Altered metaplastic response of waved-2 EGF receptor mutant mice to acute oxyntic atrophy

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Departments of 1Surgery and 2Cell and Developmental Biology, Vanderbilt University School of Medicine, and 3Nashville Department of Veterans Affairs Medical Center, Nashville, Tennessee; 4Department of Gastrointestinal Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; 5Physiological Laboratory, University of Liverpool, Liverpool, United Kingdom; and 6Columbia-Presbyterian Medical Center, New York, New York

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Ogawa, Masako, Sachio Nomura, Andrea Varro, Timothy C. Wang, and James R. Goldenring. Altered metaplastic response of waved-2 EGF receptor mutant mice to acute oxyntic atrophy. Am J Physiol Gastrointest Liver Physiol 290: G793–G804, 2006.—Metaplastic cell lineages are putative precursors for the development of gastric adenocarcinoma. The loss of parietal cells (oxyntic atrophy) is the initiating step in the evolution of gastric fundic mucosal lineage changes including metaplasia and hyperplasia. However, the intrinsic mucosal factors that promote and modulate the emergence of metaplastic phenotypes remain obscure. Over the past several years, we have studied pharmacologically induced, reversible oxyntic atrophy in rodents treated with DMP-777, a drug that acts as a parietal cell secretory membrane protonophore (21). Loss of parietal cells in response to DMP-777 leads to reversible oxyntic atrophy in rodents treated with DMP-777, a drug that acts as a parietal cell secretory membrane protonophore (21). The loss of parietal cells is associated with a number of lineage changes including foveolar hyperplasia, loss of chief cells, and mucous cell metaplasia (21, 39, 48).

Metaplastic cell lineages are considered precancerous precursors for the development of gastric adenocarcinoma. While intestinal metaplasia has received the most consideration as a precursor to neoplasia (9, 14), we and others have described SP-expressing metaplasia (SPEM) as an antralizing metaplastic lineage found in the fundic mucosa in the setting of oxyntic atrophy (41, 48). SPEM cells appear to arise from the bottom of fundic glands and replace the normal mucosal repertoire with an aberrant mucous cell lineage expressing SP/TFF2 (39).

Previous studies (11, 23, 41, 50) with gastric cancer resections from humans have shown that SPEM is more commonly associated with gastric cancer than intestinal metaplasia. While humans demonstrate both SPEM and intestinal metaplasia in the setting of atrophic gastritis and Helicobacter pylori infection, rodents only develop SPEM in the face of oxyntic atrophy and Helicobacter sp. infection (17, 18, 48). Thus mice infected with either H. felis or H. pylori develop oxyntic atrophy and extensive SPEM in fundic glands (17, 18). In mice chronically infected for over a year, dysplasia and intramucosal adenocarcinoma are frequently observed (5, 25, 47).

Parietal cell loss or oxyntic atrophy in response to H. pylori infection is the primary event in the evolution of the spectrum of metaplastic and hyperplastic lineage changes (12). However, the intrinsic mucosal factors that promote and modulate the emergence of metaplastic phenotypes remain obscure. Over the past 5 years, we have studied pharmacologically induced, reversible oxyntic atrophy in rodents treated with DMP-777 (21, 39). DMP-777 is a drug that selectively ablates parietal cells by acting as a parietal cell secretory membrane protonophore (21). Loss of parietal cells in response to DMP-777 leads to fundic mucosal lineage changes similar to those detected in Helicobacter sp.-infected animals, including the emergence of SPEM and foveolar hyperplasia, an expansion of surface mu-
ous cells (21, 39). Using gastrin-deficient mice, we (39) have recently demonstrated that gastrin is required for the development of foveolar hyperplasia but not for SPEM. Nevertheless, in gastrin-deficient mice treated with DMP-777, SPEM developed over a radically accelerated time course compared with wild-type C57BL/6 mice. While SPEM did not develop in wild-type mice until after 7 days of DMP-777 treatment, we observed extensive SPEM in gastrin-deficient mice treated with just a single dose of DMP-777 (39). These studies indicated that the absence of gastrin alters the fundic mucosal milieu, which is sensitized for the more rapid induction of metaplasia in response to the loss of parietal cells.

The objective of the present study was to provide further insights into the factors promoting the metaplastic phenotype by addressing the role of other endogenous regulatory factors in the emergence of SPEM after induced oxyntic atrophy. SP/TFF2 expression is a key marker for SPEM, but SP/TFF2 has also been implicated as a mucosal protective factor (2, 45). Therefore, to examine the possible influence of SP/TFF2 as an autocrine growth factor of metaplasia, we studied lineage changes after DMP-777 administration in SP/TFF2-deficient mice. Additionally, previous investigations have noted that EGF receptors are strongly enriched in chief cells (19, 26).

