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Nardilysin regulates inflammation, metaplasia, and tumors in murine stomach

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Chronic inflammation contributes to a wide variety of human disorders. In the stomach, longstanding gastritis often results in structural alterations in the gastric mucosa, including metaplastic changes and gastric cancers. Therefore, it is important to elucidate factors that are involved in gastric inflammation. Nardilysin (N-arginine dibasic convertase; Nrdc) is a metalloendopeptidase of the M16 family that promotes ectodomain shedding of the precursor forms of various growth factors and cytokines by enhancing the protease activities of a disintegrin and metalloproteinase (ADAM) proteins. Here, we have demonstrated that Nrdc crucially regulates gastric inflammation caused by Helicobacter felis infection or forced expression of prostaglandin E 2 in K19-C2mE mice. Metaplastic changes following gastric inflammation were suppressed by the deletion of Nrdc. Furthermore, the deletion of Nrdc significantly suppressed N-methyl-N-nitrosourea (MNU)-induced gastric tumorigenesis in the murine stomach. These data may lead to a global therapeutic approach against various gastric disorders by targeting Nrdc.

Chronic inflammation contributes to a wide variety of human disorders. In the stomach, most lifelong chronic gastritis is caused by Helicobacter pylori infection 1, although other etiologies, such as non-steroidal anti-inflammatory drugs or autoimmunity, are also prevalent 2. Longstanding gastritis can result in structural alterations in the gastric mucosa, including metaplastic changes and gastric cancers. Metaplastic changes in the stomach are associated with an increased risk of gastric cancer 3. Therefore, it is important to elucidate factors that are involved in gastric inflammation for effective prevention of various gastric diseases, including gastric cancer 4.

The local microenvironment influences the development of chronic gastritis and metaplastic changes regardless of causative factors, and inflammatory cytokines participate in constructing this environment. There are a number of factors regulating inflammatory cytokines. A disintegrin and metalloproteinase (ADAM) family proteins are involved in ectodomain shedding, and regulate the biological activities of structurally and functionally diverse inflammatory cytokines in a context-dependent manner 5, 6. Indeed, dysregulation of ectodomain shedding of those factors can be profoundly involved in the pathogenesis of a serial development of gastritis, metaplasia, and gastric cancer 7.

Nardilysin (N-arginine dibasic convertase; NRDC), a zinc peptidase of the M16 family that selectively cleaves dibasic sites 8, is diffusely localized in the cytoplasm, and is secreted to the cell surface by undetermined mechanisms 9. We previously identified NRDC as a specific binding partner of heparin-binding epidermal growth factor-like growth factor (HB-EGF) 10. NRDC also enhances the shedding of tumor necrosis factor-α (TNF-α) through activation of ADAM17 11, 12. TNF-α is produced as a membrane-anchored protein, shed from the cell surface by proteolytic cleavage, and subsequently activated. The proinflammatory genotype of TNF-α is associated with more than twice the risk of non-cardia gastric cancer 13. In this respect, we previously demonstrated that NRDC regulates activation of TNF-α and subsequent production of inflammatory cytokines in gastric cancer cells 14. These findings suggest that NRDC regulates chronic inflammation and tumorigenesis in the stomach; however, the in vivo role of NRDC in the stomach is still unclear.

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In this study, we examined the role of Nrdc during the development of chronic gastritis and metaplastic changes in the stomach using Nrdc knockout mice. We also investigated the effect of Nrdc in chemically-induced gastric tumorigenesis.

Results
Nrdc did not alter the differentiation status of gastric mucosa under physiological conditions. We first examined the effects of Nrdc deletion in the stomach under physiological conditions. Nrdc−/− mice tend to maintain lower body weights than Nrdc+/+ mice. Therefore, the mean stomach size of Nrdc−/− mice was slightly smaller than that of Nrdc+/+ mice (Fig. 1A). Other than the stomach size, there were no macroscopic differences between Nrdc+/+ and Nrdc−/− mice. Although the gastric mucosa was slightly thinner in Nrdc−/− mice.
(Fig. 1B), there were no significant differences in the differentiation status of gastric mucosal cells (Fig. 1C). Immunohistochemistry showed that the percentages of pepsinogen II-positive cells within gastric glands were not significantly different between Nrdc+/− mice and Nrdc−/− mice. This indicated that chief cell differentiation was not affected by Nrdc status (Fig. 1C and D). Further, immunostaining for H+-K+-ATPase, Muc5ac, and TFF2 did not show significant differences between Nrdc+/− and Nrdc−/− mice, indicating that differentiation of parietal, pit, and mucous neck cells was not regulated by Nrdc under physiological conditions, respectively (Fig. 1C and D). Furthermore, the proportion of Ki67-positive cells in the gastric glands of Nrdc−/− mice was similar to that of Nrdc+/− mice. Therefore, deletion of Nrdc did not alter epithelial cell proliferation under physiological conditions (Fig. 1C and D).

