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Original Article

Enhancement of tracheal cartilage regeneration by local controlled release of stromal cell-derived factor 1α with gelatin hydrogels and systemic administration of high-mobility group box 1 peptide

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ABSTRACT

Introduction: This present study evaluated the effect of combination therapy with stromal cell-derived factor 1α (SDF-1α) and high-mobility group box 1 (HMGB1) peptide on the regeneration of tracheal injury in a rat model.
Methods: To improve this effect, SDF-1α was incorporated into a gelatin hydrogel, which was then applied to the damaged tracheal cartilage of rats for local release. Furthermore, HMGB1 peptide was repeatedly administered intravenously. Regeneration of damaged tracheal cartilage was evaluated in terms of cell recruitment.
Results: Mesenchymal stem cells (MSC) with C-X-C motif chemokine receptor 4 (CXCR4) were mobilized more into the injured area, and consequently the fastest tracheal cartilage regeneration was observed in the combination therapy group eight weeks after injury.
Conclusions: The present study demonstrated that combination therapy with gelatin hydrogel incorporating SDF-1α and HMGB1 peptide injected intravenously can enhance the recruitment of CXCR4-positive MSC, promoting the regeneration of damaged tracheal cartilage.

1. Introduction

Tracheobronchomalacia (TBM) is characterized by congenital or acquired deficiency of tracheal cartilage, which can lead to airway collapse, dyspnea, acute life-threatening events, or death. The estimated incidence of congenital TBM is 1 in 2100 newborn infants [1]. In infancy, severe TBM sometimes necessitates intervention, including tracheostomy and ventilator support. Moreover, a poor clinical response often requires escalation of surgical intervention including aortopexy, tracheal stenting, or tracheoplasty [2]. However, these surgical results are not always effective because tracheal cartilage, which constitutes the airway, is poorly regenerates. Once damaged or subjected to surgical invasion, tissue healing progresses in the form of replacement of fibrotic scarring, resulting in the formation of newly acquired stenosis.

Our study group has previously prepared gelatin hydrogels that can maintain a constant concentration of chemokines and other signaling factors locally *in vivo* for several weeks by heating and cross-linking bio-applicable gelatin under various conditions, and has demonstrated that the gelatin hydrogels can induce regeneration at the site of tissue injury [3–6]. Moreover, we have reported that when a high concentration of stromal cell derived factor-1α (SDF-1α), a chemokine (cell migration factor), is maintained at the site of injury for a certain period, the migration of bone marrow-derived mesenchymal stem cells (BM-MSC) to the site of injury is promoted, which in turn promotes tissue healing [7–10].

Abbreviations: TBM, tracheobronchomalacia; SDF-1α, stromal cell-derived factor 1α; HMGB1, high-mobility group box 1; PDGFRα, platelet-derived growth factor receptor alpha; CXCR4, C-X-C motif chemokine receptor 4.

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The objective of this study was to investigate whether the controlled release of SDF-1α by gelatin hydrogel could be applied to the induction of tracheal cartilage regeneration by creating a rat tracheal injury model mimicking TBM. In addition, we loaded high-mobility group box 1 (HMGB1), a type of inflammatory cytokine, into the circulating blood to promote the mobilization of MSC from the bone marrow to the peripheral blood, and simultaneously examined whether this has a synergistic effect on tracheal cartilage repair.

HMGB1 is generally considered to be an aggravating factor for inflammation, and its systemic administration is unlikely to promote healing of tissue damage under normal circumstances [11]. However, from the results of skin regeneration experiments, HMGB1 released from necrotic epithelial cells reportedly enhances the *in vivo* migration of platelet-derived growth factor receptor alpha (PDGFRα) +/C-X-C motif chemokine receptor 4 (CXCR4) + cells (Pα cells) from the bone marrow leading to their migration into circulation. Subsequently, Pα cells home to the damaged skin through the SDF-1α/CXCR4 axis and consequently facilitate tissue repairs [7,12,13]. Moreover, recent studies have shown that a peptide fragment of the A-box domain of the two DNA-binding domains of the HMGB1 protein (referred to as the A-box and B-box) (HMGB1 peptide) promotes the mobilization of MSC from the bone marrow and may not contribute to inflammation [14–19].

In this study, we aimed to investigate the possibility that the simultaneous combined SDF-1α-containing gelatin hydrogel sheet and systemic administration of HMGB1 peptide to the regeneration of tracheal chondrocytes which are considered to be difficult to induce differentiation and regeneration by conventional methods, would further promote regeneration.

2. Methods

2.1. Preparation of gelatin hydrogels incorporating SDF-1α

Gelatin hydrogels incorporating SDF-1α were prepared as reported previously [9]. The gelatin sample prepared by acidic treatment of pig skin collagen (iso electric point [IEP] = 9) was kindly supplied by Nitta Gelatin Inc. (Osaka, Japan). In brief, an aqueous gelatin solution (5% [w/v], IEP = 9) in double distilled water (DDW) was cast into a polytetrafluoroethylene mold, followed by freeze-drying to obtain gelatin hydrogels. The freeze-dried hydrogels were crosslinked by dehydrothermal treatment at 140 °C for 24 h in a vacuum oven and then cut into 3 × 8 mm pieces using scissors. After ethylene oxide sterilization, an aqueous solution of SDF-1α (recombinant human SDF-1α, 350-(NS/CF), R&D Systems Inc., Minneapolis, MN, USA; 1μg/10 μl phosphate-buffered saline [PBS], pH 7.5) was infused into 0.8–1 mg of the gelatin hydrogels and left at 4 °C overnight to obtain SDF-1α-incorporated gelatin hydrogels. This gelatin hydrogel sheet is biodegradable and is absorbed over approximately one week while gradually releasing SDF-1α [5].

