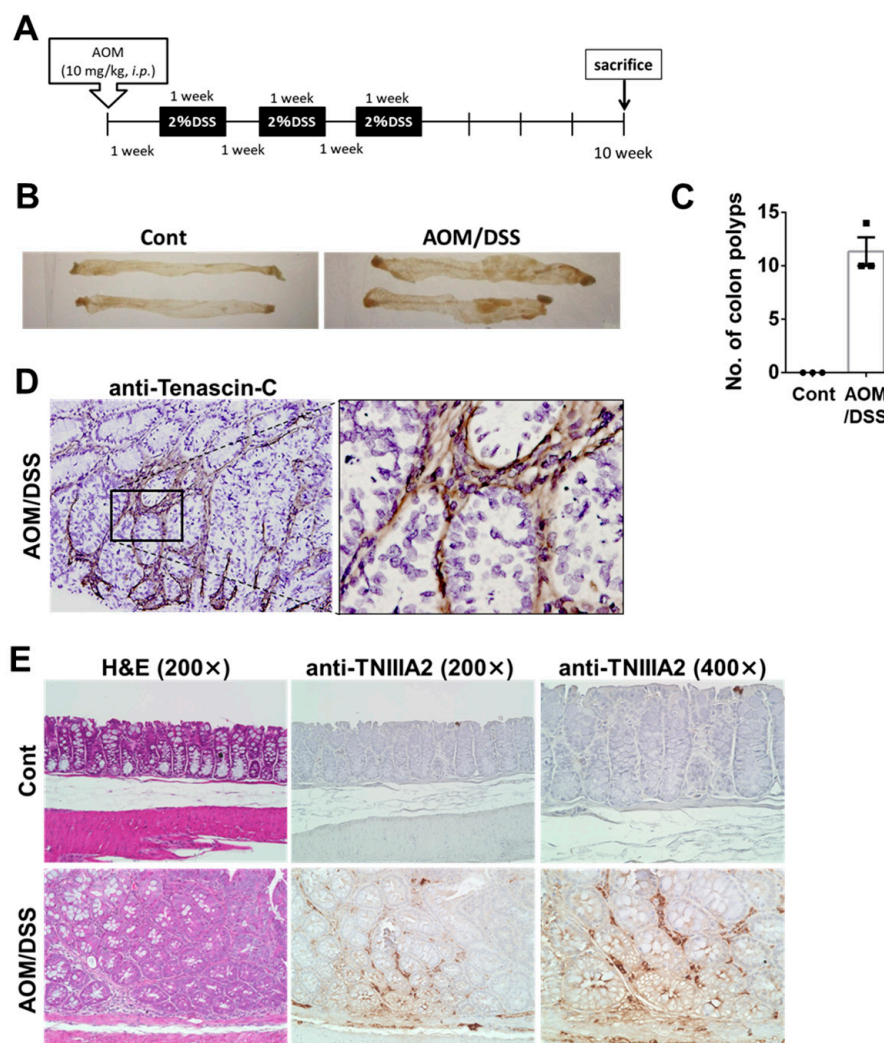
In the present study, immunohistochemical analysis first showed high expression of TNIII A2-containing TNC peptides/fragments in the stromal area of dysplastic lesions in AOM/DSS mice. Based on this observation, *in vitro* experiments focused on the effects of  $\beta$ 1-integrin activation on both preneoplastic epithelial cells and stromal fibroblasts. Interestingly, the results suggested that the activation of  $\beta$ 1-integrins by peptide TNIII A2 does not have a significant direct effect on preneoplastic epithelial cells. However, stromal fibroblasts stimulated by peptide TNIII A2 secreted a humoral factor(s) that induced malignant transformation in premalignant cells. Peptide FNIII14 suppressed both the *in vitro* effects of peptide TNIII A2 and polyp formation in the AOM/DSS model, suggesting that the activation of  $\beta$ 1-integrins is an important target for the prevention of IBDs including CAC. Peptide FNIII14, a unique factor that inactivates  $\beta$ 1-integrins, may be a promising agent for the chemotherapeutic treatment of CAC.

## 2. Results

### 2.1. TNC/TNIII A2 Expression in a Murine AOM/DSS Model

A well-characterized CAC model in ICR mice was generated by the administration of AOM and DSS, according to conventional methods [18] (Figure 1A). This model displayed dysplasia and adenomatous polyps 10 weeks after the first AOM injection (Figure 1B,C). We analyzed the expression levels of TNC and TNC fragments in the colon tissues of both untreated control mice and AOM/DSS-treated mice. In this immunohistochemical analysis, we used two kinds of antibodies: anti-TNC mAb recognizing the epidermal growth factor-like repeats present in all variants of TNC and anti-TNIII A2 polyclonal Ab raised against the proadhesive amino acid sequence (YTITIRGV), which can detect TNIII A2-containing TNC peptides/fragments. As expected, a high expression of TNC was detected in colonic tissues of AOM/DSS mice, using anti-TNC mAb (Figure 1D) [19]. TNIII A2-containing TNC peptides/fragments recognized by anti-TNIII A2 Ab were also detected in dysplastic lesions in the mucosa of AOM/DSS-treated mice (Figure 1E). It appeared that immunostaining for both TNIII A2

and TNC show prominent localization around colon stromal cells. Thus, TNIIIA2-containing TNC peptides/fragments were shown to be expressed in dysplastic lesions of AOM/DSS mice. It is possible that  $\beta$ 1-integrin activation induced by TNIIIA2-containing TNC peptides/fragments influences the development and progression of CAC.



