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Gastritis Promotes an Activated Bone Marrow-Derived Mesenchymal Stem Cell with a Phenotype Reminiscent of a Cancer-Promoting Cell

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Abstract

Background—Bone marrow-derived mesenchymal stem cells (BM-MSCs) promote gastric cancer in response to gastritis. In culture, BM-MSCs are prone to mutation with continued passage but it is unknown whether a similar process occurs *in vivo* in response to gastritis.

Aims—To identify the role of chronic gastritis in the transformation of BM-MSCs leading to an activated cancer-promoting phenotype.

Methods—Age matched C57BL/6 (BL/6) and gastrin deficient (GKO) mice were used for isolation of stomach, serum and MSCs at 3 and 6 months of age. MSC activation was assessed by growth curve analysis, FACS and xenograft assays. To allow for the isolation of bone marrow-derived stromal cells and assay in response to chronic gastritis, IRG/Vav-1^{Cre} mice that expressed both EGFP-expressing hematopoietic cells and RFP-expressing stromal cells were generated. In a parabiosis experiment, IRG/Vav-1^{Cre} mice were paired to either an uninfected Vav-1^{Cre} littermate or a BL/6 mouse inoculated with *H. pylori*.

Results—GKO mice displayed severe atrophic gastritis accompanied by elevated gastric tissue and circulating TGFβ by 3 months of age. Compared to BMMSCs isolated from uninfamed BL/6 mice, BM-MSCs isolated from GKO mice displayed an increased proliferative rate and elevated phosphorylated-Smad3 suggesting active TGFβ signaling. In xenograft assays, mice injected with BM-MSCs from 6 month old GKO animals displayed tumor growth. RFP+ stromal cells were rapidly recruited to the gastric mucosa of *H. pylori* parabionts and exhibited changes in gene expression.

Conclusions—Gastritis promotes the *in vivo* activation of BM-MSCs to a phenotype reminiscent of a cancer-promoting cell.

Keywords

cancer stem cell; Sonic Hedgehog; TGFβ; *Helicobacter pylori*

INTRODUCTION

Bone marrow-derived mesenchymal stem or stromal cells (BM-MSCs) were first identified as a progenitor population capable of differentiating along the adipogenic, osteogenic and chondrogenic cell lineages [1]. While recent work has focused on the regenerative capacity of BM-MSCs, numerous investigations highlight the potential for MSCs to promote cancer progression [2-4]. Gastric cancer is associated with the recruitment of bone marrow-derived cells [4]. These bone marrow-derived cells have since been confirmed to be mesenchymal stem cells (MSCs) that have undergone transformation into cancer-promoting cells [2,3]. When isolated from healthy mice and injected by tail vein into recipients chronically infected with *H. felis*, MSCs reverse the preneoplastic morphological changes present in the gastric epithelium and return normal histological appearance [5]. In contrast, studies show that endogenous MSCs exposed to the local and systemic pro-inflammatory milieu for the duration of *H. felis* infection contribute to a population of cancer-associated fibroblasts (CAFs) and acquire a phenotype that promotes the progression of gastric cancer development [2,6]. *In vivo* data on the mechanism initiating this transformative process is limited. However, collectively these investigations implicate the role of cytokines produced with chronic inflammation in this transformative process.

Although studies propose that during the early stages of inflammation-induced gastric cancer, the bone marrow undergoes remodeling in which MSC transformation is partly mediated by TGF β [2], the precise mechanism is unknown. What is known is that TGF β directly induces the expression of the family of Sonic Hedgehog (Shh) transcription factors Gli1 and Gli2 via Smad-3 [7]. In addition, in human BM-MSCs, Shh plays a role in the differentiation, clonogenicity and proliferation of these cells [8]. This suggests that Shh signaling may be upregulated in BM-MSCs through the convergence of inflammatory signaling pathways that in turn contributes to their aberrant proliferation. Here we investigate the role of TGF β and Shh as mediators of MSC transformation in response to chronic gastritis *in vivo*.

We tested the hypothesis that inflammatory signals produced during chronic gastritis act on MSCs within the bone marrow compartment *in vivo* to induce their aberrant proliferation and transformation. To test the hypothesis, two models of chronic gastritis were used. The first was the gastrin-deficient (GKO) mouse model. Prior studies in the GKO mouse revealed that these animals develop severe inflammation and mucous gland metaplasia as a consequence of bacterial overgrowth [9,10]. In fact, histological changes observed in the GKO mice are similar to the precursor lesions progressing to gastric cancer in human subjects [11]. GKO mice are hypochlorhydric from birth [12] and develop severe inflammation by 4 months of age and distal cancer within 12 months [9,10]. Parabiosis, the surgical joining of 2 mice to facilitate a shared blood supply, was then used to test the hypothesis that circulating signals play a key role in the alterations observed within the MSCs during chronic gastritis induced by *Helicobacter pylori* (*H. pylori*) infection. The current study demonstrates that in response to chronic gastric inflammation, BM-MSCs acquire a transformed phenotype that is characterized by the aberrant proliferation and

activation of the Hedgehog signaling pathway that may subsequently contribute to the tumor microenvironment.

