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ORIGINAL ARTICLE

# Stromal cell-derived factor-1 overexpression induces gastric dysplasia through expansion of stromal myofibroblasts and epithelial progenitors

Wataru Shibata,[1] Hiroshi Ariyama,[1] Christoph Benedikt Westphalen,[1] Daniel L Worthley,[1] Sureshkumar Muthupalani,[2] Samuel Asfaha,[1] Zinaida Dubeykovskaya,[1] Michael Quante,[3] James G Fox,[2] Timothy C Wang[1]

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[1]Department of Medicine, Division of Digestive and Liver Disease, College of Physicians and Surgeons, Columbia University, New York, New York, USA
[2]Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
[3]II. Medizinische Klinik, Klinikum rechts der Isar, Technische Universität München, Munich, Germany

**Correspondence to**
Dr Professor Timothy C Wang, Chief, Division of Digestive and Liver Diseases, Silberberg Professor of Medicine, Department of Medicine and Irving Cancer Research Center, Columbia University Medical Center, 1130 Street Nicholas Avenue, Room #925, New York, NY 10032-3802, USA; tcw21@columbia.edu

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## ABSTRACT

**Objective** Stromal cell-derived factor-1 (SDF-1/CXCL12), the main ligand for CXCR4, is overexpressed in human cancer. This study addressed the precise contribution of SDF-1 to gastric carcinogenesis.

**Design** SDF-1 transgenic mice were created and a *Helicobacter*-induced gastric cancer model was used in combination with H/K-ATPase-IL-1β mice. Gastric tissue was analysed by histopathology and cells isolated from the stomach were analysed by molecular biological methods.

**Results** Analysis of the H/K-ATPase/SDF-1 transgenic (SDF-Tg) mice showed that SDF-1 overexpression results in significant gastric epithelial hyperproliferation, mucous neck cell hyperplasia and spontaneous gastric dysplasia (wild-type mice 0/15 (0%) vs SDF-Tg mice 4/14 (28.6%), p=0.042, Fisher exact test) but has minimal effects on inflammation. SDF-Tg mice also showed a dramatic expansion of α-smooth muscle actin-positive myofibroblasts and CXCR4-expressing gastric epithelial cells in the progenitor zone, both of which preceded the development of significant gastritis or dysplasia. Gremlin 1-expressing mesenchymal stem cells, the putative precursors of myofibroblasts, were also increased within the dysplastic stomachs of SDF-Tg mice and showed chemotaxis in response to SDF-1 stimulation. SDF-1 overexpression alone resulted in minimal recruitment of haematopoietic cells to the gastric mucosa, although macrophages were increased late in the disease. When SDF-Tg mice were crossed with H/K-ATPase-IL-1β mice or infected with *Helicobacter felis*, however, there were dramatic synergistic effects on recruitment of bone marrow-derived cells and progression to preneoplasia.

**Conclusion** Activation of the SDF-1/CXCR4 axis can contribute to early stages of carcinogenesis primarily through recruitment of stromal cells and modulation of the progenitor niche.

## Significance of this study

### What is already known on this subject?

- Stromal cell-derived factor-1 (SDF-1) and its cognate receptor CXCR4 mediate many biological functions such as embryonic development, organ homeostasis, angiogenesis and immune system modulation.
- Studies in murine cancer models have shown that SDF-1 produced by myofibroblasts recruits endothelial cells and contributes to tumorigenesis.
- SDF-1 mRNA and serum SDF-1 are upregulated in both mice and human gastric cancer and blocking SDF/CXCR4 reduces tumour growth and the development of ascites.

### What are the new findings?

- Overexpression of SDF-1 in the stomach caused a significant increase in gastric epithelial cell proliferation, induced inflammation and promoted gastric tumour development, particularly in combination with inflammatory stimuli such as *Helicobacter* infection or interleukin 1β (IL-1β).
- SDF-1 recruited F4/80-positive macrophages and CD11b-positive myeloid cells and induced gastric epithelial proliferation partly through the activation of SDF/CXCR4 signalling and its downstream Erk/PI3kinase.
- SDF-1 overexpression recruited CXCR4-positive mesenchymal stem cells and the expansion of myofibroblasts in the gastric stem cell niche. This was associated with an increase in K19-positive epithelial cells.
- SDF-1 promoted gastric epithelial proliferation partly through CXCR4-positive gastric tissue stem/progenitor cells.

## INTRODUCTION

Gastric cancer is the second leading cause of cancer worldwide, and it is now well established that *Helicobacter pylori*-associated chronic inflammation plays a pivotal role in triggering the sequence from chronic gastritis to cancer.[1] The active stromal remodelling that accompanies chronic inflammation is critical to the initiation and progression of cancer.[2] Thus, there has been increasing interest to identify the key chemokines and cytokines that mobilise inflammatory and mesenchymal cells, leading to cancer.[3]

The chemokine stromal cell-derived factor-1 (SDF-1), also known as CXCL12, is a constitutively expressed and inducible chemokine that plays a fundamental role in embryonic development, organ homeostasis, angiogenesis and immune system modulation. SDF-1 binds to and initiates

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**Significance of this study**

**How might it impact on clinical practice in the foreseeable future?**

- SDF-1 drives cancer through cytokine-related recruitment of macrophages and chemotaxis of bone marrow-derived mesenchymal stem cells/myofibroblasts.
- In addition, the SDF-1/CXCR4 pathway plays a key role in the development of gastric carcinogenesis through direct modulation of CXCR4-positive stem/progenitor epithelial cells. SDF/CXCR4 signalling is an attractive target for the prevention and treatment of gastric cancer.

signalling through two G-protein-coupled receptors, CXCR4 and CXCR7. In the bone marrow (BM), SDF-1 plays a critical role in the initial localisation, retention and support of CXCR4 haematopoietic stem cells (HSCs).[4] SDF-1 is highly expressed in BM stromal cells and contributes to the BM niche. Gene knockout of either SDF-1 or CXCR4 results in impaired haematopoiesis and embryonic lethality,[5–7] and inactivation of the SDF/CXCR4 signalling pathway promotes the mobilisation of HSCs into peripheral blood[8] or mobilisation of neutrophils.[9] Taken together, there is strong evidence that SDF-1/CXCR4 interactions modulate the engraftment, retention and release of haematopoietic cells from the BM.

It is not known whether SDF-1 also promotes the mobilisation and recruitment of haematopoietic cells into peripheral tissues. Indeed, SDF-1 is often upregulated in damaged tissues as a result of hypoxia or cellular apoptosis and increased SDF-1 plasma levels correlate with mobilisation of proangiogenic BM cells.[10] The cognate receptor for SDF-1, CXCR4, is expressed on most BM-derived haematopoietic cells, including HSCs, endothelial precursor cells, immature myeloid cells, macrophages and lymphocytes[11] and, through activation of phosphoinositide 3-kinase, can regulate chemotaxis. While release of local SDF-1, such as following ischaemia, can mobilise haematopoietic cells,[12] the recruitment of inflammatory cells is usually limited in the absence of injury, suggesting that SDF-1 is not sufficient for BM-derived cell recruitment.[13 14]

SDF-1 is one of the major chemokines consistently overexpressed in most solid tumours,[15 16] where it contributes to carcinogenesis as an autocrine growth factor as well as promoting angiogenesis and the recruitment of BM cells to the tumour microenvironment.[17–19] While the CXCR4 receptor is primarily localised to the cancer cells,[11 20] the major source of the SDF-1 ligand in solid tumours appears to be stromal cells, particularly cancer-associated fibroblasts (CAFs).[17] CAFs exhibit properties of myofibroblasts, including expression of α-smooth muscle actin (αSMA), and promote the growth of tumours through their ability to secrete SDF-1.[17] Recent studies indicate that BM-derived mesenchymal stem cells (MSCs) represent one potential source for CAFs, and MSCs recruited into the cancer microenvironment are able to differentiate into CAF-like myofibroblasts.[21–23] SDF-1 secreted by CAFs is important for both the migration and survival of MSCs in vitro.[19] The overexpression of interleukin 1β (IL-1β) as well as chronic gastric *Helicobacter* infection both increase SDF-1 expression in the gastric mucosa. Furthermore, the recruitment of MSCs and CAFs, mediated by both SDF-1 and transforming growth factor (TGF) β, resulted in hyperproliferation of gastric epithelial cells.[19] Thus, SDF-1 released by CAFs potentially recruits both MSCs and CAFs, constituting the functional mesenchymal niche from the BM to sites of chronic injury. This recruited mesenchymal niche is likely to promote local stromal remodelling as well as cancer development.[10 19 24] Nevertheless, the full contribution of SDF-1 in gastric carcinogenesis is still unknown. In order to better define the precise role of SDF-1 in gastric carcinogenesis, we generated mice overexpressing SDF-1 in gastric parietal cells and examined its relevance to epithelial and stromal events in cancer.

## MATERIALS AND METHODS

All animal studies were approved by the Institutional Animal Care and Use Committee at Columbia University. SDF transgenic mice were generated as described in the online supplement. CXCR4-EGFP mice were provided by Richard J Miller (Northwestern University Medical School, Chicago, Illinois, USA). Details of the mice used in this study, all protocols for bacterial culture, chronic *H felis* infection model, histological evaluation, immunohistochemical studies, ELISA, real-time qRT-PCR assay of *H felis* infection in mouse stomachs, proinflammatory CC chemokines and cytokines are given in the online supplement.

## RESULTS

### Gastric overexpression of SDF-1 results in development of spontaneous gastric cancer

We generated H/K-ATPase/hSDF-1α transgenic mice (SDF-Tg) that expressed murine SDF-1α specifically in gastric parietal cells (supplementary figure 1A). Two independent lines (lines 3 and 6) of SDF-Tg mice were identified by ELISA of gastric mucosa for SDF-1 or v5 tag (supplementary figure 1B–E). We confirmed that the murine Met-SDF-1V5x6His protein derived from the construct was biologically active and could induce lymphocyte chemotaxis in a dose-dependent manner, and the tagged SDF-1 protein bound surface proteoglycans with only slightly less efficiency than commercial recombinant SDF-1 (PeproTech Inc., Rocky Hill, NJ, USA.) lacking initial Met (data not shown). We backcrossed SDF-Tg mice to C57B6/J mice at least six times prior to further studies.

SDF-Tg mice exhibited markedly elevated expression of SDF-1 mRNA in the gastric mucosa (supplementary figure 1E), which was greater than the expression level observed in the BM. Interestingly, SDF-Tg mice aged between 3 and 12 months showed minimal inflammation in the stomach but nevertheless exhibited gastric hyperplasia and metaplasia. Young SDF-Tg mice (2–6 months) showed only a slight increase in the number of inflammatory cells determined by immunohistochemistry and FACS (supplementary figure 2A,B). By 18 months of age, however, the SDF-Tg mice exhibited dysplasia with marked cystic dilation of glands in corpus and antral tumours (figure 1). Furthermore, we observed a significant increase in chronic inflammatory cells at these later time points. While not evident in younger mice (not shown), there was a significant increase in the number of F4/80-positive cells and myeloperoxidase activity in older SDF-Tg mice (>12 months) compared with wild-type (WT) mice (supplementary figure 2C).

