Appendix e1
ESD procedure and injection of CHST15 siRNA

Step 1: Creation of esophageal ESD ulcers
A standard endoscope (GIF-Q260; Olympus Medical Systems, Tokyo, Japan) was used for all procedures, in combination with carbon dioxide insufflation to mitigate mediastinal emphysema in the event of perforation. First, we performed a half-circumferential and 5-cm longitudinal marking by soft coagulation at 25–30 cm and at 35–40 cm from the incisors. A solution of glycercol (10% glycercol and 5% fructose) was then locally injected to lift and separate the mucosa and submucosa from the muscularis propria. Circumferential incision on the soft coagulation marking and submucosal dissection were performed using the ESD device (Flush-knife; Fujifilm Co., Tokyo, Japan) to create the 5-cm half-circumferential defects.

Two ESD ulcers were induced in each porcine model (▶Fig. 1a). Throughout the ESD procedure, a high frequency generator (VIO 300D; ERBE Elektromedizin GmbH, Tübingen, Germany) was used as follows: marking, soft coagulation mode, 60 W, effect 5; mucosal incision, Endocut I mode, cut duration 3, cut interval 3, effect 2; and submucosal dissection, forced coagulation mode, 45 W, effect 4.

Step 2: CHST15 siRNA injection following esophageal ESD
After completion of step 1, a single endoscopic injection of CHST15 siRNA was performed, using a 25-gauge, 4-mm needle (TOP Corporation, Tokyo, Japan), in one of the two ESD ulcers in each animal. A 1-mL syringe was used to inject the CHST15 siRNA manually, which facilitated injection at a consistent pressure. The needle was inserted superficially to avoid injury to the muscularis propria, and CHST15 siRNA was injected in aliquots of 0.1 mL (1 mg) into the remnant submucosa, with an additional 0.5 mL (5 mg) into the peripheral mucosa (▶Fig. 1b).

A total volume of 100 mg (total 100 mg/10 mL) of CHST15 siRNA was injected. The same protocol was followed for all animals, as summarized in ▶Table 1. Oral intake was recommenced immediately after the procedure.

Appendix e2
Histological assessment of the ulcers treated with or without CHST15 siRNA injection
Tissue specimens were fixed in 10% buffered formalin for 24 hours and the area of the ESD ulcers was dissected along the short axis of the ulcer at 4-mm intervals. Each tissue specimen was embedded in a paraffin block, which was cut into slices of 4-μm thickness and stained with hematoxylin and eosin (H&E). Serial sections were cut for Masson trichrome staining (▶Fig. 5). Imaging analysis software (cell Sens, ver. 1.7; Olympus) was used for the subsequent visual analysis of microscopic slides.

Multicolor immunofluorescence assessment
Evaluation of the expression of vimentin and α-smooth muscle actin (α-SMA) in ESD ulcers was performed on formalin-fixed paraffin-embedded esophageal sections that were deparaffinized with xylene and ethanol, and incubated with goat polyclonal anti-vimentin antibody (SC-7557; Santa Cruz Biotechnology, Dallas, Texas, USA) and rabbit monoclonal anti-α-SMA antibody (ab5694; Abcam, Tokyo, Japan). Alexa Fluor 555-labeled donkey anti-goat IgG and Alexa Fluor 488-labeled chicken anti-rabbit IgG (Invitrogen, Carlsbad, California, USA) were next reacted with DAPI nuclear counterstain (mounting medium with DAPI; Vector Laboratories Inc., Burlingame, California, USA). The sections were evaluated by immunofluorescent microscopy (BZ-9000; Keyence, Osaka, Japan).

The localization of fibroblasts (vimentin+, α-SMA−) and myofibroblasts (vimentin+, α-SMA+) within the ulcer area was evaluated by calculating the concentration of fibroblasts and myofibroblasts at ×400 magnification in three different regions of the ulcer. The values were averaged, and the standard deviation was calculated for each ulcer.