MATERIALS AND METHODS

Animal Use

C57BL/6 (BL/6, strain #000664), IRG (strain #008705) and Vav-1^{Cre} (strain# 008610) mice used for these studies were purchased from Jackson Laboratories. IRG and Vav-1^{Cre} mice were bred in-house to obtain IRG/Vav-1^{Cre} progeny. Mice carrying the transgene(s) were genotyped using PCR of genomic DNA with specific primers for DsRed Forward: 5'-CCCATGGTCTTCTTCTGCAT-3', Reverse: 5'-AAGGTGTACGTGAAGCACCC-3' and Cre, Forward: 5'-AGATGCCAGGACATCAGGAACCTG-3', Reverse: 5'-ATCAGCCACACCAGACACAGAGATC-3'. Gastrin-deficient (GKO) mice backcrossed onto a C57BL/6 background were the gift of Dr. Linda Samuelson (University of Michigan). All mouse studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC) that maintains an American Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility.

Quantitative real-time PCR amplification of 16S rRNA gene sequences

BL/6 and GKO mouse stomachs were flushed with PBS (pH 7.4) and mucosal tissue samples were collected. The wet weight of tissue was determined and the samples were homogenized with a Tissue Tearor homogenizer (Biospec Products Inc, Bartlesville, OK) for 1 minute. The DNA was extracted and purified using QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) according to the manufactures instructions. The lysis temperature was increased to 95°C and an incubation with lysozyme was added (10mg/ml, 37°C for 30 minutes) to improve the bacterial cell rupture as previously described [13]. To quantify total bacteria, the following primers were used: Forward: ACTCCTACGGGAGGCAGCAG; Reverse: ATTACCGCGGCTGCTGG [14-16]. Total bacteria was measured by qRT-PCR using a Step One Real Time PCR machine (Applied Biosystems, Carlsbad, California USA) with SYBR Green PCR master mix (Applied Biosystems) and universal total bacteria primers in a 20µl final volume. Emitted fluorescence was measured during the annealing/extension phase and cycle of threshold (C_T) values were collected. Bacterial numbers were determined using standard curves constructed with the reference bacteria *E. coli* as previously described [17]. Briefly LB broth was inoculated with *E. coli* and grown at 37°C overnight with shaking. Serial dilutions of the cultures were plated on LB agar to quantify total colony forming units (CFU). Bacterial genomic DNA was isolated from the culture and was quantified by qRT-PCR as described above. Bacterial levels were quantified by comparing C_T values to samples of a known quantity of *E. coli* and corresponding CFU that were used to generate standard curves.

Histological Evaluation

Stomach sections spanning the fundus and antrum were collected from both BL/6 and GKO mice and fixed in 4% paraformaldehyde for 16 hours. Stomachs were then paraffin embedded and sectioned at 4 microns and stained with hematoxylin and eosin (H&E). All tissue processing and hematoxylin and eosin staining were performed by McClinchey

Histology Labs, Inc. (Stockbridge, MI). Histological score was graded on parietal cell loss (atrophy), foveolar hyperplasia, neutrophil and lymphocytic infiltration as previously performed [18]. A score of 1=5-25%, 2=26-50%, 3=51-75% and 4=76-100% of the total mucosa.

Isolation and culture of bone marrow-derived mesenchymal stem cells

Whole bone marrow was flushed from the femur and tibia of 3 and 6 month old age-matched BL/6 and GKO mice for subsequent culture and passage of the plastic adherent MSC population [19]. All cells were cultured using HyClone DMEM culture media supplemented with 15% fetal calf serum and 1% penicillin-streptomycin under normal conditions. After culture expansion, the Mouse Multipotent Mesenchymal Stromal Cell Marker Antibody Panel was used to label the 3 and 6 month MSC cell lines for Sca-1, CD106, CD105, CD73, CD29, CD44 and the hematopoietic markers CD45 and CD11b (R&D Systems, SC018). Cells were suspended at a concentration of 1×10^6 cells/ml and stained according to the manufacturer's protocol. Fluorescence intensity was measured by flow cytometry using the BD FACSCalibur and CellQuestPro software.

Growth Curve and Cell Cycle Analysis of MSCs

To determine growth rate, 3 and 6 month culture expanded MSCs isolated from BL/6 and GKO animals were plated at a density of 5000 cells/well in 24 well cell culture plates. Each day, cells were trypsinized and total cell count performed over a 6 day period. To measure proliferation, cells were stained with propidium iodide for 45 minutes for measurement of DNA content using the FACSCalibur™ system (Becton Dickinson). The measured values of peak fluorescence per total number of cells were obtained using the program CellQuest Pro (Becton Dickinson). The percentage of cells from the population in each phase of the cell cycle was calculated from the peak fluorescence measurements through analysis with ModFit LT software. All analyses were performed on a BD FACSCalibur flow cytometer and analyzed using FlowJo Software, Version 9.

Xenograft

BL/6 or GKO mice were prepared by first shaving the hind quarter to clear the site of injection. Subcutaneous injections at the hind limb with 1×10^6 6 month BL/6 or GKO MSCs were performed at Day 0. Measurements of tumor volume (mm^3) were made using digital calipers every 5 days or at the end of the 20 day period of analysis. Treated mice used in experiments were injected daily with either smoothened inhibitor Itraconazole ($2 \mu\text{g/ml}$ /mouse/day i.p.) [20] or SB-505124 ($5 \text{ mg/kg/mouse/day}$, i.p.) to maintain the desired effect of each compound on the injected MSCs.

