Mesenchymal stem cells promote healing of nonsteroidal anti-inflammatory drug-related peptic ulcer through paracrine actions in pigs

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most important causes of peptic ulcer disease in high-income countries. Proton pump inhibitors are the current standard treatment; however, safety and long-term adverse effects of using these drugs are attracting more and more concerns in recent years. Using a porcine model of NSAID-related gastric ulcer, we herein show that adipose-derived mesenchymal stem cells (ADMSCs) delivered by endoscopic submucosal injection promoted ulcer healing with less inflammatory infiltration and enhanced reepithelialization and neovascularization at day 7 and day 21 when compared with the controls (saline injection). However, only few engrafted ADMSCs showed myofibroblast and epithelial cell phenotype in vivo, suggesting the ulcer healing process might be much less dependent on the stem cell transdifferentiation. Further experiment with submucosal injection of MSC-derived secretome revealed a therapeutic efficacy comparable to that of stem cell transplantation. Profiling analysis showed up-regulation of genes associated with inflammation, granulation formation, and extracellular matrix remodeling at day 7 after injection of MSC-derived secretome. In addition, the extracellular signal-regulated kinase/mitogen-activated protein kinase and the phosphoinositide-3-kinase/protein kinase B pathways were activated after injection of ADMSCs or MSC-derived secretome. Both signaling pathways were involved in mediating the major events critical to gastric ulcer healing, including cell survival, migration, and angiogenesis. Our data suggest that endoscopic submucosal injection of ADMSCs serves as a promising approach to promote healing of NSAID-related peptic ulcer, and the paracrine effectors released from stem cells play a crucial role in this process.

INTRODUCTION
The rise in the incidence of peptic ulcer complications in elderly patients is most likely due to the increased use of nonsteroidal anti-inflammatory drugs (NSAIDs) for management of rheumatic and cardiovascular diseases (1). More than 60 million Americans aged >50 years regularly take NSAIDs (2), resulting in clinically relevant upper gastrointestinal (GI) events (gastric or duodenal perforation, upper GI bleeding, and gastric outlet obstruction) in 2 to 4% of users (3). Among the available reports in the 1990s, estimated deaths attributable to NSAID use varies from 3200 to higher than 16,500 per year in the United States (4, 5).

NSAID-related gastropathy is associated with impaired GI mucosa defensive mechanisms (6, 7) caused by inhibition of cyclooxygenase (COX) and subsequent reduction in prostaglandin synthesis (8). Inflammation (9, 10), gastric hypermotility (11), and oxidative stress (12) also play a role. Acid suppression therapy with proton pump inhibitors (PPIs) or histamine-2 receptor antagonists (H2RAs) is commonly prescribed for the management of peptic ulcer disease. About 95% NSAID-related gastric ulcers healed after 8 weeks of acid suppression if the NSAIDs were discontinued, whereas for patients continuing to take NSAIDs, the therapeutic efficacy decreased by 30% with delayed ulcer healing (13). Prophylactic administration of PPIs to prevent GI complications is necessary for most NSAIDs users. However, safety and long-term effects of using PPIs are attracting increasing concerns in recent years (14). Evidence has suggested that chronic use of PPIs may exacerbate NSAID-induced small intestinal injury (15, 16) and increase the risk of gastric cancer in patients (17).

Adipose-derived mesenchymal stem cells (ADMSCs) are adult multipotent cells isolated from adipose tissue. These cells are capable of multilineage differentiation and have potent paracrine activity of releasing cytokines, chemokines, and trophic factors (18). Studies have shown the capability of mesenchymal stem cells (MSCs) in enhancing healing of gastric pathology and perforation after surgical repair (19–21). However, the potential of ADMSCs in promoting healing of NSAID-related gastric ulcer remains unknown. Because most MSCs would be trapped in the lung upon first passage if injected intravenously (22), the approach to deliver stem cells to target area and the feasibility need to be further elaborated. This study aimed to investigate the feasibility and effectiveness of endoscopic submucosal injection of ADMSCs on the healing of NSAID-related gastric ulcer in a porcine model.

RESULTS
The porcine model simulated the clinical situation in which patients develop gastric ulcers after long-term NSAID treatment
The porcine model of NSAID-related gastric ulcer was induced by administration of indomethacin (15 mg/kg) before and after creation of artificial ulcers (5 cm in diameter) with techniques of endoscopic...
submucosal dissection (ESD) (Fig. 1A). After 10 days of indomethacin administration before ESD, 36% (4/11) of the pigs developed gastric ulcers, with endoscopy showing lesions mainly at the body of the stomach (Fig. 1B).

Compared with the normal controls in which pigs were not given indomethacin, treatment of pigs with indomethacin before and after ESD significantly delayed ulcer healing ($P = 0.018$); the healing rate reduced by about 30% in the indomethacin group compared with the normal controls at day 7 ($P = 0.044$) and day 14 ($P = 0.001$) but was not different at day 21 (Fig. 1C).

