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Activated macrophages promote Wnt signaling through tumor necrosis factor-α in gastric tumor cells

Keisuke Oguma¹, Hiroko Oshima¹, Masahiro Aoki², Ryusei Uchio¹, Kazuhito Naka³, Satoshi Nakamura⁴, Atsushi Hirao³, Hideyuki Saya⁵, Makoto Mark Taketo², and Masanobu Oshima¹*

¹Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, 920-0934 Japan. ²Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto, 606-8501 Japan. ³Division of Molecular Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, 920-0934 Japan. ⁴Department of Biomedical Research and Development, Link Genomics, Tokyo, 103-0023 Japan. ⁵Division of Gene Regulation, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, 160-8582 Japan. ⁶These authors contributed equally to this work.

*Corresponding author: Masanobu Oshima, Division of Genetics, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, 920-0934 Japan. Tel.: +81 76 265 2721; Fax: +81 76 234 4519; E-mail: oshimam@kenroku.kanazawa-u.ac.jp

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Abstract
The activation of Wnt/β-catenin signaling plays a key role in gastrointestinal tumorigenesis. It has been suggested that the promotion of Wnt/β-catenin activity beyond the threshold is important for carcinogenesis. We herein investigated the role of macrophages in promotion of Wnt/β-catenin activity in gastric tumorigenesis. We found β-catenin nuclear accumulation in macrophage infiltrated dysplastic mucosa of the K19-Wnt1 mouse stomach. Moreover, macrophage depletion in ApcΔ716 mice resulted in the suppression of intestinal tumorigenesis. These results suggested the role of macrophages in the activation of Wnt/β-catenin signaling, which thus leads to tumor development. Importantly, the conditioned medium of activated macrophages promoted Wnt/β-catenin signaling in gastric cancer cells, which was suppressed by the inhibition of tumor necrosis factor (TNF)-α. Furthermore, treatment with TNF-α induces GSK3β phosphorylation, which resulted in the stabilization of β-catenin. We also found that Helicobacter infection in the K19-Wnt1 mouse stomach caused mucosal macrophage infiltration and nuclear β-catenin accumulation. These results suggest that macrophage-derived TNF-α promotes Wnt/β-catenin signaling through inhibition of GSK3β, which may contribute to tumor development in the gastric mucosa.

Key words: gastric cancer / inflammation / macrophage / tumor necrosis factor-α / Wnt
Introduction
The canonical Wnt signaling pathway (Wnt/β-catenin pathway) operates by stabilizing β-catenin (Taketo, 2006). The phosphorylation of β-catenin by glycogen synthase kinase 3β (GSK3β) results in degradation through the ubiquitin pathway. The binding of Wnt ligands to a Frizzled receptor leads to inhibition of the β-catenin degradation complex consisting of APC, AXIN, and GSK3β thereby allowing the nuclear translocation of stabilized β-catenin followed by transcriptional activation of the Wnt target genes.

Wnt/β-catenin signaling plays a key role in the maintenance of intestinal stem cells and progenitor cells (Korinek et al., 1998; van de Wetering et al., 2002), and its activation causes gastrointestinal tumor development (Fodde et al., 2001; Oshima et al., 1995; Oshima et al., 2006).

The activation level of Wnt/β-catenin signaling has been reported to be a determining factor for the multiplicity of intestinal polyposis in several types of Apc gene mutant mice (Gaspar and Fodde, 2004; Li et al., 2005). Moreover, nuclear β-catenin accumulation is predominantly observed in the invasion front of colon cancer in comparison to the non-invasive tumor area (Brabletz et al., 1998). Furthermore, sensitivity of embryonic stem (ES) cells for differentiation is inhibited by increase of Wntβ-catenin signaling activity (Kielman et al., 2002). These results, taken together, suggest that the promotion of the Wnt/β-catenin activity beyond the threshold level is required for tumorigenesis, invasion and the maintenance of cell stemness.

On the other hand, it has been established that inflammation plays an important role in cancer development (Coussens and Werb, 2002). The activation of tumor necrosis factor (TNF)-α or NF-κB pathway is required for the development of hepatocellular carcinoma (Pikarsky et al., 2004) and intestinal tumors (Greten et al., 2004; Popivanova et al., 2008) through the induction of growth factors and suppression of apoptosis. Although several signaling pathways have been reported for promotion of Wnt/β-catenin activity (Fodde and Brabletz, 2007), it has not been elucidated yet whether the inflammatory response contributes to the promotion of Wnt/β-catenin signaling during tumorigenesis.

Gastric cancer is the second most common cancer in the world and it is closely associated with Helicobacter pylori infection which leads to chronic inflammation (Correa, 2003). Moreover, the activation of the Wnt/β-catenin signaling is found in about 30% of gastric cancer (Clements et al., 2002), suggesting that Wnt activation is one of the major causes for gastric cancer development. We recently showed that cooperation of
Wnt signaling and prostaglandin E$_2$ (PGE$_2$) pathway causes gastric cancer development in a transgenic mouse model (Oshima et al., 2006). Because the activation of PGE$_2$ signaling induces infiltration and activation of macrophages in the gastric mucosa (Oshima et al., 2004; Oshima et al., 2005), these results collectively suggest that activated macrophages promote the Wnt/β-catenin signaling activity, which thus contributes to gastric cancer.