MSC Treatment and Immunoblot Analysis

Total cell lysate was collected in protein lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl_2 , 2 mM CaCl_2 , 1% Triton X-100 in PBS, pH 7.4) supplemented with protease inhibitors (Roche 05892791001). For immunoprecipitation of Shh, media from cultured or treated cells was concentrated using Millipore Amicon Ultra-15 Centrifugal Filter Units. Concentrated media was incubated with 5E1 (Developmental Studies Hybridoma Bank, $2 \mu\text{g}$

antibody per 500 µg total protein) followed by incubation with Protein A/G Agarose Beads (Santa Cruz Biotechnology, sc2003), both for 16 hours at 4°C. Cell treatments were performed using 18 or 48 hour incubation with TGFβ inhibitor SB-505124 (Sigma S4696, 2 µg/ml) and/or rmTGFβ (R&D Systems, 5 µM).

All samples were loaded onto 4-20% Tris-Glycine Gradient Gels (Invitrogen) and run at 80 V, 3 hours. Transfer to nitrocellulose membranes (Whatman Protran, 0.45 µM) was performed at 105 V, 1.5 hours, 4°C. Membranes were blocked for 1 hour at RT using KPL Detector Block Solution (Kirkegaard & Perry Laboratories, Inc.). Membranes were incubated for 16 hours at 4°C with the following antibodies: Shh N-19 (Santa Cruz, sc-1194, 1:200), Gli1 (Cell Signaling #2534S, 1:500), Gli2 (R&D Systems AF3635, 2 µg/mL), Gli3 (R&D Systems AF3690, 3 µg/mL), SMAD-3 and pSMAD-3 (Cell Signaling, 9513S, 9520S, 1:500). An antibody against GAPDH (Millipore MAB374, 1:1500) was used for 1 hour at room temperature. The appropriate AlexaFluor 680 secondary antibodies (Invitrogen) were used at a dilution of 1:1000 for 1 hour at room temperature. Immunoblots were imaged using a scanning densitometer and the Odyssey Infrared Imaging Software System.

Luminex®-based Multiplex Assay

Tissue was homogenized in phosphate buffered saline supplemented with protease inhibitor (Roche 05892791001) and supernatant removed after centrifugation for 30 minutes at 13,000 rpm at 4°C. Whole blood was centrifuged at 3000 rpm for 15 minutes at 4°C to isolate serum. TGFβ concentrations in the tissue supernatant and serum were determined by enzyme-linked immunosorbent assay (ELISA) (Millipore, Billerica, MA) according to manufacturer's protocol. The cytokine analysis was conducted by the Cytokine and Mediator Measurement Core laboratory run by Dr. Marsha Wills-Karp (Cincinnati Children's, Digestive Health Center).

Helicobacter pylori culture, inoculation and confirmation of infection

Helicobacter pylori (*H. pylori*) SS1 (kindly donated by Dr. K.A. Eaton, University of Michigan) was grown on blood agar plates containing Campylobacter Base Agar (Fisher Scientific), 5% horse blood (BD Diagnostic Systems), 5 µg/ml vancomycin and 10 µg/ml trimethoprim. Plates were incubated for 2-3 days at 37°C in a humidified microaerophilic chamber. Bacteria were harvested and used to inoculate mouse stomachs by oral intubation over 3 consecutive days with 10⁸ *H. pylori* bacteria per 200 µl of Brucella broth. Mice were infected for 15 months prior to parabiosis surgeries.

H. pylori colonization was confirmed using the culture method previously published [21]. Briefly, tissue was homogenized in 1 ml saline and a dilution of 1:100 was spread on blood agar plates containing the components described above. Plates were incubated for 5-7 days at 37°C in a humidified microaerophilic chamber. Single colonies from the plates tested positive for urease (BD Diagnostic Systems), catalase (using 3% H₂O₂) and oxidase (DrySlide, BD Diagnostic Systems).

Parabiosis

IRG/Vav-1^{Cre} mice were paired to either an uninfected Vav-1^{Cre} littermate or a BL/6 mouse previously inoculated with *Helicobacter pylori*. Mice were anesthetized and aseptically prepared with administration of pre-emptive analgesics before surgical joining based on the protocol of Duymerman et al. [22]. Briefly, the parabiosis procedure was performed by making a mirror-image incision in each paired mouse from forelimb to hind limb and skin was freed from subcutaneous fascia. A mirror image abdominal incision was then made just below the liver in each animal and joined using sutures. The dorsal and ventral incisions were matched and secured using surgical staples. Animals were surgically paired for 21 days prior to analysis.

At post-operative days 3 and 7 peripheral blood from each parabiont was assessed for percentage of GFP+ and RFP+ cells using a BD FACSCalibur system and percentage of fluorescent cells quantified using CellQuestPro software. At postoperative day 21 whole bone marrow isolated from each parabiont was prepared for FACS to isolate the RFP+ fraction containing MSCs. Longitudinal stomach sections were fixed overnight in Carnoy's Fixative, paraffin-embedded and sectioned at 5 µm and prepared for immunofluorescence analysis.