Endoscopic submucosal injection of ADMSCs accelerated healing of NSAID-related gastric ulcer by day 7

We then investigate the therapeutic potential of ADMSCs in the promotion of ulcer healing. MSCs were harvested from pig adipose tissue (fig. S1A). Cells were verified to be negative for CD45 (hematopoietic stem cell–associated marker) but positive for CD44, CD105, and CD29 (MSC-associated markers) (fig. S1B). Differentiation in vitro indicated that cells could be induced to differentiate into adipocytes, osteocytes, and chondrocytes (fig. S1C), confirming these cells had the ability of multilineage differentiation.

Five pigs were included in this experiment. Two ulcers were made in each pig; one was randomly assigned to endoscopic submucosal injection of ADMSCs, whereas the other was designated for saline injection as the control. There was no difference in the resection square between the paired ulcers. One perforated ulcer in the control group was found at necropsy at day 21 (Fig. 2A). Follow-up endoscopic surveillance showed that endoscopic injection of ADMSCs led to accelerated ulcer healing compared with the controls ($P = 0.025$; Fig. 2, B and C). The ulcer index in the ADMSC group reduced to 43.07% of the original size by day 7 (versus 85.41% in the control group; $P = 0.042$) but was not different from that in controls at day 14 and day 21 (Fig. 2C and table S1A). Histology was evaluated in terms of inflammation and mucosal regeneration (Fig. 2D). The leukocyte infiltration at granulation tissue and the scoring of glandular architecture around the ulcer margin were not different between the ADMSC group and the control group at day 21 (Fig. 2D). Nevertheless, the ulcer margin height was reduced ($P = 0.005$), whereas the granulation tissue depth was increased after ADMSC treatment at day 21 ($P = 0.048$; Fig. 2D).

ADMSC treatment stimulated reepithelization and neovascularization and alleviated inflammation

Peptic ulcer healing involves the subidence of inflammation, cell migration and epithelial regeneration (reepithelization), neovascularization, gland reconstruction, and matrix reconstitution (23). Here, we first examined the proliferative tissue-resident cells after ADMSC injection by immunostaining for the proliferating cell nuclear antigen (PCNA; a proliferation marker of the G1/S phase). The number of PCNA$^+$ cells per field of view around the ulcer margin was significantly higher in the ADMSC group.
than in the control group at day 7 ($P < 0.01$; Fig. 3A). Treatment with ADMSCs induced higher expression of PCNA at the regions of ulcer margin and granulation tissue also at day 21 ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3A). To evaluate the impact of ADMSC treatment on the neovascularization, we then determined the capillary density in the ulcer area by immunostaining for CD31 (a marker for endothelial cells) and expression of vascular endothelial growth factor (VEGF). A significantly higher density of CD31$^+$ capillaries was observed in ulcers treated with ADMSCs than in controls at day 21 ($P < 0.01$; Fig. 3B). VEGF expression showed no difference between groups at day 7 but increased in the ADMSC group compared with controls at day 21 ($P = 0.026$; Fig. 3C). Furthermore, ADMSC injection significantly up-regulated interleukin-10 (IL-10) expression at day 7 and day 21 ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3D), whereas IL-1$\beta$ expression did not show difference between groups at both time points (Fig. 3E). However, the myeloperoxidase (MPO) activity in ulcer samples obtained at day 21 was decreased in the ADMSC group compared with the controls ($P < 0.05$; Fig. 3F).

Fig. 2. Endoscopic submucosal injection of ADMSCs facilitates healing of NSAID-related gastric ulcer by day 7. (A) Details of the endoscopic submucosal dissection (ESD) specimen and necropsy at day 21. (B and C) Endoscopy to measure the ulcer size in pigs. (D) Hematoxylin & eosin (H&E) staining and scoring in terms of ulcer margin height, glandular architecture, granulation tissue depth, and leukocyte infiltration. Scale bars, 100 $\mu$m. Pigs, $n = 5$. All data are shown as means ± SEM. Change in ulcer index was analyzed by repeated-measures ANOVA with a post hoc multivariate test for each time point. Comparison between paired ulcers was made with dependent samples $t$ test. *$P < 0.05$ and **$P < 0.01$. 