These results suggest that SDF-1 is able to induce gastric neoplasia despite minimal early effects on the recruitment of chronic inflammatory cells. In particular, the findings implied that, while SDF-1 is clearly able to recruit and retain CXCR4-positive haematopoietic cells within the BM,[25] overexpression of SDF-1 in the stomach alone is not sufficient to induce the recruitment of haematopoietic cells to the stomach. We further tested this concept by transplantation of SDF-Tg and WT mice

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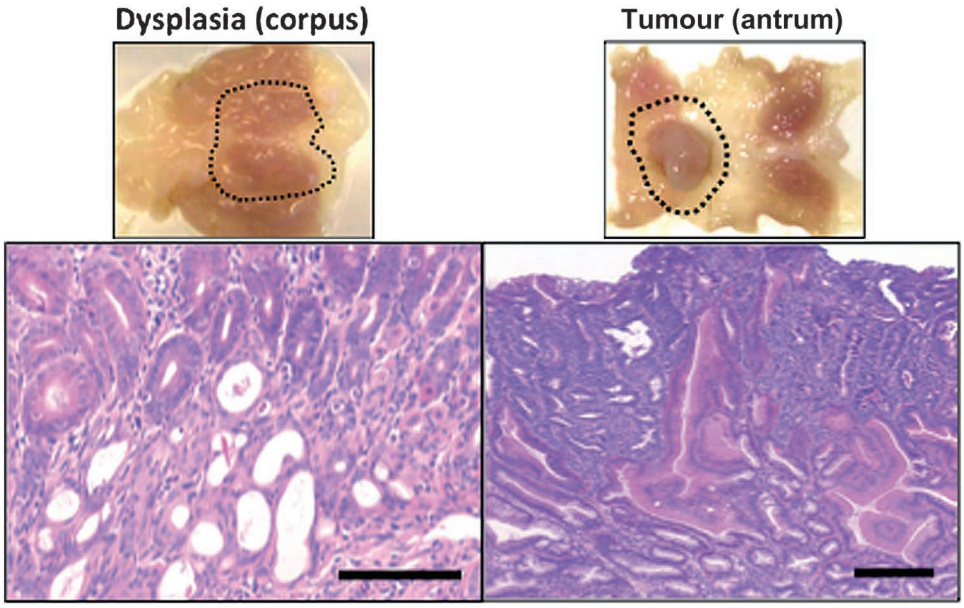


**Figure 1** SDF-Tg mice developed spontaneous gastric dysplasia. Representative macroscopic and histological micrographs from SDF-Tg mice at 18 months (original magnifications ×100).

with GFP-labelled BM and followed the mice for up to 1 year. SDF-Tg transgenic mice showed a slight increase in GFP-labelled haematopoietic cells compared with WT mice at 1 year (data not shown), indicating that overexpression of SDF-1 alone has a weak and/or indirect effect on the recruitment of haematopoietic cells to the stomach. Nevertheless, the findings suggest that SDF-1 is able to drive epithelial proliferation independent of effects on haematopoietic cells. There were no histological alterations in other organs examined including intestine, liver, lung, and kidney in SDF-Tg mice (data not shown).

### SDF-1 overexpression accelerates inflammation-induced gastric tumorigenesis

Given the absence of robust inflammation and the overall low incidence of gastric neoplastic lesions in SDF-Tg mice, we postulated that the induction of chronic inflammation in these animals might further accelerate the development of neoplasia. Thus, we employed *H felis* infection in SDF-Tg mice in order to analyse the impact of SDF-1 overexpression in an established model of inflammation-associated gastric carcinogenesis.[26] The mice were all of the same mixed genetic background (C57BL/6× CBA), and examined at time points up to 18 months after *H felis* infection (MPI). Every histopathological parameter tended to be more severe in *H felis*-infected SDF-Tg mice compared with *H felis*-infected WT mice up to 12 MPI. At time points between 15 and 18 MPI there were even more prominent histopathological changes, with significantly greater degrees of pseudopyloric metaplasia, oxyntic atrophy and foveolar hyperplasia in *H felis*-infected SDF-Tg (figure 2A,B). Interestingly, only the *H felis*-infected SDF-Tg mice exhibited gastric dysplasia and mucous metaplasia at 15–18 MPI, while no *H felis*-infected WT mice developed severe dysplasia. Additionally, SDF-Tg mice infected with *H felis* showed increased gastric expression of IL-1β, heparin-binding epidermal growth factor and amphiregulin (figure 2C) and increased serum levels of IL-6 compared with WT mice infected with *H felis* (figure 2D).

The findings regarding IL-1β were particularly interesting, given previous studies in humans that had pointed to an important role for the IL-1β gene locus in gastric cancer susceptibility.[27] Since gastric specific overexpression of IL-1β in transgenic mice was shown to be sufficient for the recruitment of myeloid cells to the stomach and the induction of gastric cancer,[18] we crossed H/K-ATPase-IL-1β mice with SDF-Tg mice in order to determine the impact on carcinogenesis. At very early time points (eg, 1–1.5 months) we found that SDF-1/IL-1β double transgenic mice showed significantly increased inflammatory cell infiltration, severe gastric atrophy and intestinal metaplasia in stomach compared with IL-1β single transgenic mice (supplementary figure 3A,B). The levels of IL-6 in gastric tissue were also significantly higher in SDF-1/IL-1β double transgenic mice compared with IL-1β single transgenic mice (supplementary figure 3C). In combination with either *H felis* infection or IL-1β overexpression, the number of F4/80 and CD11b-positive cells was significantly increased in SDF-Tg mice compared with their respective controls (figure 3A,B). These data suggest that, although SDF-1 alone is not sufficient to strongly promote chronic gastritis, it synergises with *Helicobacter* infection or IL-1β overexpression in the recruitment of myeloid cells and the induction of atrophic gastritis and gastric preneoplasia.

### SDF-1 extends survival and promotes the function of myeloid cells in vitro

Given that SDF-1 synergised with IL-1β in the induction of gastritis, we wondered whether SDF-1 overexpression could increase the abundance of macrophages and the development of dysplasia through an effect on macrophage survival, as previously suggested.[7] We isolated primary peritoneal macrophages using 4% thioglycolate and then stimulated the macrophages with either recombinant SDF-1 (rSDF-1) or gastric extract from SDF-Tg mice or WT mice. After 48 h of serum-free incubation, primary macrophages showed a significant increase in survival after administration with either rSDF-1 or gastric extract from SDF-Tg mice (supplementary figure 4A). We also analysed the effect of SDF-1 on cytokine production in primary macrophages. There was no IL-6 production after stimulation with rSDF-1 alone; however, rSDF-1 administration showed a significant synergistic effect on the production of IL-6 from primary macrophages co-stimulated with lipopolysaccharide (supplementary figure 4B). We found that the increase in cytokine production was also associated with NF-κB signalling, confirmed by experiments using NF-κB inhibitor, MG132 (data not shown). Taken together, these results suggest that, apart from

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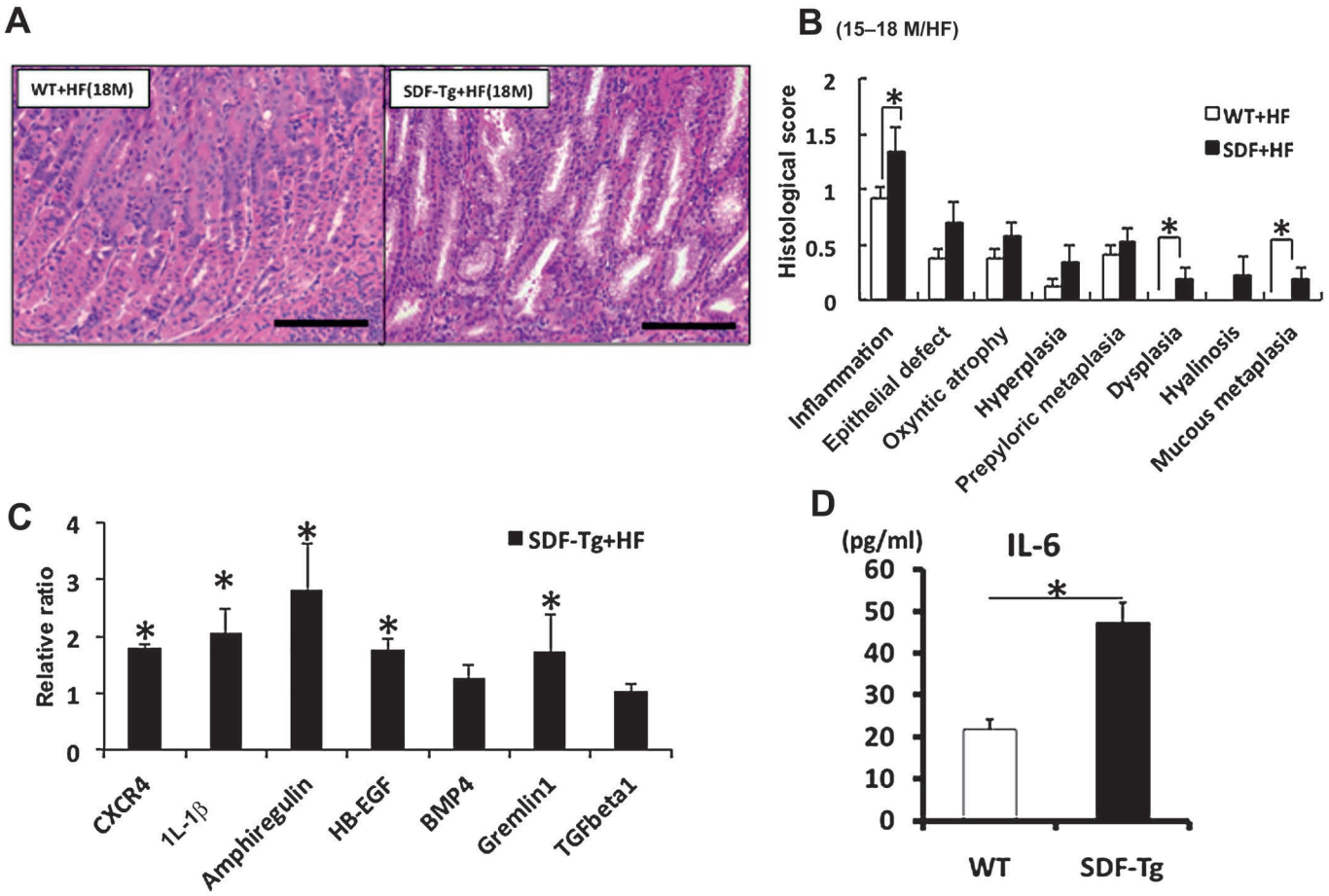


**Figure 2** Overexpression of stromal cell-derived factor-1 (SDF-1) accelerates the development of gastric inflammation and dysplasia in the setting of *Helicobacter felis* infection. (A) Representative stomach sections. (B) Pathological scores from SDF-Tg mice and WT mice infected with *H felis* for 15–18 months (n=5 per group). (C) qRT-PCR of gastric mucosa for the indicated genes. Gene expression was normalised to $10^4$ copies of GAPDH levels and the expression of each gene relative to WT mice is shown (n=5 per group). (D) Serum interleukin 6 (IL-6) levels from WT and SDF-Tg mice after 15 MPI were measured by ELISA (n=5 per group). Data shown are mean±SE. *p<0.05.

its role as a weak chemoattractant for myeloid cells, SDF-1 directly contributes to the survival and function of myeloid cells.

