The inflammatory microenvironment that promotes gastrointestinal cancer development and invasion.

Review:

The inflammatory microenvironment that promotes gastrointestinal cancer development and invasion

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Abstract

Accumulating evidence has indicated that the inflammatory response is important for tumor promotion. However, the mechanisms underlying the induction of the inflammatory response in cancer tissues and how it promotes tumorigenesis remain poorly understood. We constructed several mouse models that develop inflammation-associated gastric and intestinal tumors and examined the *in vivo* mechanisms of tumorigenesis. Of note, the activation of cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) pathway and Toll-like receptor (TLR)/MyD88 signaling cooperatively induced the generation of an inflammatory microenvironment, which is required for early-stage tumorigenesis. The inflammatory response in the stroma induces TNF-α signaling in tumor cells, and the NOX1/ROS signaling pathway is activated downstream. In addition, the inflammatory pathway induces the expression of TLR2 in tumor epithelial cells. Both the NOX1/ROS and TLR2 pathways in tumor cells contribute to the acquisition and maintenance of stemness, which is an important tumor-promoting mechanism stimulated by inflammation. We also found that inflammation promotes malignant processes, like submucosal invasion, of TGF-β signaling-suppressed tumor cells through the activation of MMP2 protease. We also showed that mutant p53 induces innate immune and inflammatory signaling in the tumor stroma by a gain-of-function mechanism of mutant p53, which may explain the "cancer-induced inflammation" mechanism. These results indicate that the regulation of the inflammatory microenvironment via the inhibition of the COX-2/PGE_2 and TLR/MyD88 pathways in combination may be an effective preventive or therapeutic strategy against gastrointestinal cancer development and malignant progression, especially those carrying p53 gain-of-function mutations.

Introduction

It has been established that inflammatory responses play a tumor-promoting role in gastrointestinal cancer development. Epidemiological studies have shown that the regular use of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, reduces the risk of gastrointestinal cancer (Thun et al., 1991; Thun et al., 2012). NSAIDs suppress the enzymatic activity of cyclooxygenase (COX)-1 and COX-2, rate-limiting enzymes for prostaglandin (PG) biosynthesis. We previously showed using genetic mouse models that COX-2 and its downstream product PGE_2 play an essential role in tumor development in the intestine and stomach (Oshima et al., 1996; Sonoshita et al., 2001; Oshima et al., 2004). In the inflammatory microenvironment generated in tumor stroma, the infiltration of tumor-associated macrophages (TAMs), which express cytokines, chemokines, growth factors and proteases, can be seen (Pollard, 2009). TAMs support not only tumor cell migration and proliferation but also metastasis through chemokine CCL2 signaling (Kitamura et al., 2015). It has also been shown that the transcription factors NF-κB and Stat3 are activated in both inflamed tissues and tumors (He and Karin, 2011), and these transcription factors as well as the COX-2/PGE_2 pathway link inflammation and cancer.

To examine the mechanism of inflammation in gastrointestinal tumorigenesis in greater detail, we have constructed genetically engineered mouse models that develop tumors in the stomach (*Gan* mice) or intestine (*Apc*$^{\Delta716}$ mice) and examined how the inflammatory microenvironment is generated in tumor tissues and how such a microenvironment promotes tumor development and malignant progression. In this chapter, we will summarize what we have learned from these mouse models.