Thus, given the important role of EGF receptor ligands in regulating epithelial mucosal cell differentiation (24), we also evaluated the role of the EGF receptor signaling pathway by treating waved-2 mice, which carry a point mutation that reduces tyrosine kinase activity in the EGF receptor (35). Our results suggest that, whereas SP/TFF2 does not impact on the development of metaplasia after an induction of parietal cell loss, attenuation of EGF receptor signaling in waved-2 mice leads to accelerated development of SPEM.

**MATERIALS AND METHODS**

**Materials**

DMP-777, formulated at a concentration of 2% as a suspension in 0.5% methylcellulose, was a gift of DuPont Pharmaceuticals. 5-Bromo-2′-deoxyuridine (BrdU) was obtained from Sigma.

**Animals**

C57BL/6 mice were obtained from Charles River Breeding Laboratories or Jackson Laboratories. SP/TFF2-deficient mice were constructed by targeted deletion of the mouse SP/TFF2 gene (13) and were maintained on a C57BL/6 background. Waved-2 homozygous mice on a C57BL/6 background were obtained from Dr. David Threadgill (University of North Carolina). Waved-2 heterozygous control mice were constructed by breeding waved-2 male homozygotes to C57BL/6 wild-type female mice. During the experiments, mice were kept in cages provided with water and regular chow ad libitum until the time of death. All procedures were in accordance with an animal protocol approved by the Vanderbilt Institutional Animal Care and Use Committee.

**Study Design**

**SP/TFF2-deficient mice.** Male C57BL/6 wild-type control mice (8 wk of age) and male SP/TFF2-deficient mice (8 wk of age) were administrated DMP-777 once daily by oral gavage (350 mg·kg⁻¹·day⁻¹). Groups of three mice were killed before the DMP-777 dose and after 1, 3, 7, and 14 days of drug administration.

**Waved-2 mice.** Female waved-2 heterozygous control mice (8 wk of age) and female waved-2 homozygotes (8 wk of age) were administrated DMP-777 once daily by oral gavage (350 mg·kg⁻¹·day⁻¹). Groups of three mice were killed before the DMP-777 dose and after 1, 3, 7, and 14 days of drug administration. Additionally, three mice each received 14 days of DMP-777 treatment and were killed after 14 days of recovery off the drug.

**Necropsy and Tissue Processing**

BrdU in saline for injection (200 mg/kg) was administered to all the mice by intraperitoneal injection 2 h before necropsy. Each mouse was anesthetized with avertin and was perfused through the left ventricle with PBS (pH 7.4) for 1 min, followed by 4% paraformaldehyde for 9 min. The stomach was excised and opened along the greater curvature. The stomach was cut into 2-mm-wide strips parallel to the lesser curvature and embedded in paraffin, and 5-μm sections were prepared.

**Histological Examination**

**Histochemistry and immunohistochemistry.** To explore the alterations in the gastric fundic mucosa, we characterized each cell lineage by performing histochemistry and immunohistochemistry. In both SP/TFF2-deficient mouse and waved-2 mouse studies, histochemistry was performed for diastase-resistant periodic acid Schiff (DR-PAS) to detect surface mucous cells. For immunohistochemistry to detect parietal cells, chief cells, and proliferating S-phase nuclei, the following primary antibodies were used: a murine monoclonal IgG raised against H-K-ATPase (a gift of Dr. Adam Smolka, Medical University of South Carolina, Charleston, SC), rabbit polyclonal anti-human intrinsic factor (a gift from Dr. David Alpers, Washington University, St. Louis, MO), and biotinylated murine monoclonal anti-BrdU IgG (Zymed), respectively.

For immunohistochemistry to detect mucous neck cells and SPEM, HIK-1083, a murine monoclonal IgM antibody that recognizes mucous neck cell and antral gland mucous cell-derived MUC6 (Kanto Chemical; Tokyo, Japan), as well as a murine monoclonal IgM antibody against human SP/TFF2, a gift of Dr. Nicholas Wright (Cancer UK, London, UK), were used because MUC6 has been known to colocalize with SP/TFF2 (33). In the waved-2 mouse study, we stained sections with both anti-SP/TFF2 antibodies and HIK1083. In the SP/TFF2-deficient mouse study, HIK-1083 was used instead of anti-SP/TFF2 antibody.

For immunohistochemistry with anti-H-K-ATPase and anti-SP/TFF2, deparaffinized sections were blocked with blocking serum provided in the HistoMouse staining kit (Zymed) and with 1.5% goat serum provided in the Vectastain ABC KIT (Vector Laboratories; Burlingame, CA) after the antigen retrieval using citrate buffer in a rice cooker. Sections were incubated with a primary antibody (1:2,000 and 1:100 for anti-H-K-ATPase and anti-SP/TFF2, respectively) overnight at 4°C followed by incubation with a biotinylated secondary antibody and alkaline phosphatase-conjugated streptavidin (Vector RED ABC KIT, Vector Laboratories). Chromogen was developed with Vector red (Vector Laboratories).

