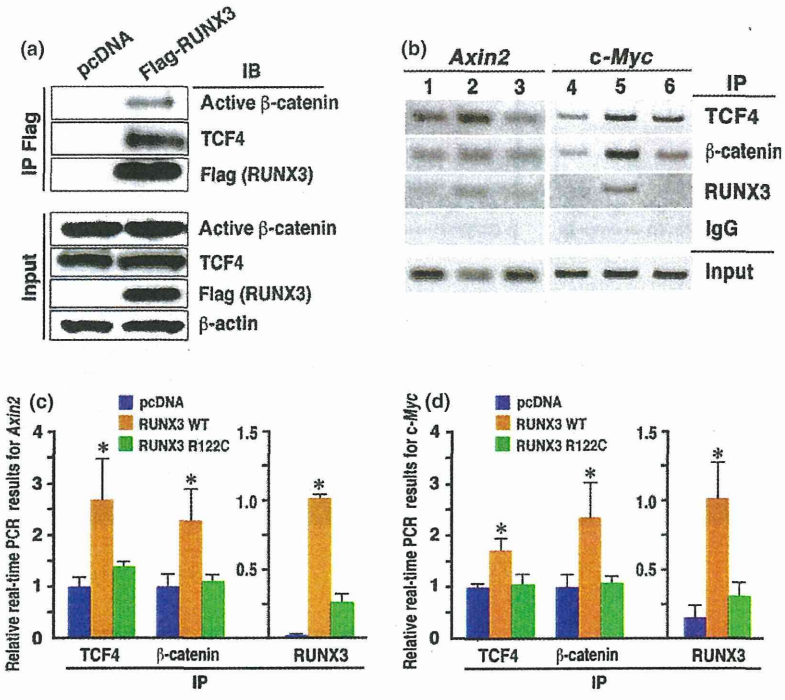

Fig. 5. Binding of the RUNX3, TCF4 and β-catenin complex to Wnt target gene promoters. (a) Immunoprecipitation for β-catenin and TCF4 with an anti-Flag (RUNX3) antibody. (b) ChIP analyses of the *Axin2* (left) and *c-Myc* (right) promoter regions using lysates precipitated (IP) with anti-TCF4, anti-β-catenin and anti-RUNX3 antibodies. Lanes 1 and 4, control KatoIII cells; Lanes 2 and 5, RUNX3-expressing KatoIII cells; and Lanes 3 and 6, R122C mutant RUNX3-expressing KatoIII cells. (c,d) ChIP-based real-time PCR for the *Axin2* (c) and *c-Myc* (d) promoters (mean ± SD). ChIP samples were precipitated (IP) with anti-TCF4, anti-unphosphor- ylated β-catenin or anti-RUNX3 antibodies from control cells (blue), RUNX3-expressing KatoIII cells (orange) or R122C mutant RUNX3-expressing KatoIII cells (green), and examined by real-time PCR. $*P < 0.05$ versus control.

TCF in the nucleus, and recruits cofactors such as Bcl9 and pygopus to the TCF4/β-catenin complex, inducing target gene transcription.[8,21,22] It has also been reported that additional factors are required for the recruitment of β-catenin to the target gene promoters. Transducin β-like protein 1 (TBL1) and its related family member, TBLR1, recruit β-catenin to TCF on the Wnt target gene promoter.[22,23] TBL1 can bind both TCF4 and β-catenin, suggesting that it strengthens their physical association, which may contribute to Wnt activation. Jerky also recruits β-catenin to chromatin, and promotes the association of β-catenin and LEF1, resulting in the induction of Wnt target gene expression.[22,24] We found that RUNX3 increases the occupancy of the TCF4/β-catenin complex in KatoIII cells, suggesting that RUNX3 plays a role in the stabilization of the TCF4/β-catenin complex on the Wnt target gene promoter like TBL1 and Jerky. It is also possible that RUNX3 is involved in the recruitment of β-catenin to TCF4 on the Wnt target gene promoter like these molecules. We have previously shown that the Wnt activation levels are oscillating in the individual KatoIII cells,[18] and in this study, we showed that the RUNX3 expression increases the ratio of the Wnt-high population. Accordingly, it is conceivable that RUNX3 maintains the Wnt activation at a high level, suppressing the decrease of Wnt activity by stabilizing the TCF4/β-catenin complex on the Wnt target gene promoters.

Another unsolved question is how the RUNX3/TCF4 complex can bind to the DNA of the Wnt target gene promoter in KatoIII cells. As previously described, RUNX3-bound TCF4 cannot bind chromatin, possibly due to the competition for the DNA binding region of TCF4 with RUNX3. It is possible that cofactor(s) in the complex affect the conformation of the RUNX3/TCF4/β-catenin complex. Genetic polymorphisms of such cofactor(s) may cause conformational changes of the complex, which allows RUNX3-bound TCF4 to bind the Wnt target gene promoter. It would be worth examining the DNA sequences of cofactors of the TCF4/β-catenin complex in KatoIII and SNU668 cells to understand the molecular mechanism(s) responsible for the RUNX3-associated Wnt regulation.

In the present study, we also confirmed that RUNX3 transfection significantly suppressed the proliferation and tumorigenicity of KatoIII cells with induction of p21 expression. Moreover, RUNX3 has been shown to induce the apoptosis of gastric cancer cells by upregulating apoptosis-related genes.[25] It is thus possible that these gene products suppressed the proliferation and survival of $RUNX3^{Hi}$-expressing KatoIII cells, and that RUNX3-induced Wnt activation is not sufficient to protect KatoIII cells from RUNX3-induced apoptosis. It remains to be investigated whether RUNX3 suppresses tumorigenicity also in SNU668 cells. However, it has been reported that RUNX3 is upregulated and functions as an oncogene in head and neck cancer cells by increasing the proliferation and sphere formation.[26,27] Accordingly, the RUNX3-associated tumor suppressing functions are likely inactivated in head and neck cancer cells, and, therefore, it is possible that RUNX3-associated Wnt activation contributes to tumorigenesis in such cancer cells.