1. COX-2/PGE2 inflammatory pathway in intestinal tumorigenesis

$Apc^{\Delta716}$ mice that carry a truncation mutation in the *Apc* gene develop intestinal tumors caused by canonical Wnt signaling activation in epithelial cells (Oshima et al., 1995). In the intestinal tumors of $Apc^{\Delta716}$ mice, the COX-2 expression is induced in stromal fibroblasts but not in tumor cells, which triggers the generation of an inflammatory microenvironment via the biosynthesis of PGE_2 (Sonoshita et al., 2002). Importantly, the deletion of the COX-2 gene or pharmacological inhibition of COX-2 in $Apc^{\Delta716}$ mice resulted in a significant decrease in polyp numbers, indicating that a COX-2-dependent inflammatory microenvironment is critical for tumor development (Oshima et al., 1996; Oshima et al., 2001). The tumor-promoting mechanism of PGE_2 remains poorly understood, but it has been reported that PGE_2 induces the cancer stem cell expansion by activating NF-κB, via signaling through its receptor EP4 (Wang et al., 2015). Based on these results, COX-2 selective inhibitors were expected to be an effective chemopreventive agent for colon cancer, and indeed, the treatment of familial adenomatous polyposis patients with celecoxib, a COX-2 inhibitor, suppressed the development of intestinal polyposis (Steinbach et al., 2000). However, COX-2 inhibitors have not been widely used for cancer prevention because of potential serious side effects on the circulation system in certain populations. Thus, other target molecules, like the PGE_2 receptor, are now thought to be potential appropriate targets for colon cancer prevention.

2. COX-2/PGE$_2$ inflammatory pathway in gastric tumorigenesis

We first constructed a gastritis mouse model, *K19-C2mE* mice, via the transgenic

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expression of COX-2 and mPGES-1, an inducible PGE converting enzyme, in gastric

mucosa. *K19-C2mE* mice develop inflammation-associated mucosal hyperplasia in the

stomach caused by increased levels of PGE$_2$ (Oshima et al., 2004). Notably, the additional

activation of canonical Wnt signaling in *K19-C2mE* stomach epithelial cells by the

transgenic induction of *Wnt1* caused the development of glandular-type tumors (Oshima et

al., 2006; oshima et al., 2009). These genetic results indicate that the cooperation of

oncogenic Wnt activation and a COX-2-induced inflammatory response induces gastric

tumorigenesis. We named this gastric tumor model "*Gan* (gastric neoplasia) mice". Later,

the Cancer Genome Atlas (TCGA) network reported the results of a genome research

analysis of human gastric cancers (The Cancer Genome Atlas Research Network, 2014).

Using the TCGA database for gastric cancer, we examined the activated signaling

pathways and found that the Wnt signaling, COX-2, and NF-κB inflammatory pathways are

activated simultaneously in intestinal-type gastric cancers (Echizen et al., 2016).

Accordingly, *Gan* mice recapitulate intestinal-type human gastric cancer development, from

the molecular mechanisms to the histological phenotypes.

We next examined the expression profiles of the gastritis and gastric tumors that

developed in *K19-C2mE* mice and *Gan* mice, respectively. On a microarray analysis, we

found that a tumor-suppressor microRNA, miR-7a, was significantly downregulated by

inflammatory responses (Kong et al., 2012). MiR-7a suppresses the expression of EGFR,

thus its downregulation may support tumor cell proliferation via the activation of EGFR

signaling (Kefas et al., 2008). By further RNA sequencing of gastritis and tumor tissues, we

found that the expression profiles of gastric tumors were similar to those of gastritis

(Echizen et al., 2016) (Fig. 1A). Of note, stem cell-related genes, such as *Cd44*, *Sox2*, *Noxo1*, *Sox9*, and *Prom1* were upregulated in both gastritis and gastric tumors, while the expression of differentiation-related genes was downregulated, suggesting that these changes in the expression of tumors were induced by an inflammation-dependent mechanism (Echizen et al., 2016) (Fig. 1B). Accordingly, the COX-2/PGE$_2$-induced inflammatory microenvironment may promote tumorigenesis by maintaining the tumor cells in an undifferentiated state.

3. TNF-α signaling in gastric tumorigenesis

Although TNF-α was isolated as a "tumor necrosis" factor, mouse genetic studies have indicated that TNF-α plays a tumor-promoting role in several solid tumors (Balkwill, 2009). In addition, a polymorphism of the TNF-α gene was linked to the gastric cancer incidence (El-Omar et al., 2002). In the *Gan* mouse gastric tumors, inflammatory cytokine levels were elevated because of constitutive activation of COX-2/PGE$_2$ pathway. To examine the role of TNF-α in gastric tumorigenesis, the TNF-α gene was deleted in *Gan* mice by crossing with *Tnf*-/- mice, which resulted in significant suppression of gastric tumorigenesis (Oshima et al., 2014). Notably, the transplantation of TNF-α-wild-type bone marrow to *Tnf*-/- *Gan* mice caused gastric tumor development, indicating that the TNF-α expression in the infiltrated cells in tumor stroma is important for tumorigenesis.