**Figure 1.** TNC/TNIIIA2 expressed in murine AOM/DSS colitis-associated carcinoma model. **(A)** Schematic of AOM and DSS administration to mice. **(B)** Macroscopic view of the resected colon in control and AOM/DSS-treated mice. **(C)** Tumor incidence in the AOM/DSS model. Dot plots show the number of colon polyps of individual mice in each group with bars representing means  $\pm$  standard deviation. **(D,E)** Paraffin-embedded colon sections were stained with hematoxylin and eosin (H&E), TNC, and TNIIIA2 (original magnification 200 $\times$  and 400 $\times$ ).

## 2.2. Paracrine Factor(s) Released from Fibroblasts in Response to Stimulation of Peptide TNIIIA2 Induces Dysregulated Proliferation in Premalignant and Malignant Cells

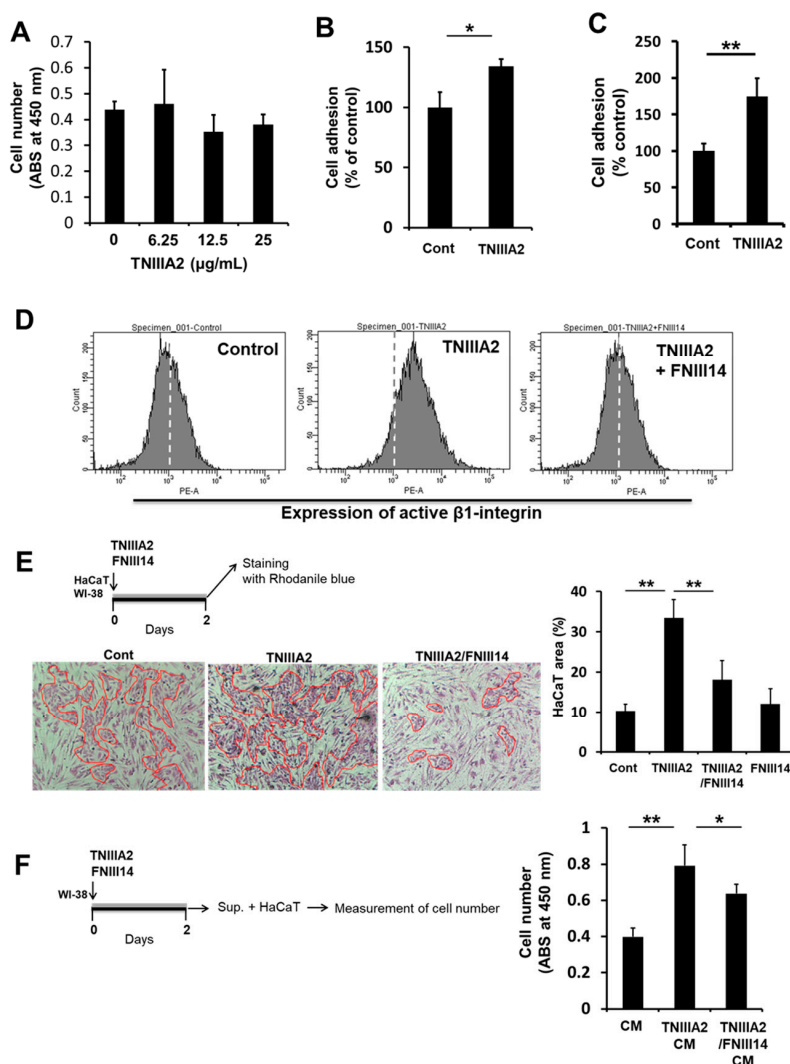
Based on the immunohistochemistry results showing that TNIIIA2-containing TNC peptides/fragments were localized within dysplastic lesions of AOM/DSS-treated mice, the effects of  $\beta$ 1-integrin activation on human preneoplastic epithelial cell line HaCaT was investigated using peptide TNIIIA2 as a representative of TNIIIA2-containing TNC peptides/fragments. However, peptide TNIIIA2 showed no remarkable effects on the proliferation of HaCaT cells (Figure 2A), although adhesion to the fibronectin substrate was significantly promoted (Figure 2B). Considering the involvement of stromal fibroblasts in cancer pathology [20,21], we next investigated whether fibroblastic cells treated