The present results, together with previous findings,[12,13] indicate that RUNX3 can either suppress or activate Wnt signaling through binding to the TCF4/β-catenin complex. Although it remains to be investigated, cofactor(s) that bind the RUNX3/TCF4/β-catenin complex may be involved in the decision regarding the direction of Wnt signaling modulation; that is, suppression or activation.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Li QL, Ito K, Sakakura C *et al.* Causal relationship between the loss of *RUNX3* expression and gastric cancer. *Cell* 2002; **109**: 113–24.
2 Blyth K, Cameron ER, Neil JC. The RUNX genes: gain or loss of function in cancer. *Nat Rev Cancer* 2005; **5**: 376–87.
3 Chuang LSH, Ito K, Ito Y. RUNX family: regulation and diversification of roles through interacting proteins. *Int J Cancer* 2013; **132**: 1260–71.
4 Chuang LSH, Ito Y. RUNX3 is multifunctional in carcinogenesis of multiple solid tumors. *Oncogene* 2010; **29**: 2605–15.
5 Ito K, Liu Q, Salto-Tellez M *et al.* RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization. *Cancer Res* 2005; **65**: 7743–50.
6 Guo WH, Weng LQ, Ito K *et al.* Inhibition of growth of mouse gastric cancer cells by *Runx3*, a novel tumor suppressor. *Oncogene* 2002; **21**: 8351–5.
7 Fukamachi H, Ito K, Ito Y. *Runx3*$^{-/-}$ gastric epithelial cells differentiate into intestinal type cells. *Biochem Biophys Res Commun* 2004; **321**: 58–64.
8 Clevers H, Nusse R. Wnt/β-catenin signaling and disease. *Cell* 2012; **149**: 1192–205.
9 Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of *Apc* heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated *Apc* gene. *Proc Natl Acad Sci USA* 1995; **92**: 4482–6.
10 Harada N, Tamai Y, Ishikawa T *et al.* Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. *EMBO J* 1999; **18**: 5931–42.
11 Oshima H, Matsunaga A, Fujimura T, Tsukamoto T, Taketo MM, Oshima M. Carcinogenesis in mouse stomach by simultaneous activation of the Wnt signaling and prostaglandin E_2 pathway. *Gastroenterology* 2006; **131**: 1086–95.
12 Ito K, Lim AC, Salto-Tellez M *et al.* RUNX3 attenuates β-catenin/T cell factors in intestinal tumorigenesis. *Cancer Cell* 2008; **14**: 226–37.
13 Ito K, Chuang LSH, Ito T *et al.* Loss of Runx3 is a key event in inducing precancerous state of the stomach. *Gastroenterology* 2011; **140**: 1536–46.
14 Proffitt KD, Madan B, Ke Z *et al.* Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. *Cancer Res* 2012; **73**: 502–7.
15 Nateri AS, Spencer-Dene B, Behrens A. Integration of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* 2005; **473**: 281–5.
16 Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* 2002; **22**: 1172–83.
17 Nojima M, Suzuki H, Toyota M *et al.* Frequent epigenetic inactivation of *SFRP* genes and constitutive activation of Wnt signaling in gastric cancer. *Oncogene* 2007; **26**: 4699–713.
18 Oguma K, Oshima H, Aoki M *et al.* Activated macrophages promotes Wnt signalling through tumour necrosis factor-α in gastric tumour cells. *EMBO J* 2008; **27**: 1671–81.
19 Sakakura C, Hasegawa K, Miyagawa K *et al.* Possible involvement of RUNX3 silencing in the peritoneal metastases of gastric cancer. *Clin Cancer Res* 2005; **11**: 6479–88.
20 Chi XZ, Yang JO, Lee KY *et al.* RUNX3 suppresses gastric epithelial cell growth by inducing $p21^{WAF1/Cip1}$ expression in cooperation with transforming growth factor β-ctivated SMAD. *Mol Cell Biol* 2005; **25**: 8097–107.
21 Cadigan KM, Waterman ML. TCF/LEFs and Wnt signaling in nucleus. *Cold Spring Harb Perspect Biol* 2012; **4**: a007906.
22 Cadigan KM. TCFs and Wnt/β-catenin signaling: more than one way to throw the switch. *Curr Top Dev Biol* 2012; **98**: 1–34.
23 Li J, Wang CY. TBL1-TBLR1 and β-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 2008; **10**: 160–9.
24 Benchabane H, Xin N, Tian A *et al.* Jerky/Earthbound facilitates cell-specific Wnt/Wngless signaling by modulating β-catenin-TCF activity. *EMBO J* 2011; **30**: 1444–58.
25 Nagahama Y, Ishimaru M, Osaki M *et al.* Apoptotic pathway induced by transduction of *RUNX3* in the human gastric carcinoma cell line MKN-1. *Cancer Sci* 2008; **99**: 23–30.
26 Tsunematsu T, Kudo Y, Iizuka S *et al.* RUNX3 has an oncogenic role in head and neck cancer. *PLoS ONE* 2009; **4**: e5892.
27 Kudo Y, Tsunematsu T, Takata T. Oncogenic role of RUNX3 in head and neck cancer. *J Cell Biochem* 2011; **112**: 387–93.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Relative RUNX3 mRNA levels in gastric cancer cell lines examined by RT-PCR.

Fig. S2. Western blotting results of RUNX3 and active form of β-catenin in KatoIII cells and KatoIII-R3 cells.

Fig. S3. Relative band intensities calculated from the Western blotting results (Fig. 2b) of active β-catenin and RUNX3 in the RUNX3 expression vector-transfected KatoIII cells.

Fig. S4. Flow cytometry analyses of β-catenin and GFP in the control KatoIII cells and GFP expression vector-transfected KatoIII cells.

Fig. S5. Cell proliferation rates of RUNX3 siRNA-transfected KatoIII-R3 cells.

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ORIGINAL ARTICLE

TNF-α/TNFR1 signaling promotes gastric tumorigenesis through induction of *Noxo1* and *Gna14* in tumor cells

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Helicobacter pylori infection induces chronic inflammation that contributes to gastric tumorigenesis. Tumor necrosis factor (TNF-α) is a proinflammatory cytokine, and polymorphism in the *TNF-α* gene increases the risk of gastric cancer. We herein investigated the role of TNF-α in gastric tumorigenesis using *Gan* mouse model, which recapitulates human gastric cancer development. We crossed *Gan* mice with TNF-α (*Tnf*) or TNF-α receptor TNFR1 (*Tnfrsf1a*) knockout mice to generate *Tnf*−/− *Gan* and *Tnfrsf1a*−/− *Gan* mice, respectively, and examined their tumor phenotypes. Notably, both *Tnf*−/− *Gan* mice and *Tnfrsf1a*−/− *Gan* mice showed similar, significant suppression of gastric tumor growth compared with control *Tnf*+/+ or *Tnfrsf1a*+/+ *Gan* mice. These results indicate that TNF-α signaling through TNFR1 is important for gastric tumor development. Bone marrow (BM) transplantation experiments showed that TNF-α expressed by BM-derived cells (BMDCs) stimulates the TNFR1 on BMDCs by an autocrine or paracrine manner, which is important for gastric tumor promotion. Moreover, the microarray analysis and colony formation assay indicated that NADPH oxidase organizer 1 (*Noxo1*) and *Gna14* are induced in tumor epithelial cells in a TNF-α-dependent manner, and have an important role in tumorigenicity and tumor-initiating cell property of gastric cancer cells. Accordingly, it is possible that the activation of TNF-α/TNFR1 signaling in the tumor microenvironment promotes gastric tumor development through induction of *Noxo1* and *Gna14*, which contribute to maintaining the tumor cells in an undifferentiated state. The present results indicate that targeting the TNF-α/TNFR1 pathway may be an effective preventive or therapeutic strategy for gastric cancer.

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Keywords: gastric cancer; inflammation; TNF-α; mouse model

INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of death from malignancy worldwide.[1] *Helicobacter pylori* infection induces chronic gastritis, which is related to gastric cancer development.[2,3] It has been established that inflammation has an important role in cancer development through a variety of mechanisms.[4,5] Genetic polymorphisms in proinflammatory cytokine genes, interleukin (IL)-1β and tumor necrosis factor (TNF)-α are associated with an increased risk of gastric cancer.[6–8] Moreover, a combination of specific polymorphisms in IL-1β, IL-1RN, TNF-α and IL-10 increases the odds ratio for gastric cancer by 27-fold, indicating an important role for these inflammatory cytokines in gastric tumorigenesis.[7,9] It has been demonstrated that transgenic expression of IL-1β in the stomach causes development of gastritis-associated gastric cancer, with the recruitment of myeloid-derived suppressor cells.[10] Moreover, mutation in the IL-6 and IL-11 coreceptor, gp130, results in gastric tumor development through activation of Stat3.[11,12] On the other hand, the role of TNF-α in gastric tumorigenesis has not yet been investigated using a genetic mouse model.

Accumulating evidence has indicated that TNF-α is an important cytokine involved in cancer development in a variety of organs. TNF-α production is associated with advanced cancers and a poor prognosis.[13,14] Mouse genetic studies indicated that disruption of the TNF-α or TNFR1 receptor genes resulted in significant suppression of chemically induced tumorigenesis in the mouse skin and colon.[15–17] These results indicate that TNF-α/TNFR1 signaling has a key role in cancer development. Thus, in the present study, we examined the role of TNF-α signaling through its receptor TNFR1 in gastric tumorigenesis.