### SDF-1 induces epithelial proliferation and hyperplasia in part through CXCR4

SDF-1 overexpression increases gastric epithelial proliferation. Immunostaining for Ki67 was increased in both the corpus and antrum of SDF-Tg mice compared with WT mice, irrespective of *H felis* infection (figure 4A and supplementary figure 5A). Compared with WT mice, the increase in gastric proliferation in SDF-Tg mice led over time to gastric hyperplasia, particularly in the gastric pit and mucous neck regions. The number of K19-positive cells, previously suggested to be gastric pit and progenitor cells,[28] was significantly increased in SDF-Tg mice compared with WT mice (supplementary figure 5B). The number of parietal cells, however, was not significantly different in SDF-Tg mice and WT mice, suggesting that overexpression of SDF-1 somehow promoted the differentiation of the gastric epithelial stem cells towards the K19-expressing pit cell lineage, but not the glandular lineage.[29]

In order to confirm that the CXCR4 receptor mediated the proliferative response to SDF-1, we administered the CXCR4 antagonist AMD3100 to a cohort of SDF-Tg mice and WT controls. AMD3100 was administered to mice for 2 weeks. The mice were then killed and gastric epithelial proliferation assessed by Ki-67 immunostaining. AMD3100 significantly reduced the gastric epithelial proliferation index of the SDF-Tg mice to a level approaching that of untreated WT mice (figure 4B), consistent with a CXCR4-dependent pathway. Moreover, phosphorylated-Akt (p-Akt) and -Erk (p-Erk) could be detected in the bottom third of the antral glands where CXCR4-expressing cells reside, and p-Akt and p-Erk positive cells were significantly increased both in the antrum and the corpus of SDF-Tg mice (figure 4C and supplementary figure 6). The increase in p-Akt or p-Erk was abrogated by the administration of AMD3100 (figure 4C and supplementary figure 6). Activation of CXCR4 by SDF-1α has previously been shown to result in the activation of ERK and PI3K/Akt pathways, and downstream effectors of these pathways are reported to include IL-6.[30] Taken together, these data suggest that SDF-1α stimulates gastric epithelial proliferation through Erk and PI3K/Akt-dependent mechanisms.

We have recently shown that the trefoil factor 2 (TFF2) functions as a partial antagonist to the CXCR4 receptor, with inhibitory effects on chemotaxis in response to SDF-1 through competitive inhibition of CXCR4.[31] We therefore crossed SDF-Tg mice with TFF2 null mice to test whether endogenous TFF2 might dampen the SDF-1-dependent proliferation and inflammation. SDF/TFF2-/- mice at 12 months of age showed increased proliferation and a dramatic increase in histopathological scores for gastric inflammation, oxyntic atrophy,



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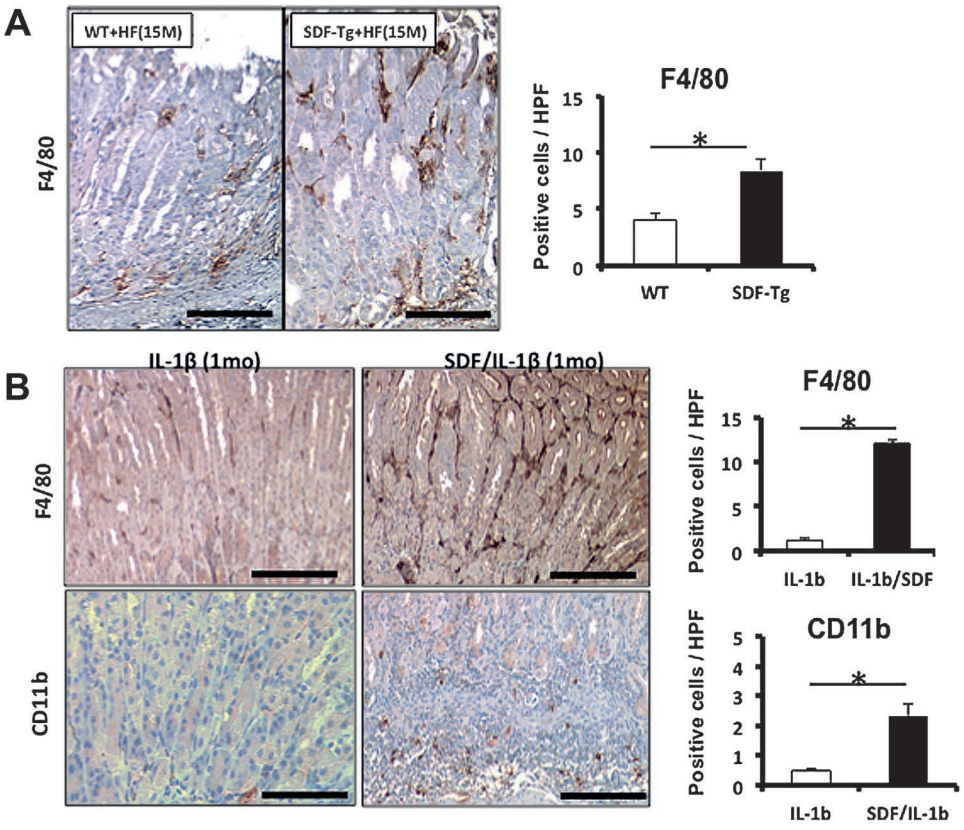


**Figure 3** SDF-Tg mice showed increase in F4/80- and CD11b-positive macrophages in stomach with *Helicobacter felis* infection or interleukin 1β (IL-1β) overexpression. (A) Number of F4/80-positive cells per high power fields in WT and SDF-Tg mice determined by counting positive cells per high power fields (n=5 per group, 15-month-old mice). (B) F4/80- or CD11b-positive cells were increased in SDF/IL-1β-Tg mice; representative micrographs from stained paraffin sections from IL-1β and SDF/IL-1β mice (n=5 per group). Original magnification ×100. Data shown are mean±SE. *p<0.05 in each comparison indicated.

hyperplasia and metaplasia compared with SDF-Tg alone (supplementary figure 7A,B). In addition, we noted that SDF-1/TFF2-/- mice showed markedly increased epithelial proliferation compared with age-matched SDF-1 transgenic mice as assessed by Ki67 immunohistochemistry (supplementary figure 7C,D). These results suggest that the development of gastric preneoplasia in SDF-Tg mice involves activation of the SDF/CXCR4 signalling pathway.

To determine whether overexpression of SDF-1 directly induced gastric epithelial proliferation, we identified potential SDF-1 target cells expressing the CXCR4 receptor in the stomach mucosa using CXCR4-EGFP BAC transgenic mice (supplementary figure 8A).[32] In the corpus these CXCR4-EGFP (+) cells were limited to the gastric isthmus, the known progenitor zone of the oxyntic mucosa. Furthermore, CXCR4-EGFP (+) cells were increased when CXCR4-EGFP mice were crossed with SDF-Tg mice compared with WT-CXCR4-EGFP mice (supplementary figure 8B). In the gastric antrum, however, the bottom third of the glands showed strong GFP-positive signals that appeared to overlap with the known location of antral stem and progenitor cells (supplementary figure 8C).[33] Taken together, these data are consistent with a model in which overexpression of SDF-1 in the stomach could promote gastric epithelial cell proliferation directly, at least in part, through activation of CXCR4-expressing gastric progenitor cells.

### SDF-1 induces expansion of stromal myofibroblasts through recruitment of CXCR4-expressing MSCs

While there were some gastric epithelial cells in the corpus that were positive for CXCR4-EGFP, there were clearly multiple stromal cells in the region of the gastric progenitor zone (isthmus) that were also positive for CXCR4-EGFP (supplementary figure 8A). Many of these had the appearance of stromal fibroblasts or myofibroblasts. Recent studies have suggested that αSMA-positive myofibroblasts contribute to the gastric stem cell niche,[34] and that expansion of these cells contributes to the development of gastric neoplasia.[19] Consequently, we examined the effects of SDF-1 overexpression on of αSMA-positive myofibroblasts by crossing SDF-Tg mice with αSMA-RFP (red fluorescent protein) reporter mice[19] to generate SDF-Tg/αSMA-RFP double transgenic mice. We found that αSMA-positive cells were significantly increased in SDF-Tg mice compared with WT mice as early as the age of 4 months (figure 5A). Similar results were obtained using immunostaining for αSMA in both SDF-Tg mice and SDF-Tg/IL-1β double transgenic mice (data not shown). Taken together, these findings suggest that overexpression of SDF-1 is able to induce the early expansion of αSMA-positive myofibroblasts within the isthmus region of the gastric corpus.

Given the expansion of stromal cells resulting from the overexpression of SDF-1 in the stomach, we analysed mRNA expression of CXCR4 and Gremlin 1 (*Grem1*). Gremlin 1, a bone morphogenetic protein antagonist, is a recently identified marker of MSCs and is highly expressed by cancer-associated stromal cells.[19 35] While SDF-Tg mice alone showed a small but significant increase in *Grem1* mRNA expression, SDF-Tg mice infected with *H felis* showed significant upregulation of both *Grem1* and CXCR4 mRNA expression in the stomach (figure 2C). These findings are consistent with SDF-1-dependent expansion and recruitment of MSCs to the stomach as recently described.[19] A subset of MSCs has been shown to strongly express CXCR4 capable of promoting migration to BM.[36] We confirmed *Grem1* expression on CXCR4 positive stromal cells, by *Grem1* immunostaining of our CXCR4-EGFP mouse (supplementary figure 9B) and that these *Grem1* positive cells were BM-derived (figure 5B). We also showed a positive correlation of mRNA expression of CXCR4 (Spearman rank correlation test, ρ=0.9, p=0.002). mRNA expression of *Grem1* was upregulated



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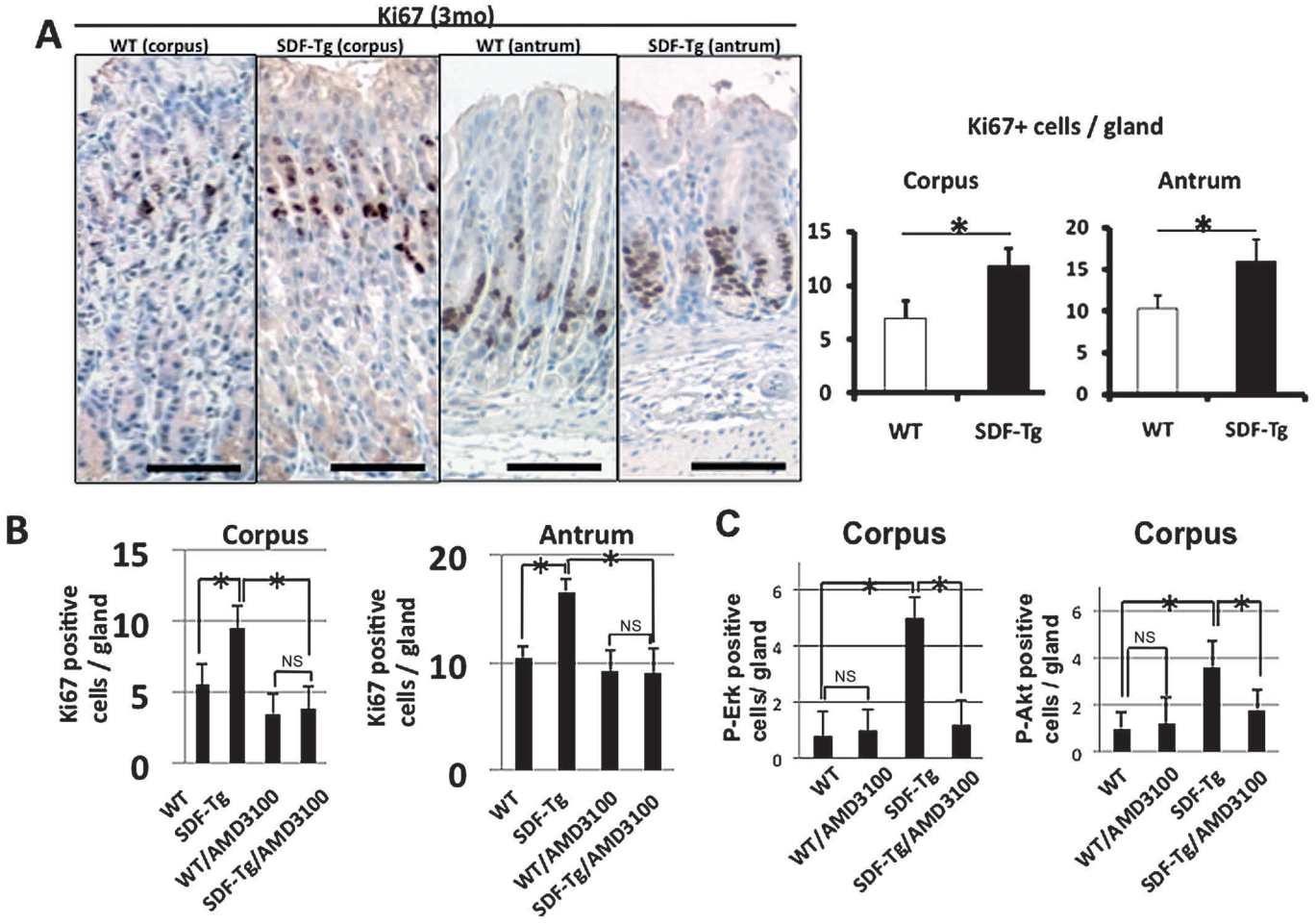