For immunohistochemistry with HIK1083, the antibody against MUC6, deparaffinized sections underwent antigen retrieval using citrate buffer in a rice cooker after blockade of endogenous peroxidase activity with 3% H₂O₂. After the retrieval, sections were blocked as for the immunohistochemistry for SP/TFF2. Sections were incubated with a primary antibody (1:50) overnight at 4°C followed by incubation with a biotinylated secondary antibody and alkaline phosphatase-conjugated streptavidin. Chromogen was developed with diaminobenzidine (Biogenex; San Ramon, CA).

For immunohistochemistry with anti-intrinsic factor, deparaffinized sections were blocked with 1.5% normal goat serum and incubated with a primary antibody (1:1,000) overnight at 4°C followed by incubation with a biotinylated secondary antibody and alkaline phosphatase-conjugated streptavidin. Chromogen was developed with Vector red.
For immunohistochemistry of BrdU, a BrdU staining kit (Zymed) was used following the recommended instructions. In brief, sections underwent retrieval in 0.25% trypsin for 10 min after blockade of endogenous peroxidase activity with 3% H2O2. After an incubation with a blocking serum, sections were incubated with biotinylated murine monoclonal anti-BrdU overnight at 4°C followed by incubation with horseradish peroxidase-conjugated streptavidin. Chromogen was developed with diaminobenzidine.

For all staining, sections were counterstained with Gill’s hematoxylin and mounted. Sections were viewed and photographed on a Zeiss Axioshot bright-field microscope equipped with an Axiosiovision digital-imaging system.

Immunofluorescence. To detect SPEM, we performed double staining with anti-SP/TFF2 and anti-intrinsic factor in the waved-2 mouse study, because SPEM lineages were associated with the presence of intrinsic factor and SP/TFF2 dual-immunostaining cells at the base of fundic glands (39). In the SP/TFF2-deficient mouse study, anti-MUC6 antibodies were used instead of anti-SP/TFF2 antibodies.

To investigate the colocalization of SP/TFF2 with MUC6 during the development of SPEM, sections from waved-2 heterozygous and homozygous mice treated with DMP-777 for 0, 1, 3, 7, and 14 days were double labeled with anti-SP/TFF2 antibodies and anti-MUC6 antibodies.

For double staining with anti-intrinsic factor and either anti-SP/TFF2 or anti-MUC6, deparaffinized sections were blocked with blocking serum provided in the M.O.M. staining kit (Vector Laboratories) and incubated with anti-intrinsic factor (1:1,000) and anti-SP/TFF2 (1:100) or anti-MUC6 HKI-1086 (1:50) at the same time overnight at 4°C, followed by incubation with Cy2-labeled anti-rabbit IgG and Cy3-labeled anti-mouse IgM. After being washed with PBS, sections were washed in sodium phosphate for 3 min and mounted using Prolong antifade with DAPI (Molecular Probes).

For double staining with anti-SP/TFF2 and anti-MUC6, sections were deparaffinized and blocked as for the immunofluorescent double labeling with intrinsic factor and SP/TFF2. For this experiment, SP/TFF2 antibodies developed in the rabbit (a gift from Dr. Daniel Podolsky, Massachusetts General Hospital, 1:500) and anti-MUC6 antibody HKI-1086 (1:50) were detected with Cy2-labeled anti-rabbit IgG and Cy3-labeled anti-mouse IgM, respectively. After being washed, sections were mounted using Prolong antifade with DAPI.

All of the sections were viewed and photographed on a Zeiss Axioshot fluorescence microscope, and digital images were captured using a SPOT digital charge-coupled device camera.

Cell indexes. To quantify cell numbers in the gastric fundic mucosa, a section on the anterior side of the stomach that showed the fundic glands was chosen from each animal. Ten fundic glands were chosen from each section, and H-K-ATPase-positive cells, DR-PAS-positive cells, SP/TFF2-positive cells (in the waved-2 mouse study) or MUC6-positive cells (in the SP/TFF2-deficient mouse study), intrinsic factor-positive cells, and BrdU-positive nuclei were counted per ×200 field on a Zeiss Axioshot microscope. Intrinsic factor and SP/TFF2 double-immunofluorescent staining cells (in the waved-2 mouse study) or intrinsic factor and MUC6 double-immunofluorescent staining cells (in the SP/TFF2-deficient mouse study) were counted in overlayed images per ×200 field assembled in Adobe Photoshop.

Statistics. Differences in cell numbers were evaluated by ANOVA with post hoc comparison of significant means with Scheffé’s test using Statview-J 5.0.

Plasma gastrin measurement. Blood was collected from the retro-orbital plexus (SP/TFF2-deficient mouse study) or right ventricle (waved-2 mouse study) of three mice treated with DMP-777 for 0, 1, and 3 days, when the mice were killed under anesthesia. The plasma was isolated after centrifugation of the blood and kept frozen until the time of the measurement. Plasma gastrin levels (carboxyl-terminally amidated) were measured by radioimmunoassay using rabbit anti-serum L2, which reacts similarly with gastrin-17 and gastrin-34, as previously described (7).