Gastritis caused by Helicobacter felis infection was attenuated by Nrdc deletion. Helicobacter felis infection is a well-characterized mouse model that mimics chronic Helicobacter pylori infection in the human stomach16. Based on a previous report16, we administered suspending water containing Helicobacter felis to Nrdc+/− and Nrdc−/− mice for three days. Mice were sacrificed 20 weeks after the end of administration. Regardless of Nrdc gene status, Helicobacter felis was infected successfully into the gastric mucosa (Fig. 2A and B). The thickness of the mucosa of gastric corpus in Nrdc+/− mice with Helicobacter felis infection was significantly different from mice without the infection, but was not significantly different from Nrdc−/− mice regardless of Helicobacter felis infection (Fig. 2C). Of note, formation of lymphoid folicles, a characteristic of Helicobacter felis infection, was scarce in Nrdc−/− mice (Fig. 2D), and the inflammation score was more severe in Nrdc+/− than in Nrdc−/− mice with Helicobacter felis infection (Fig. 2E). Consistent with these findings, infiltration of Gr1-positive neutrophils was not prominent in Nrdc−/− mice, and that of F4/80-positive macrophages was significantly decreased compared to Nrdc+/− mice (Fig. 2F and G). The mRNA expression of Cxcl1 and Ccl2, factors that recruit neutrophils and macrophages, respectively, was also decreased in Nrdc−/− mice (Fig. 2H). Notably, mRNA levels of interleukin (IL)-1α, IL-1β, IL-6, and IL-12, that can contribute to chronic gastritis in humans13–15, were not significantly increased in Nrdc−/− mice (Fig. 2I). Thus, the deletion of Nrdc attenuates gastric inflammation caused by Helicobacter infection.

Gastritis caused by forced expression of prostaglandin (PG)E2 was attenuated by Nrdc deletion. We examined the role of Nrdc in K19-C2mE mice, another mouse model that expresses PGE2 abundantly in the gastric mucosa and mimics human gastritis16. Similar to Helicobacter felis–induced gastritis, mucosa of the gastric corpus of Nrdc−/− mice were macroscopically thicker compared to Nrdc−/− mice at 30 weeks of age (Fig. 3A). Histologically, the mucosa of gastric corpus were remarkably hyperplastic in Nrdc−/− mice, consistent with a previous report16. However, this hyperplastic change was not prominent in Nrdc−/− mice (Fig. 3B). Consequently, mucosa were significantly thinner in Nrdc−/− mice (Fig. 3C). Infiltration of Gr1-positive neutrophils and F4/80-positive macrophages was significantly decreased in Nrdc−/− mice compared to Nrdc+/− mice (Fig. 3D and E). The expression of Cxcl1 and IL-1β mRNA was significantly decreased in Nrdc−/− mice compared to Nrdc+/− mice (Fig. 3F), although we could not demonstrate alterations of Ccl2, IL-1α, IL-6, and IL-12 mRNA between Nrdc+/− and Nrdc−/− mice in the presence of K19-C2mE alleles. Thus, like Helicobacter felis–induced gastritis, deletion of Nrdc attenuates gastric inflammation and hyperplasty caused by the forced expression of PGE2.

Metaplastic changes were attenuated by Nrdc deletion. We next investigated whether Nrdc plays a role in metaplastic changes following gastritis. Staining for TFF2 and/or staining with Alcian blue are widely used to detect metaplastic changes in the gastric corpus16. Upon Helicobacter felis infection for 20 weeks, the percentage of TFF2-stained cells was significantly lower in Nrdc−/− mice than in Nrdc+/− mice (Fig. 4A and B). The percentage of Alcian blue-stained cells was also reduced in Nrdc−/− mice compared to Nrdc+/− mice at 26 weeks of Helicobacter felis infection (Fig. 4C and D).