**Figure 4** (A) Proliferation in WT and SDF-Tg mice stomachs stained with Ki67 (n=5 per group). (B) WT and SDF-Tg mice with or without AMD3100 treatment stained for Ki67. The number of Ki67-positive cells per gland was determined by counting 10 glands per mouse (n=5 per group, 3-month-old mice). (C) WT and SDF-Tg mice treated with or without AMD3100 were stained with phospho-Akt or phospho-Erk (n=5 per group). Data shown are mean±SE. *p<0.05. Scale bars=100 μm.

in the stomach of SDF-Tg mice compared with WT mice (figure 5C). We also used immunohistochemistry to confirm the recruitment of *Grem1* (+) MSCs to the gastric mucosa of SDF-Tg mice in combination with *H felis* or IL-1β overexpression (figure 6A). Finally, we examined the direct chemotactic ability of SDF-1 on BM-MSCs in a migration assay using isolated BM-MSCs stimulated with mouse gastric extracts from WT or SDF-Tg mice in a Boyden chamber assay. Gastric protein extracts isolated from SDF-Tg mice induced a significant increase in cell migration of BM-MSCs compared with those isolated from WT mice, and this migration rate was reduced by the CXCR4 inhibitor AMD3100 (figure 6B). These findings clearly show that chemotaxis induced by the gastric microenvironment found in our SDF-Tg mice was mediated, at least in part, through the SDF1-CXCR4 pathway.

## DISCUSSION

While SDF-1/CXCR4 signalling has been linked to numerous pathophysiological processes, the consequences of upregulated expression have not been well defined. In the current study we show that SDF-1 overexpression under the control of the H/K-ATPaseβ promoter does not lead to severe inflammation but, nevertheless, is able to induce gastric dysplasia and tumour formation. Overexpression of SDF-1 directly stimulates the proliferation of gastric epithelial progenitor cells with increases in Ki67 and CXCR4-positive cells in the progenitor zone and an expansion of the K19-positive lineage. In addition, SDF-1 overexpression increased CXCR4-positive fibroblastic cells, especially αSMA-positive myofibroblasts, probably through recruitment of *Grem1*+ MSCs. Despite the limited effect on the induction of inflammation, SDF-1 overexpression appeared to synergise with either *H felis* infection or IL-1β overexpression, resulting in severe inflammation with a marked increase of F4/80-positive macrophages. Overexpression of SDF-1 also appeared to synergise with proinflammatory cytokines through the activation of macrophages and promotion of macrophage survival. Taken together, our data offer direct evidence that elevation of a single CXC chemokine, SDF-1, promotes gastric carcinogenesis through both direct effects on gastric epithelial progenitors and through modulation of the gastric progenitor niche, consistent with prior clinical evidence that high levels of SDF-1 in patients are associated with gastric cancer (figure 7).[37]

SDF-1 has been thought to be the key chemokine responsible for the mobilisation and recruitment of inflammatory cells from the BM to sites of inflammation or cancer.[17] SDF-1 is upregulated in a number of inflammatory diseases.[18] Deletion of the CXCR4 receptor in specific haematopoietic subsets clearly impairs the recruitment of these cells, indicating that the SDF-1-CXCR4 axis is required for normal leucocyte trafficking.[38] Our data, however, suggest that high levels of SDF-1 in peripheral



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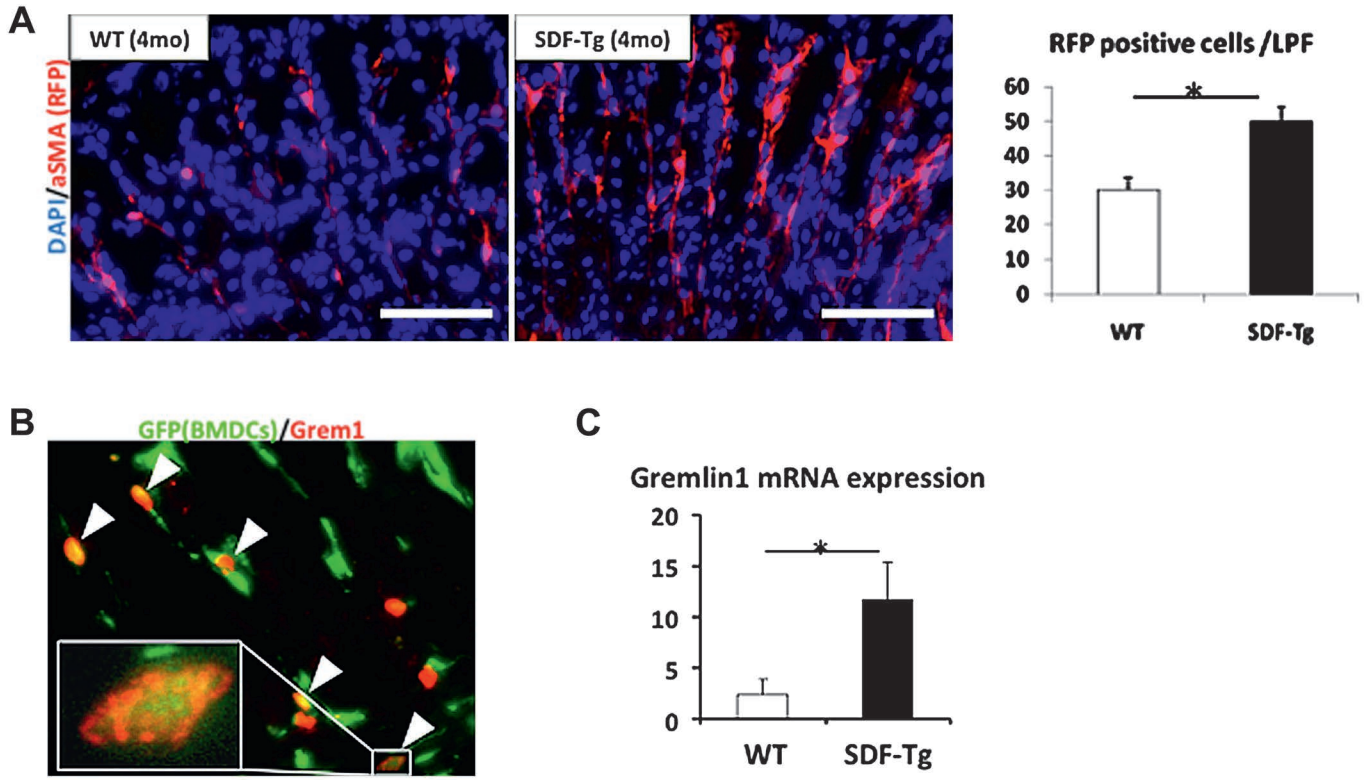


**Figure 5** (A) α-smooth muscle actin (αSMA)-positive cells were expanded in SDF-Tg mice. Frozen sections from αSMA/RFP mice crossed with either WT or SDF-Tg mice were observed under a fluorescent microscope (n=3 per group). Red: red fluorescent protein (RFP); blue: 4′,6-diamidino-2-phenylindole (DAPI). Original magnification ×100. (B) UBC-GFP traced BM-derived cells in the gastric stroma express *Grem1* by immunostaining. Gastric tissue from SDF-Tg mice transplanted with UBC-GFP bone marrow was stained with anti-Gremlin 1 antibody. Green: bone marrow-derived cells; red: Gremlin 1. (C) mRNA expression of Gremlin 1 was increased in SDF-Tg mice compared with WT mice at 6 months (n=4 per group). Results shown are mean+SE. *p<0.05. Scale bars=100 μm.

tissues such as the stomach are not sufficient for recruitment of leucocytes, since the SDF-1 Tg mice showed little inflammation at early time points. It was only at later time points that we observed a significant increase in F4/80-positive macrophages, and SDF-1 overexpression in combination of IL-1β overexpression resulted in a much more severe inflammatory response. It is likely that part of the explanation relates to the endogenous levels of BM SDF-1 which prevented the egress of BM cells.[39] The key to the recruitment of inflammatory cells from BM may depend as much on the degradation of BM SDF-1 in response to proinflammatory cytokines such as IL-1β as it does on high levels of SDF-1 in peripheral tissue.[25 40] In addition, our data would suggest that SDF-1 might contribute to inflammation by activating and stabilising macrophages, along with its effects on chemotaxis, consistent with previous reports.[41 42]

Our data support the notion that one target of SDF-1 signalling is directly on gastric epithelial progenitors. We found CXCR4-positive cells in the isthmus of the normal gastric corpus and in the lower third of the normal antral glands. These portions of the gastric corpus and antrum contain gastric stem and progenitor cells, and also the Ki67-positive progenitor cells. In addition, in SDF-1 Tg mice we observed a gradual increase in CXCR4-EGFP-positive epithelial cells, consistent with an amplification of CXCR4-positive progenitors. Thus, our findings suggest that, in the gastric epithelium, SDF-1 secreted by gastric myofibroblasts regulates the proliferation and possibly the location of gastric epithelial progenitors. CXCR4 expression has been observed in the CNS and has been postulated to be important in regulating the migration of progenitor cells in postnatal brain.[32] The downward migration of the proliferative zone commonly observed during gastric preneoplasia, for example, could in theory be related in part to SDF-1 expression by MSC-associated stromal cells which tend to expand at the base of the gastric glands. CXCR4 is also found in many cancer cell lines where it has been associated with tumour growth and metastasis,[43] and the expression of CXCR4 by gastric progenitors could account for this finding of expression in cancer cells.

