Deficiency of Stomach-Type Claudin-18 in Mice Induces Gastric Tumor Formation Independent of *H pylori* Infection

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**SUMMARY**

Stomach-type claudin-18 (*stClcln18*) deficiency induced gastric tumorigenesis in mice without *H pylori* infection. Several signaling networks, including the cytokine-, stemness-, and Wnt signaling pathways, may be activated under the *stClcln18*-deficiency-induced chronic active gastritis to accelerate the gastric tumorigenesis.

**BACKGROUND & AIMS:** Epithelial cells are joined by tight junctions (TJs) to form a cell sheet. In the stomach, epithelial cell sheet forms an essential barrier against gastric material, including gastric acid. Although the decreased expression of stomach-type claudin-18 (*stClcln18*), a TJ protein, is generally observed in human gastritis and gastric cancer, its pathological roles are not fully understood. We previously reported that mice lacking *stClcln18* (*stClcln18/-*) exhibit gastric acid leakage through TJs, which induces active gastritis at a young age. Here, we examined the gastric pathologies in mice after long-term *stClcln18* deficiency.

**METHODS:** The gastric pathologies in *stClcln18/-* mice were sequentially analyzed from youth to old age, and compared to those in humans. To examine the relationship between *stClcln18* deficiency-induced gastric pathologies and Wnt-dependent tumorigenesis, we generated *Wnt1*-overexpressing *stClcln18/-* mice.

**RESULTS:** *stClcln18/-* mice developed chronic active gastritis at middle age, with expression of the chemoattractant CCL28. At old age, 20-30% of these mice developed gastric tumors with CXCL5 expression, indicative of EMT. In this process, spasmylytic polypeptide-expressing metaplasia (SPEM) cells appeared. Increased expressions of CD44-variants, TLR2, and CXCL5 indicated age-dependent changes in cell characteristics. Some features of the *stClcln18/-* mouse gastric tumorigenesis resembled *H pylori*-infection-related human carcinogenesis. The gastric tumorigenesis was accelerated in *Wnt1*-overexpressing *stClcln18/-* mice, indicating that Wnt is involved in the *stClcln18/-* mouse gastric tumorigenesis.

**CONCLUSIONS:** *StClcln18* deficiency induced gastric tumorigenesis in mice without *H pylori* infection. Our findings
revealed that several signaling networks, including the cytokine-, stemness-, and Wnt-signaling pathways, may be activated under the stCldn18-deficiency-induced chronic active gastritis to accelerate the gastric tumorigenesis. (Cell Mol Gastroenterol Hepatol 2019;8:119–142; https://doi.org/10.1016/j.jcmgh.2019.03.003)

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See editorial on page 151.

Accumulating evidence indicates that the tissue microenvironment plays critical roles in pathophysiological processes including tumor or cancer formation. Recent studies suggest that tumor or cancer microenvironments contain immune or nonimmune tumor-related cellular components, extracellular matrix, and interstitial components, which are enclosed by epithelial cell sheets endowed with permselective transcellular and paracellular barrier functions.4-10 While the epithelial transcellular barrier functions are defined by characteristics of the epithelial cells themselves, such as their plasma membranes and transporters or channels, the paracellular barrier functions are defined by the tight junctions (TJs) between the cells.7-9

Helicobacter pylori infection is a well-known risk factor for human gastritis and gastric cancer. H pylori infects the stomach epithelia and induces gastritis, in which epithelial cell hyperproliferation and dedifferentiation are induced by the CagA-SHP2-MAPK and Wnt signaling pathways.11,12 These signaling pathways are thought to be critical causes of gastric carcinogenesis.11,12 On the other hand, epithelial cell polarity and the cell-cell adhesion system including TJs are also dysregulated, through the CagA-PAR1 interaction.13 Thus, a disruption of the TJs’ paracellular barrier function, which is thought to cause gastric material including H+ to leak into the submucosal space, might be another important factor contributing to the progression of gastritis and gastric cancer.14 However, the contribution of the paracellular barrier dysfunction of TJs to gastric pathologies has not been well analyzed.

Claudins (Cldns), the 4-transmembrane domain proteins of TJs, are indispensable for TJ formation.7,15,16 Recent studies revealed that a lack of specific claudins causes inflammation-related pathological conditions.17-21 These conditions mainly result from aberrant microenvironments generated by the inappropriate passage of water, ions, or small solutes through dysregulated TJs.7,22,23

The expression of stomach-type Cldn18, a predominant and essential Cldn for gastric TJs, is generally decreased in various types of human gastritis and gastric cancer, with or without H pylori infection.14 Recently, a Cldn18-ARHGAP fusion gene that disturbs the paracellular barrier function of TJs was also reported in human gastric cancer.24 Furthermore, we previously reported that stomach-type Cldn18 gene-knockout (stCldn18–/–) mice show acute oxyntic atrophic gastritis at a young age, induced by gastric acid (H+) leakage from the gastric lumen to the submucosal space through stCldn18–/– TJs.14,25 While it is generally accepted that tumor or cancer microenvironments can be created under chronic inflammatory conditions,26 it remains unknown whether gastric tumorigenesis or carcinogenesis can also be induced after a long period of stCldn18-deficiency-induced gastritis. Consistent with a recent report analyzing stCldn18–/– mice, here we independently revealed that gastric tumors developed in aged stCldn18–/– mice even without H pylori infection.27 Furthermore, we found that several signaling networks, including the cytokine-, stemness-, and Wnt-signaling pathways, may be activated under the stCldn18-deficiency-induced chronic active gastritis to accelerate the gastric tumorigenesis.

Results

Induction of Oxyntic Atrophic Gastritis in Young stCldn18–/– Mice and Gastric Tumor Development in Aged stCldn18–/– Mice

To examine the long-term effects of the stCldn18 deficiency on the gastric pathology, we analyzed stCldn18–/– mice at 8, 40, 60, and 100 weeks old (w.o.) (Figure 1A and B). We previously reported that stCldn18–/– mice exhibit oxyntic atrophic gastritis induced by gastric acid leakage from the gastric lumen into the submucosal space at a young age (<8 w.o.).14 The obvious histological finding in the stCldn18–/– mouse stomach was pseudopyloric metaplasia.14 In this study, we further found that gastric tumors developed in 20%-30% of the stCldn18–/– mice at older ages (>60 w.o.) (Figure 1A and B). As the gastric tumors did not appear in middle-aged stCldn18–/– mice (40 w.o.) (Figure 1A and B), the tumorigenic event might occur between the ages of 40 and 60 w.o.

Irregular Differentiation and Proliferation of Gastric Epithelial Cells in Young and Aged stCldn18–/– Mice

As gastric hyperplasia developed in the stCldn18–/– mice (>40 w.o.) (Figure 1B), we examined the age-dependent changes in the differentiation and proliferation of gastric epithelial cells. The number of H,K-ATPase-positive parietal cells was decreased in the stCldn18–/– mice at all ages (Figure 2A). Ki-67–positive proliferative cells were observed...
in the bottom region of the gastric glands in the young stCldn18−/− mouse stomach (8 w.o.) (Figure 2B). The width of the proliferative zone was increased in the middle-aged stCldn18−/− mouse stomach (40 w.o.), and the Ki-67-positive proliferative cells became sparser in the old stCldn18−/− mice (>60 w.o.) (Figure 2B). In contrast, the Ki-67-positive proliferative cells were located only in the neck region of the gastric glands in the stCldn18+/+ mice at all ages (Figure 2B). These results suggested that in the stCldn18−/− mice, oxyntic atrophic gastritis with abnormal cell differentiation and proliferation occurred at all ages.

