ORIGINAL ARTICLE

TNF- α /TNFR1 signaling promotes gastric tumorigenesis through induction of *Noxo1* and *Gna14* in tumor cellsH Oshima^{1,7}, T Ishikawa^{1,7}, GJ Yoshida², K Naoi¹, Y Maeda^{1,2}, K Naka³, X Ju¹, Y Yamada⁴, T Minamoto⁵, N Mukaida⁶, H Saya² and M Oshima¹

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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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)-1 β 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-1 β , 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-1 β 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/PGE₂ pathway. Wnt signaling activation is one of the major causes of human gastric cancer.^{18,20} On the other hand, the COX-2/PGE₂ 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 μ m. (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 μ m, 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- κ B through phosphorylation of I κ B α . 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.

I κ B α was increased in Gan mouse tumors compared with the wild-type mouse stomach (Figure 4a). In contrast, the I κ B α phosphorylation level was significantly decreased in *Tnf* $-/-$ Gan mouse tumors, indicating that NF- κ B 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- κ B 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-1 β , 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-1 β 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 $\geq$ 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 downregulated 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 I κ B α 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, CD3 ϵ (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 μ m (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 $\geq$ 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 $\geq$ 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/PGE₂-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/PGE₂ 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-1 β 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/PGE₂, IL-1 β , 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 *Ptgs*. 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 $\pm$ s.d.). Asterisks (*), P -value < 0.05 versus control. β -catenin gene (*Catnb1*) 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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- κ B pathway.^{42,43} Recently, it has been reported that activation of NF- κ B 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- κ B, 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. BMDs, including macrophages, are recruited to the inflammatory microenvironment and express TNF- α , which further activates TNFR1 receptor on BMDs 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- κ B 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 *Ptgs*, 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-ray CT analysis at 25 weeks of age ($n=9$ for *Tnfrsf1a*+/- *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' $\times$ '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, Wetzlar, 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*-/- mouse 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 μ m 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), CD3 ϵ (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 μ g of the supernatant protein sample was separated in a 10% SDS-polyacrylamide gel. Antibodies against phosphorylated I κ B α 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.

Cell culture experiments

Gastric cancer cell lines, MKN45, MKN74 and Kato-III (Riken Bioresource Center, Tsukuba, Japan) were cultured in RPMI1640 or DMEM supplemented with 10% fetal bovine serum. Knockdown experiments were performed using Silencer Select Pre-designed siRNA and negative control siRNA (Life Technologies, Carlsbad, CA, USA). For the soft agar proliferation/colony formation assay, cells were mixed in 0.4% agar and seeded in a 96-well or six-well plate and cultured for 1 or 3 weeks, respectively. Then, 96-well plates were stained with AlamarBlue reagent (Life Technologies), and the fluorescence intensity was measured to examine the cell number. Six-well plates were stained with Giemsa solution (Wako, Osaka, Japan) and colony numbers were scored. For the sphere formation assay, MKN74 cells were plated in ultra-low attachment plates (Corning, Corning, NY, USA) and were cultured under hypoxic conditions at 3% O₂ and 5% CO₂. After 10 days, the number of spheres was counted.

The primary culture of gastric epithelial cells was described previously.⁴⁵ Briefly, mouse glandular stomachs were treated with 0.1% collagenase, followed by centrifugation at 20g for 3 min to isolate gastric glands. Isolated glands were digested with trypsin and cultured on collagen-coated dishes. On day 2, the cells were passaged to induce differentiation. The primary cultured cells on day 2 and day 6 were used as undifferentiated and differentiated gastric epithelial cells, respectively.

Statistical analysis

The data were analyzed using the unpaired *t*-test and are presented as the means $\pm$ s.d. A value of *P* < 0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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