**Molecular and Cellular Pathobiology**

**K-ras Mutation Targeted to Gastric Tissue Progenitor Cells Results in Chronic Inflammation, an Altered Microenvironment, and Progression to Intraepithelial Neoplasia**

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**Abstract**

Chronic infectious diseases, such as *Helicobacter pylori* infection, can promote cancer in a large part through induction of chronic inflammation. Oncogenic K-ras mutation in epithelial cells activates inflammatory pathways, which could compensate for a lack of infectious stimulus. Gastric histopathology and putative progenitor markers [doublecortin and calcium/calmodulin-dependent protein kinase-like 1 (Dcamkl1) and keratin 19 (K19)] in K19-ras-V12 (K19-kras) transgenic mice were assessed at 3, 6, 12, and 18 months of age, in comparison with *Helicobacter felis*-infected wild-type littermates. Inflammation was evaluated by reverse transcription–PCR of proinflammatory cytokines, and K19-kras mice were transplanted with green fluorescent protein (GFP)–labeled bone marrow. Both *H. felis* infection and K-ras mutation induced upregulation of proinflammatory cytokines, expansion of Dcamkl1+ cells, and progression to oxyntic atrophy, metaplasia, hyperplasia, and high-grade dysplasia. K19-kras transgenic mice uniquely displayed mucus metaplasia as early as 3 months and progressed to high-grade dysplasia and invasive intramucosal carcinoma by 20 months. In bone marrow–transplanted K19-kras mice that progressed to dysplasia, a large proportion of stromal cells were GFP+ and bone marrow–derived, but only rare GFP+ epithelial cells were observed. GFP+ bone marrow–derived cells included leukocytes and CD45+ stromal cells that expressed vimentin or α smooth muscle actin and were often found surrounding clusters of Dcamkl1+ cells at the base of gastric glands. In conclusion, the expression of mutant K-ras in K19+ gastric epithelial cells can induce chronic inflammation and promote the development of dysplasia. *Cancer Res*; 70(21); 8435–45. ©2010 AACR.

**Introduction**

*Helicobacter*-related gastric cancer arises through a multistage progression that involves the conversion of normal gastric epithelium to regions of hyperplasia, dysplasia, and, finally, invasive adenocarcinoma. This multistep process is associated with the accumulation of genetic and/or epigenetic alterations in the target cell population that leads to their malignant transformation. *Helicobacter* infection contributes to the pathogenesis of gastric cancer in a large part through the induction of chronic inflammation (1, 2), which can induce proliferation and thus opportunity for mitotic error. In addition, *Helicobacter* infection can promote the development of cancer through mobilization and recruitment of bone marrow–derived cells (BMDC) that contribute to cancer development in diverse ways (3–5).

However, whereas chronic inflammation can likely initiate and promote oncogenic mutation and transformation, accumulating data suggest that, in many cases, the converse is also true and that oncogenic mutations can also lead to the development of chronic inflammation (6). Cytokines and soluble mediators produced by oncogene mutations in cancer cells recruit and activate inflammatory cells to develop a tumor microenvironment, which further stimulates tumor progression (6, 7).

One oncogene that has been strongly linked to the development of chronic inflammation has been the Ras oncogene (8). Expression of a mutant K-ras oncogene induces strong immune responses through activation of cytokines and experiment. Z. Dubeykovskiy: Conception, provision of study materials. W. Shibata: Conception, experiment. K.S. Betz: Conception, experiment. S. Muthupalani: Pathological diagnosis. A.B. Rogers: Pathological diagnosis. J.G. Fox: Pathological diagnosis. A.K. Rustgi: Conception, provision of study materials. T.C. Wang: Conception and design, manuscript writing. T. Okumura and R.E. Ericksen contributed equally to this work.

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T cells (9–11). In transgenic mice modeling pancreatic cancer, based in part on K-ras mutations in PDX1+ cells, significant inflammatory and stromal responses correlate with cancer progression (12). Expression of the mutant K-ras-V12 oncogene in keratin 19 (K19)–expressing pancreatic cells also results in pancreatic ductal hyperplasia with lymphocytic infiltration (13). Studies from Sparmann and Bar-Sagi showed that many of the key downstream targets of K-ras were chemokines and cytokines (10, 14). Further studies showed that transformed cell survival and tumor progression are dependent on these induced cytokines (15).