Transition From Active to Chronic Active Gastritis in Middle-Aged stCldn18−/− Mice

It is generally accepted that tumor microenvironments can be created under chronic inflammatory conditions.14 In the young stCldn18−/− mice (8 w.o.), Gr1-positive neutrophils predominantly infiltrated the stomach (Figure 3A and B), and only low numbers of CD3+ T lymphocytes were observed (Figure 3C), suggesting that acute but not chronic gastritis was present. However, infiltrating CD3+ T lymphocytes were observed in the vicinity of the gastric glands in the stomach of stCldn18−/− mice from middle age (>40 w.o.) (Figure 3C). Consistent with this result, the number of infiltrating CD4+ and CD8+ T lymphocytes was increased in the stomach of stCldn18−/− mice from middle age (>40 w.o.) (Figure 3D). Gr1-positive-neutrophils were continuously detected in the stomach of stCldn18−/− mice from middle age (>40 w.o.) (Figure 3A and B), similar to H pylori infection–related gastritis.28 These data indicated that in the stCldn18−/− mice, active gastritis transitioned to chronic active gastritis between youth and middle age.
Activation of Inflammatory Signaling including CCL28 and CXCL5 in Middle-Aged stCldn18–/– Mice

As active gastritis transitioned to chronic active gastritis in the stCldn18–/– mice with age, we further analyzed the inflammatory signaling associated with the gastritis and the gastric tumorigenesis. The expression levels of general inflammatory cytokine such as interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), and CXCL1 were upregulated in the stomach of stCldn18–/– mice (Figure 4A).

In H pylori infection–related gastritis and gastric cancer, some cytokines are reported to be related to the disease severity or survival rate. Among them, CXCL5, a multifunctional cytokine with neutrophil-attracting, angiogenic, and epithelial-mesenchymal transition (EMT)–inducing activities, is related to the severity of H pylori infection–related human gastric cancer. We observed CXCL5-positive gastric epithelial cells in the stCldn18–/– mice from middle age (>40 w.o.) (Figure 4B). Consistent with this finding, the expression level of CXCL5 was significantly increased in the stomach of stCldn18–/– mice at old ages (>60 w.o.) (Figure 4B).

The expression levels of other inflammation-related genes, including STAT3, IKKβ, and NFκB, were also upregulated in the stomach of stCldn18–/– mice (Figure 4C). IKKβ/NF-κB activation and STAT3 activation are known to have tumorigenic roles, such as in hyperproliferation, anti-apoptosis, and angiogenesis.

CCL28, a chemokine with a chemoattractant activity for lymphocytes, is upregulated in H pylori infection–related human gastritis, and of 6 genes that are reported to be significantly correlated with the aggressiveness of gastric cancer. We observed CCL28-positive cells in the stomach of stCldn18–/– mice from middle age (>40 w.o.) (Figure 4D). The expression level of CCL28 was also increased in the stomach of stCldn18–/– mice, especially at old age (>60 w.o.) (Figure 4D). To further examine CCL28’s contribution to the stCldn18–/– mouse gastritis, POL7085, an inhibitor of the CCL28-CCR10 cascade, was administered to the stCldn18–/– mice. After 1 week of POL7085 administration, the expression levels of inflammation-related genes such as CXCL5, NF-kB, and IKKβ were dramatically decreased in the middle-aged stCldn18–/– mice (around 50 w.o.) (Figure 4E). Thus, CCL28 may be a key cytokine for inducing chronic active gastritis in the stCldn18–/– mice.

Collectively, our results showed that CCL28 may be involved in the transition from active gastritis to chronic active gastritis in the stCldn18–/– mice. In addition, the...
### A

|           | 8 week | 40 week | 60 week | 100 week |
|-----------|--------|---------|---------|----------|
| Mu        |        |         |         |          |
| SM        |        |         |         |          |
| G-I/actin/DAPI stCldn18+/+ |        |         |         |          |
| stCldn18+/- |        |         |         |          |
| G-I/actin/DAPI Ly-6G   |        |         |         |          |
| stCldn18+/- |        |         |         |          |
| G-I/actin/DAPI CD11b  |        |         |         |          |
| stCldn18+/- |        |         |         |          |
| G-I/actin/DAPI CD8    |        |         |         |          |
| stCldn18+/- |        |         |         |          |

### B

|           | 0.56 | 0.08 | 0.70 | 0.02 | 0.63 | 0.01 | 4.53 | 0.07 |
|-----------|------|------|------|------|------|------|------|------|
| stCldn18+/- |     |      |      |      |      |      |      |      |
| stCldn18+/- | 0.26 | 0.24 | 0.15 | 0.04 | 0.13 | 0.17 | 10.1 | 1.09 |
| stCldn18+/- | 12.8 | 0.01 | 5.26 | 0.15 | 8.78 | 0.17 | 10.1 | 1.09 |

### C

|           | 0.03 | 0.00 | 0.01 | 0.00 | 0.11 | 0.01 | 0.50 | 0.00 |
|-----------|------|------|------|------|------|------|------|------|
| stCldn18+/- |     |      |      |      |      |      |      |      |
| stCldn18+/- | 0.04 | 0.31 | 0.05 | 0.05 | 0.41 | 0.00 | 11.1 | 0.01 |
| stCldn18+/- | 0.09 | 1.23 | 0.02 | 1.80 | 0.00 | 11.1 | 0.01 | 5.60 |

### D

|           | (×10^10) | (×10^10) | (×10^10) | (×10^10) |
|-----------|----------|----------|----------|----------|
| neutrophils |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
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| stCldn18+/- |          |          |          |          |
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| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
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| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |

### E

|           | (×10^10) | (×10^10) | (×10^10) | (×10^10) |
|-----------|----------|----------|----------|----------|
| CD4^+ T cell |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
| CD8^+ T cell |          |          |          |          |
| stCldn18+/+ |          |          |          |          |
| stCldn18+/- |          |          |          |          |
upregulation of other inflammatory signals such as CXCL5, STAT3, and IKKβ/NF-κB could be indicative of tumordirected changes, such as EMT, hyperproliferation, apoptosis, and angiogenesis, in the stCldn18−/− mice.

**Induction of Spasmodlytic Polypeptide–Expressing Metaplasia in Young and Aged stCldn18−/− Mice**

Recently, spasmodlytic polypeptide–expressing metaplasia (SPEM) was suggested to be the origin of cancer stem cells.50 We previously reported that young stCldn18−/− mice exhibit SPEM, which was probably caused by the acute decrease in the number of parietal cells due to gastric acid leakage.14 In the present study, we found that aged stCldn18−/− mice (around 50 w.o.) also showed SPEM, which was double-positive for TFF2 and Pepsin C, near the bottom of the gastric glands (Figure 5A). Ki-67 staining showed that the TFF2 and Pepsin C double-positive SPEM cells were proliferative (Figure 5A).