with TNIII A2 can affect the malignant transformation of preneoplastic HaCaT cells. Human normal fibroblasts, WI-38 cells, were used in the following experiments. First, it was verified that peptide TNIII A2 promoted the adhesion of WI-38 cells to a fibronectin substrate (Figure 2C), in response to activation of  $\beta$ 1-integrins by TNIII A2 (Figure 2D), as determined by flow cytometry using a monoclonal antibody recognizing an active conformation-specific epitope of the  $\beta$ 1-integrins. Therefore, HaCaT cells were then co-cultured with WI-38 cells in the presence or absence of peptide TNIII A2, and the proliferation of HaCaT cells was evaluated by selective staining of epithelial cells with Rhodanile blue. As shown in Figure 2E, co-culture with WI-38 fibroblasts markedly increased the area occupied by HaCaT cells in the presence of peptide TNIII A2 (represented as “TNIII A2” in Figure 2E), compared to when it was absent (represented as “Cont”). To clarify the involvement of  $\beta$ 1-integrin activation in the promotion of cell growth by peptide TNIII A2 in this co-culture system, the effect of peptide FNIII 14, which induces inactivation in  $\beta$ 1-integrins (Figure 2D), was examined. As expected, the promotion of HaCaT cell proliferation by peptide TNIII A2 in the co-culture system was prevented by the addition of FNIII 14 (represented as “TNIII A2/FNIII 14” in Figure 2E). Peptide FNIII 14 alone showed no noticeable effect on HaCaT cell proliferation in the co-culture system (represented as “FNIII 14” in Figure 2E). To investigate the role of WI-38 cells in promoting HaCaT cell proliferation in the co-culture system, the effects of WI-38 fibroblasts in conditioned medium (CM) on HaCaT cell proliferation were analyzed. WI-38 fibroblasts were cultured with or without peptide TNIII A2. After 48 h, HaCaT cells were cultured in CM (Figure 2F). CM from WI-38 cells stimulated with peptide TNIII A2 (represented as “TNIII A2 CM”) remarkably enhanced the proliferation of HaCaT cells, compared to CM from WI-38 cells cultured without peptide TNIII A2 (represented as “CM”) (Figure 2F). This peptide TNIII A2-mediated cell proliferation via WI-38 fibroblasts was also partially suppressed by peptide FNIII 14 (Figure 2F).

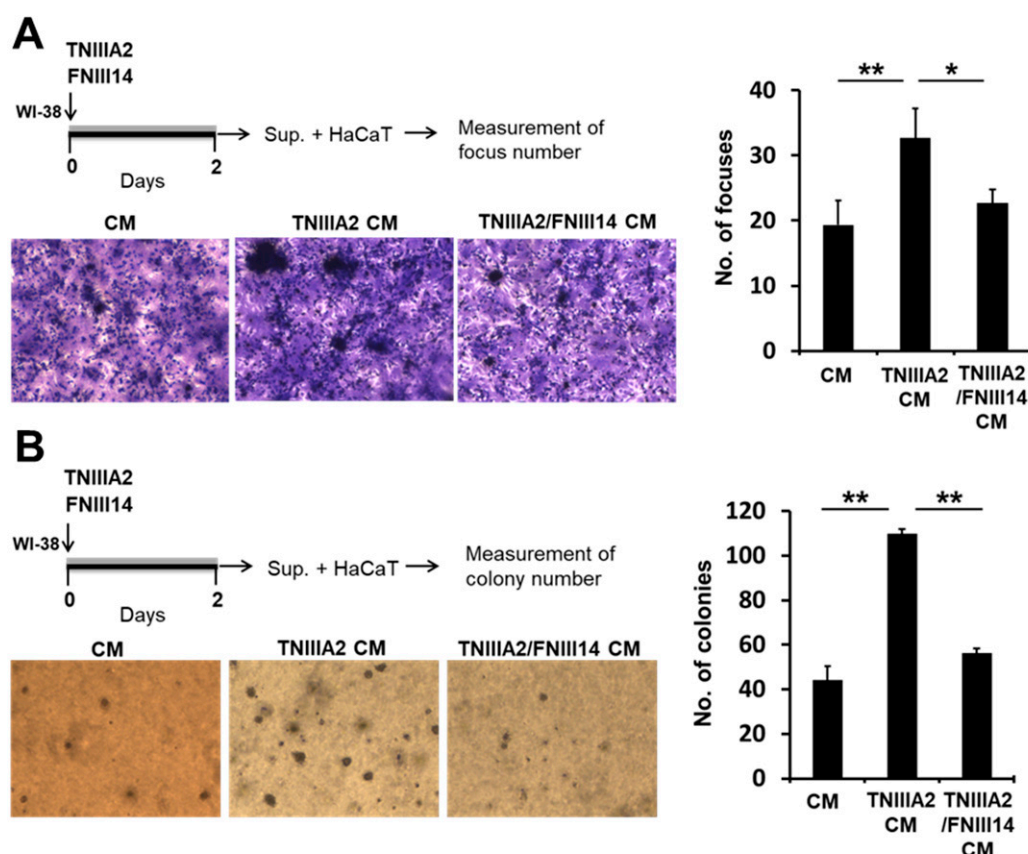
Noticeably, CM from peptide TNIII A2-stimulated WI-38 cells induced the formation of cell foci consisting of multilayered HaCaT cells (Figure 3A). Furthermore, CM from peptide TNIII A2-stimulated WI-38 cells also promoted anchorage-independent proliferation in HaCaT cells, as shown by a soft agarose colony formation assay (Figure 3B). This phenotypic change in HaCaT cells was also suppressed by peptide FNIII 14 (Figure 3A,B). These results indicated that peptide TNIII A2 acts on WI-38 to stimulate the release of a paracrine factor(s), which induces malignant transformation of preneoplastic epithelial HaCaT cells. Since the effect of peptide TNIII A2 was reversed by peptide FNIII 14 with an opposing effect on integrin activation, the enhanced proliferation of HaCaT cells in both anchorage-dependent and -independent manners by peptide TNIII A2 seems to be due to the activation of  $\beta$ 1-integrin.