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Fig. 3. Treatment with ADMSCs promotes cellular proliferation and angiogenesis and reduces inflammation. (A and B) Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and CD31. Scale bars, 100 µm. (C to F) The expression of vascular endothelial growth factor (VEGF), IL-10, and IL-1β in ulcer samples was measured by Western blot. Myeloperoxidase (MPO) activity was determined by ELISA. (G and H) Representative images (pig 1) of engraftment of stem cells (red) in vivo and fluorescence counterstaining for α-smooth muscle antigen (α-SMA) (green) and pan-cytokeratin (green). Scale bars, 200 µm. Pigs, n = 5. All data are shown as means ± SEM. Comparison between paired ulcers was made with dependent samples t test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. DAPI, 4’,6-diamidino-2-phenylindole. *P < 0.05 and **P < 0.01.
The ulcer healing process did not depend on the engraftment and transdifferentiation of stem cells in vivo
The function of stem cells for tissue repair may partly rely on the migration of MSCs to the injured site and then differentiation into specialized resident cells that subsequently replace the damaged tissues. To track the stem cells in vivo, we labeled ADMSCs with PKH-26 before endoscopic injection. Immunofluorescence demonstrated that stem cells engrafted at the lamina propria, submucosa, and muscularis externa of the stomach at day 21 (Fig. 3, G and H, and fig. S2). However, we did not find obvious colocalization of the engrafted cells with the staining for α-smooth muscle actin (α-SMA) and pan-cytokeratin, indicating that these cells did not express vascular smooth muscle cell, myofibroblast, and epithelial cell phenotype in vivo (Fig. 3, G and H). This evidence hence suggests that the role of differentiatedMSCs in promoting ulcer healing is minimal.

MSC-secreted factors alleviated indomethacin-induced cytotoxicity and enhanced angiogenesis and cell migration in vitro
Accumulating evidence in the literature suggests that the effects of stem cells in promoting tissue regeneration might not be mainly due to their differentiation properties but on their paracrine effects (24, 25). In this study, RT²Profiler PCR (polymerase chain reaction) array was used to demonstrate the secretome profile of ADMSCs at transcriptional level. To illustrate the cytokines and chemokines with relatively high expression, we set IL-13 as a control because the cycle threshold (CT) value of IL-13 was at the median among all the identified genes; cytokines and chemokines with a CT value lower than IL-13 were then considered as high expression. The relative fold gene expression was calculated using the 2−ΔΔCT method (26). The result showed that M-CSF (macrophage colony-stimulating factor) (362.54-fold), VEGFA (45.48-fold), CCL-19 (C-C motif chemokine ligand 19) (30.38-fold), MCP-1 (monocyte chemotactrant protein 1) (116-fold), MIF (macrophage migration inhibitory factor) (931.24-fold), TGF-β1 (transforming growth factor-β1) (49.59-fold), and TGF-β2 (62.16-fold), which were considered the key factors involved in tissue healing, had transcriptional expression of more than 30 times higher compared with IL-13 (Fig. 4A).

To investigate the role of paracrine activity of ADMSCs in enhancing ulcer healing, we collected ADMSC-derived conditioned medium (MSC-CM) for the following experiments. Cytotoxicity assay was performed by culturing GES-1 cells (normal human gastric epithelial cell line) with indomethacin, followed by treatment with MSC-CM in vitro. Results showed that indomethacin induced cell death as the drug concentration increased to 200, 400, and 800 μM (Fig. 4B). Coculture with MSC-CM improved cell survival; 19 and 37% increase in viability were determined when cells were incubated with 10 and 20% MSC-CM, respectively (Fig. 4C).

To explore the angiogenic activity of MSC-CM, we seeded the growth-arrested human microvascular endothelial cells (HMVECs) in a Matrigel-coated plate and incubated with phosphate-buffered saline (PBS), MSC-CM (10%), or VEGF (20 ng/ml) for 8 hours. Quantification analysis demonstrated that the mesh counts, the number of junctions and branches, and total length of branches per field of view were higher in the MSC-CM group than in the control (Fig. 4D). Scratch assay on the GES-1 monolayer showed that MSC-CM (10%) treatment facilitated wound closure; the percentage of the healed area was smaller in the MSC-CM group than in the control group at 12 and 24 hours (Fig. 4E).

Treatment with MSC-CM facilitated healing of NSAID-related gastric ulcer in pigs
We further examined the potential of MSC-CM in promoting the healing of NSAID-related gastric ulcer in the porcine model. Twelve ulcers were created in six pigs (n = 6), followed by treatment with endoscopic submucosal injection of MSC-CM (10 ml) or Dulbecco’s modified Eagle’s medium (DMEM; control) (Fig. 5, A and B). Results showed that although MSC-CM treatment did not induce an overall reduction in ulcer size (Fig. 5A), the percentage change in ulcer index was reduced in the MSC-CM group compared with the control group at day 7 (P = 0.012; Fig. 5A and table S1B). Histological assessment revealed that MSC-CM treatment resulted in a lower ulcer margin height as well as a deeper granulation tissue than the control at day 21, whereas the scoring of inflammation infiltration and glandular architecture remained similar between groups (Fig. 5C).