In K19-C2mE mice at the age of 30 weeks, Alcian blue staining showed that the development of metaplastic changes was significantly less prominent in Nrdc−/− mice compared with Nrdc+/− mice (Fig. 4E and F). Together, these data indicate that the deletion of Nrdc leads to the suppression of metaplastic changes in the stomach.

Formation of gastric tumors was suppressed by Nrdc deletion. Chronic gastritis and metaplastic changes are associated with the development of gastric cancers. In both Helicobacter felis infection and the K19-C2mE chronic gastritis mouse models, we noticed that the increase in the number of Ki67-positive cells in the gastric glands of Nrdc+/− mice was not remarkable in Nrdc−/− mice (Fig. 5A). Based on this finding, we hypothesized that the formation of gastric tumors may also be suppressed in Nrdc−/− mice. Because it requires a long period to develop tumors in Helicobacter felis–infected mouse stomach, we administered a chemical carcinogen to rapidly induce mouse gastric tumors. Administration of N-methyl-N-nitrosourea (MNU) causes polypoid tumors in the gastric antrum with inflammatory reactions in the stroma19–21. In Nrdc+/− mice, MNU treatment resulted in polyp formation in the gastric antrum (Fig. 5B and C). In Nrdc−/− mice, the number and burden of gastric tumors was dramatically reduced compared with that in Nrdc+/− mice (Fig. 5B, D and E). Thus, deletion of Nrdc attenuates gastric tumorigenesis induced by MNU.

Discussion
In the present study, we demonstrated that Nrdc crucially regulates gastric inflammation caused by Helicobacter felis infection or forced expression of PGE2. Metaplastic changes following gastric inflammation were suppressed by the deletion of Nrdc. The deletion of Nrdc significantly suppressed chemically-induced tumorigenesis of the stomach.

Helicobacter pylori–induced gastritis is a primary inflammatory disorder of the human stomach, affecting about half of the global population1. Recent improvements in the hygienic environment have reduced the rate of Helicobacter pylori infection; however, other causative factors such as non-steroidal anti-inflammatory drugs
Figure 2. Stomachs of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice infected with Helicobacter felis. (A) Representative macroscopic views of the stomachs of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice infected with Helicobacter felis. (B) Detection of Helicobacter felis in the mucosae of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice. Note that Helicobacter felis is detected in gastric glands of both Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice. Bars = 100 μm. (C) Mucosal thickness of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice with or without Helicobacter felis infection. (D) Formation of lymphoid follicles (arrowheads) in Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mouse gastric mucosae. Bars = 100 μm. (E) Inflammation scores of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mouse gastric mucosae. *P < 0.05. (F) Immunohistochemistry for Gr1 and F4/80 in Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mice. Bars = 100 μm. (G) Number of epithelial cells immunostained for Gr1 and F4/80 in Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mouse stomachs. *P < 0.05. (H) mRNA expression of Cxcl1 and Ccl2 in the gastric mucosae of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mouse stomachs. *P < 0.05. (I) mRNA expression of IL-1α, IL-1β, IL-6, and IL-12 in the gastric mucosae of Nrdc<sup>+/+</sup> and Nrdc<sup>−/−</sup> mouse stomachs. *P < 0.05.
or autoimmunity also contribute to the development of acute and chronic gastritis. Regardless of the causative factors, chronic inflammation of the gastric mucosa can slowly progress to mucosal atrophy, induce metaplastic changes, and finally cause gastric cancer. In addition to the treatment of acute gastritis that may result in hemorrhagic complications, detection and therapeutic intervention in the early stages of the developmental cascade from chronic gastritis to gastric cancer is important. Therefore, it would be helpful to understand the molecular basis of chronic inflammation in the stomach.

Chronic inflammation is regulated by a feedback loop consisting of diverse inflammatory cytokines, and is closely associated with the recruitment of inflammatory cells in tissues. We previously demonstrated that the deletion of Nrdc critically suppresses ectodomain shedding, activation of TNF-α, and the production of other inflammatory cytokines in gastric cancer cells. We also showed that the deletion of Nrdc significantly suppresses mouse steatohepatitis with attenuated inflammatory cytokine production and reduced infiltration of inflammatory cells. Therefore, in this study, we examined the undetermined in vivo role of Nrdc during the developmental process of chronic gastritis, metaplastic changes, and gastric tumors.