However, the most prominent effects of SDF-1 appeared to be the expansion of αSMA-positive myofibroblasts in the gastric mucosa. At early time points, much of this expansion appears to be due to proliferation of resident tissue mesenchymal cells, since we found only a small number of BM-derived αSMA-positive myofibroblasts in our BM transplantation studies. Nevertheless, the data are consistent with the likely presence of MSCs in most peripheral tissues such as the stomach and the slow time course for BM-derived cell recruitment prior to the development of dysplasia.[19] In previous studies we established Gremlin 1 as a putative marker for MSCs that give rise to myofibroblasts.[19] Indeed, in the current study we found that *Grem1* mRNA expression was significantly upregulated in SDF-Tg mice when compared with WT mice, particularly in the setting of *H felis*. We also showed that *Grem1*+ MSCs express CXCR4 and migrate in response to SDF-1 expression. In previous studies we also demonstrated that CXCR4 function was integral to the development of myofibroblasts as well, since TGFβ can induce myofibroblastic differentiation from MSCs through upregulation of SDF-1 production and is inhibited by



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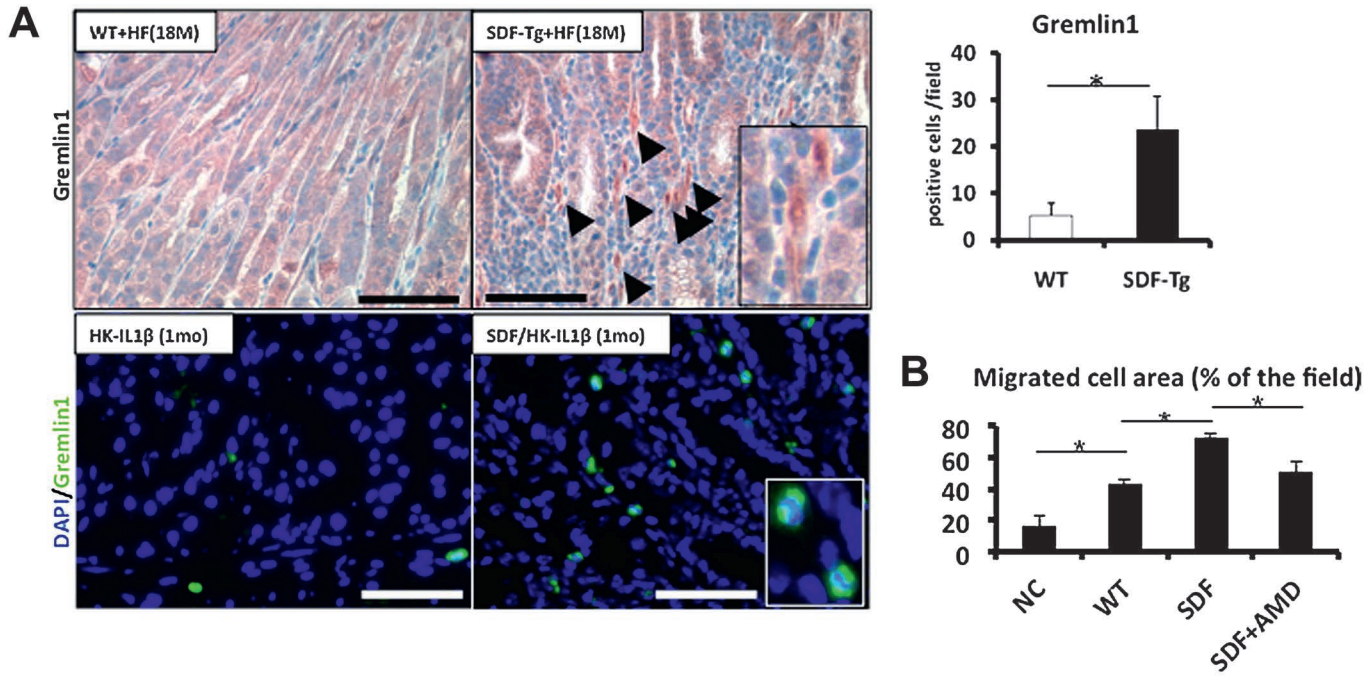


**Figure 6** (A) Gremlin 1-positive cells were increased in SDF-Tg mice compared with WT mice infected with *Helicobacter felis*. Representative paraffin sections from stomachs are shown (top). Gremlin 1-positive cells were increased in SDF/H/K-ATPase-IL-1β double transgenic mice compared with H/K-ATPase-IL-1β mice (bottom). Frozen sections from SDF/H/K-ATPase-IL-1β or H/K-ATPase-IL-1β mice were stained (n=3 per group). Green: Gremlin 1; blue: 4′,6-diamidino-2-phenylindole (DAPI). Original magnification ×100. (B) Transwell migration assay demonstrated the migration of bone marrow mesenchymal stem cells (BM-MSCs) in response to stromal cell-derived factor-1 (SDF-1) in 3-month-old mice. BM-MSCs were incubated with the gastric mixture from WT or SDF-Tg mice. SDF-1-induced BM-MSC migration was significantly inhibited by AMD3100. Results shown are mean +SE. *$p<0.05$ in each comparison indicated.

the CXCR4 antagonist AMD3100.[19] While CXCR4 antagonism could also inhibit non-SDF-1 ligands such as ubiquitin, we believe that, in our SDF1-Tg driven model, the consequences of CXCR4 antagonism are primarily due to the loss of SDF1-related signalling.

In conclusion, the results presented here show that SDF-1/CXCR4 signalling is important to the gastric epithelial niche. SDF-1 is normally produced by MSC-associated stromal cells, and increased SDF-1 promotes the proliferation of both epithelial cells and the expansion of stromal cells. While SDF-1 is a weak inflammatory chemokine, upregulation of SDF-1/CXCR4 pathway can synergise with other proinflammatory molecules (such as IL-1β) and thus contribute to neoplasia. Taken together, the SDF/CXCR4 signalling pathway represents a promising target for future cancer prevention and treatment.

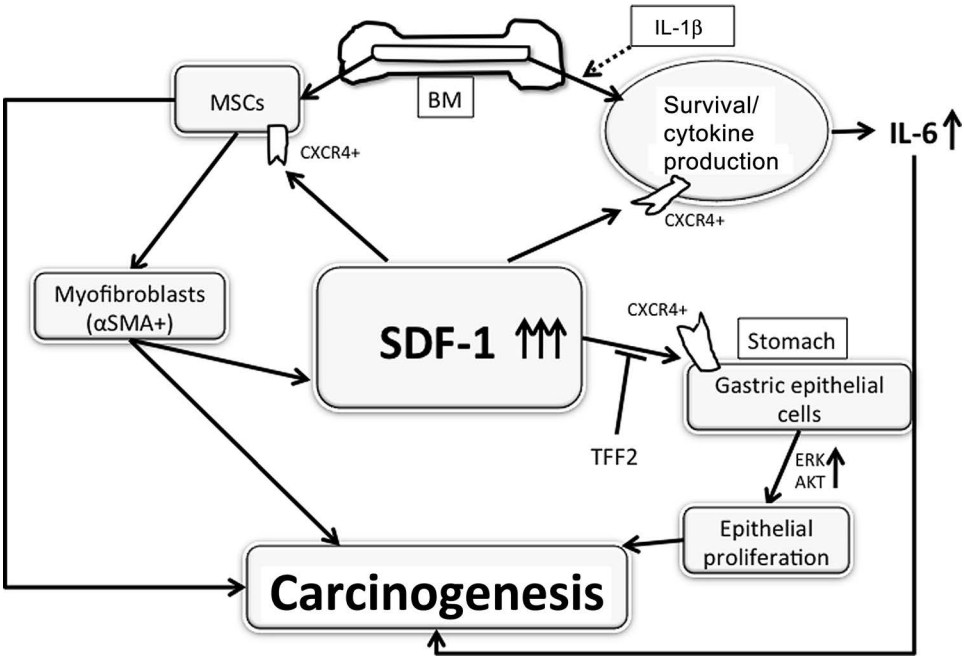


**Figure 7** Schematic hypothesis of stromal cell-derived factor-1 (SDF-1)-induced carcinogenesis. IL, interleukin; MSCs, mesenchymal stem cells; αSMA, α-smooth muscle actin.

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**Contributors** Study concept and design, obtained funding and study supervision: TCW. Acquisition of data: WS, HA. Analysis and interpretation of data: WS, HA, CBW, DW, JF, SM, SA, ZD, MQ, TCW. Drafting of manuscript: WS, HA, CBW, DW, MQ, TCW.

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**Supplementary Information**

**Supplementary Materials and Methods**

***Generation of SDF-1/CXCL12 transgenic mice***

We first generated an H/K-ATPaseβ/SDF-1 transgene that targeted constitutively secreted murine SDF-1, specifically to parietal cells of the stomach. To generate SDF-1 transgenic mice, we amplified ORF of human SDF-1/CXCL12 (BC039893) using the plasmid isolated from clone# 5729604 (Open Biosystems, AL, USA), and then introduced it in frame to create a fused SDF-1-V5-Hisx6 protein into pEFV5x6HisC (Invitrogen, CA, USA). We used this construct to amplify a shorter version of fused protein SDF-V5 (lacking the his tag but flanked with BamHI sites). The BamHI sites were used to introduce SDF-V5 into the construct with $H^+K^+$-ATPaseβ promoter. The final construct was sequenced before injection to exclude mutations (data not shown). Potential founders were screened using PCR 3 different primer pairs. Two lines exhibiting high levels of SDF-1-expression (Line 3 and 6) were selected and, initially on a mixed C57BL/6 x CBA background, were later backcrossed to C57BL/6J mice. There were no histological alterations in other organs examined, including intestine, liver, lung, and kidney in SDF-Tg mice (data not shown). Line 6 SDF-Tg mice were crossed to HK-IL-1b mice to generate SDF; IL-1b double transgenic

mice (C57BL/6J background). To generate SDF/TFF2-/- mice, Line 6 SDF-Tg mice were crossed to TFF2 knockout mice (C57BL/6J). The HK-IL-1beta transgenic mice and TFF2 knockout mice were described previously (8, 15). UBC-GFP mice were purchased from Jackson laboratory and used for bone marrow transplantation as described previously (16). All animal studies were performed in Institutional Animal Care and Use Committee (IACUC)-approved facilities at Columbia University (protocol#AAAA-8153).

***Recombinant SDF-1 and chemotaxis assay***

SDF-1V5x6His fusion gene was cloned into the pET-21D vector and expressed in *E. coli* cells. Met-SDF1-V5x6His containing inclusion bodies were isolated from bacterial cultures, solubilized in guanidinium chloride (6M) and purified using affinity Talon columns (Clontech). Purified protein was refolded by gradual dialysis against 8M, 6M, 4M, 2M, and 0M urea in 100mM Tris-HCl (pH7.5). The final product was tested for purity (>95%) and degree of refolding (>50%) by electrophoresis in reducing and non-reducing conditions (data not shown). For the migration assay, we used splenocytes or MSCs isolated from the bone marrow of wild type mice. Cells were washed, and $3x10^6$ cells/ml were suspended in RPMI 1640 medium with 1% bovine serum albumin.

100μL aliquots of cell suspension were applied to the upper chambers 24-well Transwell plates (Boyden chamber, Costar 3422, 5μm-diameter pore size). 600μL of RPMI 1640 medium supplemented with the indicated concentrations of recombinant SDF-1 or the gastric mixture from either wild-type and SDF-Tg mice were loaded into lower chambers. Mesencult medium was used as an experimental control. Cells were allowed to migrate for 3h at 37°C. After incubation, the porous inserts were removed carefully, and the viable cells were counted using a hemocytometer. The experiments were performed three times in duplicate. The results were expressed as the percentage of cells that migrated to the bottom chamber. Cells were fixed and stained with crystal violet, and positive staining area was calculated by Image J (NCI.).

***Bacterial Culture***

The *H. felis* strain (ATCC 49179) used in this study has been previously described (17). The organism was grown for 48 hours at 37°C under microaerobic conditions on 5% sheep blood agar supplemented with antibiotics. Bacteria were harvested and aliquoted at a titer of $10^{10}$ organisms/mL in Trypticase Soy broth with 10% glycerol, and bacterial suspension was stored at -70°C. Before use, aliquots were

thawed, analyzed for motility, and cultured for evidence of aerobic or anaerobic microbial contamination.