We next examined the effects of peptide TNIII A2 on the neoplastic cell lines Caco-2 and colon26-M3.1. Similar to the effects on preneoplastic epithelial HaCaT cells, peptide TNIII A2 promoted cell adhesion to the fibronectin substrate (Figure 4A) but did not directly affect the proliferation of human colorectal adenocarcinoma cell line Caco-2 (Figure 4B). CM from peptide TNIII A2-stimulated WI-38 fibroblasts increased the proliferation of Caco-2 cells on the fibronectin substrate (represented by “TNIII A2 CM” in Figure 4C). Furthermore, CM from peptide TNIII A2-stimulated WI-38 fibroblasts strongly stimulated Caco-2 cell proliferation, also in an anchorage-independent manner (Figure 4D, left panel). Similar phenomena were observed for another colon cancer cell line, colon26-M3.1 (Figure 4D, right panel). Importantly, peptide FNIII 14 suppressed the effects of peptide TNIII A2-stimulated WI-38 fibroblasts (Figure 4E). These results suggest that a paracrine factor(s) from peptide TNIII A2-stimulated WI-38 fibroblasts induces malignant progression in premalignant epithelial HaCaT cells as well as in overtly malignant cells.





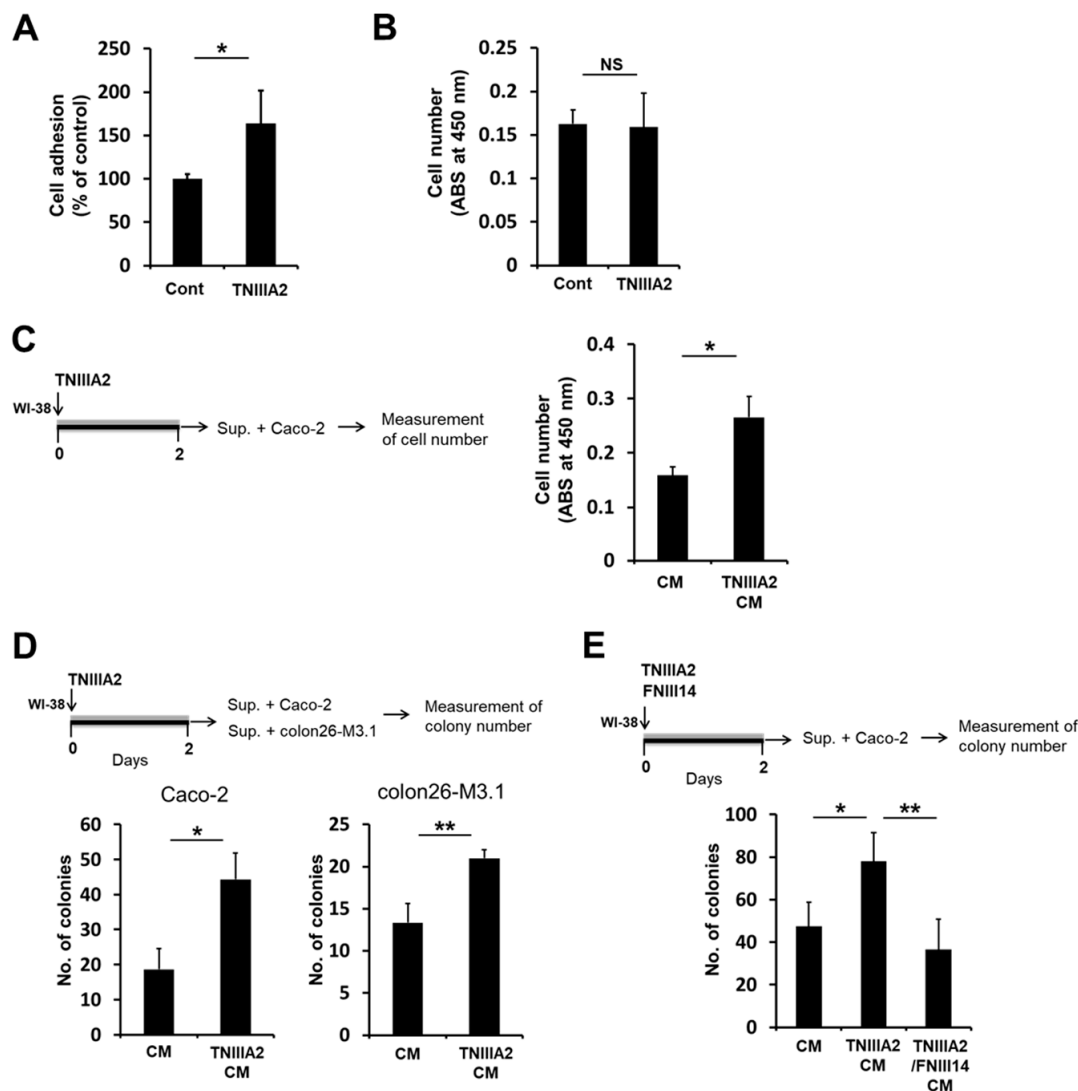
**Figure 2.** Peptide TNIII A2 indirectly accelerated the proliferation of preneoplastic epithelial HaCaT cells via stimulation of WI-38 fibroblasts. **(A)** Effect of peptide TNIII A2 (25 µg/mL) on the proliferation of HaCaT cells. Cells were treated with peptide TNIII A2 (25 µg/mL) at the indicated concentrations. After 2 days, the number of cells was evaluated by WST-8 assay. **(B)** HaCaT cells were seeded with or without peptide TNIII A2 (25 µg/mL) into 96-well plates coated with fibronectin (1.0 µg/mL). Adhered cells were measured by crystal violet staining and counted under a microscope. **(C)** WI-38 cells were seeded with or without peptide TNIII A2 (25 µg/mL) into 96-well plates coated with fibronectin (1.0 µg/mL). Adhered cells were measured by crystal violet staining and counted under a microscope. **(D)** WI-38 cells were incubated with or without peptide TNIII A2 (25 µg/mL) in the presence or absence of peptide FNIII 14 (50 µg/mL) for 30 min. Activation status of  $\beta$ 1-integrin was evaluated by flow cytometry using antibody AG89 recognizing the active  $\beta$ 1-integrin conformation-specific epitope. **(E)** HaCaT cells were co-cultured with WI-38 in the presence or absence of peptide TNIII A2 (25 µg/mL), peptide FNIII 14 (50 µg/mL) or their combination for 2 days as described in Materials and Methods. Cells were stained with 1% Rhodanile blue for epithelial cells staining. Proliferation of HaCaT cells were evaluated as described in Materials and Methods. **(F)** WI-38 cells were treated in the absence or presence of peptide TNIII A2 (25 µg/mL) and peptide FNIII 14 (50 µg/mL) for two days, and the culture supernatants were collected as conditioned medium (“CM”: control, “TNIII A2 CM”: cultured with TNIII A2, “TNIII A2/FNIII 14 CM”: cultured with TNIII A2 in the presence of FNIII 14). Preneoplastic HaCaT cells were then cultured with each of these conditioned media. Two days later, the number of viable cells was evaluated by WST-8 assays, as described in the ‘Materials and Methods’. Data represent the means  $\pm$  standard deviation of triplicate determinations, \*  $p < 0.05$ , \*\*  $p < 0.01$ .