2.2. An in vitro migration assay of MSC with SDF-1α and HMGB1 peptide

The chemokinetic migration of rat MSC (RMSC) was assayed using an 8.0-μm Costar Transwell (Corning Inc., Cambridge, MA, USA). In brief, freeze-dried gelatin hydrogels prepared with a diameter of 8 mm using a biopsy punch (Kai Industries Co., Ltd.0, Gifu, Japan) were dropped with 100 or 500 ng of SDF-1αsolution (10 μl) and left at 4 °C overnight to allow the solution to incorporate. Next, the gelatin hydrogels were placed in 100 μl PBS containing 30 μg/ml collagenase D at 37 °C overnight to obtain the hydrogels lysis solution as reported previously [5,8,20]. The MSC recruitment domain derived from human HMGB1 was generated as 'HMGB1 peptide' by solid-phase synthesis. HMGB1 peptide was kindly supplied by StemRIM Inc. (Osaka, Japan) and was dissolved in distilled water and normal saline (NS/Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to a concentration of 1 mg/ml. The hydrogel lysis solution, 100 or 500 ng of SDF-1α solution and/or NS, 100 or 1000 μg of HMGB1 peptide in 600 μl of Dulbecco's modified Eagle's medium (DMEM/Gibco-BRL, Grand Island, NY, USA) containing 0.1% (w/v) BSA and 100 U/ml penicillin and streptomycin (PS) were added to the lower chambers, and 10^6 RMSC/mL (Cell Applications Inc., San Diego, CA, USA) suspended in 300 μl of DMEM containing 0.1% (w/v) BSA and 100 U/ml PS were added to the upper chamber. Therefore, NS, 100, 500 ng of SDF-1α, 500 ng of SDF-1α/1000 μg of HMGB1 peptide, 100, 500 ng of SDF-1α gelatin, 500 ng of SDF-1α gelatin/1000 μg of HMGB1 peptide and 100, 1000 μg of HMGB1 peptide were set. The Transwell was placed in a 37 °C, 5% CO2 humidified incubator for 4 h. The cells on the lower side of the membrane were stained with hematoxylin. Images were captured using a microscope (BZ-X710; Keyence Co., Ltd., Osaka, Japan). The number of cells in 5 randomly chosen fields was counted at 200× magnification. The experiment was performed independently with five samples per experimental group.

2.3. Animals

All animal experiments were conducted in compliance with institutional regulations and ARRIVE guidelines, and all animal care and experimental protocols used in this study were approved by the local ethics committee of the Kyoto University Graduate School of Medicine. The experiments were conducted using three-week-old female Wistar rats from Shimizu Laboratory (Shimizu Laboratory Supply Co., Ltd., Kyoto, Japan). All rats were maintained under specific pathogen-free conditions in wire cages at 22–24 °C and 60 ± 5% humidity and were provided standard laboratory food (Oriental Yeast Co., Tokyo, Japan).

2.4. Preparation of a tracheal cartilage injury model

A new TBM model was created based on a tracheal stenosis model [21]. Three-week-old female Wistar rats were anesthetized by intraperitoneal injection of a combination anesthetic with 0.375 mg/kg medetomidine, 2.0 mg/kg midazolam, and 2.5 mg/kg butorphanol. The rats were placed in the supine position. The anterior cervical area was prepped with 70% ethanol. A 1-cm vertical incision was made in the midline of the neck between the level of the hyoid bone and suprasternal fossa. The underlying muscles were split to expose the trachea to below the level of the cricoid cartilage. A bipolar device (Conmed Sabre Genesis; CONMED Corporation, Largo, CA, USA) was set at 10 W. A malacic segment was made on the anterior of the 1–2 to 4–6 tracheal rings by electric coagulation using 2-s exposures. When the tracheal wall became concave (Fig. 1A and B), the intended depth of injury to the lateral surface of cartilage was reached. This model was used to treat tracheal cartilage injury. Rats were closed after cauterization. In contrast, rats with closed wounds without cauterization of the trachea were used in the sham group. To minimize variations in intervention, all operations were performed by a single surgeon.

2.5. The in vivo evaluation of the expression of SDF-1α at tracheal cartilage injury

Rats were used to create a tracheal cartilage injury model. The rats were sacrificed at 3 h, 3 days, and 1, 2, and 4 weeks after the operation. To determine the expression of SDF-1α at the site of

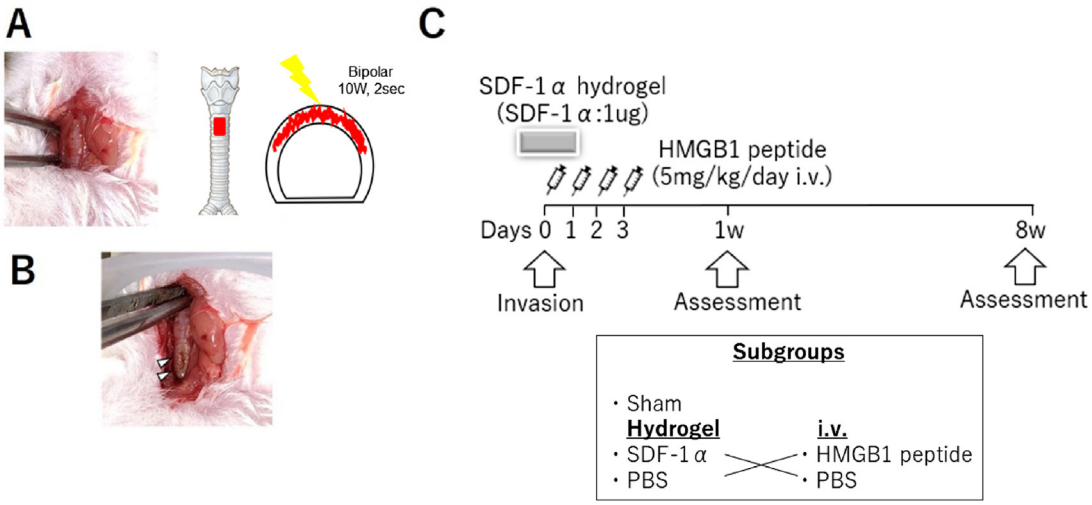


Fig. 1. Experiment summary of a rat tracheal cartilage injury model.
(A) The anterior surface of the trachea of 3-week-old Wister rat was exposed, and thermal injury with 20 J (10 W/2 s) was induced using bipolar forceps. (B) The damaged sites are indicated by white arrows. (C) Administration schedule of SDF-1α-containing gelatin hydrogel and HMGB1 peptide and the time schedule for the evaluation.

tracheal cartilage injury, real-time polymerase chain reaction (RT-PCR) and immunohistochemical staining were performed.