Submucosal injection of MSC-CM promoted angiogenesis and alleviated inflammation and oxidative stress
Given that stem cell transplantation activated processes crucial to ulcer healing including reepithelization, angiogenesis, and anti-inflammation, we hypothesized that these cellular cascades might also participate after MSC-CM injection. Results showed that MSC-CM injection promoted the formation of new capillary blood vessels; the density of CD31+ capillaries was significantly higher in the MSC-CM group than in the control group at day 7 and day 21 (P < 0.05 and P < 0.01, respectively; Fig. 5D). More CD31+ capillaries were also observed at day 21 than at day 7 in the MSC-CM group (Fig. 5D), whereas this trend was not shown in the control group. Western blot only found an increase in VEGF protein expression at day 21 after ESD (Fig. 5E). At day 7, the VEGF protein expression remained similar between the two groups (Fig. 5E); however, the VEGF mRNA expression at the same time point was up-regulated after MSC-CM injection (Fig. 5F).

The IL-1β expression in ulcer tissue was not affected by MSC-CM treatment (Fig. 5G), whereas IL-10 expression was increased in the MSC-CM group than in the control group at day 7 and day 21 (Fig. 5H). Enzyme-linked immunosorbent assay (ELISA) assay further indicated that MPO activity was decreased after MSC-CM injection at day 7 but returned at control level at day 21 (Fig. 5I).

Real-time quantitative PCR indicated that the expression of catalase, superoxide dismutase 1 (SOD1), and SOD2 in ulcer samples obtained at day 7 increased by 5.7-, 10.0-, and 18.9-fold, respectively, after MSC-CM treatment compared with controls (Fig. 5, J to L). ELISA assay further showed that the activities of catalase and SOD in ulcer tissue were higher in the MSC-CM group than in the control group at day 7 (Fig. 5, M and N).

Submucosal injection of MSC-CM induced proliferation of wound-resident cells
To determine the cellular proliferation after MSC-CM injection, we performed immunostaining for PCNA on the sections of ulcer samples. It revealed that the number of PCNA+ cells per field of view around the ulcer margin, as well as at granulation tissue, was significantly higher in the MSC-CM group than in the control group at day 7 (P < 0.01 and P < 0.05, respectively) and day 21 (P < 0.01 and P < 0.01, respectively) (Fig. 6A).

We then performed double immunofluorescence staining in an attempt to investigate the identity of these proliferative cells. In the mucosa area, 90.58% of PCNA+ cells in the MSC-CM group and 89.18% in the control group were coabeled with pan-cytokeratin (the epithelial cell marker) at day 7 (Fig. 6B and
the proportion remained high in each group (85.54 and 91.50%, respectively) at day 21, suggesting the epithelial cells actively proliferated throughout the ulcer healing process (Fig. 6B and table S2A).

In the submucosa area, the proliferative endothelial cells were identified by coexpression of CD31 and PCNA; it accounted for 25.12% of PCNA+ cells in the MSC-CM group and 16.18% in the control group at day 7, as well as 29.47 and 22.85%, respectively, at day 21 (Fig. 6C and table S2B). Similar result of dual staining with α-SMA was also seen in the PCNA+ cells with a spindle shape in granulation tissue, which were indicated to be myofibroblasts (white arrows, Fig. 6D). The amount in the control group was 11.43% at day 7 and 13.35% at day 21, compared with 6.84 and 6.89% in the MSC-CM group at each time point (table S2C1). α-SMA also labeled the small arteries (yellow arrows, Fig. 6D). There were 15.27% α-SMA+PCNA+ vascular smooth muscle cells showing in the MSC-CM group and 2.76% in the control group at day 7, compared with 3.51 and 5.59%, respectively, at day 21 (table S2C2). These results together demonstrated that the population of CD31+ endothelial/endothelial progenitor cells and α-SMA+ myofibroblasts and vascular smooth

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**Fig. 4. Stem cell–secreted factors alleviate indomethacin-induced cytotoxicity and enhance angiogenesis and cell migration in vitro.** (A) RT2 Profiler PCR array for MSC-derived cytokines and chemokines. (B and C) MTS assay testing the cell viability upon exposure to indomethacin (IND), with or without MSC-CM treatment. (D) Representative photographs of tube formation of human microvascular endothelial cells (HMVECs) on the Matrigel-coated 24-well plate after 8 hours of exposure to phosphate-buffered saline (PBS), MSC-CM, or VEGF. The number of meshes, junctions, and branches formed per high-power field (HPF), and the total length of branches per HPF were calculated by ImageJ software. (E) Representative images of vertical scratch made on the monolayer of human gastric epithelial cells GES-1 at 0, 12, and 24 hours posttreated with PBS or MSC-CM. The wound area was quantified using ImageJ software, and the percentage change was calculated as 100× (open wound area at 12 or 24 hours)/(open wound area at 0 hour). All data are shown as the means ± SEM. Statistical significance was analyzed by independent samples t test or one-way ANOVA followed by a Tukey post hoc analysis between two or multiple groups, respectively. Please refer to table S3 for the full gene names. *P < 0.05 and **P < 0.01.

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muscle cells consisted of the PCNA+ cells in granulation tissue within the submucosa.