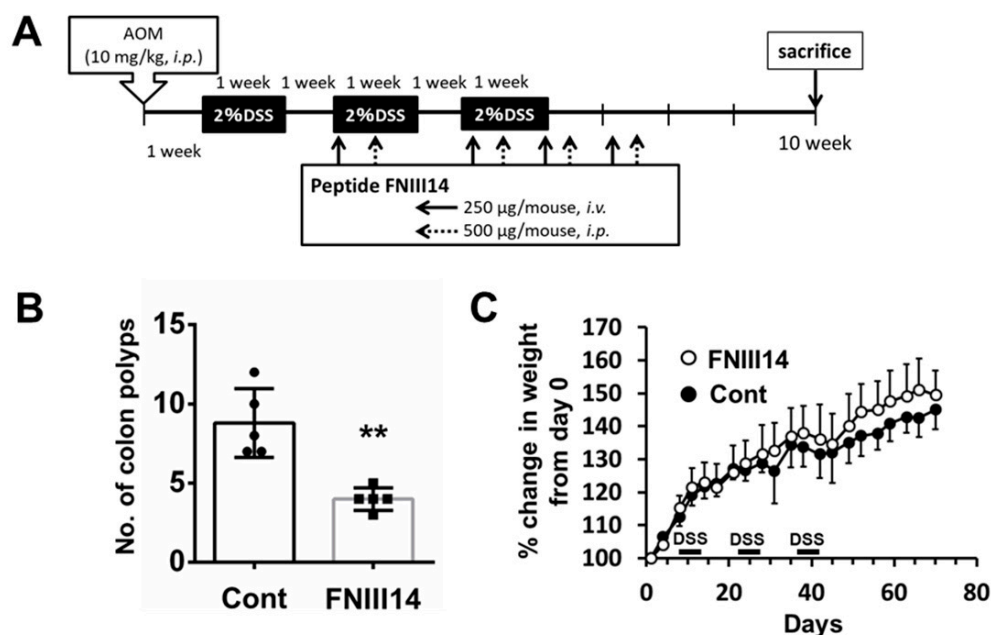
**Figure 3.** Peptide TNIII A2-induced malignant progression in preneoplastic epithelial HaCaT cells. Conditioned medium from peptide TNIII A2-stimulated WI-38 fibroblasts enhanced the proliferation/survival in preneoplastic epithelial HaCaT cells. In panels (A) and (B), WI-38 cells were treated for 2 days in the absence or presence of peptide TNIII A2 (25  $\mu\text{g}/\text{mL}$ ) and peptide FNIII 14 (50  $\mu\text{g}/\text{mL}$ ), respectively, and the culture supernatants were collected as conditioned medium, as described in the legend of Figure 2F. Preneoplastic HaCaT cells were subjected to the focus formation assay (A) or colony formation assay (B) using these conditioned media, as described in the “Materials and Methods”. Data represent the means  $\pm$  standard deviation of triplicate determinations, \*  $p < 0.05$ , \*\*  $p < 0.01$ .