Total RNA was extracted from tracheal cartilage injury samples (n = 5/time point) and reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). The resulting cDNA was used for RT-PCR with the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Division of Life Technologies Corporation, Carlsbad, CA, USA) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Rat-specific primers were used for SDF-1 (Assay ID: Rn00573260_m1; Thermo Fisher Scientific), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Assay ID: Rn01775763_g1; Thermo Fisher Scientific). Each sample was analyzed in duplicate for each gene studied. The data were normalized to the GAPDH expression levels. The delta-delta Ct (ddCt) method was used for relative expression analysis, and samples from the control rats were used as a reference.

Immunohistochemical staining was performed using an anti-SDF-1 antibody that cross-reacted with the tracheal cartilage injury obtained at each time point. For immunohistochemical analysis, specimens were fixed with 4% phosphate-buffered paraformaldehyde for 24 h at 4 °C. The tissues were equilibrated in PBS containing 10%, 15%, and 20% sucrose for each 12 h and embedded in Tissue-Tek OCT Compound (Sakura Finetek Inc., Tokyo, Japan). A 7-μm-thick section was obtained from the most damaged part. After washing, the sections were treated with HistoVT One (Nacalai Tesque) for 20 min at 70 °C to enhance the antigen-antibody reaction. The sections were treated with BLOXALL (Vector Laboratories, Burlingame, CA, USA) for 10 min at room temperature. The sections were then blocked with ImmunoBlock (KAC Co., Ltd., Kyoto, Japan) for 30 min at room temperature, and incubated with anti-SDF-1 antibody (1:100; ab9797, Abcam Cambridge, MA, USA) at 4 °C overnight. The VECTASTAIN Elite ABC Kit (PK-6101) was used as the secondary antibody. The color was developed by 3,30 -diaminobenzidine tetrahydrochloride (DAB) for 20 s at room temperature. Methyl green was used as the counterstain.

2.6. Spontaneous healing process of tracheal cartilage injury model in morphology

We evaluated the spontaneous healing process in a tracheal cartilage injury model. The rats were euthanized at 3 h, 3 days, and 1, 2, 4, 8, and 12 weeks after surgery for histological analyses (sham n = 4/time point, model n = 6/time point). As with the tissue fixation, described above, tracheal specimens were fixed in 4% phosphate-buffered paraformaldehyde and equilibrated in PBS containing sucrose. The samples were embedded in Tissue-Tek OCT compound and frozen, after which 7-μm-thick sections were obtained, and histochemical hematoxylin-eosin, Alcian blue and collagen II staining were performed. For collagen II staining, those sections were incubated with anti-collagen II antibody (1:100; ab34712, Abcam Cambridge, MA, USA) at 4 °C overnight. The VECTASTAIN Elite ABC Kit (PK-6101) was used as the secondary antibody. The color was developed by DAB for 2 min at room temperature. Hematoxylin-eosin staining was performed for the counterstaining.

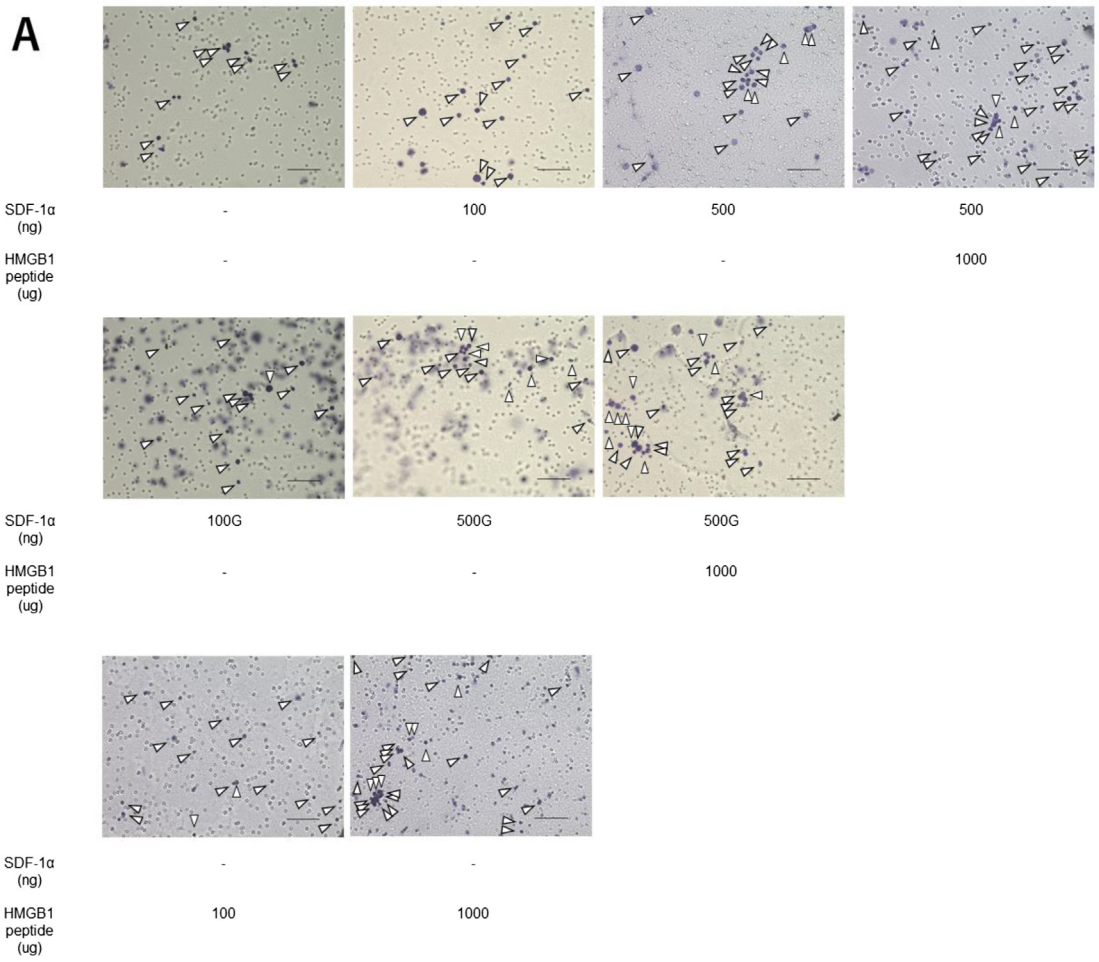
Images were captured using a BZ-X710 microscope, and histological changes were examined. Using the BZ-X Analyzer, the tracheal luminal area, vertical length, horizontal length, horizontal-to-vertical ratio of the tracheal lumen, and maximum thickness of the tracheal cartilage were measured from the stained images. Three sections were analyzed per specimen and averaged.