A microarray analysis using TNF-α wild-type and knockout *Gan* mouse tumors revealed that stem cell-related genes, such as *Cd44*, *Noxo1*, *Prom1*, and *EphB3*, were upregulated in tumor tissues in a TNF-α-dependent mechanism (Fig. 2). These genes

overlap with the upregulated genes in *K19-C2mE* gastritis. We therefore performed functional screening for TNF-α-dependent genes using gastric cancer cell lines and found that the inhibition of *Noxo1* expression significantly suppressed soft-agar colony formation of all examined cell lines.

Noxo1 is a component of the NADPH oxidase 1 (NOX1) complex, which produces reactive oxygen species (ROS). It has been shown that NOX1 complex is important for tumorigenesis through the suppression of protein tyrosine phosphatases, which results in activation of oncogenic pathway (Block and Gorin, 2012). Furthermore, the expression of Rac1, another component of the NOX1 complex, is induced in *Apc^{Min}* mouse intestinal tumor cells, and NOX1-induced ROS production is responsible for the maintenance of the stemness of tumor cells (Myant et al., 2013). We also found that *Noxo1* gene knockdown in gastric cancer cells resulted in the suppression of sphere formation, suggesting the suppression of the stemness (Block and Gorin, 2012). Consistently, a low level of ROS signaling is important for maintaining stem cells' inherent properties (Gorrini et al, 2013). It is therefore possible that NOX1/ROS signaling is an important mechanism that links TNF-α-associated inflammation and tumor cell stemness (Fig. 2).

4. The TLR/MyD88 innate immune pathway in gastric tumorigenesis

When *Gan* mice were raised in germ-free conditions, both gastritis and gastric tumorigenesis were suppressed significantly, although the COX-2/PGE$_2$ pathway was constitutively activated (Oshima et al., 2011). Furthermore, infection of *Helicobacter felis*, a close relative to *H. pylori*, to germ-free *Gan* mouse stomach induced gastric tumor

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development. These results suggest that innate immune signaling for commensal bacteria is required for COX-2/PGE$_2$-induced inflammation in the stomach. To assess this possibility, we disrupted gene encoding MyD88, an effector molecule for Toll-like receptors (TLRs) in *Gan* mice by crossing with *Myd88-/-* mice (Maeda et al., 2016). As expected, the inflammatory responses were completely suppressed by the shutdown of TLR/MyD88 signaling, while COX-2 and mPGES-1 were constitutively expressed in the gastric mucosa. Moreover, gastric tumorigenesis in *Gan* mice was also suppressed when *Myd88* was deleted in bone marrow cells. These results indicate that TLR/MyD88 signaling and the COX-2/PGE$_2$ pathway in the inflammatory cells cooperatively induce the generation of an inflammatory microenvironment in tumor tissues through the induction of cytokines and chemokines (Fig. 3).

Interestingly, the TLR2 expression is highly increased in tumor epithelial cells, and its level was decreased significantly when the TLR/MyD88 pathway was suppressed, indicating that stromal TLR/MyD88 signaling induces the epithelial TLR2 pathway (Fig. 3). Interestingly, it has been shown that signaling through TLR2 and its coreceptor CD14 through MyD88 in the intestinal and mammary "epithelial cells" is essential for the maintenance of normal stem cell properties (Scheeren et al., 2014). Furthermore, the disruption of *Myd88* in *Apc^{Min}* mouse intestinal epithelia caused a significant decrease in the numbers of intestinal polyps. These results are consistent with the original finding that epithelial cell intrinsic signaling of TLR2/4 receptors is required for homeostasis and the regeneration of intestinal mucosa (Rakoff-Nahoum and Medzhitov, 2007). It is therefore possible that the TLR2 pathway in the epithelia induced by inflammatory cytokines from the