Immunofluorescence

Longitudinal stomach sections were subjected to antigen retrieval after deparaffinization by heating the slides for 10 min at 100°C in 0.01 M sodium citrate. Non-specific antigenic sites were blocked with 20% normal goat serum in Tris buffered saline/0.1% tween 80 (TBS-T) for 1 hour at room temperature before incubating with antibodies specific for RFP (Abcam ab62341, 1:500) and CD4 (Abcam ab51312, 1:100) for 16 hours at 4°C. Sections were then incubated with AlexaFluor anti-rabbit 488 and AlexFluor anti-mouse 633 (Invitrogen, 1:100) for 1 hour at room temperature. Stained sections were mounted using VectaShield (Vector Labs) and imaged using a Zeiss LSM510 META Confocal Microscope.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from cultured cells using Trizol reagent according to the manufacturers protocol (TriReagent, Molecular Research Center, Inc.). RFP+ cells isolated by FACS from whole bone marrow of the IRG/Vav-1^{Cre} parabiont were collected in lysis buffer and RNA extracted using the Qiagen kit (RNEasy Mini Kit, cat. no. 74104) according to the manufacturer's protocol. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis following the recommended protocol. Prior to qRT-PCR analysis of cDNA from the RFP+ sorted population, pre-amplification was performed using TaqMan Preamp Master Mix (Invitrogen 4391128) with appropriate TaqMan primers. Gene expression analysis by qRT-PCR was performed using the Applied Biosystems StepOne Real Time PCR System and the following TaqMan Gene Expression Assays according to the recommended protocol: Shh, Mm00436528_m1; Gli1, Mm00494645_m1; Gremlin 1, Mm00488615_s1; Acta2, Mm00725412_s1; HPRT, Mm01545399_m1. The fold change of relative gene expression was determined relative to BL/6 MSCs or MSCs isolated from bone marrow of uninfected IRG/Vav-1^{Cre} parabionts using the C_T method [23].

Statistical Analysis

Results were analyzed using commercially available software (GraphPad Prism, GraphPad Software, San Diego, CA). A P value <0.05 was considered significant. Comparisons between two groups were made with the unpaired t -test and comparisons between more than two groups were done with ANOVA.

RESULTS

Gastrin-deficient (GKO) mice develop chronic gastritis, atrophy and metaplasia within 6 months of age

In the hypochlorhydric mouse stomach, the chronic gastritis, atrophy, metaplasia, dysplasia paradigm originally proposed by Correa et al. [11] can be recapitulated in gastrin-deficient (GKO) mice [9]. As previously documented [9] GKO mice developed inflammation, parietal cell atrophy and metaplasia (**Figure 1B, D**) when compared to the normal gastric morphology of age matched non-transgenic C57BL/6 (BL/6) controls (**Figure 1A, C**). Histological scores were recorded and supported that GKO mice developed significant inflammation and parietal cell atrophy by 3 months and metaplasia by 6 months of age when compared to the age matched non-transgenic controls (**Figure 1E**). Our prior studies in the GKO mouse revealed that these animals develop severe inflammation and mucous gland metaplasia as a consequence of bacterial overgrowth [10]. Consistent with these initial findings, we observed significantly elevated bacterial numbers in the gastric mucosa collected from the GKO mice at both 3 and 6 months of age compared to the BL/6 control mice (**Figure 1F**).

Bone marrow-derived mesenchymal stem cells (MSCs) isolated from inflamed GKO mice exhibit cell growth changes

To establish that the cultures of whole bone marrow contained a homogenous population of MSCs, the plastic-adherent population isolated from inflamed (GKO) versus non-inflamed (BL/6) mice were labeled for the representative CD markers (CD29, CD106, CD105, CD44 and CD73) and Sca-1 before analysis by flow cytometry. On average, greater than 90% of the MSCs isolated and expanded in culture from both BL/6 (**Supplemental Figure 1A**) and GKO (**Supplemental Figure 1B**) mice expressed CD29, CD106, CD105, CD44 and CD73 and Sca-1 and were negative for the hematopoietic markers CD45 and CD11b.

We first compared the proliferation rates of bone marrow-derived MSCs isolated from BL/6 (BL/6 MSC) and GKO (GKO MSC) mice. Independently of the cell culture vessel used, GKO MSCs isolated from 6 month-old animals reached confluency within 2 to 3 days of culture (**Supplemental Figure 2B**) compared to the BL/6 MSCs isolated from age-matched control animals (**Supplemental Figure 2A**). A cell growth curve comparing BL/6 MSC and GKO MSCs harvested from 3 and 6 month-old mice clearly demonstrated that GKO MSCs collected from 6 month-old animals proliferated at a faster rate compared to the other MSCs (**Figure 2A**). Increased proliferative rate was further confirmed by flow cytometric analysis of cell cycle progression that demonstrated a significant increase in the percentage of cells in S-phase within the GKO MSC population (**Figure 2C**) compared to the BL/6 MSCs (**Figure 2B**).

We then investigated whether GKO MSCs could generate tumors in vivo through a xenograft assay. GKO MSCs harvested from 4 individually inflamed GKO mice were subcutaneously injected into the hind flank of BL/6 recipient mice. All of these GKO MSCs formed tumors while no tumor development was observed in BL/6 recipients receiving BL/6 MSCs (**Figure 2D**), thus supporting a malignant transformation of these cells in response to chronic gastric inflammation. The BL/6 MSC and GKO MSC collected from 3 month-old mice were also tested for transformation by xenograft assay. While one out of the 4 GKO MSC lines did display tumor formation this was in comparison to all other groups in which tumors were not detected (data not shown). Final tumor volume in this 3 month GKO MSC line was also less than that observed across all GKO MSCs collected from 6 month old mice (data not shown). Therefore, for further analysis we assessed the BL/6 and GKO MSC lines collected from the 6 month old groups.