### 2.3. Peptide FNIII 14 Suppresses Polyp Formation in the AOM/DSS Model

Using the AOM/DSS model, we investigated whether peptide FNIII 14 exhibits a prophylactic or therapeutic effect on CAC (Figure 5A). The administration of peptide FNIII 14 in mice significantly reduced the total number of polyps formed in the inner epithelial walls of the large intestinal tracts of the AOM/DSS mouse model (Figure 5B). There were no significant changes in body weight between the groups (Figure 5C). Taken together,  $\beta$ 1-integrin activation by TNIII A2-containing TNC peptides/fragments generated in inflammatory dysplasia lesions may be involved in the malignant transformation of preneoplastic cells and the malignant progression of colon cancer cells via activation of fibroblasts in the tumor microenvironment, and peptide FNIII 14, which has the ability to neutralize TNIII A2 activity, might lead to the suppression of CAC development and progression.



**Figure 4.** Conditioned medium from peptide TNIII A2-stimulated WI-38 fibroblasts enhanced proliferation/survival in intestinal epithelial carcinoma cells. (A) Caco-2 cells were seeded with or without peptide TNIII A2 (25 µg/mL) into 96-well plates coated with fibronectin (1.0 µg/mL). Adhered cells were measured by crystal violet staining and counted under a microscope. (B) The effect of peptide TNIII A2 (25 µg/mL) on the proliferation of Caco-2 cells. Caco-2 cells were treated with peptide TNIII A2 (25 µg/mL) for two days, and the number of cells was evaluated by WST-8 assay. In panels (C), (D), and (E), WI-38 cells were cultured for 2 days in the absence or presence of peptide TNIII A2 (25 µg/mL) and peptide FNIII 14 (50 µg/mL), and the culture supernatants (conditioned medium) were collected as described in the legend of Figure 2F. (C) Caco-2 cells were cultured for 2 days with “CM” or “TNIII A2 CM”, and the number of viable cells was evaluated by WST-8 assay. (D) Caco-2 cells or colon26-M3.1 cells were subjected to the colony formation assay using “CM” or “TNIII A2 CM”, as described in the ‘Materials and Methods’. (E) The effect of peptide FNIII 14 on enhanced ability of colony formation by “TNIII A2 CM”. Caco-2 cells were subjected to the colony formation assay using “CM”, “TNIII A2 CM” or “TNIII A2/FNIII 14 CM). Data represent the means ± standard deviation of triplicate determinations, \*  $p < 0.05$ , \*\*  $p < 0.01$ , NS not significant.



**Figure 5.** Peptide FNIII14 is capable of suppressing the incidence of colon polyps in the murine AOM/DSS colitis-associated carcinoma model. (A) Schematic of the administration of AOM, DSS, control peptide, and peptide FNIII14 to the mice. (B) The effect of peptide FNIII14 on the development of colitis-associated colorectal cancer in the AOM/DSS mouse model. Dot plots show the numbers of colon polyps of individual mice in each group with bars representing the means  $\pm$  standard deviation, \*\*  $p < 0.01$ . (C) Body weight profile of the animals. Data represent the means  $\pm$  standard deviation.

### 3. Discussion

TNC is highly expressed during inflammation and several malignant cancers and its expression levels are associated with a poor prognosis in cancer patients [6]. Furthermore, a high expression of TNC is considered to be a poor prognostic factor in colorectal cancer tissues [22]. Elevated expression of TNC has been reported in areas of ulceration in ulcerative colitis as well as in areas of stricture in Crohn's disease [23]. Thus, it seems that TNC contributes to the development and/or progression of CAC. Identification of the role and the functional sites of TNC molecule responsible for CAC would enable the design of inhibitors with therapeutic potential for these diseases. More recently, TNC derived from intestinal myofibroblasts was shown to promote the onset of CAC in an AOM/DSS model [19]. In addition, extracellular matrix (ECM) remodeling is often enhanced in these pathological regions and proteolytic cleavage of ECM proteins including TNC is carried out by several inflammatory proteinases [24–26]. Indeed, elevated levels of several matrix metalloproteinases (MMPs) have been observed in IBD and are associated with IBD disease activity [27], indicating that the degradation and remodeling of the ECM, including TNC, may occur at a high level in IBD and during the development of CAC. This study supports earlier findings by demonstrating that high expression levels of TNC and the TNIII A2-containing TNC peptides/fragments are present in dysplastic lesions in the AOM/DSS mouse model. Furthermore, our results suggest that TNIII A2-containing TNC peptides/fragments are involved in the pathogenesis of CAC via potentiated and sustained activation of  $\beta$ 1-integrin. Thus,  $\beta$ 1-integrin activation by TNIII A2-containing TNC peptides/fragments is an important target process for the prevention of CAC development at the cellular level. Furthermore, peptide FNIII14 suppressed not only dysregulated survival/proliferation of preneoplastic epithelial HaCaT cells but also the incidence of polyps in an AOM/DSS mouse model. Peptide FNIII14, which has the ability to neutralize the detrimental effects of TNIII A2-containing TNC peptides/fragments by  $\beta$ 1-integrin activation, would be a promising agent for the prevention of CAC development and progression.