stroma plays a role in the acquisition of stem cell properties by tumor cells. Indeed, it has been shown that the suppression of TLR2 signaling in a gp130F/F gastric tumor mouse model resulted in the significant suppression of tumor development (Tye et al., 2012). In the gp130F/F model, constitutive activation of gp130 induced Stat3 activation-dependent inflammation and gastric tumorigenesis, and TLR2 gene deletion suppressed tumorigenesis without suppression of inflammation. Accordingly, innate immune pathways through TLR/MyD88 are important in stromal cells, possibly in macrophages, and tumor epithelial cells, and their interplay is important for tumor promotion (Fig. 3). Although ligands for these TLRs have not been identified, tumor cell-derived molecules may function as danger signal ligands for TLRs.

These results suggest that the simultaneous inhibition of COX-2/PGE$_2$ and TLR/MyD88 pathways in combination will be an effective preventive strategy against gastric cancer development.

5. Inflammatory pathway for malignant progression (TGF-β signaling suppression)

As described above, it has been established that the COX-2/PGE$_2$, NF-κB and Stat3 pathways play a role in the early stage of intestinal tumorigenesis. To explore this concept, most studies were performed using *Apc* mutant mice or azoxymethane (AOM)/dextran sodium sulfate (DSS)-treated mice that develop benign adenomas. Although chronic inflammation is associated also with malignant colon cancer, whether or not an inflammatory microenvironment promotes malignant progression, including submucosal invasion and metastasis, has been unclear.

TGF-β signaling induces the differentiation of undifferentiated gastrointestinal epithelial cells; therefore, the suppression of TGF-β signaling promotes the malignant progression of gastrointestinal cancers. Consistently, genes encoding TGF-β type-II receptor (*TGFBR2*) and Smad4 are frequently mutated in human colon cancer, suggesting that these genes are tumor suppressors (The Cancer Genome Atlas Network, 2012). Moreover, it has also been shown that glycogen synthase-3 (GSK3) induces invasion and EMT through negative regulation of TGF-β signaling (Cervello et al, 2017). We previously showed that the disruption of *Tgfbr2* or *Smad4* in *Apc*$^{\Delta 716}$ mice resulted in the induction of submucosal invasion of intestinal tumors, confirming the tumor-suppressor role of the TGF-β pathway (Kitamura et al., 2007; Oshima et al., 2015). An intensive histological analysis of *Apc*$^{\Delta 716}$ *Tgfbr2*-/- compound mice showed that only large tumors (>1 mm in diameter) had an invasive phenotype, with smaller ones in the same mice never demonstrating invasion. Over this size point (1 mm in diameter), COX-2 expression is induced in the stromal fibroblasts, and the production of inflammatory cytokines and growth factors is induced (Seno et al., 2002). These results suggest that an inflammatory microenvironment significantly contributes to the induction of submucosal invasion in intestinal tumors of TGF-β signaling-suppressed epithelial cells.

To assess this hypothesis, we treated *Tgfbr2* knockout mice with DSS, which induces ulcerative colitis in the colon (Oshima et al., 2015). Disruption of the *Tgfbr2* gene in non-inflamed intestinal mucosa did not cause any morphological changes, indicating that TGF-β signaling is not required for intestinal homeostasis. However, in the colonic mucosa of DSS-treated *Tgfbr2*-/- mice, regenerating epithelial cells from ulcers showed submucosal

invasion. Invaded epithelial cells continued to proliferate for over 10 months, resulting in the development of colonic invasive adenocarcinoma with collagen fiber deposition in the stroma. In the stroma of invasive tumors, macrophages infiltrate and express MT1-MMP, which further activates MMP2. MMP2 is an important protease for the promotion of tumor invasion because it degrades basement membrane components, such as type IV collagen and laminin.