**Upregulation of CD44 Splicing Variants in Middle-Aged stCldn18−/− Mice**

SPEM cells are known to express splicing variants of CD44, which are often found at high expression levels in cancer stem cells.50–53 In this respect, conventional real-time polymerase chain reaction (RT-PCR) showed that the CD44 v8–v10 variants were highly expressed in the stomach of stCldn18−/− mice from middle age (>40 w.o.) (Figure 5B), and CD44-positive SPEM cells were observed in the stomach of aged stCldn18−/− mice (Figure 5C). These results suggested that the tumor-directed stemness of the SPEM cells increased in the stCldn18−/− mice. Supporting this hypothesis, the expression level of TLR2, an innate immune receptor with a role in maintaining stemness,50 increased in the stCldn18−/− mice with age (Figure 6A). We also found CD44 and TLR2 double-positive cells in the stomach of stCldn18−/− mice (Figure 6B). In addition, the expression levels of other stemness markers such as SOX2, c-Myc, Ascl2, and matrix metalloproteinase-7 (MMP7) were increased in the stomach of the aged stCldn18−/− mice (Figure 6C).51 The expression level of MMP7 was especially increased in the stomach of stCldn18−/− mice at 100 w.o., which is notable because MMP7 is known to be involved in cell invasion and in the stemness of cancer stem cells.51

**Development of Intestinal Metaplasia and Ectopic Gastric Glands in Aged stCldn18−/− Mice**

Gastric intestinal metaplasia is known to be a precancerous change in which the gastric mucosa is replaced by intestinal epithelium.52 In this respect, the expression levels of Cldn2, Cldn4, and Cldn7 were significantly increased in the stomach of stCldn18−/− mice (Figure 7A), and these Cldns are known to be highly expressed in intestinal epithelial cells.53 Consistent with this finding, Cldn2- or Cldn7-positive cells were clearly observed in the stomach of stCldn18−/− mice (Figure 7B). Furthermore, villin, a marker for intestinal epithelial cells, was detected in the stomach of stCldn18−/− mice (Figure 7B). These results suggested that gastric intestinal metaplasia developed in the stCldn18−/− mice. Importantly, the changes in the expression levels of these Cldns could also be associated with the gastric pathologies of the stCldn18−/− mice.

In the old stCldn18−/− mice (100 w.o.), ectopic gastric glands formed outside the gastric mucosa (Figure 7C). This ectopic gastric gland was histologically similar to the “gastritis cystica profunda” or to the “invasive submucosal gland,” which are observed in H pylori–related gastritis,54 and it was recently reported that the “invasive submucosal gland” represents the stage between intestinal metaplasia and gastric cancer in a study in Mongolian gerbils.55 Although the ectopic gastric gland we observed had features of intestinal epithelium, which is villin−, Cldn2−, Cldn4−, or Cldn7-positive (Figure 7E), in addition to being CD44− or Ki-67−positive (Figure 7D), it did not show any obvious dysplastic changes such as nuclear atypia.

**Activation of Wnt Signaling in Aged stCldn18−/− Mice**

Wnt signaling is known to be activated in 30%–50% of human gastric cancers.56 Considering that CXCL5, CD44, and MMP7, which were upregulated in the aged stCldn18−/− mouse stomach, are related to be Wnt signaling,57–59 Wnt...
signaling might be involved in the stCldn18–/– mouse gastric tumorigenesis. In this respect, the nuclear localization of β-catenin, a marker for Wnt signaling activation, was observed in the stomach of stCldn18–/– mice from middle age (>40 w.o.) (Figure 8A). Furthermore, the expression level of Wnt1a was especially increased in the stomachs with gastric tumors of 100-w.o. stCldn18–/– mice (Figure 8B). The expression levels of Wnt4 and Wnt5 gradually increased in the stomach of stCldn18–/– mice with age (Figure 8C). These results showed that Wnt signaling was increasingly activated in the stomach of stCldn18–/– mice with age. Thus, the activation of Wnt signaling could be related to the gastric tumorigenesis in stCldn18–/– mice.

**Acceleration of Gastric Tumorigenesis Under Chronic Active Gastritis in Wnt1-Overexpressing Transgenic stCldn18–/– Double-Mutant Mice**

As previously reported, Wnt1-overexpressing transgenic (Wnt1-Tg) mice developed small gastric polyps without severe gastritis. To examine the relationship between stCldn18-deficiency–induced chronic gastritis and Wnt-dependent gastric tumorigenesis, we crossed Wnt1-Tg with stCldn18–/– mice to obtain double-mutant (DM) mice. The Wnt1-Tg and DM mice were born, and grew with a macroscopically normal appearance, similar to the stCldn18–/– mice. At a young age (8 w.o.), the Wnt1-Tg mice showed no severe gastric pathologies, in contrast to the stCldn18–/– and DM mice (Figure 9A–C). The DM mouse stomach also showed increased expression levels of inflammatory cytokines such as IL-1β, TNF-α, CXCL1, and CCL28, similar to the stCldn18–/– mouse stomach (Figure 9B). A few CD3+ T lymphocytes were observed in the DM mouse stomach (Figure 9C). These results indicated that at the young age (8 w.o.), the DM mice developed gastritis similarly to the stCldn18–/– mice.

At middle age (40 w.o.), all of the DM mice developed gastric tumors, while none of the stCldn18–/– mice did (Figure 10A). Hematoxylin and eosin–stained images of the DM mouse stomach showed that the density of the gastric glands was increased in a back-to-back manner (Figure 10B), suggesting that dysplastic changes were induced. Inflammatory cytokines such as IL-1β, TNF-α, CXCL1, CCL28, and CCL5 were upregulated in both the stCldn18–/– and DM mouse stomachs (Figure 10C). The number of CD3+ T lymphocytes was also increased in the stCldn18–/– and the DM mouse stomach compared with the stCldn18+/+ mouse and Wnt1-Tg mouse stomach (Figure 10D). At this age, the Wnt1-Tg mice did not show severe gastritis/gastric tumor formation but small foveolar hyperplasia, the phenotype of which was reported previously (Figure 10A–D).

Collectively, the extent of gastritis in the DM mice was similar to that in the stCldn18–/– mice, except for the level of CCL5, which is a target of Wnt signaling. (Figure 10A–D). These findings suggested that the Wnt-dependent gastric tumorigenesis might be accelerated under the severe gastritis induced by the stCldn18 deficiency.