We have previously generated a gastric tumor mouse model, *Gan* mice, which develop intestinal-type gastric tumors by the transgenic expression of *Wnt1*, *Ptgs2* and *Ptges*, encoding Wnt1, COX-2 and mPGES-1, respectively.[18,19] Simultaneous expression of these three genes in the glandular stomach activates both canonical Wnt signaling and the COX-2/PGE_2 pathway. Wnt signaling activation is one of the major causes of human gastric cancer.[18,20] On the other hand, the COX-2/PGE_2 pathway is induced in a variety of cancers, including gastric cancer, and is important for promoting tumor development.[21] Accordingly, *Gan* mice recapitulate human gastric cancer development at the molecular level and host responses. Notably, the gene expression profiles of *Gan* mouse tumors are similar to those of human intestinal-type gastric cancer.[22] Therefore, it is rational to use *Gan* mice for studies of the role of microenvironment and host responses, such as inflammation, in gastric tumorigenesis.

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In the present study, we crossed *Gan* mice with *Tnf*−/− and *Tnfrsf1a*−/− mice, and found that gastric tumorigenesis was significantly suppressed by disruption of the TNF-α/TNFR1 signaling. Bone marrow (BM) chimera experiments indicated that activation of TNF-α/TNFR1 signaling in BM-derived cells (BMDCs) is important for gastric tumor promotion. Moreover, we found that NADPH oxidase organizer 1 (*Noxo1*) and *Gna14* are induced in gastric tumors by a TNF-α-dependent mechanism, and that these molecules are important for the tumorigenicity and stemness of gastric cancer cells. Accordingly, the present results suggest that activation of TNF-α/TNFR1 signaling promotes gastric tumorigenesis through induction of these tumor-promoting factors in tumor epithelial cells.

RESULTS

Suppression of gastric tumorigenesis in *Tnf*−/− *Gan* mice

To examine the role of TNF-α in gastric tumorigenesis, we crossed *Gan* mice with *Tnf* knockout mice and examined the tumor phenotype. Notably, the gastric tumor development was significantly suppressed in *Tnf*−/− *Gan* mice (Figures 1a and b), and the mean tumor size decreased to 18.0% of that observed in the littermate *Tnf*+/+ *Gan* mice (Figure 1c). These results indicate that TNF-α has an important role in gastric tumorigenesis. In *Tnf*+/+ *Gan* mice, 5-bromo-2′-deoxyuridine (BrdU)-labeled proliferating cells were found in the entire tumor tissue, whereas the BrdU-incorporated cells were mostly limited to the proliferating zone at the neck area in *Tnf*−/− *Gan* mouse tumors (Figure 1d). Therefore, it is possible that cell differentiation was induced outside of the proliferating area in *Tnf*−/− *Gan* mouse tumors.

K19-Wnt1 mice did not develop gastric tumors, but they developed small preneoplastic lesions consisting of dysplastic and Ki-67-positive epithelial cells in the glandular stomach (Supplementary Figure 1), which was consistent with previous results.[18,19] Notably, *Tnf*−/− *K19-Wnt1* mice developed a similar number of preneoplastic lesions to the *Tnf*+/+ *K19-Wnt1* mice (Figure 1e). Taken together, these results indicate that TNF-α signaling is not required for the early initiation stage, but has an important role in the promotion stage of gastric tumorigenesis.

Role of TNF-α expressed by BMDCs in tumorigenesis

In *Gan* mouse gastric tumors, macrophages are infiltrated and activated,[23] suggesting that macrophage-derived TNF-α is important for tumor promotion. To assess this possibility, we examined the expression of TNF-α and its receptors, TNFR1 and TNFR2, in tumor epithelial cells and stromal cells that were separately obtained by using laser microdissection (Supplementary Figure 2). Expression levels of TNF-α, TNFR1 and TNFR2 were significantly higher in stromal cells compared with epithelial cells, although their expression was also detected in the tumor epithelial cells (Figure 2a).

To examine the role of TNF-α expressed by BMDCs, we performed BM transplantation from *Tnf*+/+ green fluorescent protein (GFP) transgenic mice or *Tnf*−/− mice into *Tnf*−/− *Gan*

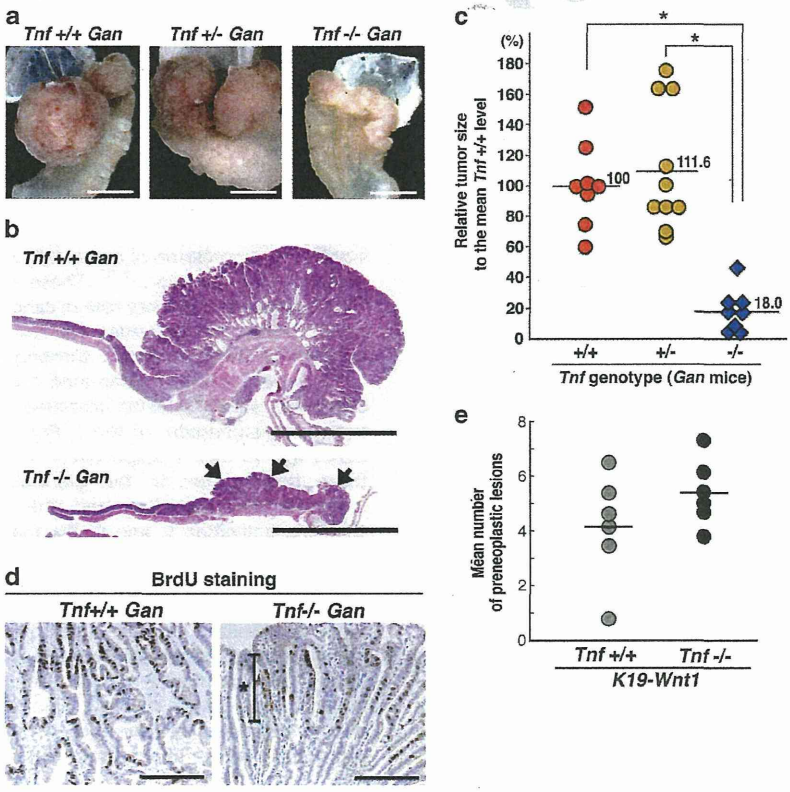

Figure 1. Suppression of gastric tumor development by *Tnf* disruption. (**a**) Representative macroscopic photographs of *Tnf*+/+, *Tnf*+/− and *Tnf*−/− *Gan* mouse gastric tumors at 50 weeks of age. Scale bars indicate 5 mm. (**b**) Representative histological photographs of whole views of *Tnf*+/+ *Gan* (top) and *Tnf*−/− *Gan* mouse (bottom) gastric tumors (H&E). The arrows indicate suppressed tumor lesions in *Tnf*−/− *Gan* mouse. Scale bars indicate 5 mm. (**c**) The gastric tumor size of *Tnf*+/+ *Gan*, *Tnf*+/− *Gan* and *Tnf*−/− *Gan* mice relative to the mean level of *Tnf*+/+ *Gan* mouse tumors (set at 100%). Asterisks (*), $P<0.05$. (**d**) Representative photographs of anti-BrdU immunostaining of *Tnf*+/+ *Gan* (left) and *Tnf*−/− *Gan* (right) mouse tumors. The asterisk (*) indicates a limited proliferating zone in a *Tnf*−/− *Gan* mouse tumor. Scale bars indicate 200 μm. (**e**) The mean number of preneoplastic lesions per section of *Tnf*+/+ *K19-Wnt1* and *Tnf*−/− *K19-Wnt1* mouse glandular stomachs.