In a previous report, K-ras-V12 was expressed in the gastric epithelium using the K19 promoter and resulted in mucous metaplasia and parietal cell loss (13). However, a detailed investigation of the inflammatory responses and development of dysplasia in this model has not yet been reported. K19 is a member of the family of intermediate filaments that is highly expressed in the proliferative zone of the gastrointestinal tract (13, 16–18). In the stomach, K19 is expressed in the neck/isthmus region of the glandular unit, in the same area where stem/progenitor cells reside (13, 16). In theory, induction of cancer is most likely to occur when oncogenic mutations accumulate in long-lived stem or progenitor cells. Whereas an authentic stem or progenitor cell for the oxyntic glands of the stomach has not yet been identified, one candidate progenitor marker that has emerged is double-cortin and calcium/calmodulin-dependent protein kinase-like 1 (Dcamkl1), a gene shown to be upregulated in an enriched gastric epithelial progenitor cell population from the murine stomach glands and do not express biomarkers associated with differentiated lineages (19, 20). The aim of the current study was to compare the effects of mutant K-ras expression to chronic Helicobacter felis infection on the gastric epithelium. We show that mutant K-ras can substitute for Helicobacter infection in the induction of chronic inflammation, leading to expansion of putative gastric progenitors and induction of gastric intraepithelial neoplasia.

Materials and Methods

Mice

The K19-K-ras-V12 (K19-kras) transgenic mice were generated previously (13), using a 2.1-kb genomic fragment of the 5′ flanking region/promoter of the mouse K19 gene linked to the full-length mutant human K-ras (Val 12) gene. K19-kras transgenic mice were backcrossed to C57/B6 mice for at least three generations under the Columbia University Institutional Animal Care and Use Committee. Chicken β-actin enhanced green fluorescent protein (EGFP) transgenic mice (8–10 weeks old) were purchased from The Jackson Laboratory.

H. felis infection, histopathology, and immunohistochemistry

H. felis infection was carried out by gavage as previously described with H. felis (American Type Culture Collection 49179; ref. 4). Stomachs were fixed in 10% neutral buffered formaldehyde, and routine H&E sections were reviewed by board-certified veterinary pathologists and graded according to previously described criteria (21, 22). Immunohistochemistry was performed on 4-μm sections with avidin-biotin-peroxidase complex kits (Vector Laboratories) and counterstained with Mayer’s hematoxylin. For primary antibodies, mouse anti-hydrogen/potassium ATPase (H/K-ATPase) β (Abcam), rabbit anti-Ki67 (Abcam), rabbit anti-K19 (Abcam), rabbit anti-Dcamkl1 (Abagent), rabbit antimouse-TFF2 (established in our laboratory), rabbit anti-TFF1 (Abcam), and mouse anti-Muc5AC (Abcam) or rabbit anti-GFP (Invitrogen) were used.

PCR and quantitative real-time-PCR analysis

Real-time PCR reactions with QuantiTect SYBR green PCR kit (QIAGEN) and primers (Supplementary Table S1) were run on an ABI Prism 7300 (Applied Biosystems). mRNA quantities were analyzed in duplicate and normalized against glyceraldehyde-3-phosphate dehydrogenase as an internal control. Results are expressed as relative gene expression using the ΔΔCt method.

Bone marrow transplantation

Bone marrow transplantation was carried out as previously described with 5 million cells per animal (3). Mice were euthanized at 3, 6, and 12 months posttransplantation.

Flow cytometry and mesenchymal stem cell culture

Freshly isolated bone marrow cells were processed for flow cytometry as previously described (4, 6) and after incubation with phycoerythrin (PE)–conjugated antimouse CD45 antibody (BD Pharmingen) or PE-conjugated rat IgG2a antibody (isotype control; Jackson ImmunoResearch) and 4′,6-diamidino-2-phenylindole (DAPI) were analyzed using BD LSRII (Becton, Dickinson). Mesenchymal stem cells (MSC) were cultured as previously described (23).

Y chromosome and K19 in situ hybridization

In situ hybridization for Y chromosome or K19 were performed using a Texas Red–labeled Y-chromosome paint (Star-FISH, Cambio) or a DIG-labeled antisense riboprobe (Roche) following the manufacturer’s protocols, as previously described (24).