RESULTS

Acute Oxyntic Atrophy in SP/TFF2-Deficient Mice

We have previously demonstrated that expression of SP/TFF2 characterizes the gastric metaplasia designated as SPEM. However, because SP/TFF2 might function as a local protective factor, it was unclear whether SP/TFF2 played a role as an autocrine or paracrine regulator in the emergence and maintenance of metaplasia. We therefore sought to evaluate the onset of gastric lineage changes in response to administration of DMP-777 to SP/TFF2-deficient mice treated for up to 14 days.

Parietal cells. Murine monoclonal antibodies against the α-subunit of H-K-ATPase were used to identify parietal cells. In both C57BL/6 wild-type control mice and SP/TFF2-deficient mice, >70% of parietal cells were labeled after a 1-day treatment with DMP-777 (Fig. 1A). In both groups, as previously reported (39), parietal cell numbers appeared to recover to an extent after mice were dosed with DMP-777 for 14 days. However, the parietal cell mass was still significantly reduced compared with untreated mice throughout the DMP-777 treatment period (Fig. 1A).

Surface mucous cells. We (39) have previously noted that DMP-777 treatment leads to foveolar hyperplasia, characterized as the rapid expansion of surface cell numbers, likely due to rapid elevation of serum gastrin levels in response to parietal cell loss. To assess foveolar hyperplasia, we stained mouse stomach sections with DR-PAS and quantified the numbers of surface mucous cells (Fig. 1B). Wild-type control mice demonstrated marked foveolar hyperplasia in the fundus after only 1 day of treatment with DMP-777 (Fig. 1B). In SP/TFF2-deficient mice, foveolar hyperplasia was also detected after 1 day of treatment with DMP-777, although it did develop more slowly compared with wild-type control mice. The extent of foveolar hyperplasia throughout the administration of DMP-777 was similar between both groups of animals (Fig. 1B).

Plasma gastrin levels. Because elevated gastrin levels are associated with foveolar hyperplasia (21, 39), we also examined plasma gastrin levels in C57BL/6 wild-type control mice and SP/TFF2-deficient animals treated with DMP-777 (Table 1). Wild-type animals dosed with DMP-777 developed marked hypergastrinemia with levels of 95–125 pM after only one dose of DMP-777 secondary to the hypochlorhydria in the face of potent inhibition of acid secretion and severe parietal cell loss. Although untreated SP/TFF2-deficient animals showed levels at the lower range of detection (13.3 ± 10.52 pM) with treatment of DMP-777, we did observe a six- to sevenfold increase in gastrin at both 1 and 3 days of treatment (91.2 ± 46.1 and 75.4 ± 8.64 pM, respectively). Mean plasma gastrin levels in SP/TFF2-deficient mice treated with DMP-777 for 1 and 3 days showed no significant differences compared with wild-type control mice treated for the same periods, which correlates with the similarity of the pattern of foveolar hyperplasia that wild-type control mice and SP/TFF2-deficient mice showed after DMP-777 treatment.

BrdU-positive proliferating cells. In our previous studies (21, 39), DMP-777 treatment induced a rapid increase in BrdU-positive cells in the fundic mucosa. To detect proliferating cells, mice were injected with BrdU before being killed.
Fig. 1. Effects of acute oxyntic atrophy on gastric lineages in wild-type and spasmolytic polypeptide (SP)/trefoil factor 2 (SP/TFF2)-deficient mice [SP/TFF2 knockout (KO) mice]. Cell lineage alterations were quantified in wild-type mice and SP/TFF2-deficient mice treated with DMP-777 for 0, 1, 3, 7, and 14 days. The mean numbers of cells per gland were determined for H-K-ATPase-positive parietal cells (A), diastase-resistant periodic acid Schiff (DR-PAS)-positive surface mucous cells (B), 5-bromo-2′-deoxyuridine (BrdU)-positive proliferating cells (C), mucin 6 (MUC6)-positive mucous cells (D), and intrinsic factor (IF)-positive cells (E). Data are represented as means ± SE. **P < 0.001 compared with untreated wild-type mice; #P < 0.001 compared with untreated SP/TFF2-deficient mice; +P < 0.05 vs. wild-type mice treated with the same dose of DMP-777. ++P < 0.01 compared with wild-type mice treated with the same dose of DMP-777.