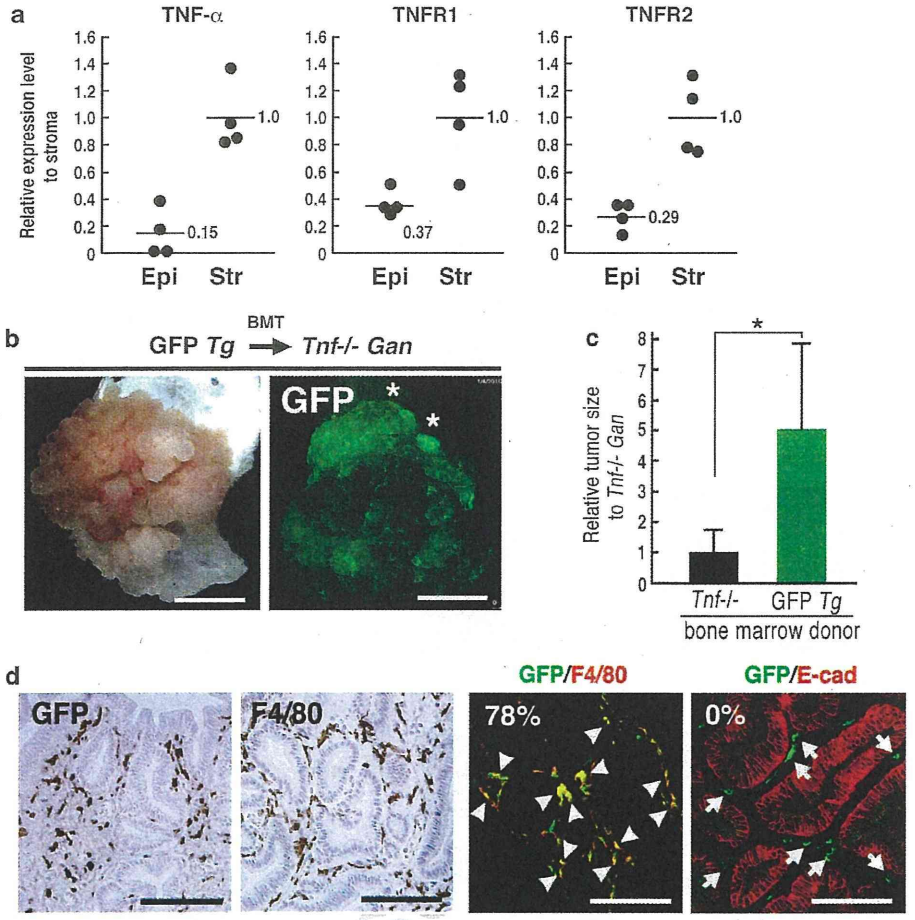

Figure 2. Promotion of gastric tumorigenesis by TNF-α expressed by BMDCs. (**a**) The expression levels of TNF-α, TNFR1 and TNFR2 examined by RT–PCR in epithelial cells (*Epi*) and stromal cells (*Str*) of *Gan* mouse tumors relative to the mean levels in the stromal cells (*Str*). (**b**) A representative macroscopic photograph (left) and GFP expression (right) of gastric tumors in a *Tnf*+/+ GFP BM-transplanted *Tnf*−/− *Gan* mice. The asterisks (*) in the GFP photograph (right) indicate a GFP-negative non-neoplastic forestomach. (**c**) The mean gastric tumor size of *Tnf*+/+ GFP BM-transplanted *Tnf*−/− *Gan* mice relative to that of *Tnf*−/− BM-transplanted *Tnf*−/− *Gan* mice. Asterisk (*), $P<0.05$. (**d**) Immunohistochemical staining for GFP and F4/80 (left), and double fluorescent immunostaining of GFP (green) with F4/80 or E-cadherin (red; right). The arrowheads in the GFP/F4/80 fluorescent immunostaining image indicate double-positive merged cells, whereas arrows in the GFP/E-cadherin immunostaining image indicate GFP single-positive stromal cells. Bars in **b** and **d** indicate 5 mm and 100 μm, respectively.

mice. Notably, gastric tumor phenotype was rescued significantly in the *Tnf*+/+ GFP BM-transplanted *Tnf*−/− *Gan* mice (Figure 2b), and the mean tumor size increased about fivefold compared with the control *Tnf*−/− BM-transplanted *Tnf*−/− *Gan* mice (Figure 2c). Strong GFP expression was detected in the gastric tumors of the *Tnf*+/+ GFP BM-transplanted *Tnf*−/− *Gan* mice, indicating extensive infiltration of BMDCs into the tumor tissues. The accumulation of GFP-positive BMDCs, as well as F4/80 positive macrophages, was found in the tumor stroma of BM chimeric mice (Figure 2d). Double fluorescent immunostaining indicated that 78% of GFP-expressing BMDCs were macrophages, whereas GFP expression was not detected in the E-cadherin-positive epithelial cells. These results indicate that TNF-α expressed by BMDCs, including macrophages, is important for gastric tumorigenesis.

Suppression of gastric tumorigenesis in *Tnfrsf1a*−/− *Gan* mice

It has been shown that TNF-α signaling through TNFR1, encoded by *Tnfrsf1a*, is important for skin and colon cancer development.[16,17] To examine the role of TNFR1 signaling in gastric tumorigenesis, we crossed *Gan* mice with *Tnfrsf1a* knockout mice and examined the tumor phenotype by X-ray computed tomography (CT) analyses. Notably, the gastric tumor development was significantly suppressed in *Tnfrsf1a*−/− *Gan* mice (Figure 3a), and the mean tumor area on CT images was decreased to 40.6% of that of the littermate *Tnfrsf1a*+/+ *Gan* mice (Figure 3b). Accordingly, it is possible that TNFR1 is the major receptor for TNF-α involved in the gastric cancer promotion.

Because the expression level of TNFR1 in tumor tissues was higher in stromal cells compared with epithelial cells (Figure 2a), we next examined the role of TNFR1 signaling in BMDCs for gastric tumorigenesis by BM transplantation from *Tnfrsf1a*−/− mice into *Gan* mice. By X-ray CT analyses, all control *Gan* mice that were transplanted with wild-type mouse BM showed a significant increase in tumor size during the 8 weeks of the study (Figures 3c and e). In contrast, *Gan* mice that received BM from *Tnfrsf1a*−/− mice showed suppression of gastric tumor growth (Figures 3d and e), indicating that TNF-α signaling through TNFR1 in BMDCs has a role in gastric tumor growth.

Inflammatory responses in *Tnf*−/− *Gan* mouse tumor tissues

TNF-α signaling leads to the activation of NF-κB through phosphorylation of IκBα. As expected, the level of phosphorylated