Immunofluorescence

Immunofluorescence was performed following in vitro fixation-perfusion with 4% paraformaldehyde followed by freezing and embedding in OCT, frozen 4-μm sections were prepared and blocked with 1% fetal bovine serum (FBS) in PBS for 1 hour at room temperature, and then rat anti-E-cadherin antibody (Zymed), rat anti-CD45 (eBioscience), rabbit anti-α-smooth muscle actin (αSMA; Abcam), rabbit anti-k19 (Abcam), rabbit anti-Dcamkl1 (Abagent), and mouse anti-vimentin (Abcam), diluted in PBS with 1% FBS (1:100 dilution), were applied. Following overnight incubation at 4°C, Texas Red–conjugated antirat, rabbit, or mouse IgG antibodies (Jackson Immunoresearch) were applied, respectively, with 1% FBS in PBS (1:300 dilution) and incubated for 1 hour at room temperature.
Nuclei were stained with DAPI and mounted using Vectashield (Vector Laboratories) for microscopy.

**Statistical analysis**

Statistical analysis was performed using Student's t test, and P values of <0.05 were considered significant. Data are expressed as mean ± SEM.

**Results**

**K19-kras mice developed gastric hyperplasia and histologic inflammation**

To assess the effects that the 2.1-kb K19 promoter-driven mutant K-ras-V12 oncogene has on the induction of chronic inflammation, we first investigated the gross appearance, gastric histopathology, and inflammatory responses in K19-kras transgenic mice compared with H. felis–infected wild-type (WT) mice over time. Colonization of H. felis organisms in pyloric and fundic glands of WT mice was confirmed by examination of H&E-stained sections and PCR amplification of H. felis–specific DNA sequence (Supplementary Fig. S1). Wet stomach weight and the ratio of wet stomach weight to body weight of K19-kras mice were significantly higher than those of control mice, regardless of H. felis infection (Fig. 1A). This was consistent with the gross appearance of the stomach, where severe mucosal thickness and enlarged folds were consistently observed in K19-kras mice (Fig. 1B).

Histopathologic scoring of H&E-stained gastric tissue sections from mice in each group using previously published criteria (21, 22) showed that H. felis–infected WT mice had significantly higher scores for gastric lesions than control mice (Fig. 1D).

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**Figure 1.** Gastric histology of WT and K19-kras mice. A–C, from mice in 12-mo cohort: body and stomach weights (A), representative gross presentation (B), and H&E-stained and Alcian blue-stained gastric tissue sections (C). Representative Alcian blue+ cells in WT H. felis–infected mice are indicated by arrows; magnified insets are of boxed regions (original magnification, 100× and 150×, respectively). D, histopathology scores for gastric lesions of the mice at 3, 6, and 12 mo after H. felis infection (*, P < 0.01; n = 3 for each group).
mice exhibited much greater chronic active gastritis, oxyntic atrophy, intestinal metaplasia, hyperplasia, and dysplasia that progressed over time (Fig. 1C), consistent with the multistage model of Helicobacter-related gastric carcinogenesis (22). Inflammation scores in K19-kras mice were similar to those of WT mice with H. felis infection. K19-kras mice also developed oxyntic atrophy, pseudopyloric metaplasia, hyperplasia, and dysplasia by 3 months, and the lesions persisted until the end of the study (Fig. 1D). Mucous metaplasia, characterized by replacement of parietal cells with foamy Alcian blue staining H/K-ATPase− cells without glandular dysplasia, was observed in the oxyntic mucosa of K19-kras mice, but not in WT mice, even with H. felis infection (Fig. 1C), consistent with the previous reports (13, 22). The K19-kras mice also showed a marked lymphocytic infiltrate with hyperplastic lymphoid follicles, similar in appearance and frequency to those found in H. felis–infected WT mice (Supplementary Fig. S2A). These results show that K-ras mutation in epithelial cells is sufficient to induce chronic inflammatory responses in the gastric epithelium.