Table 1. Plasma gastrin levels in DMP-777-treated mice

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<th>Day 0</th>
<th>Day 1</th>
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<td>C57BL/6 wild-type and SP/TFF2-deficient mice</td>
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<td>Wild-type</td>
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<td>116.33±20.17*</td>
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<td>SP/TFF2-KO</td>
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<td>91.20±4.60†</td>
<td>75.40±8.64†</td>
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<td>Waved-2 heterozygous and homozygous mice</td>
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<td>Waved-2 heterozygotes</td>
<td>49.33±21.14</td>
<td>175.00±15.28*</td>
<td>238.33±13.02*</td>
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<tr>
<td>Waved-2 homozygotes</td>
<td>17.00±4.36</td>
<td>53.33±8.41‡</td>
<td>230.00±15.00‡</td>
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Values are means ± SE (in pM); n = 3 mice/group. Plasma gastrin levels were measured for C57BL/6 wild-type mice and spasmolytic polypeptide/trefoil factor 2 (SP/TFF2) mice and for waved-2 heterozygous mice and waved-2 homozygous mice treated with DMP-777 for 0 (no treatment), 1, and 3 days of treatment. *P < 0.05 compared with untreated wild-type or heterozygous control mice; †P < 0.05 vs. untreated SP/TFF2-deficient or waved-2 homozygous mice; ‡P < 0.05 vs. control mice treated with the same dose of DMP-777.
dual-immunofluorescence labeling for intrinsic factor and MUC6 in wild-type mice and SP/TFF2-deficient mice. In both groups, animals treated with DMP-777 for 0 and 3 days showed a complete separation of intrinsic factor-staining and MUC6-staining cells (Fig. 3, *top*). However, intrinsic factor and MUC6 double-labeled cells were detected at the very bottom of the fundic glands after 7 and 14 days treatment. Also, the numbers of intrinsic factor and MUC6 double-positive cells significantly increased after 7 days of treatment with DMP-777 (Fig. 3, *bottom*). The appearance and increase of intrinsic factor and MUC6 double-positive cells at the bottom of the fundic glands after treatment with DMP-777...
suggest that intrinsic factor-positive chief cells may transdifferentiate into MUC6-positive metaplastic cells (Fig. 3).

All of these results suggested that, although in wild-type mice SPEM was characterized by the presence of SP/TFF2-positive cells at the bottom of the fundic glands, the onset of SPEM was not affected directly by expression of SP/TFF2, indicating that SP/TFF2 is not a paracrine or autocrine regulator of the metaplastic response to oxyntic atrophy.

Response of Waved-2 EGF Receptor Mutant Mice to Acute Oxyntic Atrophy

We and others (4, 22, 34, 38, 42) have previously noted the important role for the EGF receptor and its ligands in the regulation of both acid secretion and lineage determination in the gastric fundus. We therefore sought to evaluate the role of the EGF receptor in the regulation of foveolar hyperplasia and SPEM in response to oxyntic atrophy. We studied the effects of DMP-777 treatment on the emergence of lineage changes in waved-2 homozygous and heterozygous mice. Waved-2 mice carry a mutation in the EGF receptor that markedly reduces receptor tyrosine kinase activity. Although there is a reduction in EGF receptor signaling in these mice, previous investigations by Lee and colleagues (35) have demonstrated that the numbers of EGF receptors (EGF binding sites) are not altered significantly in waved-2 mice.

Parietal cells. To identify parietal cells, we stained mouse stomach sections with murine monoclonal antibodies against the γ-subunit of H-K-ATPase. Untreated waved-2 heterozygous control mice and homozygous mice showed no significant difference in mean parietal cell numbers (Fig. 4A). In both groups, >70% of parietal cells were ablated after a 1-day treatment of DMP-777. As with wild-type mice, parietal cell numbers partially recovered after DMP-777 treatment for 14 days but remained significantly reduced compared with untreated animals. Similar to the results seen previously in wild-type mice (39), 14 days after the cessation of DMP-777 treatment, both waved-2 heterozygous control mice and homozygous mice showed a rebound increase in parietal cell numbers to 162% and 150% of untreated levels, respectively (Fig. 4A).

Surface mucous cells. Histochemistry was performed for DR-PAS staining to assess surface mucous cell numbers. Whereas heterozygotes demonstrated marked foveolar hyperplasia in the fundus with prominent expansion of DR-PAS-positive surface mucous cells after only a 1-day treatment with DMP-777 (Figs. 4B and 5), waved-2 homozygote mice showed a delay in significant foveolar hyperplasia until 3 days of treatment. Waved-2 homozygotes showed foveolar hyperplasia similar to heterozygotes only after 7 days of treatment (Figs. 4B and 5). In both groups, foveolar hyperplasia was completely reversible, and withdrawal of the drug led to a decline in DR-PAS-positive cells to numbers similar to those in untreated mice 14 days after the cessation of treatment (Figs. 4B and 5).