***Murine gastric cancer model (Chronic H. felis infection and IL-1b transgenic mice)***

Mice from each group (SDF-Tg and littermate WT controls) were inoculated with *H. felis* or sterile broth as a control as described previously (18). In brief, three inoculas (0.2mL of *H. felis*, $10^{10}$ colony-forming units/mL) were delivered every other day by oral gavage using a sterile gavage needle. Animals (n=8 to 10 per each group) were sacrificed 3, 6, 12, and 18 months post-infection by $CO_2$ asphyxiation and necropsied. In order to inhibit SDF/CXCR4 pathway, AMD3100 was administered 5mg/kg every day and mice were sacrificed at day15. SDF-Tg mice were crossed to HK-IL-1b mice to generate SDF/IL-1b double transgenic mice. Blood was obtained in $CO_2$-anesthetized mice by cardiac puncture, and sera were collected and frozen at -80°C. At necropsy, stomachs were removed *en bloc* and opened. The animals were examined for gross changes, and the tissue specimens collected consisted of the gastric mucosa beginning at the gastroesophageal junction through the gastroduodenal junction. Stomach tissue specimens were fixed in neutral buffered 10% formalin, processed by standard methods, embedded in paraffin, sectioned at 5μm, and stained with H&E.

Infection status was confirmed by microscopy, and by qRT-PCR assays of gastric corpus tissue at necropsy as described previously (18). Additional sections were cut for immunohistochemistry, and additional samples were processed for qRT-PCR.

***Histological Evaluation and Immunohistochemical Studies***

Inflammation, atrophy, hyperplasia, and dysplasia in the gastric corpus were each scored on an ordinal scale from 0 to 4 in increments of 0.5 as previously described (19) by a single veterinary pathologist (S.M) blinded to genotyping groups. Three sections through the squamocolumnar junction at the lesser curvature were evaluated for each mouse, and the worst score was recorded for each section evaluated. Cell proliferation was assessed by Ki67 immunohistochemistry. Ki67 positive cells were scored per high power field (20 corpus glands). All immunohistochemistry was performed with Vectastain ABC Kit (Vector Laboratories, Inc, Burlingame, CA, USA) according to the manufacturer's instructions. Antigen retrieval was achieved by boiling sections for 15 min in 0.01M citrate buffer, pH 6.0 (or 0.8M urea for F4/80 staining). We used alpha-smooth muscle actin antibody to stain myofibroblasts (1:500, Abcam, Cambridge, USA), F4/80 (1:100, Abcam, Cambridge, USA) to stain mature macrophages, CD11b (1:100, BD Bioscience, USA) to stain myeloid cells, p-Akt (1:100,

cell signaling technology, USA) to stain p-Akt, p-Erk (1:400, cell signaling technology, USA) to stain p-ERK, CXCR4 (1:200, Acris Antibodies, Germany) to stain the CXCR4 receptor. Binding of the primary Ab was detected with corresponding HRP-conjugated anti-rabbit/mouse IgG Abs (1/500 dilution; GE Healthcare, Piscataway, NJ) followed by visualization with 3, 3'-diaminobenzidine (DAB) and counterstaining with hematoxylin.

***Single-Cell Preparation and FACS Analysis***

For single-cell suspension preparation from stomach tissues, the mucosa of whole stomach was gently scraped free from the serosa; minced; digested for 1 hr in 1 mM DTT, 1 mM EDTA, 5% FBS in PBS at 37 degree; filtered through a 40 mm nylon mesh strainer; and resuspended in Dulbecco's PBS (D-PBS). For FACS analysis, single-cell suspensions were stained with fluorescence-labeled FITC-CD45, PE-CD3, APC-CD19, PE-Cy7-CD8, Alexa 700-CD4, APC-CD11b, or PerCP-Ly-6G antibodies (BD Pharmingen) and detected using a LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo7 software (Tree Star).

***Myeloperoxidase (MPO) activity assay***

Fresh gastric tissue was isolated and rinsed with chilled PBS, and homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) solution (1 ml per 50 mg of tissue). The homogenate was centrifuged at 11,000g for 15 min at 4 °C and supernatant was stored at -80 °C until further processing. The MPO activity was determined using a myeloperoxidase substrate solution as described previously (20).

***qRT- PCR, and ELISA of Proinflammatory CC-Chemokines, and Cytokine***

Total RNAs were extracted from a sample of whole stomach from each animal with TRIzol reagent (Invitrogen, Carlsbad, CA), and 4 μg of total RNA were used for first-strand complementary DNA synthesis using the Superscript III cDNA Amplification System (Invitrogen) following the manufacturer's instructions. qRT-PCR was performed as previously described using qRT-PCR primers for each gene. All primers were designed using Lasergene® version 5.0 software (DNASTAR Inc, Madison, WI). Sequences of qRT-PCR primers are available upon request. Results were expressed by the copy number of each gene versus $10^4$ GAPDH. Cytokine levels were measured by ELISA as manufacturer's instructions.

***Isolation of primary macrophages and cell proliferation assay (WST-8)***

Isolation of Macrophages were generated as described (21). To isolate macrophages, mice were i.p. injected with 1 ml of 4% thioglycollate (DIFCO) and peritoneal cells were flushed out 4 days later. The anti-apoptotic effect of SDF-1 on cells was assessed by WST-8 cell proliferation assay, using manufacturer's instructions (DOJIN, Kumamoto, Japan). In brief, the WST-8 assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. 100 µl of cell suspension of macrophages was seeded into a 96-well plate at a density of $2.5x10^3$ cells/well. After 2hr incubation, medium was changed to 100 µl RPMI without FBS, then either gastric mixture extracted from mice stomach or recombinant SDF-1 was added (final concentration at 50nM). After incubation for 48 hrs at 37°C, 10 µl of Cell Counting Kit-8 (CCK-8) solution was added to each well, and the plate was incubated for further 3 hrs at 37°C. Then the optical density was measured at 450nm using MULTISKAN EX spectrophotometer (MTX Lab Systems, Inc., VA, USA). Each experiment was performed using six replicate wells for each stimulant exposure.

***Statistical Analysis***

Results are expressed as mean ± SE unless otherwise stated. Student *t* test was used to evaluate statistical significance. Values of $p<0.05$ were considered statistically significant.

**Supplementary Figure Legends**

**Supplementary Figure 1** Derivation of Gastric Epithelial Cell SDF-1 transgenic Mice (A) Generation of gastric epithelial (parietal) cell specific SDF-1 transgenic Mice (SDF-Tg). The construct pBS/H/K-ATPaseβ/SDF-1α contains the mouse H+/K+-ATPase β subunit gene promoter and human SDF-1 cDNA. (B) Expression of SDF-1 protein in the gastric tissue of the 4-months–old SDF-Tg and WT mice confirmed by ELISA. (C) Expression of SDF-1 in SDF-Tg mice serum. (D) The correlation of SDF-1 level over the detecting methods between SDF-1 ELISA and v5 ELISA. (E) SDF-1 level in bone marrow and stomach from the SDF-Tg and WT mice determined by the SDF-1 qRT-PCR.

**Supplementary Figure 2** (A) No differences in the recruitment of immune cells in young mice. Immunohistochemistry for F4/80 or CD11b in the WT and SDF-Tg mice at the age of 6 months. The number of F4/80 or CD11b positive cells per HPFs was determined by counting 10 HPF per mice. n=5 per each groups. Data represent means + S.E. *$p<0.05$. *Scale bars*, 100μm. (B) Immune cells recruited to the stomach were analyzed by FACS. (C) SDF-1 promoted infiltration of F4/80 positive macrophages and neutrophil activity in older mice. Immunohistochemistry for F4/80 in the WT and

SDF-Tg mice at the age of 15 months. The number of F4/80 positive cells per HPF was determined by counting 10 HPFs per mice. n=5 per each groups. (D) Myeloperoxidase (MPO) activity in the stomach from WT or SDF-Tg at 12 mo was analyzed. N=5 per each group. Data represent means ± S.E. *p<0.05. *Scale bars*, 100μm.

**Supplementary Figure 3** SDF/IL-1beta-Tg developed severe gastric inflammation and metaplasia (A) Histology from the IL-1beta, or SDF/IL-1beta-Tg mice at the age of 1 month. (Original magnifications, x100) (B) Summary of histological scoring. (C) IL-6 mRNA level in the stomach was upregulated in SDF-Tg mice compared to WT mice, determined by qRT-PCR. *Scale bars*, 100μm.

**Supplementary Figure 4** SDF-1 promoted survival/cytokine production in macrophages (A) Primary macrophages were plated in 96 wells and incubated with either rSDF-1 (lt) or gastric extract from WT or SDF-Tg mice (rt) for 2 days; and then WST-8 assay was performed. (B) Cell culture supernatant after LPS stimulation was isolated and was measured IL-6 protein using ELISA kit as manufacturer's instructions. Data represent three experiments.

**Supplementary Figure 5** (A) WT and SDF-Tg mice infected with *H. felis* were stained with Ki67. The number of Ki67 positive cells per gland was determined by counting 10 glands per mice. n=5 per each groups, 3 mo of mice. (B) SDF-Tg showed significant increase of K19-positive cells in the pit cell area compared to the WT. WT and SDF-Tg mice were stained with CK19 antibody. The number of CK19-positive cells per gland was determined by counting 10 glands per mice. n=5 per each groups, 4mo of mice. Data represent means ± S.E. *$p<0.05$. *Scale bars*, 100μm.

**Supplementary Figure 6** Immunohistochemistry for phosphor-Erk, and phosphor-Akt WT and SDF-Tg mice treated with or without AMD3100 were stained with either phosphor-Erk, or phosphor-Akt.

**Supplementary Figure 7** Loss of TFF2 accelerates the gastric atrophy and dysplasia mediating the SDF-CXCR4 signaling (A) Histology from the SDF-Tg and SDF-Tg/TFF2KO mice infected with *H. felis* for 12 months. (Original magnifications, x100) (B) Summary of the histological scoring from SDF-Tg and SDF-Tg/TFF2KO mice. (C, D) SDF-Tg and SDF-Tg/TFF2KO mice infected with *H. felis* were stained with Ki67. The number of Ki67 positive cells per gland was determined by counting 10

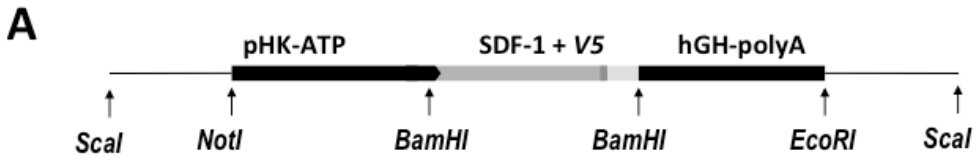
glands per mice. n=5 per each groups, 12 mo after *H. felis* infection. Data represent means ± S.E. *p<0.05 in each comparison indicated. *Scale bars*, 100μm.

**Supplementary Figure 8** Localization of CXCR4-EGFP positive cells in stomach. CXCR4-EGFP was detected in stromal lesion in corpus, and in epithelial lesion in antrum (A, top) CXCR4-EGFP BAC transgenic mice were analyzed by fluorescent microscopy. CXCR4-EGFP expression was detected at the bottom of the gland in antrum, whereas only few cells were positive in corpus. Green; GFP (CXCR4), and blue; DAPI. (A, middle) E-cadherin immunofluorescent staining on CXCR4-EGFP transgenic mice. Red; E-cadherin, Green; GFP (CXCR4), and blue; DAPI. (A, bottom) alpha-SMA immunofluorescent staining on CXCR4-EGFP transgenic mice. Red; alpha-SMA, Green; GFP (CXCR4), and blue; DAPI. (B, C) The number of CXCR4-EGFP (+) cells in the stomach within CXCR4-EGFP mice or CXCR4/SDF-Tg double transgenic mice; (B) corpus, and (C) antrum. Original magnification x100. Data represent means ± S.E. *p<0.05. *Scale bars*, 100μm.