Leucine-rich repeat–containing G protein–coupled receptor 5 (Lgr5) marks resident stem cells in antral part of the stomach (27). In this study, a single Lgr5+ cell was detected in the pyloric compartment of the glandular stomach in pigs (yellow arrows, Fig. 6E), whereas it only accounted for a small proportion of PCNA+ cells in the MSC-CM group and control group at each time point (table S2D).

The ulcer-associated cell lineage (UACL) occurring at sites of chronic GI ulceration appears to mediate the ulcer healing through coexpression of multiple growth factors (28, 29). In the current study, periodic acid–Schiff (PAS) staining identified the UACL as magenta-colored buds or glandular structures at the base of the ulcer margin (Fig. 7). Colabeling experiment further showed that PCNA colocalized with UACL glands in the MSC-CM group and the control group at day 7 (31.51 and 21.39%, respectively) as well as day 21 (26.28 and 23.76%, respectively) (table S2E).

Profiling analysis demonstrated up-regulation of genes related to wound healing after MSC-CM treatment

RT2 Profiler PCR array detected the expression of 84 genes related to wound healing in ulcer samples obtained at day 7 (Fig. 8A). A total of 46 genes were significantly up-regulated and 1 gene was...
Fig. 6. Treatment with MSC-CM induces the proliferation of wound-resident cells. (A) IHC for PCNA. Scale bars, 50 μm. (B to E) Immunofluorescence double staining for pan-cytokeratin, CD31, α-SMA (white arrows indicate the proliferative myofibroblasts; yellow arrows indicate the proliferative vascular smooth muscle cells), and leucine-rich repeat–containing G protein–coupled receptor 5 (Lgr5) (yellow arrows indicate the proliferative Lgr5+ cells) with PCNA. Scale bars, 75 μm. Pigs, n = 6. All data are shown as means ± SEM. Comparison between paired ulcers was made with dependent samples t test. *P < 0.05 and **P < 0.01.
down-regulated (collagen, type IV, alpha 3) after MSC-CM treatment compared with the control (Fig. 8B). Specifically, fold change in the gene expression related to the functions of inflammation {CD40LG (CD40 ligand), CXCL12 [chemokine (C-X-C motif) ligand 12], IL-1β, IL-2, and IL-10}, reepithelization [ANGPT1 (angiopoietin 1), CSF2 (colony stimulating factor 2), EGF, VEGFA, TGF-α, FGF7 (fibroblast growth factor 7), EGFRR (epidermal growth factor receptor), FGF10, FGF2, and TGF-β1], and extracellular matrix remodeling [PLAU (urokinase plasminogen activator surface receptor-like), MMP1-3 (matrix metalloproteinase 1–3), MMP7, MMP9, PLAT (plasminogen activator, tissue), PLAU, F13A1 (coagulation factor XIII, A1), F3, and SERPINE1 (serpin peptidase inhibitor, clade E, member 1)] are shown in Fig. 8 (C to E). A plot linking the identified genes to the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signaling in the (C to E). A plot linking the identified genes to the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signaling in the

**Stem cell therapy did not reverse the indomethacin-induced inhibition of the COX2–PGE2 axis but activated the ErK/MAPK and the PI3K/Akt pathways**

The COX2–prostaglandin E2 (PGE2) axis protects the mucosal integrity in the GI tract (30). In this study, ADMSC transplantation increased the expression of COX2 in ulcer samples (fig. S4A), whereas the PGE2 expression at day 21 was not different between the ADMSC group and the control group (fig. S4A), suggesting that ADMSC treatment did not reverse the NSAID-induced inhibition of the COX2–PGE2 axis.

Other signaling pathways including the extracellular signal–regulated kinase 1/2 (ErK1/2)–MAPK and phosphoinositide-3-kinase (PI3K)/Akt are known to be critical for cell survival, migration, and angiogenesis, all of which are involved in the regulation of the ulcer healing process (31, 32). Here, our results demonstrated that endoscopic injection of ADMSCs or MSC-CM increased the ErK phosphorylation in ulcer tissue obtained at both day 7 and day 21 compared with controls (fig. S4B). However, Akt phosphorylation in ulcer samples was only observed at day 21 after ADMSC injection as well as at day 7 after MSC-CM injection compared with controls (fig. S4C).

Then, we performed a series of studies in vitro to verify these findings. The time-course experiment with GES-1 cells showed that MSC-CM treatment induced ErK phosphorylation at 10 min (fig. S5). The phosphorylation peaked about three times higher than that in the control group at around 30 min (P < 0.01; fig. S5). MSC-CM treatment also activated the Akt signaling; increased phosphorylation of Akt was observed at 5 min, which rapidly peaked to nearly sevenfold increase compared with the control group at 30 min (fig. S5). Both ErK and Akt phosphorylation subsequently returned to control level at 3 and 6 hours (fig. S5).