**Oncogenic K-ras mutation in gastric epithelial cells upregulates cytokine and chemokine expression**

The finding of chronic inflammation in K19-kras mice suggested that K-ras mutation in gastric epithelial cells triggered the activation of specific immune responses that may be similar to H. felis–related chronic gastritis. Reverse transcription–PCR (RT-PCR) analysis of mice 6 to 12 months of age revealed increased gastric mRNA expression of interleukin-6 (IL-6) and IL-1β in both H. felis–infected WT and K19-kras mice compared with WT uninfected controls (Fig. 2A). Additionally, CXCL1 was upregulated in both these groups, although the expression of CXCL5 was upregulated only in K19-kras mice (Fig. 2B). Interestingly, CXCL1 and IL-6 expression retained the highest degree of correlation with dysplasia scores in K19-kras mice (Fig. 2C). Similar trends were observed for IL-1β and CXCL5, albeit with smaller $R^2$ values. There were no differences among the three cohorts in the expression of other detected cytokines and chemokines, including IFNγ, tumor necrosis factor α, CCL2, CCL3, and CCL2 (data not shown). In younger (3 months) mice, expansion of pit cells was moderate (Supplementary Fig. S2B). Consequently, at this point, the aforementioned inflammatory cytokines were minimally elevated, whereas the chemokine CXCL1 was significantly upregulated (Supplementary Fig. S2B), supporting an important role for CXCL1 in phenotype development. Although K-ras is known to induce cyclooxygenase-2 (COX-2) expression, which can drive tumorigenesis (25), we were unable to detect any upregulation of COX-2 expression in younger mice (<12 months); in contrast, expression was nearly 3-fold higher in K19-kras versus WT mice over 12 months of age, correlating with progression to dysplasia (Supplementary Fig. S3).

Expression of growth factors heparin-binding epidermal growth factor (HB-EGF) and amphiregulin was significantly upregulated in the stomachs of K19-kras mice when compared with WT mice, regardless of H. felis infection (Fig. 2D). These results suggest that progression of gastric preneoplastic lesions in K19-kras mice occurs in the setting of an inflammatory environment similar to that found in H. felis–dependent

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**Figure 2.** Expression of chemokines/cytokines/growth factors. A, B, and D, quantitative RT-PCR analysis of 12-mo cohort (*, $P < 0.1$; §, $P < 0.05$; $n > 3$ in each group). C, relationship between dysplasia score and mRNA expression of CXCL1 and IL-6 in K19-kras mice at 12 mo.
chronic gastritis, characterized by expression of CXCL1, IL-1β, and IL-6. However, the unique phenotype of the K19-kras mouse is associated with the expression of additional inflammatory chemokines and growth factors that are relatively specific to the K-ras-V12 mutation, which include CXCL5, HB-EGF, and amphiregulin.

**Dcamkl1**^+** putative tissue stem/progenitor cells are expanded in the preneoplastic gastric lesions of K19-kras mice

The progression to metaplasia and dysplasia in the stomachs of K19-kras mice was associated with changes in epithelial differentiation and proliferation. In the stomach of normal WT mice, K19 staining was observed primarily in pit cells, which comprised the upper one sixth of the gastric glands (Fig. 3A). Ki67^+ proliferating cells reside in the isthmus and upper neck region (Fig. 3C), in close proximity to rare Dcamkl1^+ cells found in this region (Fig. 3D). Throughout and below the neck region are H/K-ATPase^+ parietal cells, which make up the majority of glands (Fig. 3B).

The gastric epithelium of K19-kras mice at 12 months of age displayed obvious parietal cell loss, with only a small number of H/K-ATPase^+ cells remaining at the base of the gastric glands (Fig. 3F). Excluding these rare parietal cells, a majority of the remaining cells were mucus-producing K19^+ pit cells (Fig. 3E), which had expanded to comprise nearly two thirds of the entire gland, consistent with previous reports (13). Expression of TFF1 and Muc5AC in the K19-kras mouse stomach indicated that the majority of K19^+ cells in the upper middle region of glands were expanded surface pit cells (Supplementary Fig. S4A and B). Ki67^+ proliferating cells shifted over time from the neck region to the bottom of gastric glands, slightly above the remaining parietal cells. Interestingly, the number of Dcamkl1^+ cells increased markedly to many cells in each glandular unit, located just below the proliferative zone and just above the residual parietal cells (Fig. 3G and H). It should be noted that Dcamkl1 is strictly a putative progenitor marker based on its homeostatic location and frequency throughout the gastric epithelium. Lgr5 can give rise to gastric antral glands in the murine adult, but Lgr5 is not expressed in the adult gastric corpus (26). We confirmed that Lgr5-EGFP is expressed at the base of gastric antral glands, whereas Dcamkl1^+ cells reside slightly above, never colocalizing (Supplementary Fig. S4C). Therefore, these results suggest that the Dcamkl1^+ putative epithelial progenitor pool expands independent of Lgr5.