We herein show that TNF-α derived from activated macrophages promotes the Wnt/β-catenin activity in gastric cancer cells through the suppression of GSK3β. Moreover, Helicobacter infection in the Wnt1 transgenic mice resulted in macrophage infiltration and the activation of Wnt/β-catenin signaling in the gastric mucosa, which thus led to gastric tumorigenesis. These results suggest that activated macrophages in inflammatory microenvironment play an important role in gastric tumorigenesis through the promotion of the Wnt/β-catenin activity.
Results

Macrophage infiltration and β-catenin nuclear accumulation in gastric dysplasia of K19-Wnt1 transgenic mice

K19-Wnt1 transgenic mice expressing Wnt1 in the gastric epithelial cells develop sporadic dysplastic lesions in the glandular stomach (Oshima et al., 2006). In these dysplastic lesions, inflammatory cells infiltrated the submucosa, whereas cell infiltration was rarely detected in the adjacent normal mucosa (Figure 1A). By immunostaining, we found macrophage infiltration in the dysplastic mucosa, while tissue macrophages were only sparsely scattered in the normal mucosa (Figure 1B and H). We also found strong nuclear staining of β-catenin in the epithelial cells of the dysplastic mucosa (Figure 1C). Notably, β-catenin accumulated epithelial cells were physically associated with stromal macrophages in the dysplastic lesions (Figure 1E). In contrast, mild β-catenin accumulation was found only in the cell proliferation zone of the normal mucosa (Figure 1I). We confirmed the increase in the β-catenin positive index in the dysplastic lesion to be associated with the increased number of infiltrated macrophages (Figure 1F).

Moreover, the number of Ki-67-positive proliferating cells increased in the dysplastic mucosa when compared with that in the adjacent normal region (Figure 1D and J). The mean Ki-67 labeling indices in the dysplastic lesions and normal mucosa were 43% and 15%, respectively (p = 0.007). We also confirmed by BrdU incorporation analysis that cell proliferation was increased in dysplastic lesions (Figure 1G). Based on these results, we hypothesized that macrophage infiltration by inflammatory responses causes promotion of Wnt/β-catenin signaling activity in gastric epithelial cells, which probably leads to dysplastic changes in the gastric mucosa.

Requirement of macrophages for intestinal tumor development in ApcΔ716 mice

To examine whether infiltrated macrophages are required for tumorigenesis, we performed crossing experiments using op/op mice, in which the number of macrophages significantly decreased in the majority of tissues because of Csf1 mutation (Cecchini et al., 1994). We first examined whether op/op mutation suppressed macrophage infiltration in the inflamed stomach using K19-C2mE mice, which develop gastritis with heavy macrophage accumulation (Oshima et al., 2004). However, mucosal macrophages were still found in the stomach of the K19-C2mE op/op compound mice (Supplementary Figure 1). We therefore crossed op/op mice with ApcΔ716 mice, a model for intestinal
polyposis caused by Wnt activation (Oshima et al., 1995). ApcΔ716 mice develop numerous polyps in entire intestinal tract (Figure 2A), and macrophages were infiltrated in the polyp stroma (Figure 2B). Interestingly, ApcΔ716 op/op compound mice showed dramatic suppression of intestinal polyposis (Figure 2C and E), and macrophages were not found in the polyp tissues (Figure 2D). Moreover, the number of polyps > 1 mm significantly decreased in ApcΔ716 op/op compound mice by 80% compared with ApcΔ716 mice. Although it remains to be elucidated as to whether the loss of the CSF-1 function affects tumorigenesis, these results suggest that macrophages were required for the growth of Wnt/β-catenin-activated tumor cells. Consistently, the BrdU labeling index also decreased in ApcΔ716 op/op mouse tumors (Figure 2F).

**Fluctuation of Wnt/β-catenin signaling activity in gastric cancer cells**

We next examined the regulation of the Wnt/β-catenin signaling activity by *in vitro* experiments using human gastric cancer cells, AGS and Kato-III. AGS cells harbor a heterozygous mutation in the β-catenin gene (CTNNB1) at codon 34 (Caca et al., 1999; Supplementary Figure 2), whereas CTNNB1 is amplified in Kato-III cells (Suriano et al., 2005). Consistent with the previous results (Nojima et al., 2007), both cell lines showed an elevated β-catenin/TCF transcriptional activity as detected by a TOPFLASH assay (Supplementary Figure 3). By immunocytochemistry, the cytoplasmic and nuclear accumulation of β-catenin was found in both AGS and Kato-III cells (Figure 3A, left). We also found the nuclear localization of active form of β-catenin, unphosphorylated on Ser37 and Thr41 (van Noort et al., 2002; Figure 3A, right), thus indicating the activation of Wnt/β-catenin signaling. However, several cells showed a weak β-catenin staining intensity (Figure 3A, arrowheads), suggesting that the level of Wnt/β-catenin signaling activity varies in populations.

To examine the Wnt/β-catenin activity in the live cells, we transfected a TOPEGFP vector that expressed a green fluorescent protein (GFP) in response to β-catenin/TCF transcriptional activity (Figure 3B), and established AGS-GFP and Kato-III-GFP cell lines. We confirmed that GFP expression was induced in β-catenin accumulated cells after the transient transfection of mutant CTNNB1 (S33A) expression vector (Figure 3C). Importantly, we directly found by time-lapse video analysis that the Wnt/β-catenin activity was not constant but fluctuated in individual AGS-GFP cells (Supplementary Video 1).
To confirm the fluctuation of Wnt/β-catenin activity, we isolated by cell sorting the high-GFP (top 5%) and low-GFP (bottom 5%) populations corresponding to high and low in Wnt/β-catenin signaling activity, respectively, from AGS-GFP and Kato-III-GFP cell (Figure 3D, left). Immediately after isolation, both populations showed distinct GFP intensities (Figure 3D, center). Importantly, however, the GFP intensities of the isolated populations returned to a similar distribution to the unsorted cells after culturing for 5 days (Figure 3D, right). These results confirmed that the activation level of Wnt/β-catenin signaling fluctuates in each cell.

We next examined the level of Wnt/β-catenin activity in cell cycle-synchronized Kato-III-GFP cells. However, the ratio of a high GFP population did not change during cell cycling, thus indicating Wnt/β-catenin fluctuation to be independent of the cell cycle status (Supplementary Figure 4).