2.7. The evaluation of the effects of SDF-1α gelatin hydrogel and HMGB1 peptide injection

Female 3-week-old Wistar rats were used to establish a tracheal cartilage injury model. Gelatin hydrogels incorporating SDF-1α or PBS were wrapped around tracheal cartilage injury. After the incisions were closed, rats received either HMGB1 peptide (5 mg/kg/day) or the same volume of PBS as a control by injection into the tail veins for 4 consecutive days under 2% sevoflurane inhalation. After complete hemostasis, rats were housed in temperature-controlled

Table 1
Subgroups receiving a combinaion of gelatin hydrogel and systemic administraion.

Code	Scheme	
	Hydrogel	i.v.
PgPi	PBS	PBS
SgPi	SDF-1α	PBS
PgHi	PBS	HMGB1 peptide
SgHi	SDF-1α	HMGB1 peptide



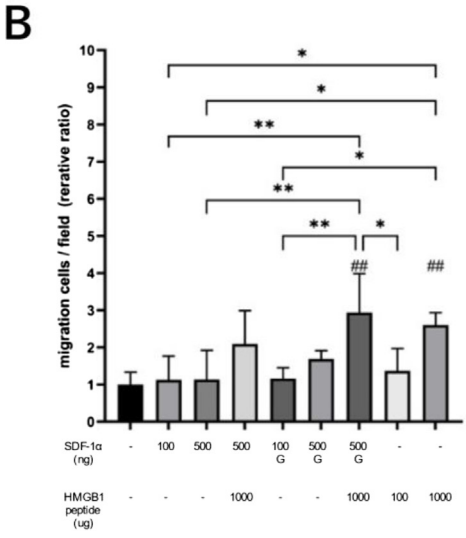


Fig. 2. Chemotaxis experiment of BM-MSC using a Transwell.
(A) Representative images of migrated MSC in each additive. White arrows indicate the migrated cells. Scale bar = 100 μm. G: gelatin. (B) Number of migrating cells compared to NS. *P*-values were calculated using a one-way ANOVA followed by Tukey's test. $P < 0.01^{\#\#}$ significant against the value of the NS group, $P < 0.05$*, $P < 0.01$** significant against two groups. G: gelatin.

cages. The rats were euthanized if markedly poor weight gain, average body weight −2 standard deviations (SD), or cyanosis was observed. One week after the operation, the accumulation of Pα cells was assessed by RT-PCR. Eight weeks after the operation, tracheal cartilage regeneration was assessed using histological analyses. The rats were euthanized (Fig. 1C) and divided into four subgroups (PBS gelatin hydrogel and PBS injection; PgPi, SDF-1α gelatin hydrogel and PBS injection; SgPi, PBS gelatin hydrogel and HMGB1 peptide injection; PgHi, SDF-1α gelatin hydrogel and HMGB1 peptide injection; SgHi) (Table 1).

2.7.1. The in vivo evaluation of the gene expression of PDGFRα and CXCR4 at tracheal cartilage injury

Specimens were obtained one week postoperatively (n = 4/group). Using rat-specific primers for PDGFRα (Assay ID: Rn01399472_m1, Thermo Fisher Scientific) and CXCR4 (Assay ID:

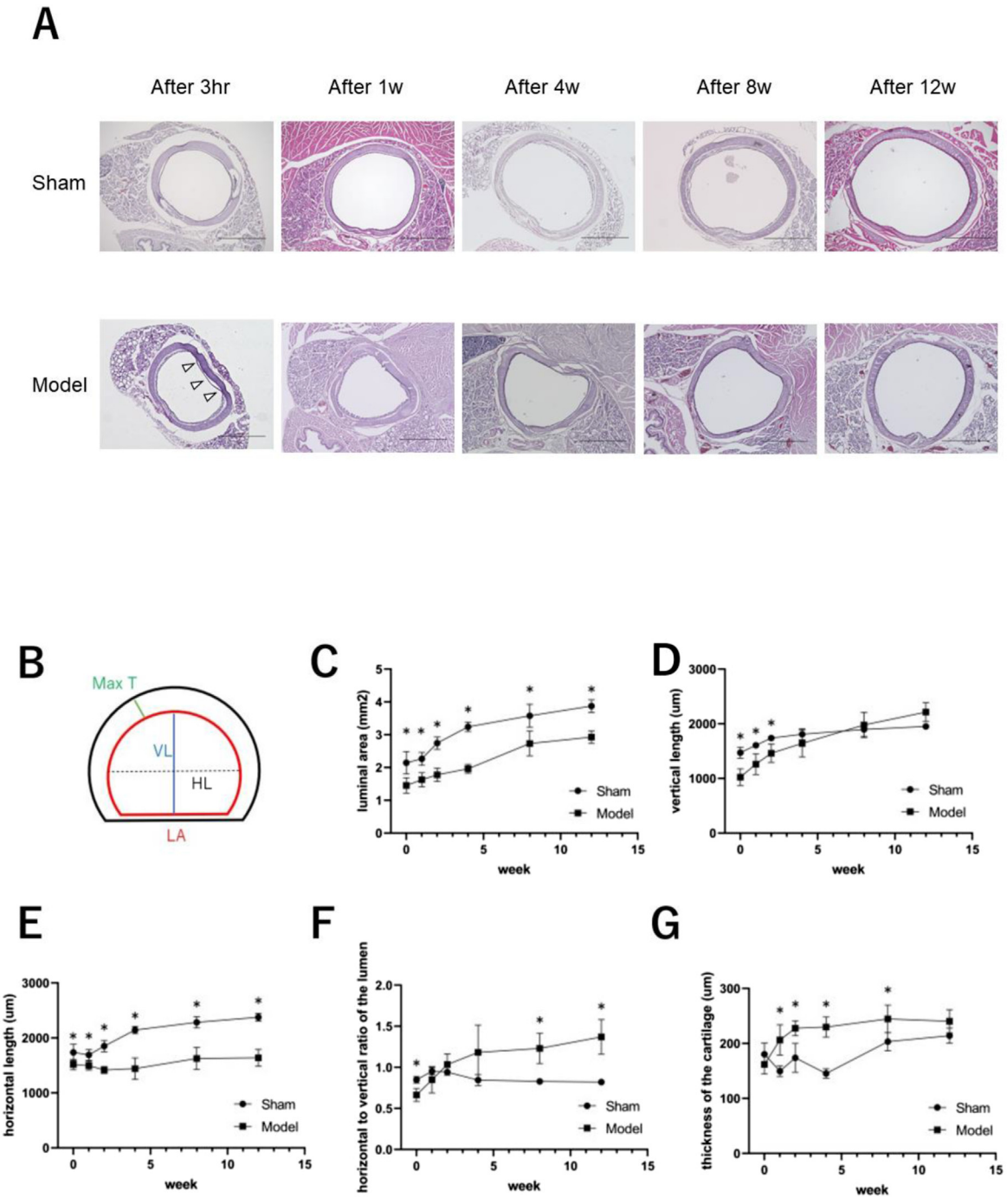


Fig. 3. Observation of the morphological regeneration process of cross-section of trachea in rat tracheal cartilage model.
(A) Temporal changes in tracheal cross-section after injury induction. Representative photomicrographs (top row: scale bar = 1 mm, bottom row: scale bar = 500 μm) of hematoxylin and eosin staining. White arrows indicate the site of heat injury. (B) The measurement sites are shown below. LA, tracheal luminal area; VL, vertical length; HL, horizontal length; Max T, maximum thickness of the tracheal cartilage. Tracheal cross-sectional luminal area (C), vertical length (D), horizontal length (E), horizontal-to-vertical ratio of the lumen (F), and maximum thickness of the tracheal cartilage (G) over time after injury change. *P*-values were calculated using the Mann-Whitney *U* test. $P < 0.05$*.

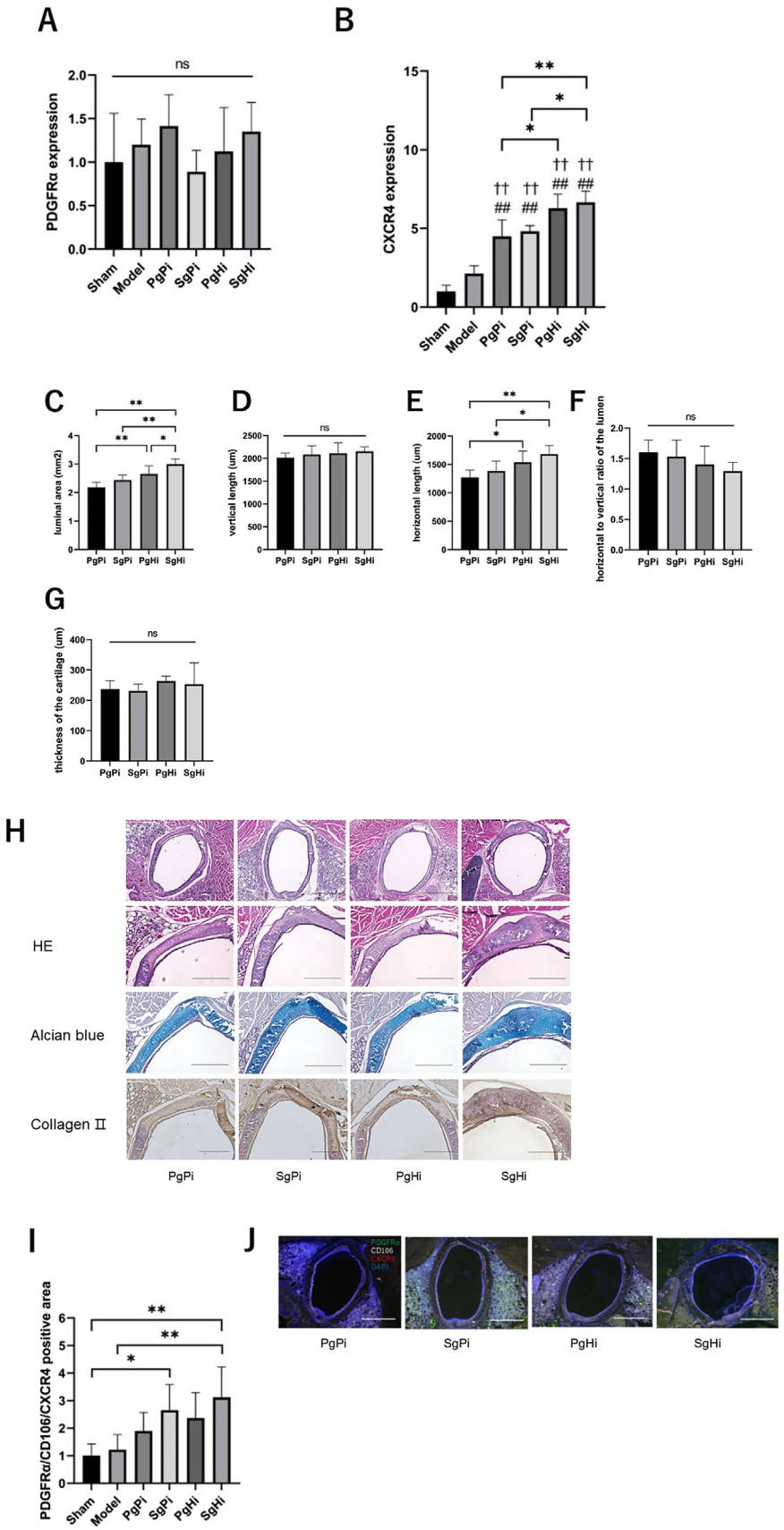


Fig. 4. Changes in subgroups in tracheal regeneration experiments.
Expression of PDGFRα (A) and CXCR4 (B) mRNA in the trachea one week after injury. Tracheal cross-sectional luminal area (C), vertical length (D), horizontal length (E), horizontal-to-vertical ratio of the lumen (F), and maximum thickness of tracheal cartilage (G) in each group eight weeks after the intervention. *P*-values were calculated using a one-way

Rn00573522_s1, Thermo Fisher Scientific), the samples were subjected to RT-PCR in the same manner as described above.