Fig. 4. Effects of acute oxyntic atrophy on gastric lineages in heterozygous and homozygous waved-2 mice. Cell lineage alterations were quantified in control heterozygous waved-2 (wa-2) mice and homozygous waved-2 mice treated with DMP-777 for 0, 1, 3, 7, and 14 days or treated for 14 days and killed after 14 days of recovery off the drug (R14). Mean numbers of cells per gland were determined for H-K-ATPase-positive parietal cells (A), DR-PAS-positive surface mucous cells (B), BrdU-positive proliferating cells (C), SP/TFF2-positive mucous cells (D), IF-positive cells (E), and cells double labeled with IF and SP/TFF2 (F).
As noted above, our previous investigation using gastrin-deficient mice suggested that gastrin was the major factor that drove foveolar hyperplasia (39). Stimulation of the EGF receptor signaling pathway is known to promote gastrin expression and secretion (15). We therefore examined plasma gastrin levels in waved-2 heterozygous control mice and waved-2 homozygotes treated with DMP-777 (Table 1). In waved-2 heterozygotes, DMP-777 elicited a rapid rise in the serum gastrin level from 49 pM in untreated mice to 175 and 238 pM in mice treated for 1 or 3 days, respectively. In contrast, waved-2 homozygotes demonstrated only a small rise in plasma gastrin initially after one dose of DMP-777 (from 17 to 53 pM). However, after 3 days of DMP-777 treatment, homozygous waved-2 mice developed hypergastrinemia to a similar level compared with heterozygotes.

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**BrdU-positive proliferating cells.** To detect proliferating cells, mice were injected with BrdU before being killed, and S-phase nuclei were detected by murine monoclonal anti-BrdU. In waved-2 heterozygous control mice, BrdU-immunoreactive cell numbers started to increase after 1 day of DMP-777 treatment. In waved-2 homozygotes, BrdU-immunoreactive cell numbers started to increase after 3 days of DMP-777 treatment. The number of BrdU-positive cells increased further after 7 and 14 days of treatment. In contrast, waved-2 homozygotes demonstrated only a small rise in plasma gastrin initially after one dose of DMP-777 (from 17 to 53 pM). However, after 3 days of DMP-777 treatment, homozygous waved-2 mice developed hypergastrinemia to a similar level compared with heterozygotes.

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treatment and were significantly elevated by 3 days of treatment and throughout the rest of the drug treatment period (Fig. 4C). In waved-2 homozygotes, the increase in BrdU-positive cell numbers was slightly blunted at 1 day of treatment but also reached significantly increased levels after 3 days of treatment. As previously reported (39), BrdU-positive cells were detected not only in the normal progenitor zones in the gland neck but also in the deep regions of the gland during the dosing with DMP-777 (data not shown), suggesting the activation of proliferative cells in the face of parietal cell loss. Fourteen days after cessation of the drug, the BrdU-labeling index decreased back toward control levels, and there was a complete loss of BrdU-labeled cells from the basal portion of the fundic glands in both groups.

**SP/TFF2-expressing cell lineages.** In the normal fundic mucosa, SP/TFF2 is expressed in mucous neck cells located in the midportion of the fundic gland (20). Wild-type mice display SP/TFF2-immunoreactive cells at the base of the glands, consistent with the SEM cell phenotype, after 7 days of DMP-777 treatment and a loss of normally staining mucous neck cells (39). After withdrawal of drug treatment for 14 days, both the number of SP/TFF2-staining cells and the pattern of staining returned to the morphological appearance of normal mucous neck cells. Similarly, waved-2 heterozygote control mice developed SP/TFF2-immunostaining SEM after 7 days of DMP-777 treatment (Fig. 5). In contrast, waved-2 homozygous mice showed accelerated SEM development with a significant increase in the numbers of SP/TFF2-positive cells by 3 days of treatment with DMP-777 (Figs. 4D and 5). In both groups, the number of SP/TFF2-staining cells and the pattern of staining returned to the morphological appearance of those in untreated animals after withdrawal of the drug treatment for 14 days (Figs. 4D and 5).

**Intrinsic factor-immunoreactive cells.** In both waved-2 heterozygotes and waved-2 homozygotes, as in wild-type mice and SP/TFF2-deficient mice, the numbers of intrinsic factor-immunoreactive cells showed a significant decrease after 1 day of treatment with DMP-777 (Fig. 4E) and then subsequently increased after 3 days of dosing. However, waved-2 homozygous mice showed an earlier recovery of intrinsic factor-immunoreactive cells by 3 days of treatment.

**Intrinsic factor and SP/TFF2 double-immunostaining cells.** To evaluate the onset of SEM, we performed dual-immunofluorescence labeling for intrinsic factor and SP/TFF2 in waved-2 heterozygous control mice and waved-2 mice (Fig. 4F). In heterozygotes, there was a significant increase in the numbers of intrinsic factor and SP/TFF2 double-positive cells after 7 days of DMP-777 treatment, whereas homozygotes showed a significant increase in double-positive cells after only 3 days, correlating with the accelerated appearance of SEM.