To investigate the possible impact of ErK and Akt activation on the cell survival, migration, and angiogenesis in vitro, we exposed GES-1 cells or HMVECs to PD98059 (PD, 20 μM; an inhibitor of MEK1 activation and MAPK cascade) (24), LY294002 (LY, 15 μM; a highly selective inhibitor of phosphatidylinositol-3-kinase) (33), or both for 2 hours before MTS assay showed that MSC-CM treatment prevented the cell death induced by indomethacin, whereas this effect was abrogated by treatment with ErK inhibitor, Akt inhibitor, or both (fig. S6A). Inhibition of both pathways also induced a lower cell viability than blocking ErK/MAPK alone (fig. S6A). Scratch wound healing assay demonstrated that inhibition of ErK and Akt activation suppressed the MSC-CM–induced cell migration for wound closure in vitro; the percentage of the unhealed area was larger in the PD, LY, or PD + LY group than in the MSC-CM group at 12 and 24 hours (fig. S6B). In addition, blocking ErK or Akt activation substantially impaired the ability of vascular endothelial cells to form the capillary-like structures after MSC-CM treatment (fig. S6C).

**Coadministration of MSC-CM did not enhance the therapeutic efficacy of PPI on NSAID-related gastric ulcer**

PPI is commonly prescribed for patients with NSAID-related peptic ulcer; it is not clear whether coadministration of MSC-CM would improve efficacy of PPI treatment. In addition to the submucosal injection of MSC-CM at days 1, 7, and 14, respectively, pigs (n = 4) had oral pantoprazole (40 mg) daily after ESD until euthanasia at day 21. There was no difference in the resection square between the MSC-CM plus PPI treatment group and the DMEM plus PPI treatment group (fig. S7, A and B, and table S1C). Within the course of PPI therapy, additional injection of MSC-CM did not further enhance the healing of NSAID-related gastric ulcer; there was no overall difference in the percentage of ulcer size reduction between the two groups (fig. S7C). Multivariate test for each time point also showed no difference between groups (fig. S7C).

**DISCUSSION**

This study sought to investigate the therapeutic potential of MSCs on NSAID-related peptic ulcer in a porcine model. Here, we found that ADMSCs delivered by endoscopic submucosal injection promoted ulcer healing at day 7, with enhanced reepithelization and angiogenesis and reduced inflammation in the ulcer area. This healing process rarely depended on the engraftment and transdifferentiation of stem cells in vivo. Further experiment showed that endoscopic injection of MSC-derived secretome achieved comparable ulcer healing effects, with up-regulation of genes central to the inflammation,
granulation, and tissue remodeling. In addition, the COX-2-PGE2 axis inhibited by indomethacin did not respond to stem cell therapy, whereas the ErK/MAPK pathway, as well as the PI3K/Akt pathway, was activated after injection of stem cells or stem cell–derived secretome (fig. S8).

Here, we introduced a porcine model of NSAID-related gastric ulcer created by indomethacin administration plus ESD. Our data suggest that indomethacin administration for 10 days induced scattered ulcerations in the stomach of pigs, and continuous oral indomethacin lowered the healing of ulcers created by ESD. This animal model

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**Fig. 8.** Treatment with MSC-CM up-regulates the transcriptions of genes related to wound healing in ulcer tissue obtained at day 7. (A) Heatmap of the expression of 84 genes in the MSC-CM group and the control group \((n = 2 \text{ ulcers per group})\). (B) Volcano plot of the differentially regulated genes between the MSC-CM group and the control group. (C to E) Heatmaps of differentially regulated genes involved in the processes crucial to gastric ulcer healing, including inflammation, reepithelization, and extracellular matrix remodeling. Please refer to table S4 for the full gene names. Pigs, \(n = 6\). All data are shown as means ± SEM. Statistical significance was analyzed by independent samples \(t\) test. *\(P < 0.05\) and **\(P < 0.01\).
specifically appear in the proliferative (PCNA+) compartment. These, which were reported as the endogenous gastric stem cells, were also actively after injection of MSC-CM at day 7 and day 21. Lgr5+ cells, blasts, and smooth muscle cells at granulation tissue proliferated in the mucosa of the ulcer margin, and the endothelial cells, myofibroblasts, and smooth muscle cells at granulation tissue proliferated actively after injection of MSC-CM at day 7 and day 21. Lgr5+ cells, which were reported as the endogenous gastric stem cells, were also detected in the antral glands of the stomach, although they did not specifically appear in the proliferative (PCNA+) compartment. These cells, which secrete a variety of growth factors, cytokines, and transcription factors, can exert different biological activities in terms of promoting reepithelization, gland reconstruction, formation of granulation tissue, neovascularization, and matrix reconstitution, finally restoring the epithelial continuity (23).