Promotion of Wnt/β-catenin activity in gastric cancer cells by activated macrophages and TNF-α

We next examined whether the activity of Wnt/β-catenin signaling is promoted by activated macrophages using the GFP reporter transfected cells. We prepared a conditioned medium of macrophage cell line, RAW264, activated with lipopolysaccharide (LPS) for 24 hours [hereafter, CM-LPS (+)]. A conditioned medium from unstimulated RAW264 cells was used as the control [CM-LPS (−)]. When AGS-GFP and Kato-III-GFP cells were stimulated with CM-LPS (+), the high-GFP population (corresponding to top 2% in the untreated control cells) significantly increased in a CM concentration-dependent manner (Figure 4A and B, left and top, red area). In contrast, the stimulation of the cells with CM-LPS (−) or LPS alone did not affect the Wnt/β-catenin activity in either cell line (Figure 4A and B, middle and bottom). These results indicate that the soluble factor(s) derived from activated macrophages promote the Wnt/β-catenin activity in gastric cancer cells.

TNF-α is a key mediator for inflammation and is produced by activated macrophages. Notably, the treatment of the cells with a neutralizing antibody against TNF-α significantly suppressed the effect by CM-LPS (+) to enhance the Wnt/β-catenin activity in both cell lines (Figure 4C). Furthermore, direct treatment with TNF-α increased the level of Wnt/β-catenin activity (Figure 4D). We detected the expression of both TNF R1 and TNF R2 on AGS and Kato-III cells as well as in the primary cultured
gastric epithelial cells (Supplementary Figure 5A, C and data not shown). Importantly, the blockade of either receptor by neutralizing antibody significantly suppressed the TNF-α-induced Wnt/β-catenin activation (Supplementary Figure 5B). These results suggest that TNF-α signaling through both TNF R1 and TNF R2 is thus involved in the promotion of the Wnt/β-catenin activity. By immunohistochemistry, we found most TNF-α-expressing cells in gastric tumors to be macrophages, while CD11c-positive dendritic cells were rarely detected (Supplementary Figure 6A). Importantly, the treatment of the K19-Wnt1 mice with anti-TNF-α neutralizing antibody resulted in a decrease of the β-catenin immunostaining intensity in the dysplastic lesions (Supplementary Figure 6B).

Moreover, we examined the Wnt-promoting effect of other inflammatory cytokines, IL-1β, IL-6 and IL-11, which play a role in gastric tumorigenesis (Fox and Wang, 2008; Howlett et al., 2005). However, these cytokines did not promote Wnt/β-catenin activity in gastric cancer cells, although IL-1β slightly increased the high-GFP population in AGS-GFP cells (Supplementary Figure 7). These results, taken together, suggest that TNF-α is one of the important macrophage-derived factors that promote the Wnt/β-catenin activity in gastric epithelial cells and cancer cells.

**Suppression of β-catenin phosphorylation by TNF-α through the inhibition of GSK3β in gastric cancer cells**

By a flow cytometry analysis, we found that the level of unphosphorylated β-catenin increased significantly in Kato-III cells when cells were treated with CM-LPS (+) or TNF-α (Figure 5A). By western blotting, we confirmed the increased level of unphosphorylated β-catenin in Kato-III cells stimulated with CM-LPS (+) or TNF-α (Figure 5B). These results suggested that CM-LPS (+) or TNF-α suppressed the phosphorylation of β-catenin, resulting in Wnt/β-catenin promotion. Significantly, treatment with TNF-α resulted in an increase of GSK3β phosphorylation on Ser9 both in AGS and Kato-III cells (Figure 5C), which caused the suppression of the GSK3β activity (Cross et al., 1995). The mean relative band intensities of the phosphorylated GSK3β to the control level were 2.03 ± 0.14 \( (p = 0.009) \) and 1.68 ± 0.31 \( (p = 0.03) \) in the AGS and Kato-III cells, respectively. Interestingly, a strong immunostaining signal for phosphorylated GSK3 was found in a subpopulation of Kato-III cells that also showed nuclear accumulation of β-catenin at the same time (Figure 5D). These results suggest that
the promotion of Wnt/β-catenin signaling is regulated by the GSK3β phosphorylation status in gastric cancer cells. Akt is an important kinase for phosphorylation of GSK3β at Ser9 (Sharma et al., 2002). Importantly, the active form of Akt, phosphorylated on Ser473, increased significantly after TNF-α stimulation in both AGS and Kato-III cells (Figure 5C). The mean relative band intensities of the phosphorylated Akt to the control level were 2.08 ± 0.16 (p = 0.005) and 1.81 ± 0.19 (p = 0.002) in the AGS and Kato-III cells, respectively. These results suggest that TNF-α accelerates the GSK3β phosphorylation through the activation of the Akt pathway.

**NF-κB independent promotion of Wnt/β-catenin activity in gastric cancer cells**

To examine whether NF-κB is involved in Wnt/β-catenin promotion by activated macrophages, we transfected IκB-superrepressor (IκBSR) expression vector into AGS-GFP cells and established two sublines, in which TNF-α-induced NF-κB activation was suppressed (Figure 6A). Notably, the TNF-α- or CM-LPS (+)-induced promotion of the Wnt/β-catenin activity did not change in either of IκBSR transfected AGS-GFP cells (Figure 6B), thus suggesting the existence of an NF-κB independent mechanism for the TNF-α-induced Wnt/β-catenin promotion.

When Kato-III cells were treated with the protein synthesis inhibitor cycloheximide, the unphosphorylated β-catenin-positive population decreased gradually in an incubation time-dependent manner, probably due to the phosphorylation of β-catenin by GSK3β (Figure 6C, black lines). However, the simultaneous treatment with CM-LPS (+) significantly suppressed the cycloheximide-induced decrease of this population (Figure 6C, red lines). Therefore, it is also possible that activated macrophages suppress β-catenin phosphorylation independent of protein synthesis, which is consistent with the results that the transcription factor NF-κB pathway is not involved in the suppression of β-catenin phosphorylation.