**MUC6 staining and dual immunofluorescence for SP/TFF2 and MUC6.** To evaluate the colocalization of SP/TFF2 with MUC6 during the development of SEM, sections from waved-2 heterozygous and homozygous mice treated with DMP-777 were stained for MUC6. We also performed the double-immunofluorescent labeling with SP/TFF2 and MUC6. MUC6-immunoreactive cells were detected in mucous neck cells at the midportion of the fundic glands in untreated mice (Fig. 6A). Whereas MUC6-immunoreactive cells were detected at the bottom of the fundic glands after 7 days of DMP-777-treatment in waved-2 homozygous control mice (data not shown), homozygous mice displayed MUC6-immunoreactive cells at the base after 3 days (Fig. 6A). Thus both waved-2 heterozygotes and homozygotes showed MUC6-positive metaplasia along with a similar time course to SEM displayed by SP/TFF2 immunostaining (Fig. 5). Furthermore, SP/TFF2 staining and MUC6 staining showed a complete colocalization throughout the dosing with DMP-777 in waved-2 heterozygotes (data not shown) and homozygotes (Fig. 6B). All of these results support the conclusion that SEM expresses both SP/TFF2 and MUC6.

**DISCUSSION**

The loss of fundic parietal cells is a seminal event in the evolution of atrophic gastritis and the attendant changes of foveolar hyperplasia and gastric metaplasia. A number of genetic manipulations of the fundic mucosa have developed various aspects of oxyntic atrophy. Recently, Katz and colleagues (29) noted that Kru¨ppel-like factor 4-deficient mice have profound deficiency in parietal cells with prominent expansion of SEM throughout the fundus (29). These mice demonstrate that SEM can develop in the absence of infection and inflammation, although chronic inflammation induced mainly by Helicobacter sp. infection is thought to be the potent exogenous promoter of lineage changes in the gastric fundus. However, the endogenous factors regulating the onset of gastric metaplasia remain more obscure. For several years, we have studied the model of DMP-777-induced metaplasia, which provides both a readily inducible and readily reversible metaplastic response to parietal cell loss. The model of DMP-777 administration in rodents provides a unique opportunity to study the effectors involved in metaplasia, because the drug-induced development of SEM does not involve either infection or inflammation (21). Indeed, DMP-777-treated mice show little inflammatory response in the mucosa (39), likely secondary to the action of the compound as a potent neutrophil elastase inhibitor. We (39) have previously noted that SEM develops more rapidly in gastrin-deficient mice treated with DMP-777, indicating the importance of gastrin as a hormonal regulator of gastric lineage differentiation. We have now sought to evaluate the role of putative intrinsic gastric growth factors in regulating the emergence of SEM. While the present study indicates that SP/TFF2, a critical marker of SEM, does not influence the emergence of SEM after DMP-777 treatment, attenuation of EGF receptor signaling in waved-2 mice does elicit a more rapid emergence of SEM. The results support a role for EGF receptor signaling in the emergence of SEM after parietal cell loss.

Several previous studies (1, 2, 8, 28, 45) have associated TFFs with regulation of mucosal integrity throughout the gastrointestinal tract. Three TFFs exist in the mammalian gastrointestinal tract (40). TFF1 is secreted in concert with the release of MUC5AC by surface mucous cells of the gastric fundus and antrum. TFF1-deficient mice develop hyperplastic polyps and gastritis cystica profunda in their antral mucosa with relative sparing of the fundus (32). TFF3 is found in goblet cells of the intestinal mucosa, where it is cosecreted with MUC2. TFF3-deficient mice are phenotypically normal but show increased susceptibility to colonic damage (46). SP/TFF2 is present in mucous neck cells in the gastric fundus,
deep antral gland cells, and duodenal Brunner’s gland cells. In all cases, SP/TFF2 is cosecreted with MUC6. SP/TFF2-deficient mice are phenotypically normal but demonstrate elevated gastric acid secretion and an increased susceptibility to gastric mucosal damage (13). Given the putative role of SP/TFF2 in regulating mucosal protection, we sought to evaluate whether SP/TFF2 could act as a requisite autocrine or paracrine factor in the emergence of SPEM after a loss of parietal cells. To study the SPEM lineage in SP/TFF2-deficient mice, we analyzed the emergence of a MUC6-staining antral type metaplastic lineage, consistent with the phenotype of SPEM. SP/TFF2 and MUC6 showed a complete colocalization throughout the development of SPEM in waved-2 as well as wild-type mice (data not shown). Our results here indicate that SPEM emerges after a parietal cell loss along a similar time course in wild-type and SP/TFF2-deficient mice. Thus SP/TFF2 and MUC6 are markers of lineage derivation in SPEM rather than secreted factors required for the emergence of SPEM.

We also evaluated the role of the EGF receptor signaling pathway, because it influences a number of aspects of gastric lineage differentiation including cell proliferation, cell differentiation, and acid and mucous secretion (24). Parietal cells are known to secrete several EGF receptor ligands including TGF-α, amphiregulin, and HB-EGF (3, 30, 37). TGF-α is a paracrine and autocrine regulator both inhibiting acid secretion and promoting parietal cell differentiated function (6, 34). Overexpression of TGF-α leads to expansion of the foveolar surface mucous cell compartment in both metallothionein (MT)-TGF-α transgenic mice (22, 42) and in Ménétrier’s disease patients (10, 38). Although overexpression of TGF-α does yield an antralizing phenotype, the process appears to be separable from that of oxyntic atrophy associated with either
demonstrated in expression (15), the modified response to DMP-777 treatment EGFR receptor signaling pathway is known to stimulate gastrin influence for foveolar hyperplasia and that the absence of treatment by studying gastrin-deficient mice. DMP-777-treated a rapid rise in plasma gastrin in response to hypochlorhydria days of treatment compared with a normal pattern of SPEM hand, they displayed an accelerated emergence of SPEM by 3 showed a delayed onset of foveolar hyperplasia. On the other 777.

heterozygous mice, SP/TFF2-deficient, and waved-2 homozygous mice treated with DMP-777 showed a delayed emergence of SPEM by 3.