2.7.2. Histological analyses of tracheal cartilage regeneration

Specimens were obtained at 8 weeks postoperatively (n = 6/group). Frozen sections were obtained for pathological analyses, and histochemical hematoxylin-eosin, Alcian blue and collagen II staining were performed.

For immunofluorescence staining, the frozen sections were labeled with mouse monoclonal anti-CXCR4 antibody (1:100; 4G10, sc-53534 AF594; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-PDGFRα (1:100; PA5-16571; Thermo Fisher Scientific), and rabbit monoclonal anti-CD106 antibody (1:100, ab134047, Abcam, Cambridge, MA, USA) at 4 °C overnight. PDGFRα and CD106 are expressed in BM-MSC and are commonly used markers [22–24]. Sections were then stained with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:400; Thermo Fisher Scientific) and Alexa Fluor 546 goat anti-rabbit IgG secondary antibody (1:400; Thermo Fisher Scientific) for 1 h. Sections were then mounted with an anti-fade solution, VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories) for 10 min at room temperature.

Images were captured using BZ-X710, BZ-X Analyzer, and ImageJ software program (National Institutes of Health, Bethesda, MD, USA), and the tracheal luminal area, vertical length, horizontal length, horizontal-to-vertical ratio of the tracheal lumen and maximum thickness of tracheal cartilage, and area of PDGFRα+/CD106+/CXCR4+ cells were measured from the stained images. Three sections were analyzed per specimen and averaged.

2.8. Statistical analyses

Values are expressed as mean ± SD. Between-group differences were compared using the Mann-Whitney *U* test. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's test of significance between individual groups. All data were analyzed using GraphPad Prism software, version 9.5.1 for Windows (GraphPad Software, Boston, MA, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. An in vitro migration assay of MSC with and SDF-1α and HMGB1 peptide

Fig. 2 shows the migration of RMSC induced by SDF-1α and HMGB1 peptides. Compared to NS, a slight increase in the number of migration cells was observed in the SDF-1α supplemented group, but also an increase in the number of migration cells was confirmed in the HMGB1 peptide supplemented group depending on the concentration (HMGB1 peptide 100 μg vs. NS; 1.4 ± 0.5 vs. 1.0 ± 0.3, $P < 0.01$**, HMGB1 peptide 1000 μg vs. NS; 2.6 ± 0.3 vs. 1.0 ± 0.3, $P < 0.01$**). In addition, there was no significant difference between the SDF-1α dripping and SDF-1α gelatin hydrogel groups. Furthermore, an increase in the number of migrating cells was observed in the group treated with the SDF-1α and HMGB1 peptide combination. In addition, the SDF-1α gelatin hydrogel and HMGB1 peptide group showed the largest increase in the number of migrating cells. Thus, RMSC migration was observed using SDF-1α and HMGB1 peptides.

3.2. The in vivo evaluation of the expression of SDF-1α at tracheal cartilage injury

Fig. S1 shows the expression of SDF-1α in the damaged tracheal cartilage tissue. The SDF-1 mRNA expression at 1 week was higher in the tracheal cartilage injury rat model than at other time points (1 week vs. 3 h: 6.5 ± 2.5 vs. 1.0 ± 0.5, $P < 0.01$**; 1 week vs. 3 days: 6.5 ± 2.5 vs. 1.5 ± 0.5, $P < 0.01$**; 1 week vs. 2 weeks: 6.5 ± 2.5 vs. 3.5 ± 1.0, $P < 0.01$**; 1 week vs. 4 weeks: 6.5 ± 2.5 vs. 2.5 ± 0.5, $P < 0.01$**) (Fig. S1A). Furthermore, microscopic imaging revealed SDF-1 expression at the border of the tracheal cartilage injury and regenerative area (Fig. S1B).

3.3. Spontaneous healing process of the tracheal cartilage injury model from a morphological perspective

Fig. S2 shows how the tracheal cartilage injury model healed spontaneously without treatment. After bipolar cauterization, cauterization of the ventral hemicircumference of the tracheal cartilage caused the cartilage lacuna to disappear, and the tracheal lumen was concavely deformed (Fig. S2A). The cauterized part became necrotic and began to fall off (Fig. S2B), and the boundary between the normal and necrotic areas swelled like a club. At the border, only cartilage matrix without nuclei was observed (Fig. S2C). Chondrocytes regenerated from the vicinity of the inner and outer perichondrium, forming two or three layers (Fig. S2D). Chondrocytes in the regenerating layer near the inner and outer perichondrium began to form cartilaginous lacunae (Fig. S2E). Chondrocytes in the regenerating layer separated from the perichondrium and began to form cartilaginous lacunae (Fig. S2F). These two to three layers merged into a single layer (Fig. S2G).

Fig. S3A shows images of these changes stained with Alcian blue. The layer near the perichondrium, where regeneration began first, was initially lightly stained, but this layer later became darkly stained.

Fig. S3B shows images of these changes stained with collagen II. Similar to the Alcian blue staining results, the layer near the perichondrium, where regeneration began first, was initially lightly stained, and later became darkly stained, but not with the same intensity as the normal area.

As a result, the shape of the trachea first deformed to become inwardly convex, but then the lumen expanded longitudinally forward and backward. The trachea was not horseshoe-shaped, but the lumen widened longitudinally, and the concave tracheal lumen returned to a convex shape. However, regeneration was never completed and not even at 12 weeks after the injury.