**Macrophage infiltration and Wnt/β-catenin activation in mouse gastric epithelial cells by Helicobacter infection**

We next examined whether Wnt/β-catenin signaling is activated by infection-associated inflammation in the mouse stomach. *Helicobacter felis*, a close relative to *H. pylori* was infected to *K19-Wnt1* mouse stomach. Eight weeks after *H. felis* infection, the infiltration of macrophages was found in the *H. felis*-infected gastric mucosa (Figure 7A-C), while
tissue macrophages were sparsely scattered in the non-infected mucosal area (Figure 7G-I). Such an inflamed mucosa in the infected-K19-Wnt1 mice was only detected by histological examinations, and this inflamed mucosa was distinct from the spontaneously developed dysplastic lesions (Figure 1A). Importantly, the number of β-catenin-accumulated epithelial cells increased significantly in the infected and inflamed mucosa compared with that in the non-inflamed mucosa (Figure 7D, J, and M). These results suggest that the infection-associated inflammation promotes the Wnt/β-catenin signaling activity in gastric epithelial cells in vivo. Moreover, in the inflamed mucosa, the epithelial cell proliferation detected by Ki-67 immunostaining significantly increased (Figure 7E, K, and N). Furthermore, the number of H⁺/K⁺-ATPase-positive parietal cells decreased dramatically in the H. felis-infected area, thus indicating the suppression of epithelial differentiation (Figure 7F, L, and O). These results, taken together, suggest that infiltrated macrophages in response to Helicobacter infection play a role in the activation of Wnt/β-catenin signaling, thereby enhancing the proliferation and suppression of differentiation. When ApcΔ716 mice were infected with H. felis, we did not find any β-catenin accumulated dysplastic cells in the inflamed gastric mucosa (data not shown). Therefore, it is possible that the basal activation level of Wnt/β-catenin signaling is required for its promotion.

**Tumor development in H. felis-infected K19-Wnt1 mouse stomach**

We finally examined whether H. felis infection contributes to gastric tumorigenesis in K19-Wnt1 mice. Importantly, K19-Wnt1 mice developed gastric tumors at 20 weeks after H. felis infection, while no tumors were found in the H. felis-infected wild-type mice (Figure 8A, E). Histologically, submucosal infiltration was found in both H. felis-infected K19-Wnt1 and wild-type mouse stomach (Figure 8B, F). Although macrophage infiltration was detected in both genotypes (Figure 8C, G), dysplastic epithelial cells were evident in the infected K19-Wnt1 tumors but not in the wild-type mouse stomach (Figure 8D, H). These results, taken together, suggest that infection-associated inflammation plays a role in gastric tumorigenesis through the promotion of the Wnt/β-catenin activity beyond the basal activation level.
Discussion

**Macrophage infiltration and cancer development, progression and stemness**

The activation of Wnt/β-catenin signaling by genetic alteration, such as mutation in *APC* or *CTNNB1*, is responsible for gastrointestinal tumorigenesis. However, accumulating evidence suggests that further promotion of the Wnt/β-catenin activity plays an important role in tumor growth and progression (Fodde and Brabletz, 2007). For example, increased β-catenin accumulation is found in the invasion front of colon cancer (Brabletz *et al*, 1998). We herein demonstrated direct evidence that the Wnt/β-catenin activity fluctuates in individual cancer cells that carry the same genetic alteration in *CTNNB1*. Accordingly, Wnt/β-catenin signaling can be promoted beyond the basal activated level by genetic alteration, and such promotion may play an important role in both tumor development and progression.

We have previously shown the induction of COX-2/PGE$_2$ pathway to play a key role in the intestinal tumorigenesis that is triggered by Wnt/β-catenin activation (Oshima *et al*, 1996; Sonoshita *et al*, 2001). The simultaneous induction of both Wnt and PGE$_2$ pathways is also responsible for gastric tumor development in mouse models (Oshima *et al*, 2006). Although PGE$_2$ may have multiple functions for tumorigenesis, we previously found an increased PGE$_2$ level to result in macrophage accumulation in the gastric mucosa of *K19-C2mE* mice (Oshima *et al*, 2004). Accordingly, it is possible that macrophage infiltration in dysplastic lesions of *K19-Wnt1* mice is caused by the spontaneous induction of COX-2/PGE$_2$ pathway. We herein show that Wnt/β-catenin signaling is promoted by macrophage-derived TNF-α, which contributes to gastric tumorigenesis. It is therefore possible that the induction of the COX-2/PGE$_2$ pathway promotes Wnt/β-catenin signaling through the enhancement of macrophage infiltration, which is responsible for tumor development.

On the other hand, Wnt/β-catenin signaling is not activated in the stomach of either *K19-C2mE* mice or *H. felis*-infected *Apc$^{Δ716}$* mice, thus indicating that the induction of the COX-2/PGE$_2$ pathway and subsequent macrophage infiltration are not sufficient for the promotion of Wnt/β-catenin in gastric epithelial cells. It is conceivable that the basal activation of the Wnt/β-catenin pathway by genetic alteration is required for the promotion of Wnt/β-catenin signaling.

The activation of Wnt/β-catenin pathway is also suggested to be important for stemness. Wnt/β-catenin signaling is required for the maintenance of normal intestinal
stem cells (Korinek et al, 1998), and the increase in the Wnt/β-catenin activity contributes to the maintenance of stemness in embryonic stem cells (Kielman et al, 2002). Accordingly, it is possible that the increased Wnt/β-catenin activity is also important for the maintenance of cancer stem cells. Therefore, infiltrated macrophages in tumor tissues may be an important component of niche for cancer stem cells by promoting Wnt/β-catenin signaling through TNF-α pathway. This hypothesis is supported by the results that macrophages are required for the maintenance of progenitor cells in the intestinal crypt (Pull et al, 2005).