TGF β tissue and plasma concentrations are increased in inflamed GKO mice

It was clear from our comparative analysis of the BL/6 MSCs and GKO MSCs that a localized chronic infection caused a systemic inflammatory response that induced the transformation of MSCs within the bone marrow compartment. The transformation of GKO MSCs was characterized by aberrant cell proliferation and the capacity to induce tumor formation in vivo. In vitro studies propose that during the early stages of inflammation-induced gastric cancer, the bone marrow undergoes remodeling in which MSC differentiation is partly mediated by TGF β [2]. When compared to the BL/6 controls, we observed a significant increase in TGF β concentrations in both the stomach tissue (**Figure 3A**) and plasma (**Figure 3B**) collected from 3 and 6 month-old GKO mice. In addition, there was a significant increase in phosphorylated Smad 3 (p-Smad3) protein expression in whole bone marrow harvested from the GKO inflamed mice when compared to the BL/6 bone marrow (**Figure 3C, D**) that was indicative of activated TGF β signaling within the bone marrow compartment.

Chronic inflammation correlates with induced Hedgehog signaling within bone marrow-derived MSCs

TGF β directly induces the expression of the family of Hedgehog transcription factors Gli1 and Gli2 via Smad-3 [7] and may be involved in the malignant transformation of MSCs. **Figures 4A, B and C** shows that there was a significant increase in Shh protein expression and Hedgehog signaling as indicated by an increase in Gli1 in the 6 month GKO MSCs compared to the BL/6 MSCs (**Figure 4A-C**). In the presence of Shh and activation of Smoothened, Gli2 and Gli3 processing to the repressor form is blocked [24-26]. Consistent with activation of Shh signaling in the GKO MSCs, we observed a significant increase in the full-length Gli2 and Gli3 proteins and a decrease in the repressor fragment by immunoblot (**Figure 4A-C**).

Collectively, our data demonstrates that chronic gastric inflammation is associated with TGF β induced Hedgehog signaling within bone marrow-derived MSCs. To identify the TGF β signaling pathway leading to upregulated Shh signaling in transformed MSCs, MSCs isolated from bone marrow of 6 month old BL/6 mice were treated with either TGF β alone, TGF β plus intraconazole (Smo inhibitor) or TGF β plus SB-505124 (TGF β inhibitor). The

TGF β inhibitor SB-505124 was chosen as it specifically inhibits the downstream activation of the signal transducers Smad2 and Smad3 via ALK4, ALK5 and ALK7, consistent with our observation of increased pSmad-3 in whole bone marrow of 6 month old GKO mice [27]. Treatment with intraconazole did not alter the phosphorylation of Smad3 induced by TGF β treatment alone (**Figure 4D, E**). In fact, Shh expression downstream of TGF β treatment was inhibited by both intraconazole and SB-505123 treatment and was associated with elevated Gli1 (**Figure 4F**). Cell cycle analysis of treated cells by flow cytometry confirmed that MSC-derived TGF β promoted cell proliferation through autocrine activation of the Hedgehog signaling pathway, with a significant increase in MSCs in S phase that was abrogated with inhibition of both Hedgehog and TGF β signaling (**Figure 4G**).

The BL/6 MSCs, previously shown to be benign, promoted tumor development in xenograft assays using 12 month old, chronically inflamed GKO animals as recipients. Tumor development and growth completely regressed when recipient mice were treated with intraconazole or SB-505124 for the duration of the experiment (**Supplemental Figure 3**). Final tumor volume in the BL/6 MSC/GKO experimental group exceeded that of the GKO MSC/BL/6 previously assessed. This implies that increased circulating TGF β produced by the chronic immune response and the resulting increase in Shh within MSCs may be sufficient to promote malignant transformation.

Circulating signals during chronic *H. pylori* gastritis induce altered gene expression within bone marrow-derived MSCs collected from IRG/Vav-1^{Cre} parabionts

The data collected from the GKO mice suggests that a chronic inflammatory response localized within the stomach correlates with the malignant transformation of MSCs within the bone marrow. Such data suggests that circulating signals play a key role in the alterations observed within the MSCs during chronic gastritis. To allow us to isolate bone marrow-derived stromal cells and assay changes in response to chronic gastritis, we generated the IRG/Vav-1^{Cre} mice. The IRG/Vav-1^{Cre} mice were generated by crossing the IRG mice expressing a floxed DsRed-Express red fluorescent protein (RFP), to the Vav-1^{Cre} transgenic line expressing an enhanced green fluorescent protein (EGFP) [28]. The Vav-1^{Cre} mouse expresses an optimized variant of Cre recombinase to hematopoietic progenitors and lineages [29]. Thus, when Vav-1^{Cre} mice were crossed with the IRG mice, IRG/Vav-1^{Cre} animals expressed both EGFP- (green) cells of the hematopoietic lineage and RFP-expressing (red) stromal cells (**Figure 5A, B**). Parabiosis, the surgical joining of 2 mice to facilitate a shared blood supply, was used to test the hypothesis that circulating signals play a key role in the alterations observed within the MSCs during chronic gastritis. Our experimental strategy was such that an IRG/Vav-1^{Cre} mouse was paired with either an uninfected Vav-1^{Cre} mouse or a BL/6 mouse that had been infected with *H. pylori* for 12-15 months. Bone marrow was then collected from the IRG/Vav-1^{Cre} parabiont for FACS analysis 21 days post-surgical pairing (**Figure 5C**). **Figure 5D** demonstrates the surgical procedure used for pairing the mice. **Figure 5G and H** show equivalent percentages of GFP and RFP positive cells within the blood collected from both parabionts in the pair on post-surgical day 7, thus demonstrating a joined circulation.