**Figure 3.** Immunohistochemical staining in gastric epithelium of uninfected WT and K19-kras mice at 12 mo. Expression of K19 (A and E), H/K ATPase (B and F), Ki67 (C and G), and Dcamkl1 (D and H) in the gastric epithelium of WT (A–D) and K19-kras (E–H) mice (arrows indicate Dcamkl1^+ cells; original magnification, 150×).
expression, under inflammatory responses induced by mutant K-ras expression.

**K19-kras mice develop high-grade dysplasia by 20 months of age**

We followed a cohort of aged mice, combining data from mice that survived 16 to 21 months of age, to investigate possible progression of the gastric preneoplastic lesions. As expected, K19-kras mice and *H. felis*-infected WT mice continued to exhibit higher inflammation scores when compared with WT mice without *H. felis* infection, becoming progressively more severe with longer observation periods (Supplementary Fig. S5; see also Fig. 1D). Histology scores for dysplasia were also elevated after a long-term observation, in parallel with the severity of inflammation (Fig. 4A; see also Fig. 1D). Among these mice, three of four (75.0%) *H. felis*-infected WT mice and three of eight (37.5%) K19-kras mice developed gross papillary tumors in the stomach (Fig. 4B). The lesions of three K19-kras mice were histologically defined as high-grade dysplasia or intramucosal carcinoma, with some areas invading into submucosal layers (Fig. 4C).

Immunohistochemistry on serial tissue sections revealed that the lower portions of dysplastic glands contained a mixture of TFF2+ and Dcamkl1+ cells (outlined in red in Fig. 4D; Supplementary Figs. S6B and C and S7B and D). More apical regions of these glands typically contained strongly staining K19+ cells, consistent with a surface epithelial phenotype (Supplementary Figs. S6A and S7C). K19 expression was especially strong in glands with squamous metaplasia (Supplementary Fig. S6A, bottom right). Infrequently, weakly staining K19+ regions also contained expanded Dcamkl1+ cells (outlined in black with arrowheads and as magnified insets, Fig. 4D; arrowheads in Supplementary Fig. S7A and B). However, TFF2 and K19 expression were clearly distinct from Dcamkl1, and colocalization of these markers was never observed in these serial sections or double immunofluorescence (Supplementary Fig. S7E). It appears that K19 mRNA is expressed early on as the pit cell migrates upward through the gland and before K19 protein expression. Therefore, it is likely that K-ras is expressed in a few cells that are not reactive to the K19 antibody. Whereas the transgene-derived mutant human K-ras could not be directly detected due to high homology with murine K-ras, we observed that K19 mRNA expression as assessed by in situ hybridization is present in a few cells below K19 protein+ cells, but distinctly above the region where Dcamkl1 is typically expressed (Supplementary Fig. S7F).

Similar immunohistochemical observations were made in regions of high-grade dysplasia, where Dcamkl1+ cells were expanded but never coexpressed as TFF2 or K19 (arrows in Fig. 4D). Proliferating Ki67+ cells typically resided between the aforementioned cell types, although they were occasionally found to be TFF2+ (Fig. 4D). Although we cannot completely exclude the possibility that the observed expansion of Dcamkl1+ cells is due to the expression of K-ras in these cells, the lack of colocalization with K19 mRNA and protein would suggest that it is more likely due to alterations in paracrine signals and/or their niche.