**Similarity of Gastric Tumorigenesis in stCldn18–/– Mice to H pylori Infection–Related Tumorigenesis in Humans**

To compare the mouse model and human disease, we analyzed the expression levels of Cldn18 in human gastritis...
A

TFF2/Peptic C/Ki-67 TFF2/Peptic C/Ki-67

stCldn18+/+ Corpus

stCldn18-/- Corpus

stCldn18-/- Tumor

B

stCldn18 8 40 60 100 weeks

CD44

GAPDH

C

TFF2/Peptic C/CD44 TFF2/Peptic C/CD44

TFF2

Pep C

CD44
samples and in various human gastric tumors. Gastric hyperplastic polyp (HP), which is usually associated with *H. pylori* infection in humans, develops in the context of chronic inflammation. Fundic gland polyp (FGP), a major human tumor, is a hyperplasia that is not associated with either inflammation or *H. pylori* infection, and is considered to be different from the inflammation-related process such as that seen in the *stCldn18*–/– mice. Another disease, Peutz-Jeghers polyp (PJP), is an autosomal-dominant inherited disorder characterized by hamartoma in the digestive tract, including gastric tumors, and also has a different disease process from that seen in the *stCldn18*–/– mice.

Pathologically, the tumor regions in the *stCldn18*–/– mice closely resembled human HP (Figure 11A). The expression level of *Cldn18* in HP was clearly lower than that in FGP or PJP (Figure 11B), and the expression level of CXCL5 in HP was clearly higher than that in FGP or PJP (Figure 11C). These results suggested that the expression level of *Cldn18* was inversely correlated with that of CXCL5 in HP. These data suggested that the *stCldn18*–/– mouse gastric tumors at least partly resembled human HP.

Furthermore, we noted that the *Cldn18* expression level in a gastric adenocarcinoma that originated from HP was reduced (Figure 11D). The CXCL5 expression were also upregulated in the adenocarcinoma region of the human stomach (Figure 11D).

**Toxic Effects of Gastric Acid in Aged *stCldn18*–/– Mice**

In the *stCldn18*–/– mice, the long-term gastritis continued without healing. In this respect, only a few parietal cells remained in the aged *stCldn18*–/– mice, suggesting that gastric acid secreted by these cells promoted the gastritis. After 1 week of administering the H2-blocker cimetidine, an inhibitor of gastric acid secretion, to middle-aged *stCldn18*–/– mice (around 50 w.o.), the expression levels of inflammation-related genes such as CXCL5, IKKβ, and NF-kB were dramatically decreased (Figure 4E). This result suggested that the remaining parietal cells could still secrete gastric acid to promote gastritis.

Importantly, it was still unclear to what extent the gastric acid leakage due to the *stCldn18* deficiency directly contributed to the gastritis and gastric tumorigenesis. Our present findings suggested that the activation of several signaling pathways, including the cytokine-, stemness-, and Wnt-signaling pathways, might contribute to these gastric pathologies under the severe gastritis induced by the *stCldn18* deficiency. For example, the administration of cimetidine or POL7085 dramatically inhibited the gastritis, and also decreased the expression level of Ascl2, a stemness marker, in middle-aged *stCldn18*–/– mice (around 50 w.o.) (Figure 4E). Thus, it was possible that the stemness was also increased under the chronic gastritis induced by the *stCldn18* deficiency.

**Process of Gastric Tumorigenesis in Young to Aged *stCldn18*–/– Mice Without *H pylori* Infection**

Based on our findings, we propose a scheme in which the loss of *stCldn18* creates a microenvironment that leads to chronic active gastritis and gastric tumor formation in the absence of *H pylori* infection (Figure 12). The active gastritis, which occurred in young *stCldn18*–/– mice (<8 w.o.), transitioned to chronic active gastritis with expression of the chemoattractant CCL28 in middle-aged *stCldn18*–/– mice (40 w.o.). In the old *stCldn18*–/– mice (>60 w.o.), gastric tumors formed with the expression of CXCL5, which promoted EMT downstream of Wnt. In this tumorigenesis process, SPEM cells appeared. Changes in cell characteristics were suggested by the upregulation of CD44, TLR2, and Wnt. Furthermore, the gastric tumorigenesis was accelerated in Wnt1-Tg *stCldn18*–/– DM mice.

Our analysis provides evidence that long-term *stCldn18* deficiency induces chronic active gastritis and then gastric tumors in mice even in the absence of *H pylori* infection. Several signaling pathways, including the cytokine-, stemness-, and Wnt-signaling pathways, may be activated to accelerate the gastric tumorigenesis under the *stCldn18* deficiency-induced chronic active gastritis. Notably, the process of *stCldn18*–/– mouse gastric tumorigenesis partially resembled that of *H pylori* infection–related human carcinogenesis.

**Discussion**

Disruption of the paracellular barrier function of TJs is thought to be an important factor in the progression of gastritis and gastric cancer, although its contribution to gastric pathologies has not been thoroughly analyzed. In this respect, we previously reported that in young *stCldn18*–/– mice, acute oxyntic atrophic gastritis is induced by the leakage of gastric acid from the gastric lumen to the submucosal space through *stCldn18*–/– TJs. In the present study,
we analyzed the stCldn18–/– mice over their entire lifetime. Our analysis provides evidence that long-term stCldn18 deficiency induces gastric tumors to form under chronic active gastritis in mice without H pylori infection, in a process involving several signaling pathways, including the cytokine-, stemness-, and Wnt-signaling–activated pathways.

CCL28 could be a key chemokine causing the stCldn18–/– mouse gastritis to become chronic, based on the following findings. The expression of CCL28 is reported to be induced by hypoxia-inducible factors under hypoxic conditions, suggesting that hyperproliferative tumor-related hypoxia could upregulate CCL28 expression in the stCldn18–/– mice (Figure 6C). It is also reported that the expression of CD44 v8-v10 variants is induced by hypoxia to promote cell proliferation. Thus, a positive feedback loop involving CD44 upregulation, cell proliferation, and hypoxia could increase the CCL28 expression to accelerate the transition from active to chronic active gastritis in the stCldn18–/– mice.

Recently, SPEM cells were suggested to be the origin of cancer stem cells. Evidence also suggests that prolonged gastritis eventually changes the stemness of SPEM cells. In the stCldn18–/– mice, the expression levels of stemness markers were increased from middle age, when chronic active gastritis was induced. Therefore, we speculate that the stCldn18 deficiency–induced chronic active gastritis promoted tumor-directed changes in the SPEM cells.

The EMT upregulates cell migration and invasion in cancer development. In this respect, we observed that CXCL5, a downstream target of Wnt signaling and an EMT-activated pathways, was upregulated in the stomach of our stCldn18–/– mice. Although the CXCL5 expression was not increased in the DM mice at a young age (8 w.o.), when severe gastritis was not observed. These findings suggested that the chronic active gastritis induced by stCldn18 deficiency dramatically potentiated the effect of Wnt signaling. Consistent with this possibility, the CXCL5 expression was upregulated in stCldn18–/– mice especially at old age. Thus, changes in the characteristics of the stCldn18-deficiency–induced gastritis might be required for the activation of Wnt signaling to accelerate the gastric tumorigenesis. As Wnt signaling is reported to be coordinated with Notch to regulate gastric stem cell proliferation and differentiation, it is possible that the upregulation of Notch is also involved in the stCldn18–/– mouse gastric tumorigenesis.

Fox’s group recently reported that gastric pathologies including gastritis and gastric tumors were observed in their Cldn18–/– mice, similar to our stCldn18–/– mice. They globally analyzed the signaling pathways by RNA sequencing, and, like us, found that Wnt signaling was involved in the Cldn18–/– mouse gastric tumorigenesis. In our study, we further used DM mice to examine the tumorigenic roles of Wnt signaling under the chronic gastritis induced by stCldn18 deficiency.