Compared to bone marrow harvested from a Vav-1^{Cre} mouse (0.18%), gated RFP positive cells comprised approximately 2.87% of the total cell population within the bone marrow harvested from the IRG/Vav-1^{Cre} parabionts (**Figure 6A**, cells that were sorted are shown in the lower right quadrant). RFP positive cells were isolated by FACS, RNA was extracted and gene expression for Shh and Gli1 was analyzed by qRT-PCR. Consistent with our observations made in the malignantly transformed GKO MSCs, we observed a significant increase in Shh, Gli1, alpha smooth muscle actin (α SMA) and MSC marker Gremlin 1 (Grem1) gene expression in RFP positive cells sorted from the IRG/Vav-1^{Cre} parabionts paired with *H. pylori* infected mice (**Figure 6B**). Analysis of stomachs collected from *H. pylori* infected parabionts paired with the IRG/Vav-1^{Cre} mice revealed the rapid recruitment of RFP positive cells to the gastric mucosa within 21 days post-surgical attachment (**Figure 6D, F, H**), when compared to the stomachs of the uninfected IRG/Vav-1^{Cre} parabionts (**Figure 6C, E, G**). Data presented in **Supplemental Figure A-F** demonstrated a lack of RFP positive stromal recruitment to the stomachs of the uninfected parabionts.

Data presented in **Figures 5 and 6** demonstrate that circulating signals released during chronic gastritis results in induced Hedgehog signaling within bone marrow-derived stromal cells and the rapid recruitment of the RFP+ bone marrow population containing MSCs to the inflamed stomach. Consistent with our observations in the inflamed GKO mice, these changes were correlated with a significant increase in circulating levels of TGF β (**Figure 7**).

DISCUSSION

Mesenchymal stem cells are a population of cells within the bone marrow compartment with the capability of promoting cancer progression when recruited to areas of chronic gastric inflammation [2-4]. While the role of MSCs within the tumor stroma continues to be investigated, the mechanism that triggers the transformation of this cell type to a cancer-promoting phenotype prior to recruitment is largely unknown. We show that this process begins in the bone marrow in response to chronic gastritis. The first model of chronic inflammation that was used was the GKO mouse model, which exhibits similar precursor lesions progressing to gastric cancer in human subjects [9-11]. MSCs isolated from the inflamed GKO mouse model (GKO MSCs) exhibited changes that included elevated proliferative rate, induced Hedgehog signaling as early as 6 months after the initiation of chronic inflammation, and the capacity to induce tumor formation *in vivo*. This altered MSC phenotype correlated with an increase in circulating TGF β . Our *in vitro* studies demonstrated that TGF β -induced proliferation of MSCs was mediated by Hedgehog signaling. Furthermore, parabiosis experiments confirmed that circulating inflammatory signals played a key role in inducing Hedgehog signaling within MSCs.

MSCs isolated from the bone marrow of chronically inflamed GKO mice (GKO MSCs) exhibited an altered phenotype. This altered phenotype was documented by an aberrant proliferative rate and the capacity to induce tumor development *in vivo*. While several investigations have used similar assays to confirm transformation of both murine and human mesenchymal stem cells kept in long-term culture, few have demonstrated the same result *in vivo* within the bone marrow compartment [30-35]. Collectively, these data show similar changes occurring within transformed MSCs in both species, which include increased

proliferative rate and abnormal karyotype. In vitro transformation was shown to be related to changes in expression of oncogenes/tumor suppressor genes as well as those related to cell cycle and DNA repair [30,36]. These observations support the increased proliferative rate and maintenance of an activated phenotype in our GKO MSCs that is reminiscent of a cancer-promoting transformed cell.

The increase in cell proliferation we observed in the GKO MSCs was associated with increased Shh secretion. Increased Shh secretion was accompanied by the significant increase in the full length forms of each of the Gli transcription factors in the GKO MSCs compared to the BL/6 MSCs. The repressor forms of Gli2 and Gli3 were significantly decreased in MSCs isolated from either animal and supports autocrine Shh signaling pathway activation [24,25,37]. The Shh signaling pathway targets cell cycle proteins such as cyclin B1 and cyclin D1 [38,39].

MSC activation into an altered phenotype also coincided with increased circulating TGF β concentrations in both the GKO and *H. pylori* parabionts. Both TGF β and Shh signaling can promote MSC proliferation [2,8,40]. In our studies, TGF β signaling via SMAD3 was capable of inducing Shh production and the aberrant proliferation of MSCs, a process inhibited by both TGF β and Hedgehog signaling inhibitors. Malignant transformation of MSCs was assessed in a xenograft assay and again tumor development initiated by MSCs could be blocked by inhibition of TGF β or Hedgehog signaling. This data suggested that the Hedgehog signaling pathway components, such as the Gli family of transcription factors, may be downstream targets of TGF β and mediate MSC proliferation. In support of this hypothesis, *in vitro* studies using fibroblast and cancer cell lines show that TGF β induces Gli2 via SMAD3, which in turn upregulates Gli1 expression [7]. Continued work by this group identified a SMAD3/T cell factor binding site within the human Gli2 promoter region [41]. This may also imply a mechanism in which the PI3/Akt pathway via TGF β inhibits the negative regulation of Gli2 by PKA and GSK-3 β [42,43]. We have advanced these findings by showing that the effects of TGF β on MSCs were mediated by the Hedgehog signaling pathway.