**Figure 3. Gastritis caused by forced expression of prostaglandin E2.** (A) Representative macroscopic views of the stomachs of Nrdc+/+ and Nrdc−/− mice with forced expression of PGE2, by the insertion of K19-C2mE alleles. (B) H&E staining of Nrdc+/+ and Nrdc−/− mouse stomachs with forced expression of PGE2. Bars = 1000 µm. (C) Mucosal thickness of the Nrdc+/+ and Nrdc−/− mice with forced PGE2 expression. *P < 0.05. (D) Immunohistochemistry for Gr1 and F4/80 in Nrdc+/+ and Nrdc−/− mice. Bars = 100 µm. (E) Numbers of epithelial cells immunostained for Gr1 and F4/80 in Nrdc+/+ and Nrdc−/− mice. *P < 0.05. (F) mRNA expression of Cxcl1 and IL-1β in the gastric mucosae of Nrdc+/+ and Nrdc−/− mouse stomachs. *P < 0.05.
To mimic chronic infection and inflammation in the human stomach caused by Helicobacter pylori, we used a Helicobacter felis-infected mouse model. Mucosae of the gastric corpus were thinner in Nrdc−/− mice than in Nrdc+/+ mice, and the inflammation score was less severe in Nrdc−/− mice. Infiltration of neutrophils and macrophages was decreased in Nrdc−/− mice concomitantly with attenuated expression of inflammatory cytokines. Although infiltration of inflammatory cells such as neutrophils may not be critical for the development of Helicobacter felis-induced gastritis, this pattern of cytokine expression is consistent with our previous data obtained from a human gastric cancer cell line. Notably, levels of IL-1α, IL-1β, IL-6, and IL-12, which are the main contributors to human chronic gastritis, were decreased in Nrdc−/− mice. Thus, the deletion of Nrdc attenuates gastric inflammation, indicating that Nrdc also plays a role in the intravital stomach. It is also important that the deletion of Nrdc attenuated inflammation caused by the forced expression of PGE2. This indicated that the deletion of Nrdc attenuates gastric inflammation regardless of the cause of inflammation, and a global therapeutic approach may be possible by targeting Nrdc.

We next questioned whether Nrdc plays a role in metaplastic changes and tumorigenesis. In Helicobacter felis-infected mice, metaplastic changes develop in the gastric corpus, and progress to mucous metaplasia that can be stained with Alcian blue. In the present study, metaplastic changes induced by Helicobacter felis infection, or forced expression of PGE2, were significantly attenuated by the deletion of Nrdc. More importantly, gastric

Figure 4. Metaplastic changes in Nrdc+/+ and Nrdc−/− mouse stomachs. (A) Immunohistochemistry for TFF2 in Nrdc+/+ and Nrdc−/− mouse stomachs with Helicobacter felis infection. Bars = 100 μm. (B) Areas stained for TFF2 in Nrdc+/+ and Nrdc−/− mouse stomachs with Helicobacter felis infection. *P < 0.05. (C) Alcian blue staining of Nrdc+/+ and Nrdc−/− mouse stomachs with Helicobacter felis infection. Bars = 100 μm. (D) Areas stained with Alcian blue in Nrdc+/+ and Nrdc−/− mouse stomachs with Helicobacter felis infection. *P < 0.05. (E) Alcian blue staining of Nrdc+/+ and Nrdc−/− mouse stomachs with PGE2 expression. Bars = 1000 μm. (D) Areas stained with Alcian blue in Nrdc+/+ and Nrdc−/− mouse stomachs with forced PGE2 expression. *P < 0.05.
tumor formation was remarkably suppressed by the same genetic change. It is now becoming clear that the tumor microenvironment, which is orchestrated by inflammatory cells, is an indispensable participant during a wide-range of tumor developmental processes27.

Inflammation plays decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis28. We previously reported that NRDC is highly expressed in the epithelium of human gastric cancer tissues 14. Knockdown of Nrdc on inflammation may lead to the suppression of metaplastic changes and tumor formation. In this respect, it is worth noting that metaplastic changes may be regulated at least partly by factors other than Nrdc. To determine the exact mechanisms underlying how Nrdc coordinates inflammation, metaplastic changes, and tumor formation, further studies are required.