It was recently reported that in lung-type Cldn18 gene knockout (luCldn18–/–) mice, luCldn18 loss induced the nuclear localization of YAP, which promoted the development of lung adenocarcinoma. Although the luCldn18 expression that was upregulated in the stomach of our stCldn18–/– mice, the nuclear localization of YAP could be related to the gastric tumorigenesis in our stCldn18–/– mice. Collectively, we found that the stCldn18 deficiency induced the development of gastric tumors under chronic active gastritis in the absence of H pylori infection. Age-dependent changes in several signaling networks involving inflammation-, stemness-, and Wnt-related pathways, may accelerate this gastric tumorigenesis in a multi-faceted manner.

Materials and Methods

Ethics Statement

Animal experiments were performed in accordance with protocols approved by the Animal Studies Committee of Osaka University School of Medicine. Recombinant DNA experiments were carried out in accordance with protocols approved by Osaka University. Studies using human samples were performed at Hiroshima University in accordance with protocols approved by the Hiroshima University School of Medicine Ethics Committee.

Figure 6. [See previous page]. Upregulation of TLR2 in aged stCldn18–/– mice. (A) (Left) Immunofluorescence micrographs for TLR2 (green) co-stained with α-catenin (red) and DAPI (blue) in the stomach from stCldn18+/+ and stCldn18–/– mice (8, 40, 60, and 100 w.o.). High-magnification images are shown in insets. TLR2-positive cells were clearly observed in the stomach of stCldn18–/– mice after middle age (40, 60, and 100 w.o.). At middle age (40 w.o.), the TLR2-positive cells did not show the characteristics of gastric epithelium (arrowheads), but at old age (60, and 100 w.o.), the TLR2-positive cells might have been gastric epithelial cells (arrows). Representative images from at least 2 independent experiments are shown. Scale bars = low magnification: 50 μm, high magnification: 50 μm. (Right) Expression level of TLR2 in the stomach from stCldn18+/+ (n = 4, 4, 10, 10) and stCldn18–/– mice (n = 8, 4, 12, 16) (8, 40, 60, and 100 w.o.) quantified by qRT-PCR. Results are expressed as mean ± SD. Comparisons between 2 groups were performed using Student's t test, and differences with P < .05 were considered statistically significant. **P < .01. (B) Immunofluorescence micrographs for CD44 (green), co-stained with TLR2 (red) in the stomach from stCldn18+/+ and stCldn18–/– mice (100 w.o.). High-magnification images are shown in insets. CD44-and TLR2-double-positive cells were clearly observed in the stomach of the stCldn18–/– mice (arrows). Representative images from at least 3 independent experiments are shown. Scale bars = low magnification: 50 μm, high magnification: 50 μm. (C) Expression levels of c-Myc, hypoxia-inducible factor HIF2a, MMP7, SOX2, and ASCL2 in the stomach from stCldn18+/+ (n = 5, 4, 12, 9) and stCldn18–/– (n = 9, 4, 13, 16) mice (8, 40, 60, and 100 w.o.) quantified by qRT-PCR. The expression level of MMP7 was especially increased in the stomach of the 100 w.o. stCldn18–/– mice. Gene expressions were normalized to GAPDH. Results are expressed as mean ± SD. Comparisons between 2 groups were performed using Student’s t test, and differences with P < .05 were considered statistically significant. *P < .05; **P < .001.
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A

Graph showing gene expression levels of Cldn18 in stCldn18+/+ and stCldn18−/− mice.

B

Immunofluorescence images comparing Cldn2/Ecad/Villin and Cldn7/Ecad expression in the corpus and tumor of stCldn18+/+ and stCldn18−/− mice.

C

Histological images showing stCldn18−/− tissue morphology.

D

Immunofluorescence images comparing TFF2/Pepsin C/CD44 and TFF2/Pepsin C/Ki-67 expression in stCldn18+/+ and stCldn18−/− mice.

E

Immunofluorescence images comparing Cldn2/Ecad/Villin, Cldn4/Ecad, and Cldn7/Ecad expression in stCldn18−/− mice.
Antibodies
Antibodies used for immunochemical staining and fluorescence-activated cell sorting (FACS) analyses are listed in Table 1.

Generation of stCldn18–/–, Wnt1-Tg, and Wnt1-Tg stCldn18–/– DM Mice
The method for generating stCldn18-deficient (stCldn18–/–) mice was previously reported. In brief, the first exon of the mouse stCldn18 locus was replaced with a neomycin-resistance gene by a conventional homologous recombination technique. The obtained heterogeneous, A129 R1 ES cells were injected into ICR blastocysts, and a freshly excised mouse stomach was fixed in 10% neutral buffered formalin solution at 4°C for 24 hours. After fixation, the stomach was dehydrated in a graded ethanol series and embedded in paraffin using a Spin Tissue Processor (Thermo Scientific, Tokyo, Japan). The samples in paraffin were cut into 5-μm-thick sections, deparaffinized twice by immersion in xylene at room temperature for 3 minutes, and hydrated by being passed through a series of decreasing ethanol concentrations (100%, 95%, 90%, 80%, 70%, and 50%) at room temperature for 1 minute each. The samples were then blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin at room temperature for 10 minutes, and stained with primary antibodies at 4°C overnight. After 3 washes with PBS, the samples were stained with Horseradish peroxidase (HRP)-labeled secondary antibodies for 1 hour at room temperature. After 3 washes with PBS, the samples were developed with 3,3′-Diaminobenzidine tetrahydrochloride (DAB) at room temperature for 10 minutes. The samples were then rinsed with running tap water for 5 minutes. After counterstaining with hematoxylin, the samples were dehydrated, and mounted with Marinol (Muto Pure Chemicals Co, Ltd, Tokyo, Japan; Cat. No. 20091).

For the immunofluorescence staining of frozen sections, the mouse stomach was washed with ice-cold PBS, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co, Ltd, Tokyo, Japan; Cat. No. 4583), and was frozen in liquid N2. The frozen samples were cut into 5-μm-thick sections, and air dried at room temperature for 20 minutes. The sections were fixed with methanol at −20°C for 10 minutes, or with PBS containing 2% paraformaldehyde at room temperature for 10 minutes after permeabilization with PBS containing 0.1% Triton-X100 at room temperature for 10 minutes. The samples were then blocked with PBS containing 1% bovine serum albumin at room temperature for 10 minutes. After blocking, the samples were stained with primary antibody overnight at 4°C. After 3 washes with PBS, the samples were stained with fluorescently labeled secondary antibodies and with 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. After 3 washes with PBS 1 and with deionized distilled water, the samples were embedded in fluorescence mounting medium (Dako Japan, Tokyo, Japan; Cat. No. S3023).