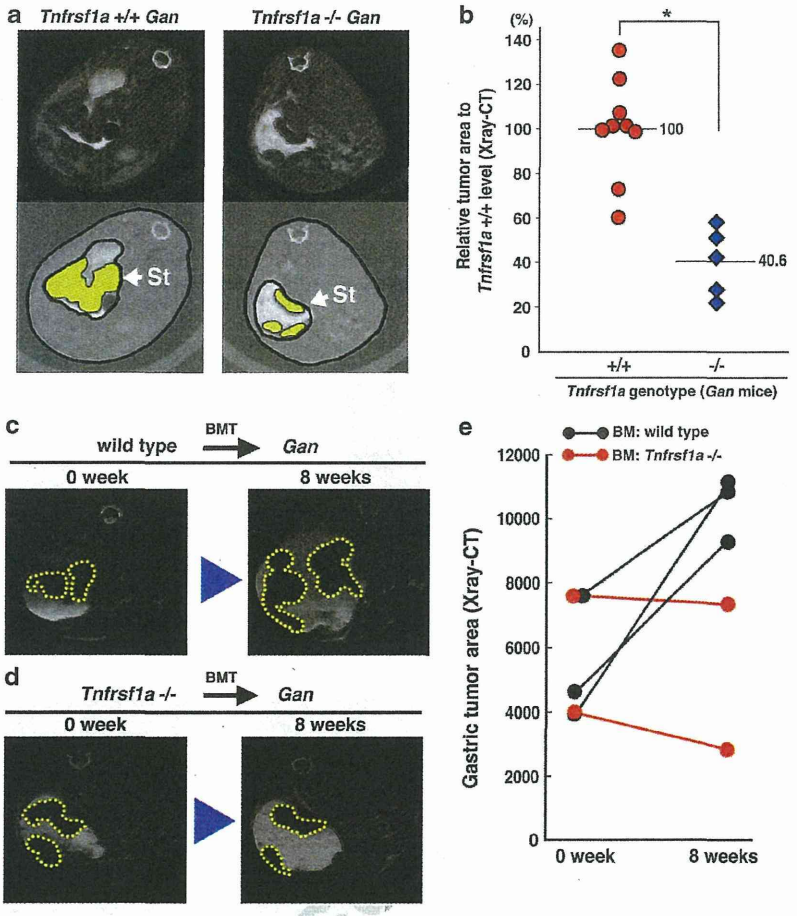

Figure 3. Suppression of gastric tumorigenesis by *Tnfrsf1a* disruption. (**a**) Representative X-ray CT slice images of *Tnfrsf1a* + / + *Gan* mice (left) and *Tnfrsf1a* − / − *Gan* mice (right). The stomach and tumor areas are indicated by lines and yellow color, respectively, in the copy CT images (bottom). (**b**) The calculated mean tumor area of *Tnfrsf1a* + / + *Gan* and *Tnfrsf1a* − / − *Gan* mice measured using slice images relative to the calculated mean tumor area of *Tnfrsf1a* + / + *Gan* mice (set at 100%). Asterisk (*), $P<0.05$. (**c**, **d**) Representative X-ray CT images of wild-type BM-transplanted *Gan* mice (**c**) and *Tnfrsf1a* − / − BM-transplanted *Gan* mice (**d**) at 0 weeks (left) and 8 weeks (right) after BM transplantation. The gastric tumor areas are indicated with yellow dashed lines. (**e**) The calculated mean tumor area from the X-ray CT slice images of wild-type BM-transplanted *Gan* mice (black line) and *Tnfrsf1a* − / − BM-transplanted *Gan* mice (red line) at 0 and 8 weeks after BM transplantation.

IκBα was increased in *Gan* mouse tumors compared with the wild-type mouse stomach (Figure 4a). In contrast, the IκBα phosphorylation level was significantly decreased in *Tnf* − / − *Gan* mouse tumors, indicating that NF-κB activation in tumors was suppressed by the disruption of *TNF-α* gene. On the other hand, the levels of phosphorylated Stat3 were increased significantly in both the *Tnf* + / + and *Tnf* − / − *Gan* mouse tumors to similar levels compared with the wild-type mouse level, suggesting that the cytokine pathways other than the TNF-α/NF-κB signaling were not suppressed in the *Tnf* − / − *Gan* mouse tumors.

Consistently, infiltration of T cells and macrophages were found in the *Tnf* − / − *Gan* mouse gastric tumors, similar to what they had in the *Tnf* + / + *Gan* mouse tumors (Figure 4b). The expression levels of IL-1β, IL-6, CXCL1 and CXCL2 in the gastric tumors were increased in the *Tnf* + / + *Gan* mouse tumors (Figure 4c). Notably, in the *Tnf* − / − *Gan* mouse tumors, the expression levels of these cytokines and chemokines significantly increased compared with those in wild-type mouse stomach, thus indicating that inflammation was not suppressed by *Tnf* gene disruption. It is therefore possible that the activation of TNF-α signaling is required for gastric tumor promotion, even if other tumor-promoting cytokines, such as IL-1β and IL-6, are induced in the tumor tissues.

Differentiation of tumor cells by *TNF-α* gene disruption

To identify the tumor-promoting factors that are induced by a TNF-α, we performed a microarray analysis using *Tnf* − / − *Gan* and *Tnf* + / + *Gan* mouse tumors and wild-type mouse stomachs (Gene Expression Omnibus (GEO) accession GSE43145). Using the microarray results, we extracted genes that were upregulated ⩾twofold in *Tnf* + / + *Gan* mouse tumors compared with wild-type mouse stomachs (Figure 5a). We next extracted the genes that were significantly downregulated in *Tnf* − / − *Gan* mouse tumors compared with *Tnf* + / + *Gan* mice. By comparing these two gene sets, 157 genes were identified that were upregulated in gastric tumors in a TNF-α-dependent manner (Figure 5b and Supplementary Table 1). Interestingly, *CD44*, *Prom1*, *Sox9* and *EphB3* were significantly donwregulated in *Tnf* − / − *Gan* mouse tumors, which are known markers of stem cells or progenitor cells in the intestine and liver,[24,25] suggesting that differentiation of tumor cells was induced by inhibition of TNF-α signaling.

We have previously demonstrated that expression of CD44 is induced in *Gan* mouse tumors.[26,27] Because CD44 is a marker of normal and cancer stem cells,[28] we examined CD44 expression and differentiation status of epithelial cells in *Tnf* − / − *Gan* mouse tumors. In the *Tnf* + / + or *Tnf* + / − *Gan* mouse tumors, CD44 mRNA levels increased significantly, by more than eightfold, the

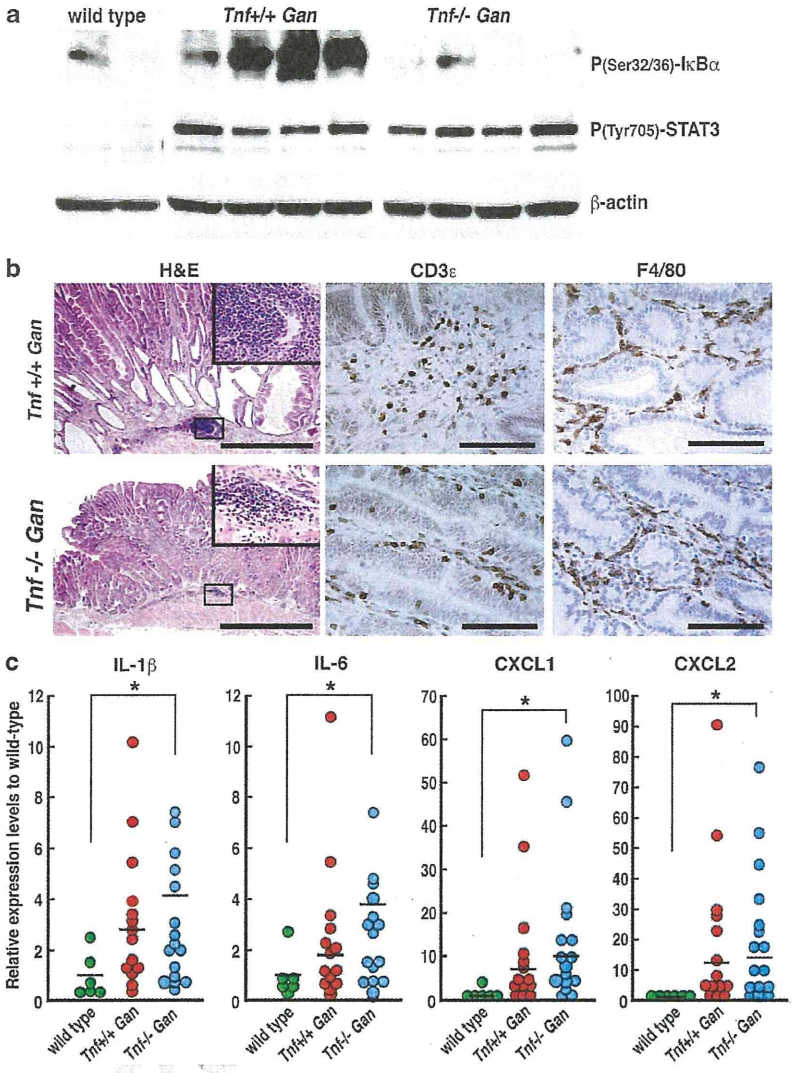

Figure 4. Inflammatory responses induced in *Tnf*−/− *Gan* mouse gastric tumors. (**a**) Immunoblotting of phosphorylated IκBα at Ser32/36 and phosphorylated Stat3 at Tyr705 in gastric tumors from *Tnf*+/+ *Gan* and *Tnf*−/− *Gan* mice ($n=4$ for each), as well as wild-type mouse normal stomach ($n=2$). β-Actin was used as an internal loading control. (**b**) Histological sections of *Tnf*+/+ *Gan* (top) and *Tnf*−/− *Gan* mouse tumors (bottom). H&E staining (left), immunostaining for a T cell marker, CD3ε (center) and a macrophage marker, F4/80 (right). The insets in the H&E staining images show submucosal mononuclear cell infiltration. Scale bars indicate 1 mm (left) and 100 μm (center/right). (**c**) The mRNA levels of the indicated cytokines and chemokines in the wild-type mouse stomach (green), gastric tumors of *Tnf*+/+ *Gan* (red) and *Tnf*−/− *Gan* (blue) mice relative to the mean level of a wild-type mouse stomach. Asterisks (*), $P<0.05$.