Methods
Animal models. Generation of the Nrdc knockout mouse with a CBA background was described previously15. Animals were housed under specific pathogen-free conditions at the Animal Facilities of Kyoto University. All animal experiments were performed in accordance with institutional guidelines. The Review Board of Kyoto University granted ethical permission for this study, and The Kyoto University Animal Experimentation Committee approved the experimental protocol. For the infection by Helicobacter felis, suspending water containing Helicobacter felis was gavaged to Nrdc+/− and Nrdc−/− mice for three days according to the previous report16. K19-C2mE mice were generated as previously described16. To analyze chemically-induced gastric tumorigenesis, mice at the age of 6 weeks were administered MNU (Sigma-Aldrich, St. Louis, MO, USA) in drinking water at 240 ppm on alternate weeks for five weeks as described previously19. Animals were housed under specific pathogen-free conditions at the Animal Facilities of Kyoto University. All animal experiments were performed in accordance with institutional guidelines. The Review Board of Kyoto University granted ethical permission for this study, and The Kyoto University Animal Experimentation Committee approved the experimental protocol. For the infection by Helicobacter felis, suspending water containing Helicobacter felis was gavaged to Nrdc+/− and Nrdc−/− mice for three days according to the previous report16. K19-C2mE mice were generated as previously described16. To analyze chemically-induced gastric tumorigenesis, mice at the age of 6 weeks were administered MNU (Sigma-Aldrich, St. Louis, MO, USA) in drinking water at 240 ppm on alternate weeks for five weeks as described previously19.

Histological and immunohistochemical analyses. Mouse stomach was resected, fixed in 4% buffered paraformaldehyde solution, embedded in paraffin, and cut into sections 5-μm thick. For immunostaining, the sections were incubated overnight with the primary antibodies at 4°C, after which the secondary antibodies were added. The primary antibodies used were rat anti-F4/80 (Abcam, Cambridge, MA, USA), rat anti–Gr-1 (eBioscience, San Diego, CA, USA), sheep anti-pepsinogen II (Abcam), mouse anti-H+/K+-ATPase α subunit (MBL,
Nagoya, Japan), mouse anti-Muc5AC (Abcam), mouse anti-spasmodolytic polypeptide (TFF2) (R&D Systems, Minneapolis, MN, USA), and rat anti-Ki67 (Dako, Glostrup, Denmark). All immunohistochemical analyses were performed with immunoglobulin isotype controls. For Alcian blue staining, deparaffinized sections were incubated with Alcian blue solution for 30 minutes, followed by counterstaining with Nuclear Fast Red. To determine the differentiation status of the gastric mucosa under physiological conditions, stained cells were counted in 10 randomly selected gastric glands per mouse in 6 Nrdc+/− and 4 Nrdc−/− mice. In Helicobacter felis infection experiments, we used 12 Nrdc+/− and 6 Nrdc−/− mice. In addition, 6 Nrdc+/−, K19-C2mE and 3 Nrdc−/−; K19-C2mE mouse samples were subjected to the analyses. Using these mice, to analyze histology and count inflammatory cells, eight high power field sections from each mouse were selected randomly. To investigate gastric tumorigenesis, 12 Nrdc+/− and 6 Nrdc−/− mice were treated with MNU. Inflammation scores were determined according to the previous report24.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA). Single-strand complementary DNA was synthesized using a First Strand SYBR Green Master Mix (Roche Applied Science) and the LightCycler 480 system (Roche Applied Science). Values are expressed as arbitrary units relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The primer sets used were: interleukin (IL)-1α-forward, TGGTGCTGAGATAGTGTTTGTC; IL-1α-reverse, CAGGGTCAAGGCAAGCCTC; Ccl2-forward, ATCCACGGCATACTATCAACATC; Ccl2-reverse, TGGTGCTGAGATAGTGTTTGTC; Gapdh-forward, AGGTCGGTGTGAACGGATTTG; and Gapdh-reverse, TGTAGACCATGTAGTGAAGTCA. In each experiment, 3–6 samples were subjected to the reactions.

Statistical analyses. Results are expressed as means ± standard error unless stated otherwise. Differences between treatments, groups, and strains were analyzed by the two-tailed Student’s t-test.

References
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Author Contributions
Y.K., E.N., and H.S. designed the studies and wrote the manuscript; Y.K. and K.I. performed experiments and were involved in data analysis; H.O. and M.O. provided essential materials; T.K. and T.C. supervised all studies.

Additional Information
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