![Figure 4](image_url)

**Figure 4.** Aged K19-kras mice develop high-grade dysplasia. A, histopathology scores for dysplasia in gastric lesions of mice between 16 and 19 mo. B, gross presentation of the stomachs with papillary tumors. Stomachs from a WT mouse at 19 mo with *H. felis* infection (7445) and K19-kras mice at 16 to 19 mo (9003 and 7456, respectively). C, H&E-stained gastric tissue section of a K19-kras mouse at 16 mo displaying submucosal penetration of intramucosal carcinoma (original magnification, 200×). D, expression of Dcamkl1, K19, Ki67, and TFF2 in low-grade and high-grade dysplasia of a K19-kras mouse (original magnification, 300×).
K19-kras transgenic mice display recruitment of BMDCs to the tumor microenvironment

To investigate the role of mutated K-ras in the recruitment of BMDCs to the tumor microenvironment, we performed bone marrow transplantation (BMT) studies. Bone marrow from donor chicken β-actin-EGFP mice (WT/GFP) was transplanted into K19-kras or uninfected WT recipients. Six months after BMT, half of the bone marrow cells from recipient mice expressed GFP, similar to donor mice, with most of the GFP+ cells CD45+ hematopoietic cells (Supplementary Fig. S8A). Successful engraftment was confirmed by demonstration of GFP expression by MSC cultures (Supplementary Fig. S8B), quantitative detection of transgene DNA sequences in BMT recipient mice (Supplementary Fig. S8C), and detection of GFP+ cells in MSC cultures established from peripheral blood of all BMT recipient K19-kras mice (Supplementary Fig. S8B). Twelve to 18 months after BMT, gastric tissues from K19-kras BMT recipient mice displayed all the phenotypic changes of nontransplanted K19-kras mice, including mucosal thickness, histologic pathologies, shift of the Ki67+ proliferation zone, and dramatic expansion of Dcamkl1+ cells (Supplementary Fig. S8B and D).

In the BMT recipient mice, only rare GFP+ cells were observed in the gastric mucosa of uninfected WT mice, whereas K19-kras mice had a >10-fold increase in the number of recruited cells (Fig. 5A–C). E-cadherin, an epithelial-specific cell surface molecule, was detected in all epithelial cells of the gastric epithelium, including dysplastic glands. More detailed analysis of the glandular areas with confocal microscopy revealed that, although a small number of GFP+ cells were E-cadherin+ epithelial cells, a majority were E-cadherin− stromal cells (Fig. 5D).

The expression of GFP was confirmed by immunohistochemical staining for GFP (Fig. 5E). In addition, because GFP was present in only half of bone marrow cells (Supplementary Fig. S8A), we performed Y-chromosome in situ hybridization (Y-FISH) on sections prepared from female recipients that had received male donor bone marrow. Gastric sections showed many Y-chromosome+ cells in lymphoid follicles (Supplementary Fig. S8E) but no Y chromosome+ cells in the gastric glands (Fig. 5F). These findings suggest that the K19-expressing dysplastic gastric epithelium does not derive from the engrafted BMDCs.

To further characterize the GFP+ BMDCs, we performed immunofluorescent staining against CD45, vimentin, and αSMA. In all gastric sections, we detected colocalization of GFP with CD45 or spindloid cells positive for vimentin and/or αSMA, indicating the BMDCs had differentiated primarily into leukocytes, fibroblasts, and myofibroblasts, respectively (Fig. 6A). By 12 months, an estimated 15% of the recruited GFP+ BMDCs in the gastric epithelium of WT mice with H. felis and K19-kras mice were myofibroblasts, as characterized by their morphologic appearance and coexpression of αSMA (Supplementary Fig. S9).

To further understand the expansion of Dcamkl1+ cells, we performed staining against GFP, αSMA, and F4/80 in K19-kras

Figure 5. Detection of BMDCs in the gastric epithelium of recipient mice. Endogenous GFP fluorescence of BMDCs in gastric tissue of WT (A) or K19-kras (B) BMT recipients (original magnification, 150×), quantified in C (*, P < 0.01; n = 3 per group). D, representative E-cadherin (red) and GFP (green) in gastric tissue of K19-kras BMT recipients (original magnification, 200×). Magnified inset of a representative double positive cell (bone marrow–derived epithelial cell) and GFP single positive cells (bone marrow–derived stromal cells). E, representative GFP in K19-kras BMT recipients detected by immunohistochemistry (original magnification, 200×). F, Y-FISH (red) in a gastric tissue section of a K19-kras female receiving male donor BMT (original magnification, 400×).
BMT recipient mice. A plethora of GFP+ cells were found surrounding most clusters of Dcamkl1+ cells. Excessive αSMA+ stromal cells, as well as inflammatory F4/80+ macrophages, were typically seen surrounding these clusters, indicating that K-ras expression in adjacent putative progenitor cells favored an altered, inflammatory microenvironment (Fig. 6B).