*Other possible pathways than TNF-α for the promotion of the Wnt/β-catenin signaling activity*

It has been shown that inflammatory cytokines, TNF-α, IL-1β, IL-6 or IL-11, play an important role in gastric tumorigenesis (Fox and Wang, 2007; Howlett et al, 2005). Among these cytokines, TNF-α is the most potent Wnt/β-catenin promoting factor, although IL-1β slightly increases Wnt signaling in AGS cells. However, the increased level of the Wnt/β-catenin activity by CM-LPS (+) is significantly higher than that by TNF-α alone in both cell lines (Figure 4), thus suggesting the effect of other macrophage-derived factors on Wnt/β-catenin promotion. It has been reported that hepatocyte growth factor (HGF) activates Wnt/β-catenin signaling through the tyrosine phosphorylation of β-catenin (Rasola et al, 2006), and platelet-derived growth factor (PDGF) also promote Wnt/β-catenin signaling through suppression of β-catenin phosphorylation (Yang et al, 2006). It is therefore possible that these factor(s) contribute to Wnt/β-catenin promotion together with TNF-α in gastric tumorigenesis.

Recently, using a colon tumor mouse model, TNF R1 signaling in bone marrow-derived cells has been shown to indirectly enhance epithelial Wnt/β-catenin signaling (Popivanova et al, 2008). Accordingly, it is possible that TNF-α signaling, not only in epithelial cells but also in stromal cells, may therefore contribute to the promotion of Wnt/β-catenin signaling in cancer cells.

*Promotion of Wnt/β-catenin signaling in cancer cells that retain wild-type CTNNB1*

We show here that TNF-α stimulation promotes the Wnt/β-catenin signaling activity through the suppression of β-catenin phosphorylation by GSK3β. Therefore, the expression of wild-type β-catenin is required for the promotion of Wnt/β-catenin signaling
in epithelial cells as well as cancer cells. Because Kato-III cells have a multicopy of the wild-type CTNNB1 (Suriano et al, 2005), TNF-α stimulation suppresses phosphorylation of these β-catenin, thus leading to the enhancement of Wnt/β-catenin signaling. On the other hand, AGS cells possess mutation in CTNNB1 close to the phosphorylation site of GSK3β (Supplementary Figure 1; Caca et al, 1999), which causes the activation of the basal Wnt/β-catenin signaling. It is therefore possible that TNF-α stimulation suppresses phosphorylation of wild-type β-catenin expressed from retained intact CTNNB1, which contributes to the promotion of the total Wnt/β-catenin activity of AGS cells. In human gastric cancer, a CTNNB1 mutation is found in about 30% of the Wnt-activated cases (Clements et al, 2002). However, either a somatic mutation or downregulation of E-cadherin (Cheng et al, 2005), secreted frizzled-related proteins (Nojima et al, 2007), or β-TrCP, a component of ubiquitin ligase complex (Kim et al, 2007) is found in gastric cancer, which leads to the basal activation of Wnt/β-catenin signaling without mutation in CTNNB1. These results, taken together, suggest that wild-type β-catenin expressed in gastric cancer cells is stabilized by inflammatory macrophages thus resulting in the promotion of Wntβ-catenin signaling, which contributes to cancer development, progression and the maintenance of cancer stem cells.

Accordingly, the present results suggest that the suppression of macrophage infiltration and its activation by anti-inflammatory drugs or inhibitors for PGE2 pathway is therefore a possible strategy for chemoprevention against gastric cancer.
Materials and Methods

Animal experiments

The construction of K19-Wnt1 and K19-C2mE transgenic mice has been described previously (Oshima et al., 2004; Oshima et al., 2006). Briefly, K19-Wnt1 mice express Wnt1, while K19-C2mE mice express COX-2 and mPGES-1 in gastric epithelial cells under transcriptional regulation by cytokeratin 19 (K19) gene promoter. K19-Wnt1 transgenic mice were euthanized at 30 weeks of age (n=10), and the glandular stomach was fixed with 4% paraformaldehyde and processed for histological analysis. Helicobacter felis (ATCC49179) was cultured as previously described (Oshima et al., 2004). H. felis bacteria were inoculated at 10^8/mouse p.o. to K19-Wnt mice, Apc^Δ716 mice (Oshima et al., 1995), and wild-type mice. The infected K19-Wnt1 mice were examined at 8 weeks and 20 weeks after the H. felis inoculation (n=3 for each mice). The infected Apc^Δ716 mice were examined at 6 weeks after infection (n=4). The localization of H. felis-infected mucosa at 8 weeks was estimated by the detection of bacteria using microscope with X100 objective (Figure 7B). Apc^Δ716 mice were crossed with op/op mice (The Jackson Laboratory) to obtain Apc^Δ716 op/op compound mice (n=3) and littermate control Apc^Δ716 mice (n=3). The intestine of Apc^Δ716 and Apc^Δ716 op/op mice was examined at 12 weeks of age, and the total number of polyps was determined using a dissecting microscope. For scoring the BrdU labeling index, the mice were injected i.p. with 200 μl of BrdU solution (Roche) and then the tissue specimens were processed for immunostaining using anti-BrdU antibody. All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University.

Histology and immunostaining

The stomach tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4 μm-thickness. These sections were stained with H&E. Serial sections were immunostained with antibodies for Ki-67 (DakoCytomation), F4/80 (Serotec), unphosphorylated (activated) β-catenin on Ser37 and Thr41 (Upstate), H^+/K^+-ATPase (MBL, Nagoya, Japan), or BrdU (Roche) as the primary antibody. The staining signals were visualized using the Vectorstain Elite Kit (Vector Laboratories). The number of positive stained cells for F4/80, β-catenin, Ki-67, or H^+/K^+-ATPase was scored in 20 randomly selected gastric glands and the mean values were calculated. The number of
BrdU positive cells and the total number of nuclei was counted in five microscopic fields and then the mean value was calculated as the BrdU labeling index. For immunofluorescence staining, Alexa Fluor 594 donkey anti-mouse IgG or Alexa Fluor 488 anti-rat or rabbit IgG (Molecular Probes) was used as the secondary antibody.