Quantitative real-time polymerase chain reaction (RT-PCR)
RT-PCR analysis was performed on tissue specimens from ESD ulcers treated with and without CHST15 siRNA and normal esophageal mucosa for each pig (total of nine lesions in three pigs). Total RNA was extracted using Trizol (Invitrogen), according to the standard protocol, and then reverse transcribed. Thereafter, complementary DNA was amplified using the ABI 7700 sequence-detector system (Applied Biosystems, Foster City, California, USA), with a set of primers and probes corresponding to CHST15, TGF-β, collagen-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of mRNA expression were normalized to that of GAPDH, as CHST15/GAPDH, TGF-β/GAPDH, and collagen-1/GAPDH. Data were expressed as the average ± standard deviation.

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Statistical analysis
Comparison between ulcers treated with or without CHST15 siRNA treatment was evaluated by Student’s t test for macroscopic assessment, with one-way analysis of variance (ANOVA) and post-hoc Bonferroni multiple comparison for immunofluorescence and RT-PCR analyses, with significance set at P<0.05. Statistical analysis was performed using GraphPad Prism, version 6.0 for Windows (GraphPad Software, San Diego, California, USA).
In untreated ESD ulcers, thin and immature epithelium covered almost all ulcer beds (▶ Fig. 5a). Beneath the regenerative epithelium, a large number of spindle-shaped cells were observed, considered fibroblasts and myofibroblasts (▶ Fig. 5a, right lower inset). Compared with normal esophageal tissues (▶ Fig. 5a, left upper inset), strong and dense deposition of ECM was observed around these cells, with an observable defect in the muscle layer. Thick fibrotic tissue was identified, partly occupying the superficial surface of the muscularis propria (▶ Fig. 5a, yellow triangle).

In contrast, in ESD ulcers treated with CHST15 siRNA, thick regenerative epithelium covered the edges of the ulcer, but not the center of the ulcer bed (▶ Fig. 5b). Beneath the regenerative epithelium and in the uncovered bed of the ulcer, round and oval-shaped cells were mainly observed, with no identifiable defect in the muscle layer (▶ Fig. 5b, right lower inset). Overall, ECM deposition was relatively weak and sparse in ESD ulcers treated with CHST15 siRNA, compared with ulcers not treated with CHST15 siRNA.

CHST15 siRNA reduced the number of mesenchymal cells post-ESD

In normal esophageal tissue, subepithelial fibroblasts (vimentin +, α-SMA−) or myofibroblasts (vimentin +, α-SMA+) were rarely observed (17.7 ± 2.1 and 0 ± 0 per high power field [HPF], respectively). In ESD ulcers not treated with CHST15 siRNA, the number of fibroblasts and myofibroblasts significantly increased beneath the regenerative epithelium (314 ± 31.0 [P < 0.001] and 191 ± 12.2 [P < 0.001], respectively), compared with those in the normal esophageal tissue. In ESD ulcers treated with CHST15 siRNA, the number of fibroblasts and myofibroblasts was significantly reduced, compared to those in ulcers not treated with CHST15 siRNA (224 ± 17.7 [P = 0.009] and 25 ± 10.5 [P < 0.001], respectively) (▶ Fig. 6).

CHST15 siRNA inhibited the expressions of CHST15, TGF-β, and collagen-1

PCR analyses were performed to investigate the expression of CHST15 mRNA and the effect of CHST15 siRNA injection. CHST15 mRNA was weakly detectable in the normal esophageal mucosa and was dramatically increased in ulcers 14 days after ESD (12.1 ± 3.0). CHST15 siRNA injection significantly reduced the expression of CHST15 mRNA (4.3 ± 1.0; P = 0.01). The expression of TGF-β and collagen-1 mRNAs was increased in esophageal ESD ulcers (8.7 ± 1.3 and 13.5 ± 2.0, respectively), compared with those in the normal esophageal tissues. CHST15 siRNA significantly decreased the expression of TGF-β mRNA (2.5 ± 0.39; P < 0.001) and collagen-1 mRNA (4.8 ± 1.2; P < 0.001) on day 14 (▶ Fig. 7).