Discussion

In this study, we show that oncogenic K-ras mutation in K19-expressing putative gastric epithelial progenitor cells activates inflammatory pathways and induces gastric atrophy, metaplasia, and dysplasia in a manner that largely parallels H. felis–induced carcinogenesis. K-ras–dependent chronic inflammation leads to recruitment of BMDCs that contribute primarily to the stromal microenvironment and correlates with the expansion of Dcamkl1+ putative progenitor cells and development of high-grade dysplasia and rarely intramucosal carcinoma. Interestingly, H. felis infection of K19-kras mice did not accelerate gastric cancer progression in these mice (data not shown), suggesting that the two models (K-ras mutation and H. felis infection) may progress through overlapping and/or nonadditive pathways. Taken together, K-ras mutation is able to compensate for a lack of infectious stimulus, such as H. felis infection, to induce inflammation and carcinogenesis.

The development of dysplasia in the K19-kras mouse was not entirely surprising, given the known role of K-ras as an oncogene for the pancreas and other organs in the mouse (11, 12, 27) and the fact that K-ras mutations have been reported rarely in gastric cancer (28, 29). Although previous reports have noted only gastric hyperplasia and mucous metaplasia in K19-kras mice up to 18 months of age (13), the current study is the first to examine the phenotype in a pure inbred (C57BL/6) background.

Previous studies by our group have modeled gastric cancer in mice using chronic Helicobacter infection or transgenic overexpression of cytokines that can drive gastric carcinogenesis independent of Helicobacter infection (4). Indeed, many cancers arise in the setting of chronic inflammation, which likely plays a role in both the initiation and promotion of tumors (30) and can lead to oncogenic mutations by inducing proliferation and increasing the mutation rate (31). However, cancers are also thought to arise in the absence of chronic inflammation through spontaneous or carcinogen-induced mutations in key oncogenes and tumor suppressor genes, with inflammation developing later on (32). In this latter model, transformed cells produce inflammatory mediators that attract tumor promoting inflammatory cells and set up

Figure 6. Characterization of BMDCs in BMT recipient mice. Gastric tissue sections from K19-kras BMT recipients detecting (A) endogenous GFP of BMDCs and stained red for CD45 (left), vimentin (middle), and αSMA (right) and (B) endogenous GFP (left, from a BMT recipient) or stained for F4/80 (middle, green) or αSMA (right, green) and stained for Dcamkl1 (red; original magnification, 400×).
cytokine networks. Accordingly, a number of studies have also shown that specific oncogenic mutations lead to a chronic inflammatory state (6, 7).

Oncogenic K-ras mutation in epithelial cells induces a number of molecular pathways that intrinsically regulate cellular processes such as proliferation, survival, and migration, which contribute to tumorigenesis (1, 12, 33). However, another consequence of oncogenic Ras signaling in carcinogenesis is the upregulation of cytokines and chemokines (9–11). Indeed, most of the cytokine/chemokines elevated in K19-kras mouse in this study have been reported to promote tumor development (4, 10, 34, 35). The development of chronic inflammation in the stomachs of K19-kras transgenic mice was associated with significant increases in CXCL1, IL-1β, and IL-6. It has previously been reported that oncogenic Ras mutations are able to induce the secretion of IL-6, which acts in a paracrine and autocrine fashion to promote tumor growth (34). K-ras has also been shown to upregulate the expression of IL-1β in epithelial cells, propagating and enhancing inflammatory networks (36). The chemokine CXCL1 is overexpressed in human gastric cancers (37) and is a known critical downstream mediator of mutant Ras that recruits neutrophils (38), supports angiogenesis (39), regulates the microenvironment (35), and directly promotes tumorigenesis (39). Moreover, CXCL1 (also known as Gro1) is associated with other epithelial cancers such as hepatocellular carcinoma, even in the absence of overt neutrophil recruitment (40). The induction of dysplasia by K-ras correlated most tightly in our study with increased CXCL1, but further studies will be required to understand the role of CXCL1 in gastric cancer progression.