Figure 7. (See previous page). Development of intestinal metaplasia and ectopic gastric gland in aged stCldn18–/– mice. (A) Expression levels of Cldns in the stomach from stCldn18+/+ (n = 6), and stCldn18–/– (n = 7), mice at 100 w.o. quantified by qRT-PCR. The expression levels of Cldn2, 4, 7, and luCldn18 were significantly increased in the stomach of stCldn18–/– mice at old age. Gene expressions were normalized to GAPDH. Results are expressed as mean ± SD. Comparisons between 2 groups were performed using Student’s t test, and differences with P < .05 were considered statistically significant. n.s., not significant. *P < .05. (B) Immunofluorescence micrographs for Cldn2 or Cldn7 (green) co-stained with E-cadherin (red) and with or without villin (blue) in the stomach from stCldn18+/+ and stCldn18–/– mice (around 50 w.o.). Representative images from at least 3 independent experiments are shown. Scale bars = 100 μm. (C) Hematoxylin and eosin–stained images of the ectopic gastric gland in aged stCldn18–/– mice. Ectopic gastric glands were found in 1 of 3 old stCldn18–/– mice by examining stomach tissue slices. Right panels are magnified images of the boxed regions in the left panels. Representative images from at least 2 independent experiments are shown. Scale bars = 100, 50, 10 μm, left to right panel. (D) (Left) Immunofluorescence micrographs for TFF2 (green) and Pepsin C (blue) as SPEM cell markers co-stained with CD44 (red) and DAPI (white) in the ectopic gastric gland from stCldn18–/– mice (around 50 w.o.). (Right) Representative immunofluorescence micrographs for TFF2 (green), and Pepsin C (blue) as SPEM cell markers co-stained with Ki-67 (red) and DAPI (white) in the ectopic gastric gland from stCldn18–/– mice (around 50 w.o.). Right panels are magnified images of the boxed regions in the left panels. Representative images from at least 2 independent experiments are shown. Scale bars = 100, 50 μm, left to right panel. (E) (Left) Immunofluorescence micrographs for Cldn2 (green) co-stained for E-cadherin (red) and villin (blue) in the ectopic gastric gland from stCldn18–/– mice (around 50 w.o.). Right panels are magnified images of the boxed regions in the left panels. Representative images from at least 2 independent experiments are shown. Scale bars = 100, 50 μm, left to right panel. (Middle) Right) Immunofluorescence micrographs for Cldn4 or Cldn7 (green) co-stained for E-cadherin (red) in the ectopic gastric gland from stCldn18–/– mice (around 50 w.o.). Right panels are magnified images of the boxed regions in the left panels. Representative images from at least 2 independent experiments are shown. Scale bars = 100, 50 μm, left to right panel.
Figure 8. Activation of Wnt signaling in aged stCldn18−/− mice. (A) Immunohistological micrographs for β-catenin as an EMT marker in the stomach from stCldn18+/+ and stCldn18−/− mice (8, 40, 60, and 100 w.o.). A nuclear localization of β-catenin, indicative of Wnt signaling activation, was observed in the stomach of stCldn18−/− mice after middle age (40, 60, and 100 w.o.) (arrows). Representative images from at least 3 independent experiments are shown. Scale bars = 50 μm. (B) Expression level of Wnt1a in the stomach from stCldn18+/+ mice (n = 11, 10), and stCldn18−/− mice with hyperplasia (n = 8, 13), with gastric tumor (n = 5, 3) (60, and 100 w.o.) quantified by qRT-PCR. Wnt1a was especially increased in the gastric tumors of the 100-w.o. stCldn18−/− mice. Gene expressions were normalized to GAPDH. (C) Expression level of Wnt2, Wnt3, Wnt4, and Wnt5 in the stomach from stCldn18+/+ (n = 5, 4, 12, 9) and stCldn18−/− (n = 9, 4, 13, 16) mice (10, 40, 60, and 100 w.o.), quantified by qRT-PCR. Gene expressions were normalized to GAPDH. Results are expressed as mean ± SD. Comparisons between 2 groups were performed using Student’s t test, and differences with P < .05 were considered statistically significant. *P < .05; **P < .001.
Figure 9. No tumor formation in young Wnt1-Tg stCldn18−/− DM mice. (A) Hematoxylin and eosin–stained images of the stomach from stCldn18+/+, Wnt1-Tg, stCldn18−/−, and Wnt1-Tg mice crossed with stCldn18−/− DM mice at a young age (8 w.o.). No obvious difference was detected in the stomachs between stCldn18−/− and DM mice at the young age. Representative images from at least 3 independent experiments are shown. Scale bars = 1 mm. (B) Expression levels of IL-1β, TNF-α, CXCL1, CCL28, and CXCL5 in the stomach from stCldn18+/+, Wnt1-Tg, stCldn18−/−, and DM mice at a young age (8 w.o.) quantified by qRT-PCR (n = 8, 4, 10, 11). No significant differences were detected between the stomachs of stCldn18−/− and DM mice at the young age. Gene expressions were normalized to GAPDH. Results are expressed as mean ± SD. Comparisons among more than 3 groups were performed using the Kruskal-Wallis 1-way analysis of variance on ranks (P < .01) followed by the Steel-Dwass test. *P < .05. (C) Immunofluorescence micrographs for CD3 or CXCL5 (green) co-stained with ZO-2 (red) and DAPI (blue) in the stomach from stCldn18+/+, Wnt1-Tg, stCldn18−/−, and DM mice at a young age (8 w.o.). High-magnification images are shown in insets. No significant differences were detected between the stomachs of stCldn18−/− and DM mice at the young age. Representative images from at least 3 independent experiments are shown. Scale bars = low magnification: 100 µm, high magnification: 10 µm.
For the immunofluorescent staining of paraffin sections, deparaffinized and hydrated 5-μm-thick sections were prepared as described above. The samples then underwent antigen retrieval in deionized distilled water containing 0.5% Immunosaver (Nissin EM, Tokyo, Japan) at 98°C for 45 minutes. After antigen retrieval, the samples were washed 3 times with PBS. The samples were then blocked, and stained with appropriate antibodies as described above.

**Microscopy**

Immunofluorescent images were captured or tiled by an LSM710 confocal laser-scanning microscope (Carl Zeiss Japan, Tokyo, Japan) equipped with a 405-nm blue diode laser, 488-nm argon laser, 561-nm DPSS laser, and 633-nm HeNe laser, and with a Plan-Apochromat 20×/0.2 M27 objective, C-Apochromat 40×/1.2 W Corr M27 objective, and C-Apochromat 63×/1.20 W Corr M27 objective. The acquired images were analyzed by ZEN 2012 (Carl Zeiss Japan).

**Flow Cytometry**

A glandular portion of a freshly excised mouse stomach was washed with cold PBS, and minced with scissors. The minced tissue was washed twice with cold PBS and centrifuged at 600 g at 4°C for 5 minutes, then incubated in 20 mL of Hank’s balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS), 50-mM EDTA, and 15-mg/mL dithiothreitol at 37°C for 10 minutes. After centrifugation at 600 g at room temperature for 10 minutes, the tissue was resuspended and incubated in HBSS containing 300 U/mL collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ; Cat. No. LS004196), 0.01/mg/mL DNase 1 (Roche Diagnostics, Tokyo, Japan; Cat. No. 10104159001), and 10% FBS at 37°C for 50 minutes with intermittent pipetting. The recovered cells were sequentially filtered using a 100-, 40-, and 10-μm-pored nylon mesh. After filtration, the single-cell suspension was blocked with HBSS containing 1% FBS (FBS/HBSS), and stained with fluorescently labeled antibodies at 4°C for 30 minutes. After staining, the cell suspension was washed 3 times with HBSS, each followed by centrifugation at 300 g at 4°C for 5 minutes. The final cell pellet was resuspended in 1% FBS/HBSS containing 1-μg/mL propidium iodide (Nacalai tesque, Kyoto, Japan; Cat. No. 29037-92). The cell suspension was analyzed using a BD FACSanto II (BD Biosciences Japan, Tokyo, Japan) and FlowJo software (FlowJo LLC, Ashland, OR).