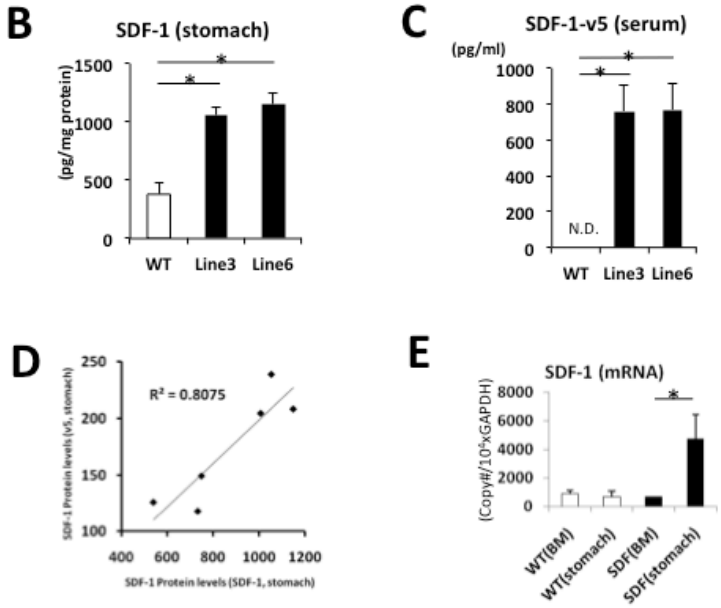
**Supplementary Figure 9** (A) Bone marrow mesenchymal stem cells isolated from CXCR4-EGFP mice were observed under fluorescent microscopy. Green fluorescence

confirmed the expression of CXCR4. (B) A section of the gastric corpus taken from a CXCR4-EGFP mouse infected with *H. felis* for 15 months, confirming co-localization (white arrows) of Gremlin 1 (immunostaining, red) with CXCR4 (expression, green) in stromal cells within the gastric corpus. Green = CXCR4, Red = Gremlin 1 immunostaining, Blue = Dapi. Original magnification 300x.