Studies on human and animal models have shown that healing of gastric ulcer follows a characteristic pattern, which consists of slow healing in the first so-called lag phase, rapid healing in the second phase, and, again, slow healing in the third so-called remodeling phase (35, 36). Schmassmann et al. (37) previously reported that indomethacin affected gastric ulcer healing predominantly at the early phase, showing profound ulcer healing delay occurring during days 7 to 15 after ulcer creation in a rodent model. This was consistent with our observation that indomethacin ingestion reduced ulcer size reduction in pigs at day 7 and day 14 after ESD compared with the normal controls, but not at day 21. Moreover, clinical trial demonstrated that acid suppression therapy with omeprazole accelerated ulcer healing mainly during the early (first 2 weeks) healing stage (38). Given that the maximum relative risk for indomethacin-induced GI complications is estimated at 14 days after initiation of treatment (39), strategy to facilitate the early healing of GI mucosal erosions will have profound implication and provide benefits for patients requiring long-term NSAID use. In the current study, we observed enhanced healing of NSAID-related gastric ulcer after injection of ADMSCs or MSC-CM at day 7, with an average ulcer size reducing to about 40% of the original size. Although the ulcer size in the stem cell treatment group was similar to that in controls at days 14 and 21, the early promotion of ulcer healing at day 7 might be of more clinical relevance.

Angiogenesis to restore the blood flow and, thus, to support oxygen and nutrient delivery to the healing site is an essential process during peptic ulcer healing (24). NSAIDs have been shown to impair angiogenesis at granulation tissue (40, 41). Basic FGF (bFGF) is the major growth factor responsible for angiogenesis; subcutaneous or intragastric treatment with bFGF was found to increase angiogenesis and the ulcer healing process despite indomethacin ingestion in the rodent model (37). VEGF is another mitogen of endothelial cells that promotes capillary regeneration (42). In the current study, we observed that MSC-CM treatment induced a higher vessel density at granulation tissue at day 7 after ESD, when VEGF protein expression remained similar between the two groups. Contradictorily, VEGF mRNA expression in ulcer tissue obtained at day 7 was higher after MSC-CM injection compared with controls as determined by RT2 Profiler PCR array and further confirmed by real-time quantitative PCR. This discrepancy might be related to different detection methods because PCR for mRNA quantitation is much more sensitive than immunoblotting for determination of relative protein expression. An alternative is that VEGF can appear as a doublet on SDS–polyacrylamide gel electrophoresis as a consequence of glycosylation; hence, the apparent discrepancy may not actually exist.

The COX-PGE2 axis exhibits a crucial role in the maintenance of mucosal integrity of the stomach. In the current study, although COX2 was up-regulated after ADMSC treatment at day 7, PGE2 expression was not increased at the same time point. Our data were similar to a previous study showing that omeprazole and bFGF treatment could not reverse the indomethacin-induced decrease in prostaglandin generation in rats (37). Although the mechanism is not elucidated, it might be related to the substantial inhibition of the functionalized COX2 by indomethacin, resulting in the limited compensatory synthesis of PGE2. We found that the activation of the ErK/MAPK and PI3K/Akt pathways was increased after injection of ADMSCs or MSC-CM. The ErK/MAPK pathway controls a broad range of cellular activities that are crucial to gastric ulcer healing, including reepithelization (31) and, to some extent, angiogenesis (43).

In addition, the PI3K/Akt pathway is known to play key roles in cell viability and migration that are independent of ErK (44). In the current study, our data determined that blocking the activation of MAPK/ErK and PI3K/Akt signal transduction abrogated MSC-CM–induced cell survival, migration, and angiogenesis in cell cultures, implying the importance of ErK and Akt activation on promoting gastric ulcer healing after treatment with ADMSCs or MSC-CM.

Whether the stem cell–assisted organ repair is primarily through cell differentiation to replace the damaged tissues or through a paracrine effect with release of trophic factors remains controversial (45). Our previous study found that only few MSCs showed a myofibroblast phenotype with expression of α-SMA at day 14 after transplantation into the stomach in rats (20), suggesting that the differentiation process is unlikely to play a role in stem cell–mediated therapeutic effects. Moreover, in the present study, the importance of paracrine effects of MSCs was supported by the observation of quick ulcer healing at day 7 after MSC transplantation, when MSC differentiation in vivo was still incomplete. Recently, an increasing number of studies have turned to investigate the potential of stem cell–derived secretome for the treatment of myocardial infarction, hepatic failure, GI neurodegenerative disorder, and diabetes mellitus (46–49). Such secretome-based therapy seems more promising and valuable in regenerative medicine, because it may bypass a series of issues that arise with stem cell therapy (50). MSC-derived secretome refers to the broad repertoire of trophic and immunomodulatory cytokines produced by stem cells (18). In this study, we observed that submucosal injection of stem cell–derived secretome achieved ulcer healing effects comparable to those of stem cell transplantation, accompanied by up-regulation of genes controlling the process of inflammation, reepithelization, and tissue remodeling. Therefore, current evidence suggests that the therapeutic action of stem cells in
promoting healing of NSAID-related gastric ulcer mainly depends on the paracrine activity of MSCs.