K-ras is known to induce COX-2 expression largely through upregulation of COX-2 mRNA stability (41), and the phenotype of K19-kras mice shows striking similarities to mice that overexpress COX-2–derived prostaglandins in K19+ cells (25); notably, both K19-kras mice and K19-C2mE mice exhibit expansion of mucous-producing cells and eventual gastric tumor development. Although we were unable to detect increased COX-2 expression before the development of moderate dysplasia, it is interesting to speculate that it may have a role in phenotype development. Furthermore, we noted lymphoid aggregates in K19-kras mice similar to those found in H. felis–infected mice. Whereas the antigen responsible for this adaptive immune response is unknown, it is possible that mutant K-ras peptides may induce the antigenic response, as previously suggested (42, 43), and contribute to phenotype development.

Additional inflammatory chemokines and growth factors, such as CXCL5, HB-EGF, and amphiregulin, were expressed relatively specific to the K19-kras mice compared with H. felis–dependent chronic gastritis. CXCL5 is an additional potent neutrophil chemoattractant (44), linked to connective tissue remodeling, and has been associated with gastric cancer progression independent of Helicobacter infection (45, 46). HB-EGF is expressed by both myeloid cells (47) and gastric cancer cells (48) and has been shown to play a role in wound healing and carcinogenesis. Amphiregulin is also a member of the EGF family that is expressed by epithelial cells and fibroblasts (49) and induced in tumor cells with Ras mutations (50). Given the link between these growth factors and EGF receptor signaling, it seems likely that they contribute to the glandular proliferation and metaplasia phenotype in the K19-kras mice.

One of the consequences of K-ras–induced chronic inflammation was the recruitment of BMDCs to the stromal microenvironment in gastric dysplasia. Many of the BMDCs in BMT recipient K19-kras mice were infiltrating immune cells, consistent with the previous observations of lymphocyte infiltration in the pancreatic periduct and gastric mucosa of K19-kras mice (13). However, BMDCs in the K19-kras mouse also comprised fibroblasts and myofibroblasts [also known as cancer-associated fibroblasts (CAF)] that expressed vimentin ± αSMA, which surrounded the base of the dysplastic gastric glands. Stromal myofibroblasts are believed to contribute to the stem cell niche in the gastrointestinal tract (51, 52), but as yet, the effect of depleting these stromal cell populations is unknown. Previous studies have reported that many CAFs present in tumors are likely to be bone marrow derived (53), and evidence for the importance of a bone marrow contribution to tumor stroma has previously been recognized (54). Thus, this is the first study to show that K-ras mutation on its own can lead to the recruitment of bone marrow–derived CAFs. Few epithelial cells were bone marrow derived, in contrast to earlier reports in the H. felis murine model (3).

Interestingly, in the K19-kras mouse stomach, there was a gradual expansion of Dcamkl1+ cells that moved down to the base of the gastric glands and clustered in a region adjacent to bone marrow–derived myofibroblastic niche cells, similar to that observed in the intestinal crypts of APC/Min mice (20). Dcamkl1-expressing cells have been proposed as a candidate progenitor cell fraction in both the stomach and intestine (19, 20), raising the possibility that K-ras overexpression in K19 cells may lead to an expansion of the gastric progenitor pool in the K19-kras mouse. It is probable that the expansion of Dcamkl1+ cells was due to paracrine signals from stromal cells and/or adjacent K19+ putative progenitors rather than a direct effect of K-ras within Dcamkl1+ cells, given that K19 and Dcamkl1 were differentially expressed. However, to date the only reported marker to lineage trace an entire gastric gland is Lgr5 (25). Because Lgr5 lineage tracing is restricted to the antrum and dysplasia in K19-kras mice is found in the corpus, it is unlikely that Lgr5-derived cells are the primary source of the corpus dysplasia. However, definitive data as to the source of dysplastic lesions will require additional lineage tracing studies.

In conclusion, K-ras mutation in K19+ putative gastric tissue progenitor cells induced proinflammatory responses, resulting in early onset of gastric dysplasia. Dcamkl1+ cells were found clustered in dysplastic glands, adjacent to expanded K19+ regions. BMDCs contributed significantly to the microenvironment of dysplastic glands as immune cells, fibroblasts, and myofibroblasts. Further characterization of the relationship between K19+ and Dcamkl1+ cells in the normal gastric epithelium and dysplastic lesions may provide
greater insight into the cellular and molecular mechanisms of carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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