Of note, *Tgfbr2* gene deletion in the regenerating mucosa resulted in expansion of CD44-positive undifferentiated epithelial cells in organoids, forming a long crypt structure, which indicates that TGF-β signaling is required for the differentiation of regenerating intestinal epithelial cells. Accordingly, in the colonic mucosa of ulcerative colitis patients, the suppression of TGF-β signaling may be sufficient to cause invasive colon cancer, as the mucosa is continuously inflamed and epithelial cells are regenerated (Fig. 4A). However, in sporadic colon cancer tissues, Wnt signaling activation is an initial step for tumorigenesis, and an inflammatory microenvironment is constructed via the induction of COX-2 expression in the stroma when tumors grow over 1 mm in diameter. In such adenoma tissues, MMP2 is activated, and the cooperation of inflammation and TGF-β signaling suppression in the Wnt-activated adenoma cells causes submucosal invasion (Fig. 4B). Accordingly, the regulation of the inflammatory microenvironment may prevent the invasion of intestinal tumors. It has been reported that bioactive sphingolipids play critical roles in cancer and inflammatory conditions in including ulcerative colitis (Pyne et al., 2016; Espaillat et al., 2017). Accordingly, it is possible that sphigolipids also promote CRC invasion through induction of inflammatory responses.

6. Inflammatory pathway for malignant progression (mutant p53 expression)

TP53 is another important tumor suppressor in most cancers, and the p53 mutation is found

in more than 60% of human colon cancers (The Cancer Genome Atlas Network, 2012).

TP53 has shown to regulate expression of oncogenic as well as tumor suppressor

microRNAs, thus targeted therapies to stabilize TP53 have been developed as possible

anti-cancer drugs (McCubrey et al., 2017). Furthermore, a majority of p53 mutations are

missense type, which results in amino acid substitutions at hot spots, and mouse genetic

study has indicated that mutant p53 protein promotes tumorigenesis via a "gain-of-function"

(GOF) mechanism (Olive et al., 2004). It was recently reported that mutant p53 protein

induces the modification of the chromatin structure via a GOF mechanism, which further

results in the activation of transcription factors by increasing the promoter accessibility

(Pfister et al., 2015; Zhu et al., 2015). To examine the GOF of mutant p53 in malignant

progression of intestinal tumors, we crossed $Apc^{\Delta 716}$ and $Trp53^{R270H}$ mice to generate

compound mice. As expected, the expression of mutant p53 R270H induced the

submucosal invasion of tumor cells, which was rarely found in p53-deleted $Apc^{\Delta 716}$ mice

(Nakayama et al., 2017). We also confirmed that promoter accessibility was increased by

the mutant p53 R270H expression in tumor cells, which was associated with the

upregulation of about 350 genes. A pathway analysis using the expression data revealed

inflammatory signaling and innate immune pathways, like NF-κB, TLRs, and cytokines.

Consistently, the levels of cytokines and chemokines secreted in the culture medium were

increased significantly in intestinal tumor cells that carried p53 mutations. Accordingly, the

generation of an inflammatory microenvironment in malignant cancer may be caused by

mutant p53 GOF at least in part, as a "cancer-elicited inflammation" mechanism.

Conclusion

Both the COX-2/PGE$_2$ pathway and TLR/MyD88 signaling play key roles in cancer

development via the generation of an inflammatory microenvironment. It is therefore

possible that targeting the COX-2/PGE$_2$ pathway together with TLR/MyD88 signaling in

combination will be an effective prevention strategy against gastrointestinal cancers. In the

inflammatory environment, TNF-α signaling induces NOX1/ROS signaling, and Stat3

activation causes TLR2 upregulation in tumor cells. These signaling pathways contribute to

the acquisition of stemness, which may be important mechanisms for inflammation-induced

tumor promotion. An inflammatory microenvironment also promotes malignant progression,

such as submucosal invasion, via the activation of MMP2 protease. We also found that

mutant p53 induces an innate immune response and inflammation by a GOF mechanism,

suggesting that targeting inflammation will be effective against cancers that carry p53

mutations.