Data collected from the parabiosis experiments suggested that circulating inflammatory signals play a key role in the gene alterations observed within the MSCs during chronic gastritis. Little is known about how increased systemic levels of the mediators of the immune response, such as TGF β , may activate signaling pathways leading to gene expression changes in MSCs. Mishra, et al., [44] show that tumor conditioned media promoted human MSC differentiation to a myofibroblastic or cancer-associated fibroblast phenotype based on the increased expression of α SMA, previously shown to be associated with TGF β . *In vivo* studies using *Helicobacter felis* infected mice transplanted with fluorescently labeled bone marrow-derived cells confirmed the differentiation of ~20% of MSCs recruited to the gastric mucosa to cancer associated fibroblasts, a population which proliferates in response to TGF β [2]. This is consistent with our findings using a chronically *H. pylori* infected parabiont paired to an IRG/Vav-1^{Cre} mouse. Analysis of BM-MSCs from the IRG/Vav-1^{Cre} parabionts showed that elevated circulating levels of TGF β correlated with gene expression changes in Shh and Gli1, the MSC marker Gremlin-1 and α SMA in the RFP+ stromal compartment. The BMP4 antagonist Gremlin-1 has recently been shown

to be a specific marker of tumor associated stromal cells and MSCs both in bone marrow and the tumor microenvironment [2] [45]. We extend our current knowledge of MSC transformation by demonstrating that circulating signals, such as TGF β , that are elevated during chronic gastritis alter the gene expression profile of MSCs within the bone marrow. These gene alterations that include induced Hedgehog signaling, Gremlin-1 and α SMA are consistent with a transformed MSC phenotype previously reported to be cancer-promoting [2].

The RFP+ stromal cells within the bone marrow of IRG/Vav-1^{Cre} parabionts were also rapidly recruited to the site of gastric inflammation. We observed RFP+ stromal cells within the stomachs of chronically *H. pylori* infected parabionts within 21 days post-surgery. In earlier reports using mice transplanted with fluorescently-labeled bone marrow, bone marrow-derived cells were recruited to the gastric inflammation only after 5-6 months of *H. pylori* infection [4]. We may account for the rapid infiltration of BM-stromal cells to the stomach by the increased expression of SDF-1 receptor CXCR4. Recent work using SDF-1 transgenic mice establishes a relationship between SDF-1-mediated recruitment of stromal cell populations within the stomach [6]. We and others have observed that the SDF1 receptor CXCR4 may be a target of the Shh signaling pathway [46](Donnelly and Zavros unpublished data). Overall, this could explain the rapid recruitment of our RFP+ stromal population but this requires further investigation.

Our study is of clinical relevance not only in gastrointestinal malignancies but also in cancer development throughout the body where MSCs have been implicated in tumor development [47-50]. This reinforces the importance of understanding how systemic inflammation induces the activation of MSCs into a cell type that has the potential to promote cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Shh	Sonic Hedgehog
GKO	gastrin-deficient mouse model
BL/6	C57BL/6 mice

TGF β transforming growth factor beta
H. pylori *Helicobacter pylori*

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Figure 1. Histological evaluation of BL/6 and GKO mouse stomachs

Hematoxylin and eosin (H&E) stains of stomach sections collected from 6 month-old (A) BL/6 and (B) GKO mice. Insets shown are a higher magnification of area in A and B (images captured at 40X magnification), dashed line indicates metaplastic region, arrow indicates inflammatory infiltrate. H&E stains of stomach sections collected from 12 month-old (C) BL/6 and (D) GKO mice. Images were captured at 10X magnification. (E) Histological score was graded on inflammation (neutrophil and lymphocytic infiltration) and parietal cell loss (atrophic gastritis). A score of 1=5-25%, 2=26-50%, 3=51-75% and 4=76-100% of the total mucosa. Each data point represents the histological score given for an individual animal. (F) Total bacterial numbers were quantified by qRT-PCR using RNA prepared from gastric tissue collected from 3 and 6 month-old BL/6 and GKO mice. Data are shown as calculated CFU/g tissue. * $P < 0.001$ compared to BL/6 group, $n = 4$ animals/group.

Figure 2. Proliferation of bone marrow-derived MSCs isolated from BL/6 and GKO mice
(A) Cell growth curve of MSCs collected from the bone marrow of 3 and 6 month-old BL/6 and GKO mice. *P<0.05 compared to the 3 month-old BL/6 MSCs. Flow cytometric graphs generated from cell cycle phase analysis by MODFitLT software showing changes in distribution of G0/G1, S and G2/M phases from MSCs isolated from (B) BL/6 and (C) GKO mouse bone marrow at 6 months of age. Table 1 shows tumor volume at day 20 of a xenograft assay using 6 month BL/6 and GKO MSCs in BL/6 recipients. *P<0.05 compared to the BL/6 group, n = 4 MSC lines isolated from the bone marrow of 4 individual mice.