DMP-777 in wild-type, SP/TFF2-deficient, and waved-2 homozygous mice, waved-2 homozygous mice did not develop foveolar hyperplasia, but the drug treatment did elicit SPEM after only a single dose (39). These results suggested that gastrin was a requisite influence for foveolar hyperplasia and that the absence of gastrin accelerates the emergence of SPEM (39). Because the EGFR receptor signaling pathway is known to stimulate gastrin expression (15), the modified response to DMP-777 treatment demonstrated in waved-2 mice could be referable to a decrease in gastrin secretion. Indeed, whereas the elevation of plasma gastrin reached its maximal levels after a 1-day treatment with DMP-777 in wild-type, SP/TFF2-deficient, and waved-2 heterozygous mice, waved-2 homozygous mice did show a delay in gastrin elevations. The delay in elevations in plasma gastrin is consistent with the slower induction of foveolar hyperplasia in waved-2 homozygous mice. In contrast, waved-2 homozygous mice developed accelerated SPEM after 3 days of DMP-777 treatment despite the presence of hypergastrinemia. These results indicate that the influence of the impaired EGFR receptor signaling pathway on the development of SPEM after DMP-777 treatment in waved-2 homozygous mice is predominantly independent of plasma gastrin levels. The results did not result from alterations in endocrine cell numbers because gastrin and somatostatin cell numbers were not altered (data not shown). We (39) have previously suggested that SPEM cells arise from the transdifferentiation of chief cells, based on the observation of cells expressing both intrinsic factor and SP/TFF2 at the bases of the fundic glands after a single dose of DMP-777 in gastrin-deficient mice. Although a previous investigation (36) has suggested that EGF receptors are enriched in human parietal cells, it is important to note that several investigators have reported that EGF receptors are enriched in rodent chief cells (19, 26). Thus alterations in EGF receptor ligands might have particular influence on the differentiation and function of chief cells. All of these results support a higher sensitivity to parietal cell loss in EGF receptor-impaired waved-2 mice.

The induction of metaplasia in the gastric fundus likely represents a physiological response to local mucosal injury, which can take on pathological consequences under the influence of chronic inflammatory influences. The DMP-777 treatment paradigm represents a unique vehicle for studying the onset of metaplasia after the loss of parietal cells in the absence of inflammatory infiltrate. With chronic injury in the presence of inflammation, the metaplastic response may progress toward neoplasia. Indeed, previous investigations have supported the concept that inflammation is required for neoplastic progression (16). As noted above, chronic infection of C57BL/6 mice with Helicobacter sp. leads to both oxyntic atrophy and SPEM (17, 47, 48). Recent investigations have demonstrated that eradication of Helicobacter at early time points of infection can reverse the progression toward metaplasia (5). However, once the metaplasia is established, metaplasia may be partially irreversible even though progression to neoplasia is inhibited by eradication. Once the metaplasia is established, it also may serve as a focal point for engraftment of bone marrow-derived cells. Houghton et al. (25) have noted that in the setting of chronic inflammation, bone marrow-derived cells engraft as SPEM and can further progress toward dysplasia. Animals treated for up to 2 yr with DMP-777 have prominent SPEM but never progress to cancer (21). The data from DMP-777-treated animals suggesting that SPEM development accrues from a loss of parietal cells, we favor a two-step model where, first, parietal cell loss would lead to the emergence of SPEM. Second, in the presence of chronic inflammation, as in the setting of Helicobacter sp. infection, SPEM may serve as a focal point for engraftment of bone marrow-derived cells. These cells could then adopt the metaplastic phenotype and prove susceptible to further neoplastic transitions. In any case, it is clear that the focal point for the process is the inciting of metaplasia by the loss of parietal cells. While this process may be influenced by cytokine release associated with chronic inflammation, the actual induction of metaplasia in the face of parietal cell loss appears to be regulated by hormonal and paracrine pathways intrinsic to the gastric mucosa.

In summary, we have demonstrated that, although SP/TFF2 is not required for the development of SPEM after the loss of parietal cells, attenuation of the EGFR receptor signaling pathway leads to the accelerated emergence of SPEM in response to DMP-777-induced oxyntic atrophy. These results demonstrate that both hormonal (gastrin) and paracrine (EGF receptor ligand) regulators influence the metaplastic response to parietal cell loss in the gastric fundic mucosa.

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