One limitation of this study is that the key therapeutic molecules in MSC-CM and their interactions with tissue-resident cells were not elucidated. A good understanding of the molecules involved in the therapeutic effect is crucial before moving to clinical practice. Furthermore, because ErK/MAPK and PI3K/Akt pathways were activated after injection of stem cells or MSC-CM, multiple injections might then lead to sustained ErK or Akt activation that may trigger adverse events such as the development of hyperplasia (51, 52); the optimal course of treatment, dosing, and long-term follow-up remain to be determined. Nevertheless, current results showed that the effects of stem cell treatment on promoting ulcer healing were primarily established at the early time point (day 7), suggesting a single dosage of stem cells or MSC-CM might be enough to trigger the ulcer healing process.

In summary, our study showed that endoscopic submucosal injection of ADMSCs facilitated healing of NSAID-related gastric ulcer through suppressing inflammatory infiltration and promoting cellular proliferation and angiogenesis. In this process, the paracrine actions of stem cells appeared to be more critical than differentiation. Our findings suggest that stem cell–based therapy might be a promising alternative for patients with NSAID-related GI ulceration, especially for those who suffer the side effects from long-term use of PPI.

MATERIALS AND METHODS

Study design
The objective of this study was to investigate whether treatment with ADMSCs would promote healing of NSAID-related peptic ulcer. We harvested the primary ADMSCs from five allogeneic porcine donors, verified the cell surface markers using fluorescence-activated cell sorting, and tested the potential of multilineage differentiation before storage in liquid nitrogen for experimental use. This study was based on a self-controlled design; two ulcers were created in each pig: one was randomized to the experimental group (MSCs or MSC-CM treatment), and the other served as the control. Follow-up endoscopic surveillance was performed every week to evaluate ulcer healing. The sample size was estimated on the basis of our experience, using this animal model in preliminary experiments and the power calculation (effect size = 1.57, α = 0.05, and power = 0.80). Cell experiments were conducted to validate key signaling transductions involved in mediating the major cellular events responsible for the enhanced ulcer healing process. All experiments in vitro were repeated at least three times. Blinded approach was used for the histopathological evaluation and analysis of immunohistochemistry (IHC) and immunofluorescence images. Original data were reported in data file S1. All animal procedures were carried out in accordance with the guidelines of the Animals Ordinance (Chapter 340), Department of Health, Hong Kong, and were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (15-025-MIS).

Statistical analysis
A self-controlled design was adopted in this study. This design alleviated individual differences between porcine models and enabled a unitive baseline of ulcer area for comparison. Results were expressed as means ± SEM or median (interquartile range) when appropriate. Normality of distributions was tested by P-P plot and Shapiro-Wilk test. Repeated-measures analysis of variance (ANOVA) was used to examine changes in the ulcer index throughout the study period, with a post hoc multivariate test for each time point. Comparison of data between the paired gastric ulcers in each pig was made with dependent samples t test or Wilcoxon signed-rank test when appropriate. Independent samples t test, or Mann-Whitney U test when appropriate, and one-way ANOVA followed by a Tukey post hoc analysis were used to determine statistical significance between two or multiple groups, respectively. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 16.0, and a two-sided P < 0.05 was considered statistically significant. A detailed methodology of the animal study and cell experiments is illustrated in the Supplementary Materials and Methods.

REFERENCE AND NOTES

View/request a protocol for this paper from Bio-protocol.


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Author contributions: X.X. conducted the animal and in vitro experiments, analyzed the data, and drafted the paper; K.F.C. and G.T.Y.W. provided assistance on the animal operation and did the primary stem cell culture; P.W. and B.P.M.Y. collected and processed the samples; L.L., E.K.W.N., and J.Y.W.L. commented on the study and interpreted the data; and P.W.Y.C. designed and supervised the study and critically revised the manuscript. All authors reviewed and approved the final manuscript.

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Data and materials availability: All data associated with this study are present in the paper or in the Supplementary Materials.

Mesenchymal stem cells promote healing of nonsteroidal anti-inflammatory drug-related peptic ulcer through paracrine actions in pigs

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Secreting healing factors
One of the main adverse events associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is the development of gastric ulcers. Proton pump inhibitors are used to alleviate this adverse event; however, they are associated with serious adverse events. Now, Xia et al. developed a porcine model of NSAID-induced gastric ulcers and showed that intragastric administration of adipose tissue–derived mesenchymal stem cells (ADMSCs) rapidly promoted healing and reduced gastric inflammation. The therapeutic effect was mediated by activation of the ErK and Akt pathways in ulcer tissue induced by stem cell–secreted factors. The results suggest that mesenchymal stem cells or their secretome could be effective in treating NSAID-induced gastric ulcers.