level observed in wild-type mice (Supplementary Figure 3a). In the *Tnf*−/− *Gan* mouse tumors, however, the CD44 induction was only about 4.5-fold than that of the wild-type stomachs. Notably, expression of differentiation markers, Muc5AC and H^+K^+/ATPase, was detected in the CD44-negative epithelial cells in *Tnf*−/− *Gan* mouse tumors, whereas CD44-positive tumor cells did not express these markers (Supplementary Figure 3b). Moreover, Ki-67-positive cells were predominantly found in the CD44-positive cell population. These results suggest that disruption of *TNF-α* gene causes differentiation of tumor epithelial cells, resulting in suppression of proliferation.

Candidate tumor-promoting factors induced by TNF-α

To select candidate genes whose products function to maintain the undifferentiated status, we compared the selected 157 genes with a gene set that was upregulated ⩾twofold in Lgr5+ gastric stem cells.[29] As a result, we found that 11 out of the 157 genes were upregulated also in gastric stem cells (Figure 5c). We next transfected small interfering RNAs (siRNAs) against these 11 genes into Kato-III cells, and examined the cell growth in soft agar. Notably, inhibition of *Noxo1*, *Gna14* and *Prom1* expression resulted in a significant decrease of cell proliferation in soft agar (Figure 6a). We further examined the tumorigenicity of Kato-III, MKN45 and MKN74 gastric cancer cells by transfection with siRNAs targeting different sequences of *Noxo1*, *Gna14*, and *Prom1*. Notably, siRNAs for *Noxo1* or *Gna14* suppressed the soft agar colony formation in all cell lines, while *Prom1* siRNAs suppressed only in Kato-III cells (Figure 6b and Supplementary Figure 4). Moreover, we found by reverse transcription-PCR (RT–PCR) that expression of *Noxo1* and *Gna14* was induced in gastric tumor epithelial cells as well as the tumor tissues of *Gan* mice (Figures 6c

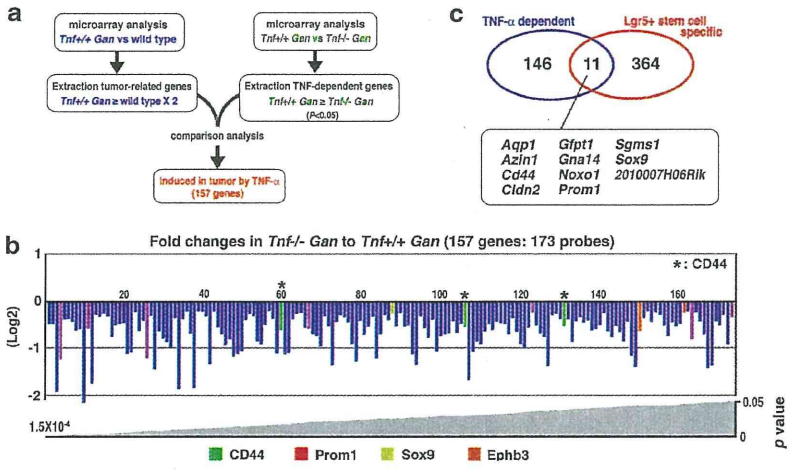

Figure 5. Extraction of candidate TNF-α-dependent tumor-promoting genes. (**a**) Strategy used for the selection of genes that were induced in gastric tumors in a TNF-α-dependent manner. (**b**) The fold-changes in the expression levels of the 157 TNF-α-dependent genes (173 probes) in *Tnf*−/− *Gan* mouse tumors compared with those in *Tnf*+/+ *Gan* mice (Log2 ratio). These genes were significantly downregulated in *Tnf*−/− *Gan* mouse tumors compared with *Tnf*+/+ *Gan* mouse tumors ($P<0.05$). Asterisks (*) indicate the three probes for CD44. Stem cell-related genes (CD44, Prom1, Sox9 and EphB3) are shown by bars with different colors. (**c**) A Venn diagram of 'induced genes in gastric tumors in a TNF-α-dependent manner (157 genes)' and 'upregulated genes ⩾twofold in Lgr5+ gastric stem cells (375 genes)'. Eleven genes were upregulated in both tumor tissues and Lgr5+ stem cells. These genes are indicated by bars in different colors in **b**.

and d). Accordingly, it is possible that TNF-α plays a tumor-promoting role through the induction of *Noxo1* and *Gna14* in tumor epithelial cells.

Role of Noxo1 and Gna14 in differentiation and cancer cell stemness

Differentiation of the primary cultured gastric epithelial cells was associated with the downregulation of a *Sox9* and induction of *Muc5AC* expression (Figure 6e). *Sox9* and *Muc5AC* are markers for undifferentiated and differentiated status, respectively. Notably, expression of both *Noxo1* and *Gna14* was decreased significantly in the differentiated epithelial cells compared with the undifferentiated cells, suggesting a role for Noxo1 and Gna14 in maintenance of undifferentiated status. We therefore examined the role of Noxo1 and Gna14 in the sphere formation of gastric cancer cells. MKN74 cells formed sphere colonies under hypoxic conditions, which were thought to reflect the characteristic of cancer stem cells. Notably, inhibition of *Noxo1* and *Gna14* expression by transfection of siRNAs resulted in a significant decrease in the number of sphere colonies, suggesting that Noxo1 and Gna14 have a role in maintaining the stemness of gastric cancer cells (Figures 6f and g).

TNF-α and CD44 in human gastric cancer tissues

Finally, we examined the expression of TNF-α and CD44 in human primary gastric cancers by real-time RT–PCR. The expression of TNF-α and CD44 was upregulated in 65% and 74% of gastric cancer tissues, respectively, and the expression of TNF-α and CD44 was positively correlated (Supplementary Figure 5). Therefore, it is possible that undifferentiated status of cancer cells is related to the level of TNF-α signaling also in the human gastric cancer.

DISCUSSION

Polymorphism of *TNF-α* gene is associated with an increased risk of gastric cancer, suggesting a role for TNF-α in gastric tumorigenesis.[7,9] In the present study, we have demonstrated, for the first time, that the induction of TNF-α signaling through TNFR1 promotes gastric tumorigenesis through inducing tumor-promoting factors, *Noxo1* and *Gna14*, in tumor epithelial cells (Figure 7).

One of the most important points of the present study is that we have successfully separated TNF-α signaling from COX-2/PGE_2-associated inflammatory responses in tumor tissues. Inflammatory cytokine signaling, including TNF-α, IL-6 and CXCL12, is induced simultaneously in tumor tissues, and the cytokine pathways activate each other by constructing a cytokine network.[30] Some of the inflammatory mediators have been shown to have a role in tumorigenesis. For example, we and other groups[31–33] have demonstrated that the COX-2/PGE_2 pathway has an essential role in gastrointestinal tumorigenesis by inducing angiogenesis and activation of Wnt signaling. Moreover, IL-6 and Stat3 are important for the development of colitis-associated colon cancer,[34,35] and constitutive activation of IL-1β signaling can induce gastric tumorigenesis.[10] It has also been reported that CXCL1/2 expression is linked to chemoresistance and metastasis.[36] Notably, the COX-2/PGE_2, IL-1β, IL-6 and CXCL1/2 pathways were still induced in the *Tnf*−/− *Gan* mouse tumors, possibly as a result of the transgenic expression of *Ptgs2* and *Ptges*. Accordingly, the present results clearly indicate that TNF-α signaling is required for gastric tumor development, even when an inflammatory network of other cytokines/chemokines is present.