To evaluate these changes, when comparing the regeneration process of damaged tracheal cartilage between the sham and model groups, recovery of the cross-sectional area was obtained, but it was found that the lumen area was significantly smaller in the model group than in the sham group, even after 12 weeks. The model group became increasingly vertically long; however, the horizontal length remained constant over time. In contrast, the tracheal cartilage at the regenerated site was significantly thickened in the model group, as it regenerated in multiple layers, but it was confirmed that the thickness in the model group returned to

ANOVA followed by Tukey's test. $P < 0.01^{\#\#}$ significant against the value of the Sham group; $P < 0.01^{\dagger\dagger}$ significant against the value of the Model group; $P < 0.05$*, $P < 0.01$** significant between the two groups. ns: not significant. (H) Tracheal histology eight weeks after injury. Representative photomicrographs (top row: scale bar = 1 mm, bottom rows: scale bar = 500 μm) of hematoxylin and eosin, Alcian blue, and collagen II staining. Recruitment of Pα cells in each experimental group during tracheal cartilage regeneration. Measurement of the relative area of PDGFRα+/CD106+/CXCR4+ (I) and PDGFRα, CD106, CXCR4, and DAPI multiple immunohistochemical staining (J). *P*-values were calculated using one-way ANOVA followed by Tukey's test. $P < 0.05$*, $P < 0.01$**.

the same level as that in the sham group at 12 weeks after injury, merging into a single layer. As there was almost no change in the length of the horizontal diameter, the aspect ratio was greater than that of the sham group (Fig. 3).

3.4. The in vivo evaluation of the PDGFRα and CXCR4 gene expression at tracheal cartilage injury

RT-PCR data for PDGFRα and CXCR4 expression are shown in Fig. 4A and B. The PDGFRα mRNA expression was not significantly different between the groups. In contrast, the CXCR4 mRNA expression in the HMGB1 peptide-administered group was significantly higher than that in the HMGB1 peptide-unadministered group (PgPi vs. PgHi: 4.4 ± 1.0 vs. 6.1 ± 0.9, $P < 0.05$*; PgPi vs. SgHi: 4.4 ± 1.0 vs. 6.5 ± 0.7, $P < 0.01$**; SgPi vs. SgHi: 4.6 ± 0.4 vs. 6.5 ± 0.7, $P < 0.05$*). The CXCR4 expression increased in the order of Sham < Model < PgPi < SgPi < PgHi < SgHi. There was no correlation between the PDGFRα and CXCR4 expression.

3.5. Histological analyses of tracheal cartilage regeneration

Eight weeks after each treatment, although no significant differences were observed in the vertical length among the groups, the horizontal length was the longest in the SgHi group, and as a result, the luminal area was also the largest in the SgHi group (Fig. 4C–G). In addition, multiple layers merged, and the tracheal cartilage became a single layer in the SgHi group, so the maximum thickness of the tracheal cartilage passed its peak. On Alcian blue and collagen II staining, the SgHi group had the largest area of regenerative cartilage with the same color intensity as the normal part (Fig. 4H). Regeneration progressed in the SgHi group.

3.6. Pα cell mobilization to tracheal cartilage

The area positive for PDGFRα+/CD106+/CXCR4+ cells in the tracheal cartilage increased in the SgHi group compared with the other treatment groups (Sham vs. SgHi: 1.0 ± 0.4 vs. 3.1 ± 1.0, $P < 0.01$**; Model vs. SgHi: 1.2 ± 0.5 vs. 3.1 ± 1.0, $P < 0.01$**; Sham vs. SgPi: 1.0 ± 0.4 vs. 2.7 ±0 .0.9, $P < 0.05$*) (Fig. 4I and J).

4. Discussion

The human body has several endogenous regenerative mechanisms to repair damaged tissues. BM-MSC play an important role in repairing damaged tissues by recruiting other host cells, secreting various growth factors, and differentiating into various cells [25–27]. They promote angiogenesis and suppress fibrosis in various injuries and diseases, including stroke [28] and myocardial infarction [29]. However, cell transplantation therapy has not yet been perfected, with remaining issues including the need for a large cell number [30] and poor cell retention [31]. These problems can be solved by selectively recruiting a large number of stem cells in the body to accelerate endogenous regeneration without the need for a supply of *ex vivo* cells, which is known as endogenous regenerative therapy.

The present study demonstrated that SDF-1α alone was effective in inducing *in vivo* recruitment of MSC to tracheal cartilage damage in this rat model. Interestingly, more effective recruitment was observed when SDF-1α and HMGB1 peptides were used. Among all the groups, the inductive effect was the largest for gelatin hydrogel-incorporated SDF-1α combined with HMGB1 peptide administration. In the combination therapy group, tracheal cartilage regeneration was observed faster than that of the other groups in the cross-section of the trachea and extracellular matrix components, such as acidic proteoglycan, was abundant on Alcian blue staining. Once damaged, hyaline cartilage often does not heal with the original hyaline cartilage and turns into fibrous cartilage; however, in this study, the regenerated cartilage was stained with collagen II, suggesting that hyaline cartilage had regenerated. (Fig. 4H). In the combination therapy group, the stained area of collagen II was larger in comparison to the other groups. Furthermore, the horizontal length was the longest, so the expansion of the luminal area was the largest in the combination therapy group (Fig. 4C). It is conceivable that the largest number of Pα cells was concentrated in the trachea in the combination therapy group, promoting tracheal cartilage regeneration (Fig. 4I and J).

The amount of SDF-1 released *in vitro* from gelatin hydrogels increases with time before eventually becoming saturated [10]. The *in vivo* time profiles of SDF-1 release and hydrogel degradation correlated. SDF-1 was immobilized in the hydrogel through physicochemical interactions with hydrogel gelatin. Immobilized SDF-1 was not released from the hydrogel unless it was enzymatically degraded to produce water-soluble gelatin fragments. SDF-1 is positively charged at physiological pH (pH 7.4) because of its IEP of 10.26. It is conceivable that electrostatic interactions with gelatin facilitated the release of SDF-1. It has been confirmed that SDF-1 is released as a result of degradation of the release carrier [10]. This indicates that SDF-1 release is not governed by the simple diffusion mechanism of SDF-1 but by the degradation of the gelatin hydrogel. The release profiles of SDF-1 can be easily controlled by varying the dehydrothermal treatment time [32]. The degradation period ranges from a few days to several months [5,6]. This gelatin hydrogel sheet degrades over approximately one week while releasing SDF-1α gradually [5,9]. Frozen cross-section examination confirmed that the hydrogel was completely degraded seven days after application in this study (data not shown). This release system for drug delivery prolongs the half-life *in vivo*, resulting in lower doses required with greater therapeutic efficacy [32].