**Plasmid vector construction**

For the construction of the β-catenin/TCF reporter plasmid with GFP (TOPEGFP vector), cDNA encoding EGFP was excised from pIRES2-EGFP (Clontech) and replaced with luciferase gene in TOPFLASH plasmid (Upstate). TOPEGFP reporter plasmid was transfected to cells using Effectene Transfection Reagent (QIAGEN), and stable cell lines were then obtained by cell sorting. The Wnt/β-catenin activity using TOPEGFP reporter was measured by flow cytometry (see below). pcDNA3-S33A-β-catenin and pBSFI-IκB-SR (IκB-Superrepressor) plasmids were kindly provided by Dr. Peter Vogt at the Scripps Research Institute. pcDNA3-S33A-β-catenin was transiently transfected to Kato-III-GFP cells for 72 hours to express stabilized mutant β-catenin. pBSFI-IκB-SR was transfected to AGS-GFP cells to establish the sublines in which the NF-κB pathway is suppressed.

**Immunocytochemistry**

Gastric cancer cells grown on cover slips were fixed with 10% neutral buffered formalin and permeabilized with 0.1% Triton X-100 in PBS. Anti-total β-catenin antibody (Sigma), anti-unphosphorylated β-catenin antibody (Upstate), or anti-phosphorylated GSK3 (Cell Signaling) was used as the primary antibody, and anti-mouse IgG Alexa 594 or anti-rat IgG Alexa 488 (Molecular Probes) were used as the secondary antibody. Next, cover slips were mounted using VECTASHIELD Mounting Medium (Vector laboratories) that contained DAPI for nuclear staining.

**Cell culture and preparation of conditioned medium**

Gastric cancer cell lines, AGS (ATCC) and Kato-III (Cell Resource Center for Biomedical Research, Tohoku Univ., Japan) were cultured in RPMI1640 medium supplemented with 10% FBS in a humidified incubator at 37°C with 5% CO₂. The mouse macrophage cell line RAW264 (RIKEN BioResource Center, Tsukuba, Japan) was cultured in RPMI1640, and treated with LPS purified from *Salmonella enterica* serotype
typhimurium (Sigma) at 100 ng/ml for 24 h. The conditioned medium, CM-LPS (+) was collected and used for the Wnt/β-catenin activation experiments. The control conditioned medium, CM-LPS (−) was prepared from non-stimulated RAW264 cell culture. For either the stimulation or inhibition of the TNF-α pathway, TNF-α (Calbiochem) or anti-mouse TNF-α neutralizing antibody (R&D systems) was added, respectively, in the culture medium at the indicated concentration.

**NF-κB ELISA assay**
The nuclear extract was prepared from AGS-GFP cells or pBSFI-IκB-SR-transrected clones (IκBSR #1 and #2) using NE-PER Nuclear Extraction Reagents (Pierce), and the NF-κB activity was measured using an NF-κB p65 Transcription Factor Assay Kit (Active Motif). These cell lines were also used for the flow cytometry analysis.

**Flow cytometry analysis**
After treatment with CM-LPS (+), CM-LPS (−), LPS, or TNF-α at the indicated concentrations for 48 h, AGS-GFP, Kato-III-GFP cells or pBSFI-IκB-SR transfected cells (IκBSR #1 and #2) were washed with PBS containing 2% FCS and stained with 5 μg/ml propidium iodide to exclude dead cells. GFP fluorescence was examined with FACSCalibur (Becton Dickinson). A fluorescence intensity corresponding to the top 2% of the control untreated cells was judged as the high-GFP population (high-Wnt/β-catenin signaling population).

For the analysis of intracellular unphosphorylated (active) β-catenin, cells were fixed in 1-2% paraformaldehyde and permeabilized using 0.05% Triton X-100 in PBS containing 2% FCS. Next, the cells were incubated with anti-unphosphorylated β-catenin antibody on Ser37 and Thr41 (Upstate) followed by anti-mouse IgG-Alexa 488 (Molecular probes), and examined with FACSCalibur. For the inhibition of protein translation, the cells were treated with cycloheximide (Sigma) at 0.5 μg/ml for the indicated time, and then the intracellular unphosphorylated β-catenin was measured by a FACS analysis as described above.

**Western blotting analysis**
For the detection of total β-catenin and unphosphorylated β-catenin, the cells were stimulated with CM-LPS (+) or TNF-α at 10 ng/ml for 24 h. For the detection of total or
phosphorylated GSK3β (Ser9), and phosphorylated Akt (Ser473), the cells were cultured in serum free conditions for 24 h followed by stimulation with TNF-α at 100 ng/ml for 24 h. Next, the cells were lysed and sonicated in lysis buffer. After centrifugation at 20,000 × g, 10 μg of the supernatant protein was separated in a 10% SDS-polyacrylamide gel. Antibodies for unphosphorylated (active) β-catenin (Upstate), total β-catenin (Sigma), total GSK3β (BD Biosciences), phosphorylated GSK3 on Ser9 and 21 (Cell Signaling), and phosphorylated Akt at Ser473 (Cell Signaling) were used as the primary antibody. β-Actin was used as an internal control. The ECL detection system (Amersham Biosciences) was used to detect specific signals.

*Statistical analysis*

The data were analyzed by the paired or unpaired *t*-test using Microsoft Excel (Microsoft). A value of *p* < 0.05 was accepted as statistically significant.