A number of studies have indicated that cells in the tumor microenvironment, such as tumor stroma fibroblasts, and senescent and immune cells, are able to influence tumor progression. Among them, fibroblasts, specifically cancer-associated fibroblasts, are key determinants of carcinogenesis and cancer progression [20,21]. Sasaki and colleagues reported that the incidence of colon tumors is suppressed in CCL3- or CCR5-deficient mice treated with AOM/DSS and coincides with lower accumulation of fibroblasts in dysplastic lesions compared with wild-type mice [28]. They also showed that fibroblasts express HB-EGF to promote the proliferation of tumor cells in colitis-associated colorectal carcinogenesis in mice [28]. Moreover, fibroblast-derived epiregulin increases the proliferation of intestinal epithelial cells via the activation of ERK signaling pathway, which enhances the growth of CAC [29]. Thus, cancer-associated fibroblasts might be responsible for the development and progression of CAC. In addition, there is increasing evidence that a high expression of TNC along with other CAF markers in tumor stroma correlates with poor prognosis in several malignancies, such as esophageal squamous cell carcinoma [30], breast ductal carcinoma [31], prostate cancer [32], and colorectal cancer [33], indicating that TNC activates CAF to promote tumor progression. Here, we have shown that conditioned medium from peptide TNIII A2-activated fibroblasts enhanced the survival/proliferation of both preneoplastic cells and colon cancer cells. Further investigations are required to identify the paracrine factor(s) that induces focus formation and anchorage-independent proliferation in preneoplastic epithelial HaCaT cells. In addition, an understanding of the signaling pathway by which  $\beta$ 1-integrin activation triggers the secretion of this paracrine factor would contribute to the development of prophylactic agents for CAC. Further studies are also required to determine whether peptide FNIII 14 actually attenuates the activation of fibroblasts in tumor microenvironment via inactivation of  $\beta$ 1-integrins.

Accumulating evidence indicates that increased ECM stiffness is associated with the malignancy of tumors. A recent study demonstrated that glioblastoma aggression correlates with the stiffness of a TNC-enriched ECM [34]. More recently, Barnes et al. reported that glioblastoma cells expressing an auto-clustering V737N mutated  $\beta$ 1-integrin exhibit potentiated mechanosignaling, increased ECM stiffness, while promoting tumor malignancy [35]. Increased ECM stiffness has also been observed in the stricture area of Crohn's disease [36]. Increased ECM stiffness augments adhesive properties, such as the formation of focal adhesion and actin stress fibers of colonic fibroblasts [36]. We previously reported that peptide TNIII A2 has the ability to induce potent and sustained activation as well as clustering of  $\beta$ 1-integrin [12,13]. Thus, the potent activation of  $\beta$ 1-integrin by the TNIII A2-containing TNC peptides/fragments may contribute to CAC development by increasing the stiffness of the ECM. Further investigations are needed to verify whether  $\beta$ 1-integrin activation by peptide TNIII A2 actually increases ECM stiffness.

## 4. Materials and Methods

### 4.1. Reagents

Human plasma fibronectin was purified as described previously [37]. Peptide TNIII A2 and FNIII 14 has been described previously [12,38]. Dextran sodium sulfate (DSS) and Rhodanile blue were purchased from Sigma-Aldrich (Tokyo, Japan). Azoxymethane (AOM) was purchased from Wako Pure Chemicals (Tokyo, Japan).

### 4.2. Murine AOM/DSS Colitis-Associated Carcinoma Model

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Tokyo University of Science. To develop colitis-associated carcinoma, we challenged 6–8-week-old male ICR mice (Sankyo Laboratory Service, Tokyo, Japan) with the organotropic carcinogen AOM (10 mg/kg body weight, intraperitoneally). Following AOM, mice were treated with 7 days of 2% DSS in drinking water, followed by recovery for 7 days with regular drinking water. The seven days of 2% DSS followed by seven days of normal drinking water was repeated three times. Mice were then analyzed 71 days after

the AOM injection, and colons were removed and flushed with PBS and cut longitudinally. The number of colon polyps was counted using a dissecting microscope. For administration of peptide FNIII14, mice were then intravenously injected with FNIII14 (250 µg/mouse) on days 22, 36, 43, and 49, and intraperitoneally injected with FNIII14 (500 µg/mouse) on days 24, 38, 45, and 51.