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Figure Legends

Figure 1. Expression analyses of *K19-C2mE* mouse gastritis and *Gan* mouse gastric

tumors.   (A) Principal component analysis of *Gan* mouse tumors (*red*), *K19-C2mE* gastritis

(*orange*), and wild-type normal stomach (*green*). Not that expression profiles of gastritis and

gastric tumors are similar. (B) Average-linkage clustering analysis of genotype-specific

genes using RNA sequencing results of the wild-type normal stomach (*WT*), *K19-C2mE*

mouse gastritis (*C2mE*), and *Gan* mouse gastric tumors (*Gan*).   Stem cell-related genes

were upregulated (red), while differentiation markers were downregulated (green) in both

gastritis and gastric tumors (*right*). (Reproduced from Echizen et al, Cancer Sci, 2016)

Figure 2. A schematic summary of the TNF-α-associated promotion of gastric

tumorigenesis.   Macrophages are recruited to tumor stroma and express TNF-α, which

further activates stromal cells including macrophages and fibroblasts as well as tumor cells.

In tumor epithelial cells, Noxo1 is induced by TNF-α signaling, and NOX1 complex is

activated, which further leads to increased ROS production. NOX1/ROS pathway may

promote tumorigenesis through acquisition of stem cell properties. (Reproduced from

Oshima et al, Oncogene, 2014)

Figure 3. Schematic drawing of interaction of inflammatory cells and cancer cells. In

macrophages, both TLR/MyD88 and COX-2/PGE$_2$ pathways are required for induction of

inflammatory cytokines and generation of inflammatory microenvironment. In tumor cells,

TNF-α signaling induces activation of NOX1/ROS signaling, and gp130 activation induces

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TLR2 pathway. Both of ROS signaling and TLR2 pathway contribute to acquisition of stem cell property, which promotes tumorigenesis. (Reproduced from Echizen et al, Cancer Sci, 2016)