It has been shown that the TNF-α receptor, TNFR1, signaling has a role in tumor development in chemically induced skin tumor and colitis-associated colon cancer mouse models.[16,17] In this study, we also showed that disruption of the *TNFR1* gene resulted in significant suppression of gastric tumorigenesis. Accordingly, it is possible that TNFR1 is the major receptor of TNF-α involved in gastric tumor promotion. In the present study, we found that TNF-α/TNFR1 signaling in BMDCs is important for gastric tumor growth. However, blocking TNFR1 signaling did not induce an effective regression of tumors even at 8 weeks after BM transplantation. It is possible that more than 8 weeks are required for established gastric tumors to regress by blocking the TNF/TNFR1 pathway. Moreover, TNFR1 is also expressed in the epithelial cells. Thus, it is conceivable that TNF-α signaling through TNFR1 on epithelial cells also contributes to gastric tumorigenesis, although it remains to be investigated.

Here, we have identified two tumor-promoting factors, Noxo1 and Gna14, which have a role in maintaining the tumorigenicity

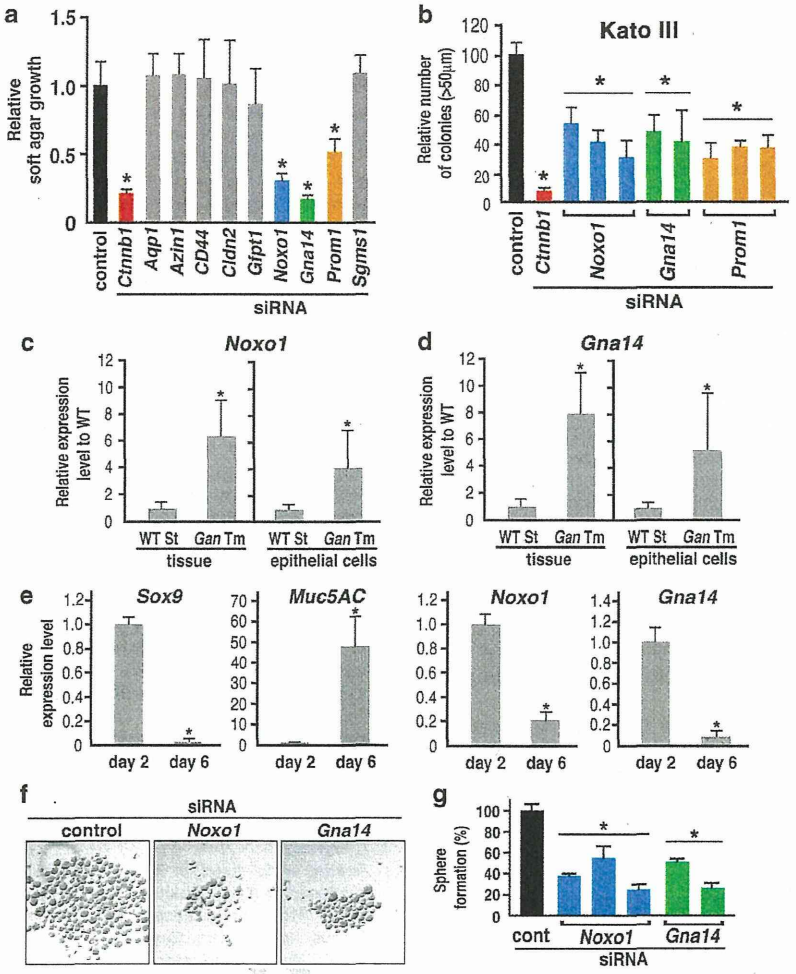

Figure 6. The roles of Noxo1 and Gna14 in tumorigenicity and stemness characteristics. (**a**) The cell growth in soft agar examined by fluorescence intensity of the indicated siRNA-transfected Kato-III cells relative to the mean value of control siRNA-transfected cells (mean ± s.d.). Asterisks (*), *P*-value <0.05 versus control. β-catenin gene (*Catnnb1*) siRNA was used as a positive control. (**b**) The numbers of soft agar colonies of the indicated siRNA-transfected Kato-III cells relative to the mean control siRNA level (mean ± s.d.). Individual bars in the same color indicate results of different siRNAs targeting different sequences for the same gene. Asterisks (*), *P*-value <0.05 versus control. (**c**, **d**) The mRNA levels of *Noxo1* (**c**) and *Gna14* (**d**) in tissues (left) or isolated epithelial cells (right) of wild-type (WT) mouse stomach (*WT St*) or *Gan* mouse tumors (*Gan Tm*) relative to the mean value of WT mouse level (mean ± s.d.). Asterisks (*), *P*-value <0.05 versus WT level. (**e**) The relative mRNA levels of the indicated genes in undifferentiated (day 2) or differentiated gastric epithelial cells (day 6; mean ± s.d.). Asterisks (*), *P*-value <0.05 versus the day 2 level. (**f**) Representative photographs of the spheres of control siRNA-transfected (left) and *Noxo1*- (center) or *Gna14*-siRNA-transfected (right) MKN74 cells. (**g**) The ratio of sphere formation by *Noxo1*- or *Gna14*-siRNA-transfected MKN74 cells relative to the mean level of control siRNA-transfected cells (mean ± s.d.). Individual bars in the same color indicate results of different siRNAs targeting different sequences for the same gene. Asterisks (*), *P*-value <0.05 versus control level.

and stemness of gastric cancer cells. It is possible that one of the tumor-promoting mechanisms of TNF-α signaling is the maintenance of the stemness of cancer cells. *Noxo1* encodes NOX-organizing protein 1, which is a component of the cytosolic regulatory subunits of NOX, and is associated with catalytic isoform NOX1.[37] NOX1 is one of the NOX family members, which are reactive oxygen species-generating enzymes that regulate the redox-sensitive signaling pathways. It has been reported that NOX1 expression is upregulated by oncogenic Ras activation and is required for transformation of cancer cells.[38,39] Moreover, a microarray analysis indicated that *Noxo1* expression is upregulated in a subpopulation of colon cancers.[40] Accordingly, it is possible that *Noxo1* is induced in cancer cells, together with NOX1, in the inflammatory microenvironment, contributing to an oncogene-induced transformation phenotype through the generation of reactive oxygen species.

In contrast to NOX1, little is known about the role(s) of Gna14 in tumor development. *Gna14* encodes guanine nucleotide-binding protein subunit alpha14 ($G\alpha_{14}$), a member of the $G_q\alpha$ subfamily of G proteins.[41] $G\alpha_{14}$ is employed by a variety of G protein-coupled receptors, including somatostatin type 2 receptor and chemokine receptor CCR1, which activate the NF-κB pathway.[42,43] Recently, it has been reported that activation of NF-κB in the Wnt-activated cells induces dedifferentiation in intestinal epithelial cells, resulting in the acquisition of tumor-initiating property.[44] Accordingly, it is possible that *Gna14* expression contributes to maintaining tumor cells in an undifferentiated status through activation of NF-κB, which leads to tumor development.