#### 4.3. Cell Culture

Human diploid lung embryonic fibroblast cell line WI-38, which was obtained from RIKEN Bio Resource Center (Ibaraki, Japan), was maintained in EMEM medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (SAFC Biosciences, MO, USA). Human preneoplastic epidermal keratinocytes HaCaT, which was kindly provided by Dr. Masuho Yasuhiko (Tokyo University of Science), was maintained in DMEM medium (Nissui Pharmaceutical) supplemented with 10% FBS. Human colorectal adenocarcinoma cell line Caco-2, which was kindly provided by Dr. Michihiro Mutoh (National Cancer Center Research Institute), was maintained in DMEM medium supplemented with 10% FBS. Mouse colon cancer cell line colon26-M3.1, which was kindly provided by Dr. Ikuo Saiki (University of Toyama), was maintained in RPMI1640 medium supplemented with 10% FBS. Cells were incubated in a 5% CO<sub>2</sub> incubator at 37 °C.

#### 4.4. Immunohistochemistry

The resected specimens were fixed in 10% formalin and embedded in paraffin. For antigen retrieval, the slides were incubated at 37 °C for 30 min in 0.2% trypsin. The paraffin-embedded tissue samples were cut to a 4 µm thickness and stained with hematoxylin and eosin. The following immunohistochemical reagents were performed with anti-TNC 4F10TT Antibody (IBL, Gunma, Japan) and anti-TNIIIA2 polyclonal antibody (IBL) and Histofine Sab-Po (Multi) kit (Nichirei Biosciences, Inc, Tokyo, Japan) according to the manufacturer's instructions. Images were captured using a light microscope Olympus BX-53 (Olympus, Tokyo, Japan).

#### 4.5. Cell Adhesion Assay

Adhesion to fibronectin was performed as described previously [13].

#### 4.6. Flow Cytometry

The activation status of β1-integrins on the cells was evaluated by flow cytometric analysis using a monoclonal antibody that recognize the active conformation of β1-integrin (clone AG89; MBL, Aichi, Japan), as described previously [12].

#### 4.7. 2D Co-Culture Experiments

WI-38 cells ( $2.5 \times 10^4$  cells/well) were seeded on 24-well culture plates. After 24 h, HaCaT cells ( $4.0 \times 10^4$  cells/well) suspended in a 1:1 mixture of DMEM-EMEM medium in the presence or absence of TNIIIA2 and/or peptide FNIII14 were overlaid. After 48 h, cells were fixed with 4% paraformaldehyde and stained with 1% Rhodanile blue for epithelial cells staining [39]. Images were captured and area of epithelial cells was measured by Motic Image Plus 2.2S (Shimadzu Rika, Kyoto, Japan).

#### 4.8. Preparation of Conditioned Medium from WI-38 Cells

WI-38 cells ( $2.0 \times 10^5$  cells/well) were seeded on 12-well culture plates coated with fibronectin (1.0 µg/mL) and then cultured with the EMEM medium without FBS in the presence or absence of peptide TNIIIA2 and/or peptide FNIII14. Two days later, culture supernatants were collected as conditioned media (CM) and stored at −80 °C until use.

#### 4.9. Cell Proliferation Assay

Cells ( $8.0 \times 10^3$  cells/well) were seeded on 96-well culture plates coated with fibronectin (1.0  $\mu\text{g/mL}$ ) and then cultured with medium in the presence or absence of peptide TNIII A2, or CM prepared under various conditions. The number of viable cells was evaluated based on the WST-8 assay, as described previously [13].

#### 4.10. Colony Formation Assay

Solution of a 1:1 mixture of 1.4% agar (BD Bioscience, NJ, USA) and  $2\times$  DMEM or RPMI 1640 growth medium was poured into 12-well plates. After solidification, HaCaT ( $1.0 \times 10^4$  cells/well), Caco-2 ( $7.0 \times 10^3$  cells/well), or colon26-M3.1 ( $4.0 \times 10^3$  cells/well) suspended in CM prepared under various conditions containing 0.7% agar were overlaid on top of a base layer. After solidification of the top agar layer, CM prepared under the corresponding conditions was added. Media were changed every 4–5 days. After 10–14 days, colonies were stained with crystal violet and the number of them was counted for five randomly selected fields under the microscope at  $20\times$  magnification.

#### 4.11. Focus Formation Assay

Cells ( $2.0 \times 10^5$  cells/well) were seeded on 24-well culture plates coated with fibronectin (1.0  $\mu\text{g/mL}$ ) and cultured with CM prepared under various conditions. After 8–10 days, cells were fixed with 4% paraformaldehyde and stained with crystal violet. Images were captured and analyzed by Motic Image Plus 2.2S.

#### 4.12. Statistical Analysis

Data are expressed as the mean  $\pm$  standard deviation. Two-tailed Student *t* test or one-way ANOVA analysis was used to determine statistical differences. Values of  $p < 0.05$  were considered significant.

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#### Abbreviations

IBD	inflammatory bowel disease
UC	ulcerative colitis
CD	Crohn's disease
CAC	colitis-associated colorectal cancer
AOM	azoxymethane
DSS	dextran sodium sulfate
<i>i.p.</i>	intraperitoneal injection
EGF	epidermal growth factor
CM	conditioned medium
MMP	matrix metalloproteinase
ECM	extracellular matrix
HB-EGF	heparin-binding EGF-like growth factor
ABS	absorbance
Sup.	supernatant

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