Figure 3. Upregulated MSC-Derived Shh Corresponds to TGFβ Signaling Pathway Activation
 Luminex-based assay was performed for analysis of TGFβ cytokine levels in the (A) gastric tissue and (B) serum of 3 and 6 month BL/6 and GKO animals used for MSC isolation. An increased concentration of TGFβ is detected by 3 months in both the stomach and serum of GKO animals compared to BL/6 mice. (C) Whole bone marrow isolated from 6 month BL/6 and GKO mice shows significantly more phospho-Smad3 in the GKO versus BL/6 group, indicating TGFβ signaling pathway activation. (D) Quantification of immunoblots shown in C. n=4 mice per group.

Figure 4. TGF β stimulated increases in MSC proliferation are mediated by the Shh signaling pathway

(A) Representative immunoblots of Gli1, Gli2 and Gli3 expression in whole cell lysates from 6 month BL/6 MSCs or GKO MSCs. (B) Quantification of immunoblots in A. * $p < 0.05$ compared to BL/6 MSCs, $n = 4$ individual cell lines per group. (C) Representative immunoblot of Shh expression in immunoprecipitated media with quantification. * $p < 0.05$ compared to BL/6 MSC, $n = 4$ individual media samples collected from cultures of 6 month BL/6 and GKO MSCs. (D-G) 6 month BL/6 MSCs were treated overnight with 1: Vehicle, 2: rmTGF β , 3: rmTGF β + Hedgehog co-receptor Smoothened inhibitor (SmoI), or 4: rmTGF β + TGF β R inhibitor (SB-505124). (D) Representative immunoblots show expression of SMAD3 and phospho-SMAD3 (E) which are quantified, * $p < 0.05$ compared to vehicle treated groups, $n = 4$ individual treated cell lines. (F) Representative immunoblots show expression of Shh and GAPDH in treated 6 month BL/6 MSCs. (G) Cell cycle analysis by flow cytometry shows activation of Shh signaling downstream of TGF β induces increased MSC proliferation based on the significant increase in the percentage of S-phase cells. * $p < 0.05$, $n = 4$ individual treated cell lines.

Figure 5. IRG/Vav-1^{Cre} mice express RFP and GFP in the bone marrow compartment for *in vivo* tracking of MSCs with parabiosis

(A) The IRG mouse ubiquitously expresses a double fluorescent reporter (floxed RFP-GFP). The Vav-1 promoter in the Vav-1^{Cre} mouse drives expression of Cre recombinase in the hematopoietic compartment of the bone marrow. By crossing these two mice, IRG/Vav-1^{Cre} progeny contain double-labeled bone marrow with RFP+ expression in the mesenchymal or stromal cell population and GFP expression within the hematopoietic/immune cell population. (B) The genotyping strategy for these animals by RT-PCR examines RFP and Cre gene expression, with IRG/Vav-1^{Cre} animals expressing both. (C) Parabiosis experiments were performed using the IRG/Vav-1^{Cre} mice paired with uninfected Vav-1^{Cre} mice or BL/6 *H. pylori* infected animals (12-15 months post inoculation). (D) At day 0 of parabiosis, symmetrical incisions are made in the skin and peritoneum of each parabiont which allows for surgical joining. (E) Healing of the surgical incision and (F) a common abdominal cavity are apparent at day 21 of parabiosis. Individual peripheral blood samples isolated from the (G) IRG/Vav-1^{Cre} and (H) *H. pylori* infected parabionts were analyzed for RFP and GFP fluorescence by flow cytometry and show that a common circulation develops by day 7 of parabiosis.

Figure 6. Circulating factors upregulate Shh Signaling in the RFP+ population of the IRG/Vav-1^{Cre} parabiont bone marrow

(A) At day 21 post-surgery, bone marrow was isolated and the RFP+ (stromal) population sorted by fluorescence-activated cell sorting (FACS) (RFP+ 2.87%) from the IRG/Vav-1^{Cre} parabionts. The inset compared the RFP and GFP cell distribution in a nontransgenic wild type (WT) mouse. (B) qRT PCR analysis of the sorted RFP+ cells showed changes in expression of Shh signaling pathway genes, and MSC marker Gremlin1 (Grem1) and αSMA (typically expressed in fibroblast cell types). Representative hematoxylin and eosin (H&E) staining of stomach sections from the (C) IRG/Vav-1^{Cre} and (D) *H. pylori* infected parabiont at Day 21 of parabiosis. The chronically infected *H. pylori* gastric mucosa (F) shows recruitment of RFP+/CD4- stromal cells from the bone marrow of the uninfected IRG/Vav-1^{Cre} parabiont which are absent in the mucosa of an uninfected control parabiont (E). Higher magnification shown in (G, H). *p<0.05 compared to the sorted RFP+ cell population of uninfected parabionts, n=3 parabiosis pairs (IRG/Vav-1^{Cre} and Uninfected Control or IRG/Vav-1^{Cre} and *H. pylori* infected C57BL/6).

Figure 7. Circulating Levels of TGFβ increase in IRG/Vav-1^{Cre} mice paired to *H. pylori* infected mice

With the establishment of a common circulation, serum levels of TGFβ increase in both the *H. pylori* parabionts and the IRG/Vav-1^{Cre} mice they are paired to. This increase is not observed in the control parabionts, and no difference in circulating TGFβ levels was detected between paired IRG/Vav-1^{Cre} and Vav-1^{Cre} mice. *p<0.05, n=3 parabiosis pairs (9 peripheral blood samples isolated from individual parabionts).