**Quantitative Real-Time Polymerase Chain Reaction**

A freshly excised mouse stomach was cut into small pieces, which were quickly immersed into 500 μL of RNAlater (Sigma-Aldrich Japan, Tokyo, Japan; Cat. No. R0901-500ML). The stomach tissue was then frozen in liquid N2, fractured using an SK-100 mill (Tokken, Inc, Chiba, Japan), and processed for RNA isolation. The total RNA was isolated using an RNAeasy Mini Kit (Qiagen, Tokyo, Japan; Cat. No. 74106), and complementary DNA was synthesized using ReverTraAce (Toyobo, Osaka, Japan; Cat. No. TRT-101) according to the manufacturers’ instructions. Quantitative RT-PCR (qRT-PCR) was performed using the Quantitect SYBR Green PCR Kit (Qiagen; Cat. No. 204143), and the 7900HT Fast Real-Time PCR System (Applied Biosystems Japan, Tokyo, Japan). The oligonucleotide sequences for qRT-PCR are listed in Table 2.

**POLT085 and Cimetidine Administration**

POLT085 (Polyphor Ltd, Allschwil, Switzerland) (15 mg/kg body weight/250 μL in saline containing 0.03% dimethyl sulfoxide, each day), or cimetidine (Nacalai tesque; Cat. No.09031-14) (20 mg/kg body weight/250 μL in saline containing 0.05% methanol, each day) were intraperitoneally administered to around 50 w.o. mice using a 1-mL syringe (Terumo, Tokyo, Japan; Cat. No. SS-01T) with a 27-gauge needle (Terumo; Cat. No. NN-27195). Saline containing 0.03% dimethyl sulfoxide or 0.05% methanol was used as the vehicle control. After 1 week of daily administration, the mouse stomach was excised and subjected to qRT-PCR analysis as described above.

**Figure 10.** (See previous page). Accelerated gastric tumorigenesis under chronic active gastritis in aged Wnt1-Tg stCldn18+/−/DM mice. (A) Macroscopic images of the stomach from stCldn18+/+, Wnt1-Tg, stCldn18+/−, and DM mice at middle age (40 w.o.). Gastric tumors were observed in all of the stomachs of DM mice but not of stCldn18+/−/DM mice at middle age (40 w.o.). Representative images from at least 3 independent experiments are shown. Scale bars = 1 cm. (B) Hematoxylin and eosin–stained images of the stomach from stCldn18+/+, Wnt1-Tg, stCldn18+/−, and DM mice at middle age (40 w.o.). Dysplastic changes were observed in the stomach of DM mice at middle age (40 w.o.). Representative images from at least 2 independent experiments are shown. Scale bars = low magnification = 1 mm, high magnification = 50 μm. (C) Expression levels of IL-1β, TNF-α, CXCL1, CCL28, and CXCL5 in the stomach from stCldn18+/+, Wnt1-Tg, stCldn18+/−, and DM mice at middle age (40 w.o.) quantified by qRT-PCR (n = 4, 4, 4, 5). The expression levels of IL-1β, CCL28, and CXCL5 were increased in the stomach of stCldn18+/−/DM mice compared with those of stCldn18+/+ and Wnt1-Tg mice at middle age. Gene expressions were normalized to GAPDH. Results are expressed as mean ± SD. Comparisons among more than 3 groups were performed using the Kruskal-Wallis 1-way ANOVA on ranks (P < .01) followed by the Steel-Dwass test. no symbol, not significant. (D) Immunofluorescence micrographs for CD3 or CXCL5 (green) co-stained with ZO-2 (red) and DAPI (blue) in the stomach from stCldn18+/+, Wnt1-Tg, stCldn18+/−, and DM mice at middle age (40 w.o.). The levels of CD3- and CXCL5-negative cells (arrows) were increased in the stomach of stCldn18+/−/DM mice at middle age (40 w.o.). High-magnification images are shown in insets. Representative images from at least 3 independent experiments are shown. Scale bars = 50 μm, 10 μm.
Figure 11. Inverse correlation between the expression levels of Cldn18 and CXCL5 in human HP and cancer. (A) Hematoxylin and eosin–stained images of human HP, FGP, and PJP. These gastric tumors are the result of H pylori infection, a side effect of proton pump inhibitor therapy, and an inherited gastric tumor of hamartoma/hyperplasia, respectively. Lower panels are magnified images of the boxed regions in the upper panels. Representative images from at least 3 independent experiments are shown. Scale bars = 1 mm and 200 μm in the upper and lower panels, respectively. (B) (Left) Immunohistological micrographs for Cldn18 in human HP, FGP, and PJP. Lower panels are magnified images of the boxed regions in the upper panels. Representative images from at least 3 independent experiments are shown. Scale bars = 1 mm and 200 μm in the upper and lower panels, respectively. (Right) In the graphs, 4+: 75%–100%, 3+: 50%–75%, 2+: 25%–50%, 1+: 1%–25%, 0: 0% of the epithelial cells were stained. The number of Cldn18-positive gastric epithelial cells was significantly decreased in human HP compared with FGP and PJP. (C) (Left) Immunohistological micrographs for CXCL5 in human HP, FGP, and PJP. Lower panels are magnified images of the boxed regions in the upper panels. Representative images from at least 3 independent experiments are shown. Scale bars = 1 mm and 200 μm in the upper and lower panels, respectively. (Right) In the graphs, 4+: 75%–100%, 3+: 50%–75%, 2+: 25%–50%, 1+: 1%–25%, 0: 0% of the epithelial cells were stained. The number of CXCL5-positive gastric epithelial cells (arrows) was significantly increased in human HP compared with FGP and PJP. (D) (Left) Hematoxylin and eosin–stained images of a human gastric adenoma. Lower panels are magnified images of the boxed regions in the upper panels. Representative images from at least 2 independent experiments are shown. Scale bars = 1 mm and 200 μm in the upper and lower panels, respectively. (Middle, Right) Immunohistological micrographs for claudin-18 and CXCL5 in the human gastric adenoma. Lower panels are magnified images of the boxed regions in the upper panels. Representative images from at least 2 independent experiments are shown. Scale bars = 1 mm and 200 μm in the upper and lower panels, respectively.
Figure 12. Schematic illustration of the signaling pathways in stCldn18−/− mouse gastric tumorigenesis. Scheme of the signaling pathways in stCldn18−/− mouse gastric tumorigenesis. In the gastric tumorigenesis of stCldn18−/− mice, several signaling pathways, including the cytokine-, stemness-, and Wnt- signaling pathways, may be activated to accelerate gastric tumorigenesis under the stCldn18 deficiency–induced chronic active gastritis.