Figure 4. A schematic drawing of the development of invasive colon cancers in regenerating and inflamed mucosa of ulcerative colitis patients (*A*) and in Wnt signaling-activated adenomas (*B*). Inflammatory microenvironment-induced MMP2 activation and TGF-β signaling suppression cooperatively induces invasive cancer in the colon. (Reproduced from Oshima et al, Cancer Research, 2015)
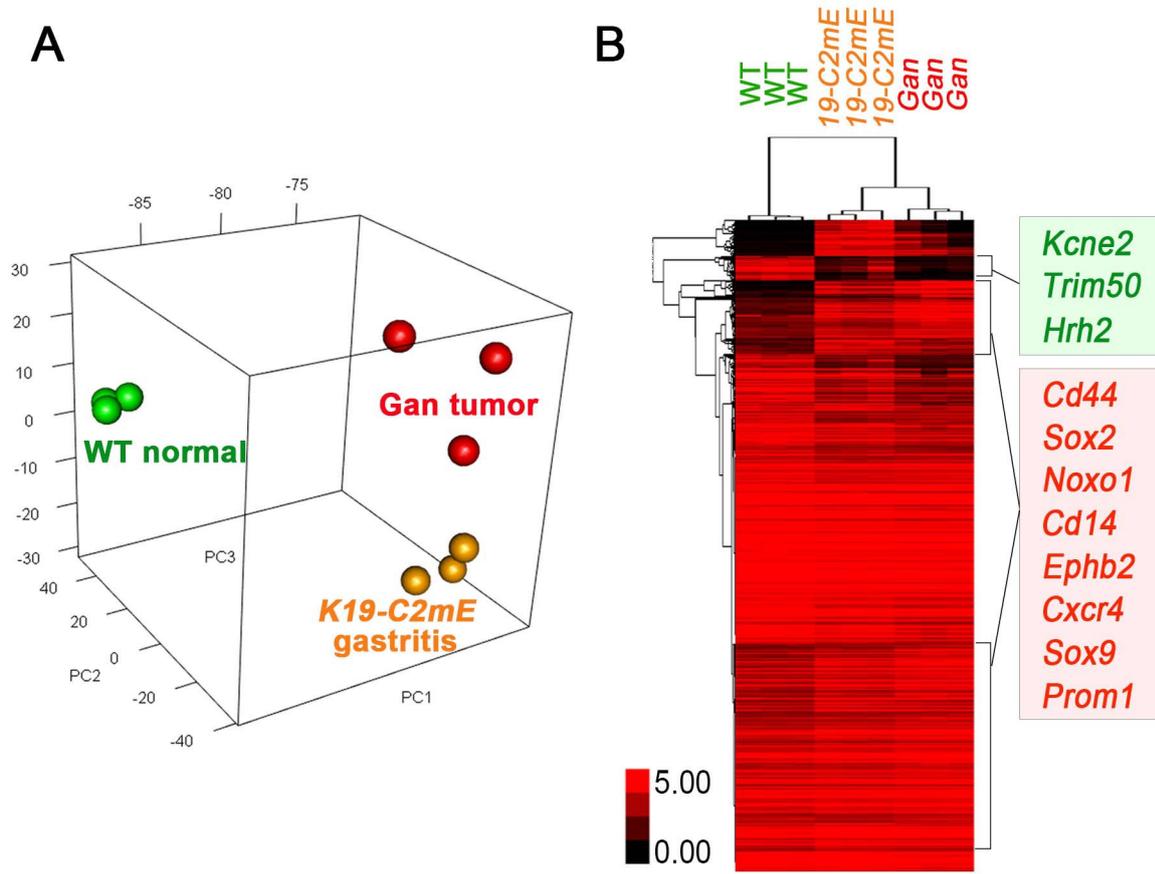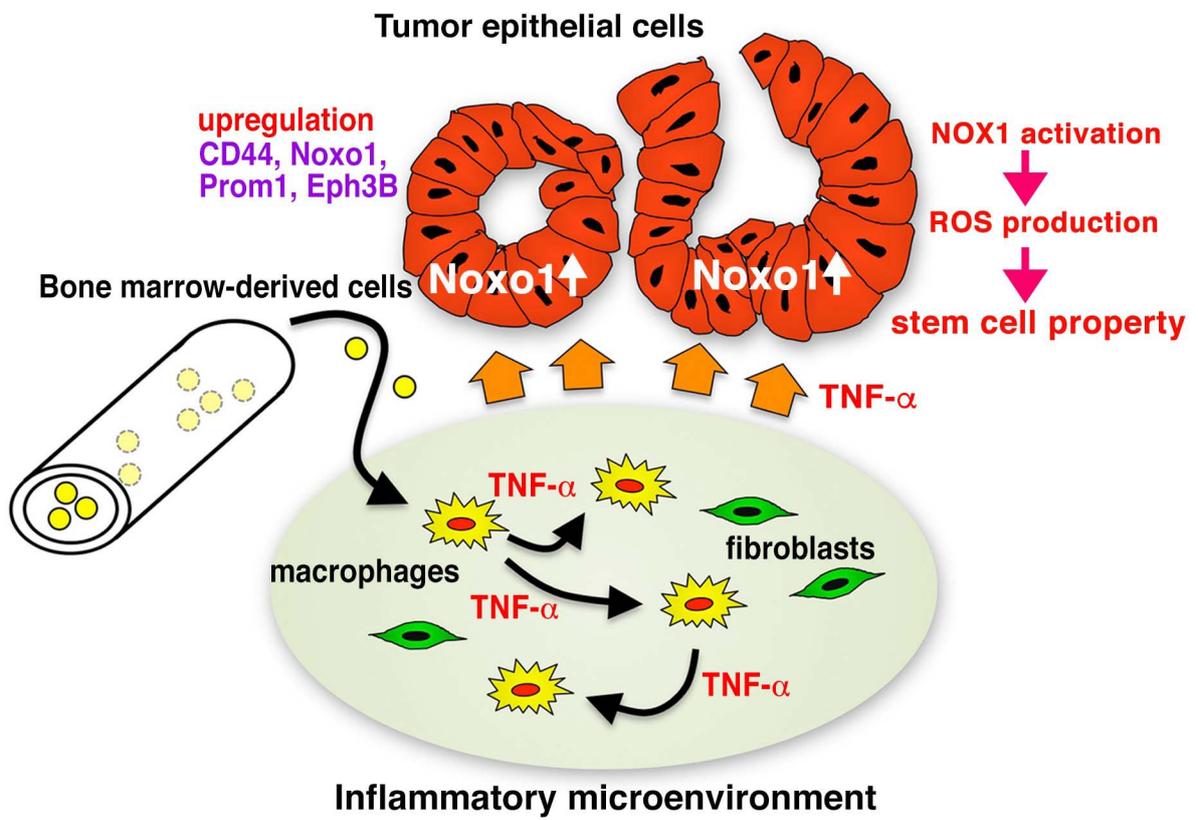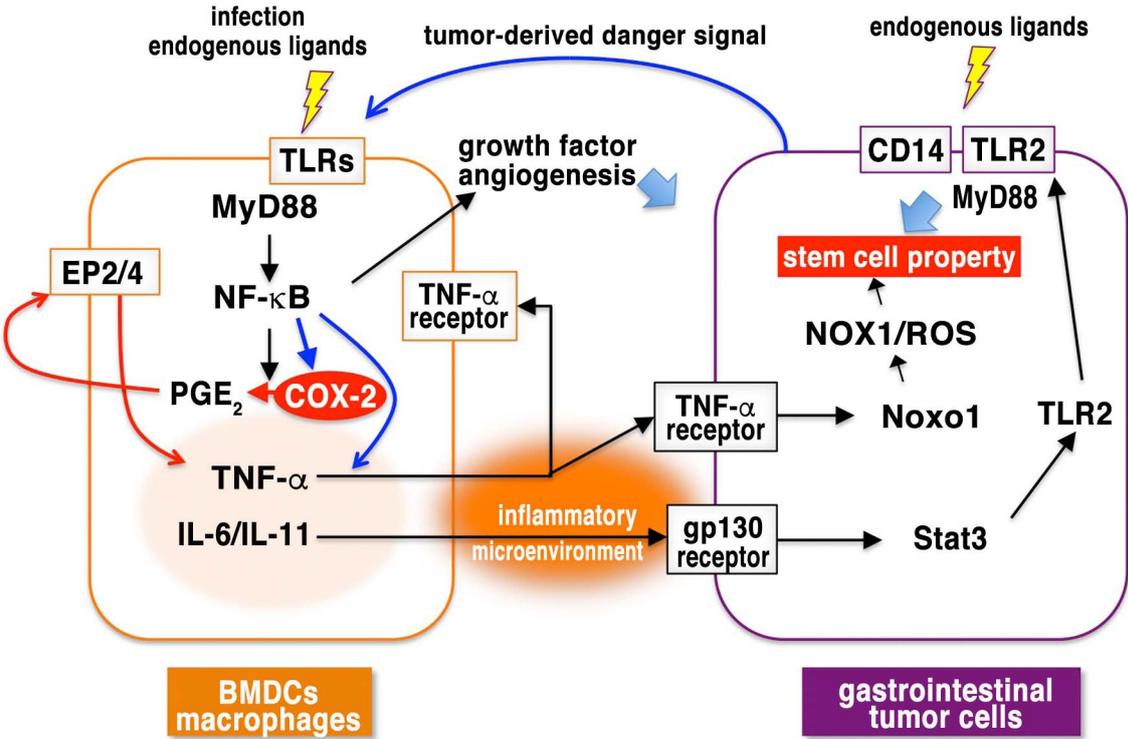
Figure 1

Figure 2

Figure 3

Figure 4

A

chronic inflammation   mucosa regeneration

MT1-MMP expression   ← TGFβ suppression

MMP2 activation   invasiveness

invasive adenocarcinoma

B

← Wnt activation

stem cell property

inflammatory microenvironment

MT1-MMP expression

MMP2 activation   adenoma

← TGFβ suppression

invasiveness

invasive adenocarcinoma