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**Figure Legends**

**Figure 1.** Macrophage accumulation and nuclear localization of β-catenin in dysplastic lesion of the K19-Wnt1 mouse stomach. (A) Representative histology of a dysplastic lesion in the K19-Wnt1 glandular stomach (H&E staining). The arrowheads indicate the dysplastic mucosal area. The arrows indicate submucosal inflammatory infiltration. High-magnifications of boxed area (D, dysplastic lesion; and N, adjacent normal mucosa) in serial sections are shown in (B-E, G) and (H-J), respectively. Immunostaining results for macrophage marker F4/80 (B, H), β-catenin (C, I), Ki-67 (D, J), and BrdU (G) are shown. The arrowheads in (B, H) indicate mucosal macrophages. The inset in (C) indicates the nuclear localization of β-catenin in epithelial cells, while the inset in (I) indicates weak cytoplasmic accumulation of β-catenin. The arrows in (D, G) indicate proliferating cells that are positive for Ki-67 and BrdU, respectively. (E) Double immunofluorescence staining for F4/80 (red) and β-catenin (green) in dysplastic lesion. Arrowheads indicate macrophages. (F) The mean ratio of β-catenin accumulated epithelial cells and the mean number of infiltrated macrophages per field in the dysplastic lesion and adjacent normal mucosa. The arrows in (I, J) indicate normal progenitor cells localized in gland neck with mild accumulation of β-catenin and positive Ki-67 staining. Bars indicate 100 μm.

**Figure 2** Suppression of intestinal tumorigenesis by depletion of macrophages. (A, C) Representative photographs of small intestine of ApcΔ716 mice (A) and ApcΔ716 op/op compound mutant mice (C) were taken using a dissecting microscope. The arrowheads indicate intestinal polyps. Bars indicate 2 mm. (B, D) F4/80 macrophage immunostaining of polyp tissues in ApcΔ716 (B) and ApcΔ716 op/op compound mice (D). The arrows indicate macrophages. Bars indicate 100 μm. (E) The number of total polyps and large polyps > 1 mm in diameter of ApcΔ716 (cont) and ApcΔ716 op/op compound mice (op/op) are shown as mean ± s.d. (F) BrdU labeling index in polyps of ApcΔ716 (cont) and ApcΔ716 op/op mice (op/op) are shown as mean ± s.d. The asterisks indicate p < 0.05 in comparison to the control.

**Figure 3** Fluctuation of the Wnt/β-catenin activity in gastric cancer cells. (A) Representative results of immunocytochemistry of AGS (top panels) and Kato-III (bottom panels) using anti-total β-catenin antibody (green) and DAPI (blue) (left panels)
or anti-unphosphorylated β-catenin antibody (red) \((\text{right panels})\) of the same specimen. The arrows indicate cells with accumulated both total and unphosphorylated β-catenin. The arrowheads indicate cells with weak or loss of β-catenin staining. (B) Construction of TOPEGFP expression vector. EGFP cDNA was inserted between TCF binding site/TK minimum promoter and SV40 pA signal. (C) Representative results of immunocytochemistry of Kato-III-GFP cells transiently transfected with pcDNA3-S33A-β-catenin for 72 h. GFP expression (green) with DAPI staining (blue) \((\text{left panel})\), and unphosphorylated β-catenin (red) of the same specimen \((\text{right panel})\) are shown. The arrows indicate the cell cluster with elevated GFP and unphosphorylated β-catenin levels. (D) Representative results of flow cytometry for GFP fluorescence of AGS-GFP \((\text{top})\) and Kato-III-GFP \((\text{bottom})\) cells. Flow cytometry results of the isolated high GFP \((\text{top 5\%, red line})\) and low GFP \((\text{bottom 5\%, blue line})\) populations at 0 h \((\text{center})\) and 5 days \((\text{right})\) after isolation by cell sorting.

**Figure 4** Enhancement of Wnt/β-catenin signaling by the activated macrophages in gastric cancer cells. (A, B) Representative results of flow cytometry of AGS-GFP \((\text{A})\) and Kato-III-GFP cells \((\text{B})\). The cells were treated with CM-LPS (+) \((\text{top})\), CM-LPS (−) \((\text{middle})\), or LPS alone \((\text{bottom})\) at various concentrations indicated on top of each panel. The red area in each panel indicates a population with a high GFP intensity corresponding to the top 2% of the control cells \((\text{left})\). The percentage of high GFP population (red area) is indicated in each panel. (C) Representative flow cytometry of AGS-GFP \((\text{top})\) and Kato-III-GFP \((\text{bottom})\) treated with CM-LPS (+) alone \((\text{center})\) or CM-LPS (+) with anti-TNF-α neutralizing antibody \((\text{right})\). The percentage of a high-GFP population (red area corresponding to top 2% in control cells, \(\text{left}\) is indicated in each panel. (D) Representative flow cytometry results of AGS-GFP \((\text{top})\) and Kato-III-GFP \((\text{bottom})\) treated with TNF-α \((\text{red lines})\) and untreated control \((\text{black lines})\). The percentage of high-GFP in TNF-α treated cells \((\text{corresponding to top 2\% in control cells, gray area})\) is indicated in each panel.

**Figure 5** Suppression of β-catenin phosphorylation by CM-LPS\((+\)) or TNF-α in gastric cancer cells. (A) Representative flow cytometry results of Kato-III cells using an antibody against unphosphorylated β-catenin on Ser37 and Thr41 are shown. The cells were treated with CM-LPS (+) \((\text{center})\) or TNF-α \((\text{right})\) for 24 hours. The red area
indicates population with high level of unphosphorylated β-catenin: top 10% in control cells (left). The percentage of red area is indicated in each panel. (B) Western blotting of Kato-III cells for total β-catenin (top) and unphosphorylated β-catenin (middle). The cells were treated with CM-LPS (+) (CM) or TNF-α at 10 ng/ml (TNF). β-Actin was used as internal control (bottom). (C) Western blotting of AGS cells (left) and Kato-III cells (right) for total and phosphorylated GSK3β on Ser9, and phosphorylated Akt on Ser473. β-Actin was used as an internal control. The cells were treated with TNF-α at 100 ng/ml (TNF) in serum free conditions. The results of three independent samples are shown for each cell line. (D) Representative immunocytochemistry for phosphorylated GSK3 (p-GSK, green) and DAPI (blue) (left) and unphosphorylated β-catenin (right) of the same specimen. The arrows indicate cells with strong staining for both p-GSK and β-catenin, while the arrowheads indicate cells with weak staining for both.