Statistical Analysis
All data were expressed as the mean ± SD. Comparisons between 2 groups were performed using Student's t test, and differences with P < .05 were considered statistically significant. Comparisons among more than 3 groups were performed using the Kruskal-Wallis 1-way analysis of variance on ranks (P < .01) followed by the Steel-Dwass test.

Table 1. Antibodies Used in Immunofluorescence Images and FACS Analysis

| Epitope          | Supplier                | Catalog number | Conjugate  |
|------------------|-------------------------|----------------|------------|
| Ki-67            | Santa Cruz Biotechnology| sc-7846        | Unconjugate|
| H,K-ATPase       | MBL                     | D031-3         | Unconjugate|
| Gr-1             | BD Pharmingen           | 553123         | Unconjugate|
| CD3              | BD Pharmingen           | 555273         | Unconjugate|
| CXCL5            | Bioss Antibodies        | bs-2549R       | Unconjugate|
| CXCL5            | Cell Signaling Technology| MAB433         | Unconjugate|
| CCL28            | BioLegend               | 525802         | Unconjugate|
| TFF2             | Bioss Antibodies        | bs-1921R       | Unconjugate|
| Pepsin C         | Santa Cruz Biotechnology| sc-51188       | Unconjugate|
| CD44             | BioLegend               | 103001         | Unconjugate|
| TLR2             | BioLegend               | 121802         | Unconjugate|
| E-cadherin       | Dr. Masatoshi Takeichi  | ECCD-2         | Unconjugate|
| ɑ-catenin        | Sigma                   | C-2081         | Unconjugate|
| ZO-2             | Santa Cruz Biotechnology| sc-8148        | Unconjugate|
| Claudin-2        | Immuno-Biological Laboratories| 18825 | Unconjugate|
| Claudin-4        | Invitrogen              | 364800         | Unconjugate|
| Claudin-7        | Invitrogen              | 349100         | Unconjugate|
| Claudin-18       | LifeSpan BioSciences    | LS-C146633     | Unconjugate|
| Villin           | Santa Cruz Biotechnology| sc-7672        | Unconjugate|
| Active β-catenin | Cell Signaling Technology| 88145         | Unconjugate|
| CD4              | BioLegend               | 100515         | APC        |
| CD8a             | BioLegend               | 100723         | Alexa488   |
| CD11b            | BioLegend               | 101217         | Alexa488   |
| Ly-6G            | BioLegend               | 127613         | APC        |
| Gene     | Forward primer         | Reverse primer         |
|----------|------------------------|------------------------|
| IL-1b    | GCCTCGTGCTGCGAGGACC    | TGTGGCTGTTGCTTCTCCITG  |
| TNF-α    | AGGAGCCCGCGACTACGT     | GACCTTCTCTGTAGAGAAGGCAA |
| CXCL1    | TGAGTCGCTGCTGACTGCTTCT | AGAAAGGAGTTCCACAGAGA   |
| CXCL5    | GCATTCTCTGCTGCTAGGCTCGT | CCTCTCCCTTTGGTATTTCTAGC |
| CCL28    | GACGCGCAGTGGACTCTGTA   | AGCTAGTGAGATCTGGTATTGG |
| STAT3    | GGTGTGGCTCATGAGTACCC   | GTCAGGGGCTCGACTCTGCT   |
| NFκB     | GAAATCTCTTGATCCAGAACAAC | ATCACCTCAATTGCGCTTGATAG |
| IKKb     | TCCTAGTGAGAAAGGTCAATCT | AGCTTGTATTCCGGAGATAG   |
| ASC1L2   | GCCTACTCTGCTGGAGGAA    | CCAACTGAGAAATAGCTAGCA  |
| TLR2     | AACCTCAGACAAAGGAGGAAA  | ACCAAGATGAGAGACTCGAA   |
| c-Myc    | TGGAGTTGCCTAGTGGAGGA   | TTGCTGATTTTCGCGAGAGA  |
| MMP7     | AGATGTGAGAGAGGAGTGTAG  | CGAGTGGACATCGAGAGGAA   |
| sox2     | GGCGAGCTAGCACTGAGGAGGA | CTGGACTGAGGTAGTACTGCT |
| Wnt1     | AAATGGGAAATTCCGAAACC   | GAAGATGAAAGCTTCTCTGCT |
| Wnt2     | AAATGGGAAATTCCGAAACC   | GAAGATGAAAGCTTCTCTGCT |
| Wnt3     | AGGTGTGGAGTGCCAGATTG   | CCAGGCTGATTTCCGAGAGA  |
| Wnt4     | CTGGAGACTGTGGACTGTA    | CACAGGACTGTTGTTGAGA   |
| Wnt5     | CAATAGGGCAAGCGAGAGAT  | CTCTGAGTGGTACAGAGCATC |
| mCldn1   | CCGGAGAGCTGAGGAGGAGAAG | CTGGACTGAGGTAGTACTGCT |
| mCldn2   | ATTACTCTCTTCTGACTGCTAGA | CCGTGAAGCTGACATTGAGAG |
| mCldn3   | CACGACTACACACTTGAGTAG  | AGACTGAGGTGAGGTACTGCT |
| mCldn4   | GTTAAGACAAGTGGGAGGAGGAGA | GTCAGGAAGCTGACATTGAGAG |
| mCldn5   | TACCTGAAAGGCGAGCTGAGAA | AGTGGAGCTGACTGAGGAGAAG |
| mCldn6   | TGCCCACTGCCTACAGAGGTTAC | AGGCCAGCTGACATGAGAGAAG |
| mCldn7   | AGGGCTGCAATATCACAGGCTAGA | CTTGCTTCTGCTGCTCCACTAC |
| mCldn8   | CCTGGAGTAAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn9   | CTTGAGTTAAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn10.1| TGGGTAAGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn10.2| TCCCACTGCCTACAGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn11  | CTTGAGACTGTTGTTGAGAAGAAG | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn12  | CAGGAGGTGAGGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn13  | CTCGAGAGGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn14  | GCCGCTGAGGTTGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn15  | GCCGAGGAGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn16  | CTGGGAGGAGGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn17  | CTTGAGGTGAGGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn18.1-1.2A| AGTGAAGAGGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn18.2-1.2A| GTATGGGAGGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn19  | CAGGAGACTGTTGTTGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn20  | GGTAACTAGTGGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn21  | CGAGGACTGTTGTTGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn22  | CTTGAGGTGAGGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn23  | TGATGGGAGGAGGAGGAGGAGGAGA | AGGCCGCAAGAGCTGACATTGAGAG |
| mCldn24  | GATGATTGACTACTTACAGAAG | TAAAGGCAGAGTGACGAGGAG |
| mCldn25  | GATGATTGACTACTTACAGAAG | TAAAGGCAGAGTGACGAGGAG |
| mCldn26  | GATGATTGACTACTTACAGAAG | TAAAGGCAGAGTGACGAGGAG |
| mCldn27  | GATGATTGACTACTTACAGAAG | TAAAGGCAGAGTGACGAGGAG |

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