Noxo1 and *Gna14* were induced in the tumor epithelial cells, whereas TNF-α/TNFR1 signaling was predominantly activated in the BMDCs of *Gan* mouse tumors. Accordingly, it is possible that the expression of *Noxo1* and *Gna14* in tumor cells is not directly

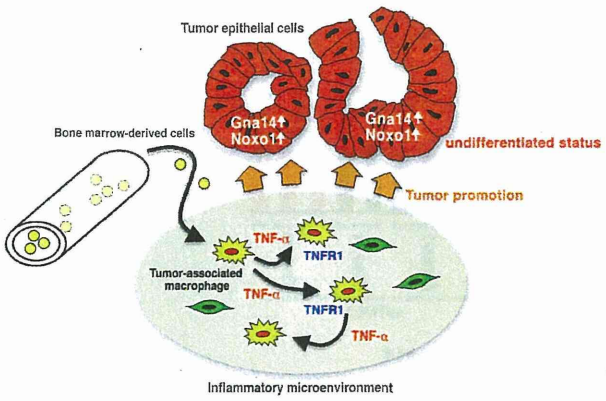

Figure 7. A schematic drawing of the role of TNF-α signaling in gastric tumorigenesis. BMDCs, including macrophages, are recruited to the inflammatory microenvironment and express TNF-α, which further activates TNFR1 receptor on BMDCs in the microenvironment, which is important for inducing the tumor-promoting factors including *Noxo1* and *Gna14* in tumor epithelial cells.

regulated by TNF-α signaling, but indirectly through BMDC-expressing molecule(s) that are induced by the TNF-α/NF-κB pathway. It is therefore conceivable that such a molecule would be an effective target for the prevention or treatment of gastric cancer.

In conclusion, we demonstrated that TNF-α/TNFR1 signaling in the tumor microenvironment promotes gastric cancer development. *Noxo1* and *Gna14* are induced in tumor epithelial cells by a TNF-α-dependent manner, which is important for gastric tumorigenesis. Accordingly, it is possible that targeting TNF-α signaling in the microenvironment, or inhibition of Noxo1 and Gna14 induction or their functions in tumor cells, may represent a preventive or therapeutic strategy against gastric cancer.

MATERIALS AND METHODS

Animal models

K19-Wnt1 mice express *Wnt1* driven by the *Krt19* gene promoter, which is transcriptionally active in gastric epithelial cells, whereas *Gan* mice express *Wnt1*, *Ptgs2* and *Ptges*, driven by the *Krt19* promoter.[18,19] *Tnf* mutant mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and *Tnfrsf1a* mutant mice were described previously.[17] For the tumor phenotype analyses, *Gan* mice were euthanized and examined at 50 weeks of age ($n=8$ for *Tnf*+/+ *Gan* mice and *Tnf*−/− *Gan* mice, and $n=10$ for *Tnf*+/− *Gan* mice) or examined by a X-rayCT analysis at 25 weeks of age ($n=9$ for *Tnfrsfr1a*+/+ *Gan* mice, and $n=5$ for *Tnfrsf1a*−/− *Gan* mice). All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University, Japan.

Measurement of tumor volume and scoring of preneoplastic lesions

The tumor area was measured by the ImageJ 1.46 software program (NIH, Bethesda, MD, USA) using photographs that were taken under a dissecting microscope. The mucosal thickness (tumor height) of the gastric tumors was measured using histology sections. The 'tumor size' was calculated by multiplying the tumor area by the tumor height ('tumor area' × 'tumor height'). The relative tumor size was calculated in comparison with the mean of the control *Gan* mouse tumor size. The X-ray CT images of the gastric tumors were examined using a LaTheta LCT-100 instrument (Aloka, Tokyo, Japan). The tumor areas of the slice images were measured using the ImageJ 1.46 software program (NIH), and three serial images, including the largest tumor image, were selected from all scanned images for each mouse, and the mean tumor area of the three images was calculated and compared with the mean value of the control *Gan* mice. The number of preneoplastic lesions in the glandular stomachs of *K19-Wnt1* mice ($n=6$ for each genotype) was counted using eight independent histology sections, and the mean number per section was calculated.

Real-time RT-PCR

Gastric tumors of *Tnf*+/+ *Gan* ($n=7$) and *Tnf*−/− *Gan* mice ($n=8$), and normal stomachs of wild-type mice ($n=6$), were used for RNA extraction. For human tissue samples, paired samples of human gastric cancer tissues and adjacent normal stomach tissues ($n=23$) were collected at Kanazawa University Hospital, Japan. For experiments using human tissue samples, approval for the project was obtained from the Kanazawa University Medical Ethics Committee, and written informed consent was obtained before specimen collection. The total RNAs of tissue samples were extracted using ISOGEN (Nippon Gene, Tokyo, Japan), reverse-transcribed using the PrimeScript RT reagent kit (Takara, Tokyo, Japan) and were PCR-amplified by a Stratagene Mx3000P instrument (Agilent Technologies, Santa Clara, CA, USA) using SYBR Premix ExTaqII (Takara). To avoid location-related differences in the differentiation and proliferation status within a *Gan* mouse tumor tissue, two samples were collected from different regions of the same tumors. The primers used for the real-time RT–PCR were purchased from Takara. For laser microdissection-based RT–PCR, epithelial cells and stromal cells were separately collected from frozen sections of *Gan* mouse tumors ($n=4$) using laser microdissection LMD7000 (Leica Microsystems, Wetzler, Germany), and the total RNAs were extracted using a RNeasy Micro kit (Qiagen, Valencia, CA, USA).

BM transplantation

BM cells were prepared from the femurs and tibias of donor mice. Recipient mice were irradiated with 9 Gy, followed by intravenous injection of 2×10^6 BM cells. *Tnf*+/+ GFP mouse BM was transplanted into *Tnf*−/− *Gan* mice and *Tnf*−/− mice BM was transplanted into *Tnf*−/− *Gan* mice as a control. *Tnfrsf1a*−/− BM was transplanted into *Tnfrsf1a*+/+ *Gan* mice and wild-type mouse BM was transplanted into *Tnfrsf1a*+/+ *Gan* mice as a control. The X-ray CT images of the gastric tumors were examined at 0 and 8 weeks after BM transplantation.

Histology and immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, paraffin-embedded and sectioned at 4 μm thickness. Sections were stained with hematoxylin and eosin (H&E) or processed for the immunohistochemistry. Antibodies against Ki-67 (Dako, Carpinteria, CA, USA), F4/80 (Serotec, Oxford, UK), GFP (Life Technologies, Grand Island, NY, USA), E-cadherin (R&D, Minneapolis, MN, USA), CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD44 (Millipore, Billerica, MA, USA), Muc5AC (Thermo Fisher Scientific, Rockford, IL, USA) and H^+K^+/ATPase (MBL, Nagoya, Japan) were used as the primary antibodies. Staining signals were visualized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA). For fluorescence immunohistochemistry, Alexa Fluor 594 or Alexa Fluor 488 antibodies (Molecular Probes, Eugene, OR, USA) were used as the secondary antibody. One milliliter of BrdU was injected intraperitoneally (1 mg/ml; Roche Diagnostics, Indianapolis, IN, USA) 1.5 h before euthanasia, and tissue sections were immunostained with an anti-BrdU antibody (Roche).

Immunoblotting analysis

Tissues were homogenized in lysis buffer, and 10 μg of the supernatant protein sample was separated in a 10% SDS-polyacrylamide gel. Antibodies against phosphorylated IκBα at Ser32/36 and phosphorylated Stat3 at Tyr705 (Cell Signaling, Danvers, MA) were used. An anti-β-actin antibody (Sigma, St Louis, MO, USA) was used as the internal loading control. The ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to detect the signals.

Microarray analysis

Total RNA was extracted from the gastric tumors of *Tnf*+/+ *Gan* mice and *Tnf*−/− *Gan* mice and wild-type mouse stomachs ($n=3$ for each) using ISOGEN (Nippon Gene). GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) were used for the expression profile analyses. The labeled cRNA was prepared using standard Affymetrix protocols, and the chips were scanned using a GeneChip Scanner 3000 7G (Affymetrix). The microarray results were deposited in the GEO as accession GSE43145.