Figure 6 NF-κB independent promotion of the Wnt/β-catenin signaling activity. (A) The level of NF-κB activity in the TNF-α-treated (TNF) or untreated control (Con.) cells (mean ± s.d.). IκBSR #1 and #2 are AGS-GFP-derived stable transfectants with IκB superrepressor, whereas IκBSR(-) indicates parental AGS-GFP. (B) Representative flow cytometry results of IκBSR(-), IκBSR #1 and #2 cells (left to right) treated with TNF-α (top) or CM-LPS (+) (bottom) at the indicated concentrations (red lines). The percentage of high-GFP populations (corresponding to top 2% in untreated cells, black lines) is indicated in each panel. (C) Flow cytometry results of Kato-III cells using antibody against unphosphorylated β-catenin. The cells were treated with cycloheximide alone (black line) or cycloheximide and CM-LPS (+) (red line) for 2, 4, 8, and 12 h. The percentages of the population with high level of unphosphorylated β-catenin are indicated in each panel (red, cycloheximide and CM-LPS (+); black, cycloheximide alone).

Figure 7 The activation of Wnt/β-catenin signaling in H. felis-infected gastric mucosa of K19-Wnt1 mice. (A-F) Representative histology of inflamed gastric mucosa (IM) of K19-Wnt1 mice infected with H. felis for 8 weeks. (G-L) Non-inflamed gastric mucosal area (NIM) of the K19-Wnt1 mice. H&E staining (A, G), high magnified H&E staining (B, H), immunostaining for F4/80 (C, I), unphosphorylated β-catenin (D, J), Ki-67 (E, K) and H⁺/K⁺-ATPase (F, L). Bars in (B, H) and other panels indicate 50 μm and 100 μm, respectively. The arrows in (A) and (C) indicate infiltrated mononuclear cells and
macrophages, respectively. The yellow arrowheads in (B) indicate Helicobacter bacteria in the gland lumen, which is not found in NIM (H). The arrows in (D) and (E) indicate β-catenin accumulated and proliferating epithelial cells, respectively. The arrows in (F) indicate \( \text{H}^+/\text{K}^+\)-ATPase-positive parietal cells that are dramatically decreased in IM compared with NIM (L). The arrows in (J) and (K) indicate progenitor cells with cytoplasmic β-catenin accumulation and Ki-67-positive proliferation. The mean numbers of positive cells per gland for unphosphorylated β-catenin (M), Ki-67 (N), and \( \text{H}^+/\text{K}^+\)-ATPase (O) in IM (red bars) and NIM (blue bars) are shown as histograms (mean ± s.d.). The asterisks indicate \( p < 0.05 \).

**Figure 8** Tumor development in *H. felis*-infected *K19-Wnt1* mouse stomach. *K19-Wnt1* (A-D) and wild-type (E-H) mouse stomach infected with *H. felis* for 20 weeks. (A, E) Macroscopic photographs. The arrows in (A) indicate gastric tumors which developed in the infected *K19-Wnt1* mice. H&E staining at low magnification (B, F) and high magnification (D, H). Immunostaining results for macrophage marker F4/80 (C, G). The arrows in (B, F) indicate submucosal infiltration. The arrowheads in (D) indicate dysplastic epithelial cells. Bars in (B, F) and (C, D, G, H) indicate 200 μm and 40 μm, respectively.
Figure 1

K19-Wnt1

Dysplastic lesion

Normal mucosa

F4/80

unphospho. β-catenin

KI-67

F4/80:β-cat

|           | β-c (+) epithel. | macrophages/field |
|-----------|------------------|-------------------|
| dysplasia | 63.8%            | 78 ±13            |
| normal    | 13.1%            | 12 ± 3            |
Figure 2

(A) Apc^{1716} and (B) F4/80

(C) Apc^{1716} op/op and (D) F4/80

(E) Number of polyps

(F) BrdU labeling index

* indicates significant difference.
Figure 5
Figure 6

A

B

AGS-GFP

\( \kappa_{BSR} \) ( )
\( \kappa_{BSR} \) #1
\( \kappa_{BSR} \) #2

TNF-\( \alpha \)
\( \times 100 \text{ ng/ml} \)

C

Kato-III (unphospho. \( \beta \)-catenin)

Cycloheximide / CM-LPS(+) (red)
Cycloheximide (black)

2 hrs
4 hrs
8 hrs
12 hrs
Figure 7

A-H: H&E and immunohistochemistry staining showing 

- A: H&E stain of inflamed mucosa (IM) showing inflammation.
- B: H&E stain of non-inflamed mucosa (NIM) showing normal tissue.
- C: F4/80 stain of IM showing macrophage infiltration.
- D: β-catenin stain of IM showing increased expression.
- E: Ki-67 stain of IM showing increased proliferation.
- F: HK-ATPase stain of IM showing increased enzyme activity.
- G: H&E stain of NIM showing normal tissue.
- H: Ki-67 stain of NIM showing normal proliferation.
- I: F4/80 stain of NIM showing low macrophage infiltration.
- J: β-catenin stain of NIM showing normal expression.

M-N: Bar graphs showing quantification of

- M: β-catenin-positive cells/gland in IM vs. NIM.
- N: Ki-67-positive cells/gland in IM vs. NIM.
- O: HK-ATPase-positive cells/gland in IM vs. NIM.