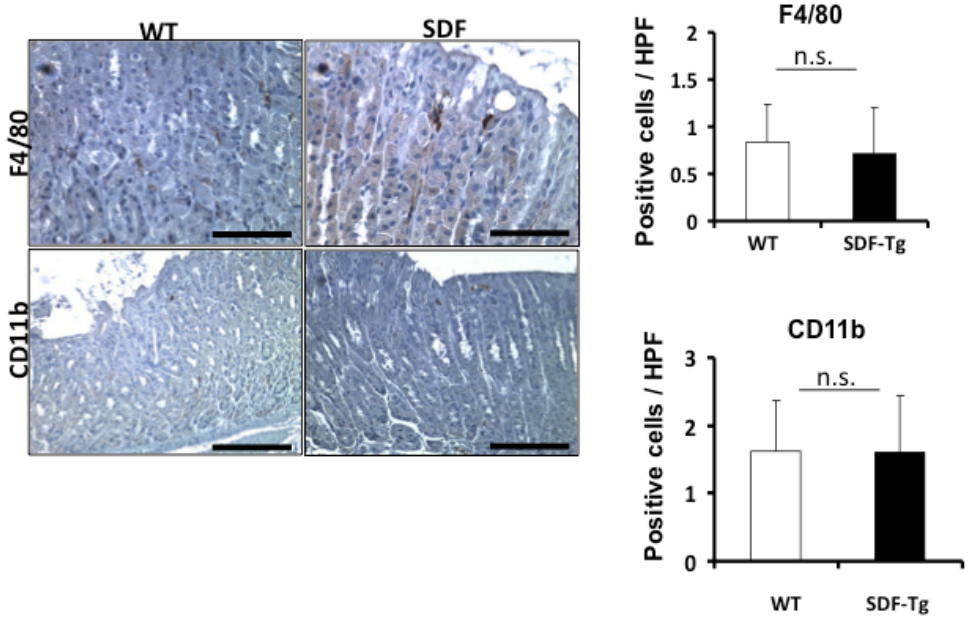
**Supplemental Figure 1A**



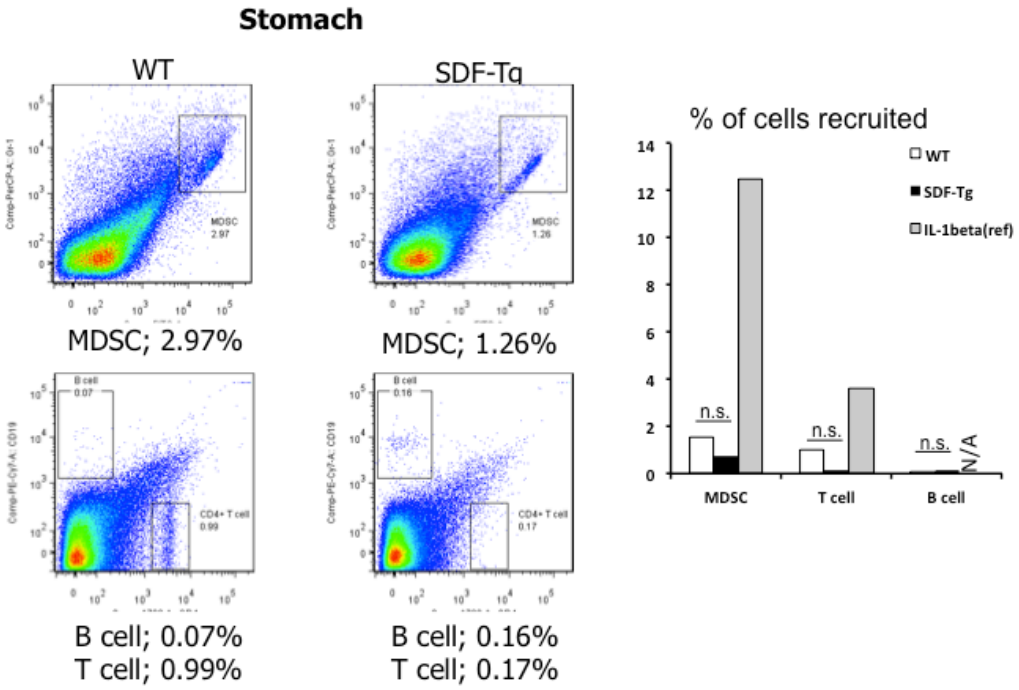
## Supplemental Figure 1B-E



## Supplemental Figure 2A

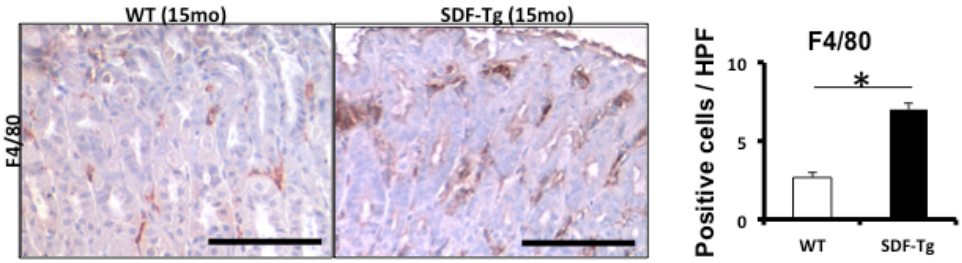


## Supplemental Figure 2B

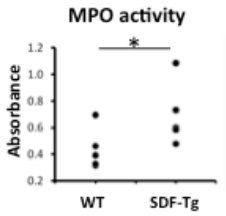


## Supplemental Figure 2 (cont.)

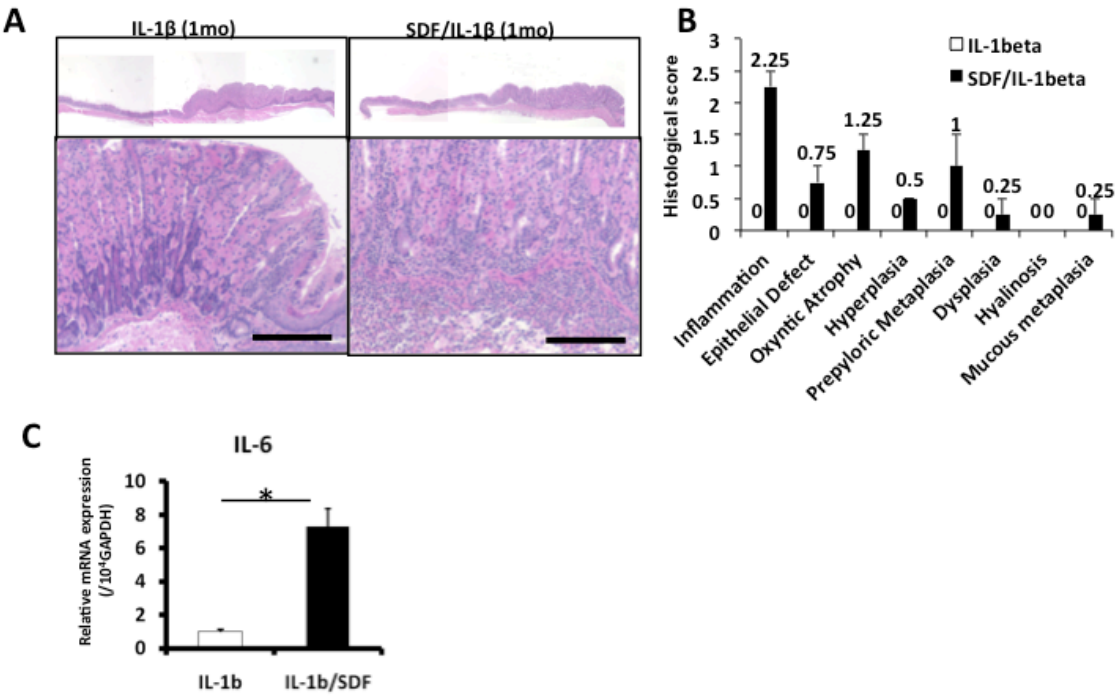
**C**



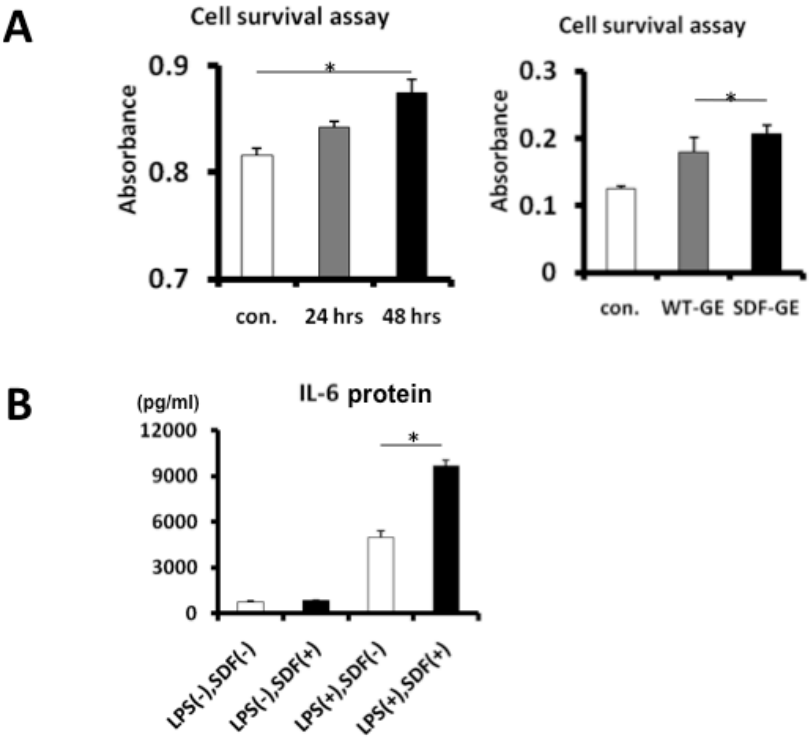

**D**



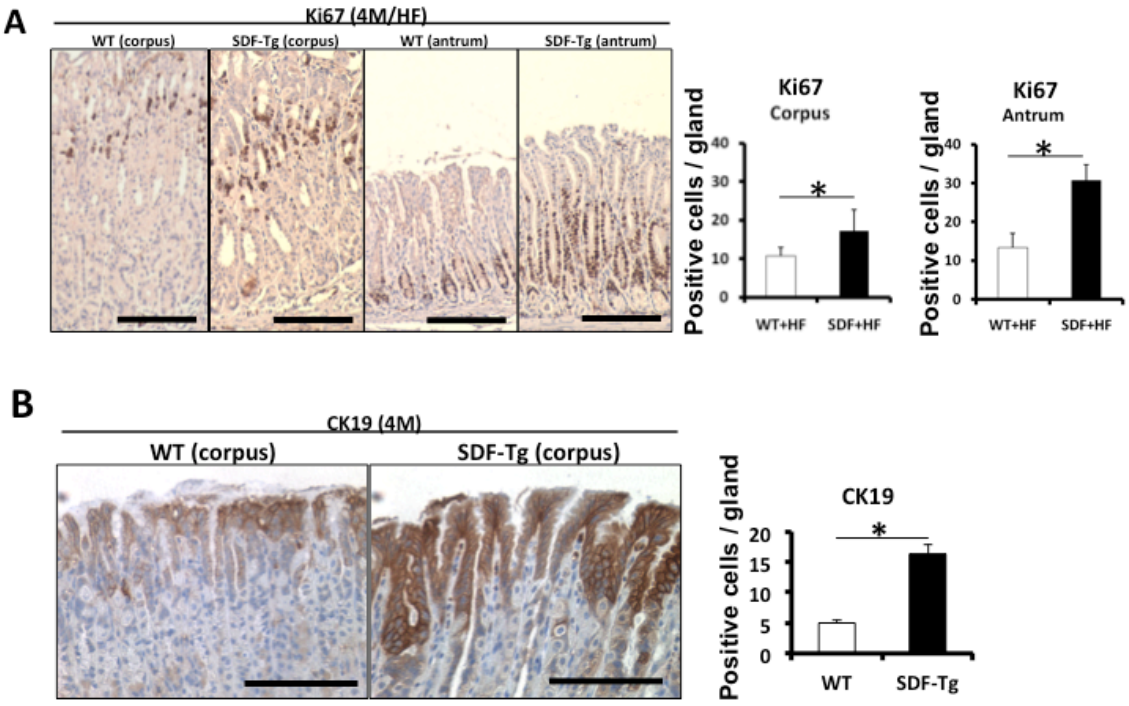
## Supplemental Figure 3



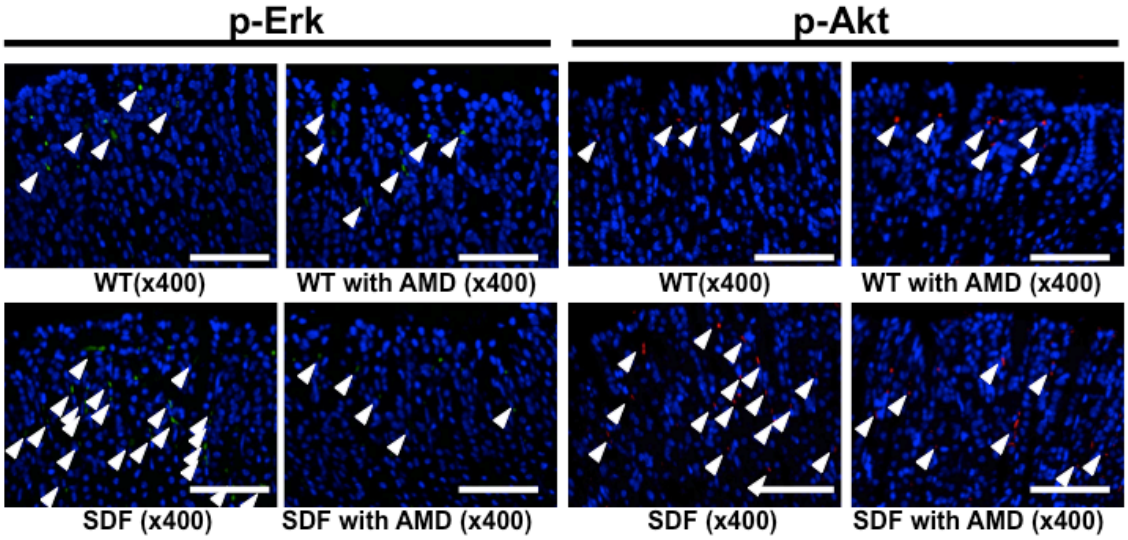
## Supplemental Figure 4



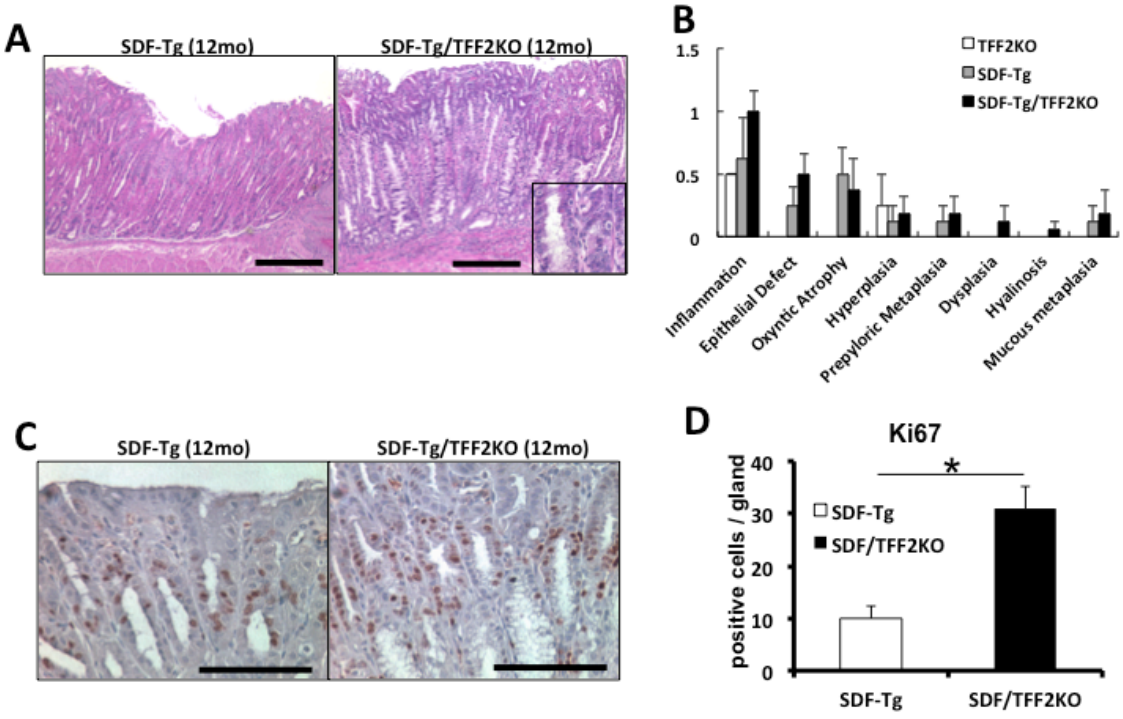
## Supplemental Figure 5



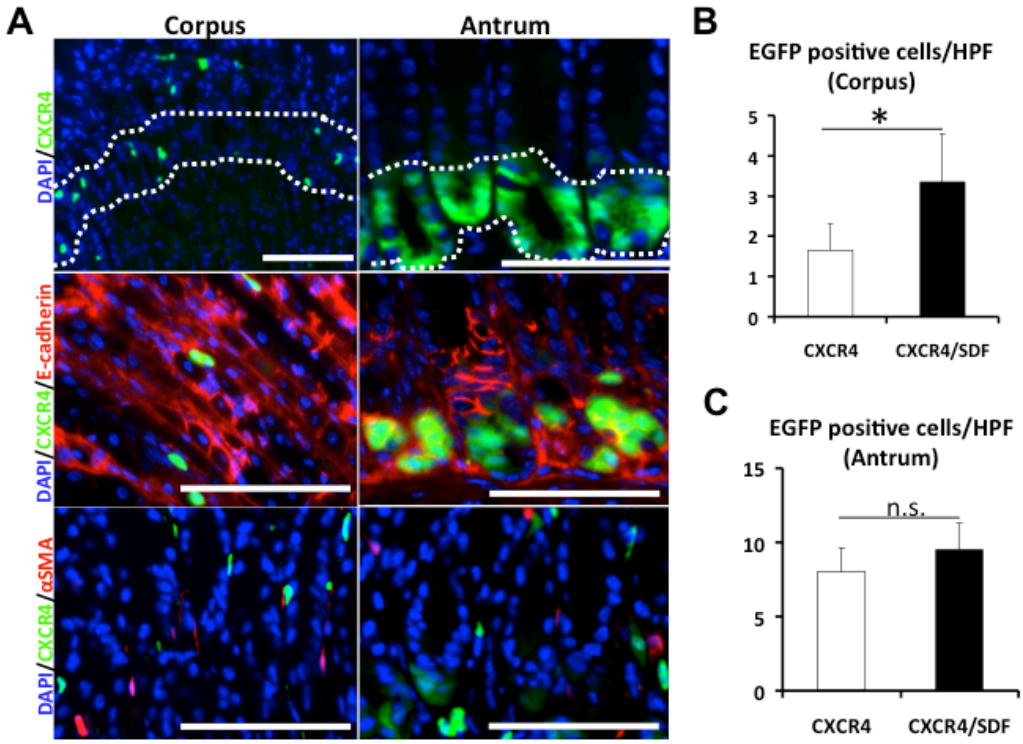
Supplemental Figure 6



## Supplemental Figure 7

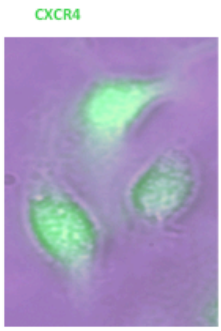


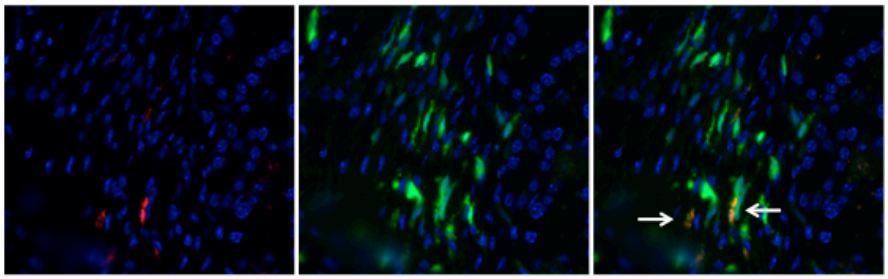
## Supplemental Figure 8



## Supplemental Figure 9

**A**




**B**