SDF-1 plays an important role in the circulation of bone marrow-derived cells [33,34]. Several studies have reported controlled release of SDF-1 [35]. In our release system, only a single implantation of biodegradable hydrogel could enhance the natural healing potential of the body and thus positively support the healing process. In contrast, several studies have reported the administration of HMGB1 peptide has been administered alone [19,22], but there have been no reports of HMGB1 peptide being used in combination therapy. Combination with other therapeutic agents is another strategy to enhance the therapeutic efficacy of tissue regeneration [36,37]. The combination of SDF-1α and HMGB1 has been reported previously [38]. Our study differs from previous studies in terms of the controlled release of SDF-1α and a combination therapy with HMGB1 peptide. It is likely that more Pα cells are released from the bone marrow into the circulating blood by HMGB1 administration home to the tissue to be repaired by the SDF-1α released, resulting in the promotion of tissue repair.

This combination therapy may be more effective than monotherapy with either HMGB1 peptide or SDF-1α in small-range disorders and chronic diseases where SDF-1α secretion is low. The plasma half-life of SDF-1α is short, 25.8 ± 4.6 min [39], as is that of HMGB1, 17 min [40]. However, when they form complexes, degradation is inhibited [41,42], prolonging their lifetime. The secretion of SDF-1α from injured tracheal cartilage peaked at one week (Fig. S1), which was consistent with the results of previous reports on articular cartilage defect models in which the maximum secretion time was one week [43]. When administering HMGB1 peptide, which has a short half-life, implanting the SDF-1α-containing gelatin hydrogel before the SDF-1α secretion peak was thought to be useful for forming a complex between SDF-1α and HMGB1 peptide. From the perspective of maximum complex formation, it was considered a good practice to supplement SDF-1α

before the peak of SDF-1α secretion, which would necessitate a slow-release administration. This combined approach of the systemic administration of HMGB1 peptide to recruit cells into the blood, while maintaining high local SDF-1α concentrations at sites where cell accumulation is required, may maximize therapeutic efficacy.

The present study, we found that the expression of SDF-1α was increased in tracheal cartilage injury rats, particularly in the border and regenerative area in the tracheal cartilage injury (Fig. S1B). This is consistent with the findings of previous studies such as the peri-infarction area of myocardial infarction in ischemic lesions [19] and articular cartilage defect [43]. This can be explained in terms of Pα cell recruitment to the border and regenerative area via the SDF-1/CXCR4 signaling complex. To ensure a proper homing effect, it is necessary to administer SDF-1α at the site where Pα cells should be localized.

The dose of 1 μg of SDF-1α per hydrogel *in vivo* was determined by converting the rat body weight based on research concerning MSC recruitment activity, which involved experiments involving SDF-1α contained in the gel. The administration dose of HMGB1 peptide was selected based on previous research on the therapeutic effects of HMGB1 peptide [18].

The migration assay results showed differences based on the presence of gelatin hydrogel (Fig. 2), with PgPi in animal experiments expressing more CXCR4 in PCR and more accumulation in fluorescence immunostaining than in the model. Although there was no significant difference, the gelatin hydrogel showed a mild effect, suggesting that the implanted gelatin hydrogel itself promotes regeneration [43]. As there have been reports on the effect of ultra-purified alginate gel as a scaffold material [42], gelatin hydrogel may have had some effect.

RT-PCR and immunohistological staining revealed that SDF-1α and HMGB1 peptide administration increased the expression of CXCR4. In terms of the expression of the MSC surface marker PDGFRα, the expression was not increased by the SDF-1α-containing gelatin hydrogel or the administration of HMGB1 peptide. Although there was no significant difference in the number of MSCs recruited between the groups, it is possible that SDF-1α and HMGB1 peptides increased the proportion of CXCR4-positive MSC among the recruited MSC. This was consistent with the immunostaining results. These results suggested an enhanced SDF-1/CXCR4 signal. However, additional experiments are required to elucidate the mechanism of regeneration promotion.

Animal models of TBM have been reported [2,44,45], in which the tracheal cartilage was fractured or the cartilage rings were peeled off. However, these studies were limited to animals large enough to support the use of such surgical methods and have been limited to Yorkshire pigs [2], piglets [44], mongrel dogs [45], and other animals, as these surgical techniques are difficult to apply to small animals. Because small animals are considered highly versatile, new TBM models in small animals (e.g., mice and rats) are needed in order to facilitate animal research on tracheal cartilage. The method used to generate the model in the present study makes it technically feasible to generate both mouse and rat models (data not shown). In our study, rats were used because the trachea size of rats changes with age, so it was easier to evaluate the size than in mice. Furthermore, the severely injured model in this study exhibited cyanosis and moaning, similar to TBM. The model in this study specifically mimicked the postoperative partial TBM of esophageal atresia. This may serve as a good step for future research on the tracheae. If a gelatin hydrogel incorporating SDF-1α is placed in the site of esophageal tracheal fistula closure during surgery for esophageal atresia, there is no need to consider invasive surgical procedures. This is advantageous for clinical application. This model differs from the previously reported large animal models that mimic the weakening of tracheal strength. However, we try to use the present animal model to evaluate cellular mechanisms which we expect to experimentally demonstrate in this study: that the promotion of cell recruitment to the injured tracheal cartilage is responsible for the regenerative repair of cartilage tissue.

No previous reports have described the spontaneous regeneration of injured tracheal cartilage. The methods for assessing tracheal cartilage regeneration, such as via the lumen area and cartilage thickness, were based on the study by Ishimaru et al. [46]. These endpoints corresponded well with the expected clinical effects of lumen enlargement and improvement in tracheal cartilage fragility. However, with respect to the thickness of cartilage, increasing the cartilage matrix and accelerating cartilage regeneration is more important than merely increasing the cartilage thickness. In the present study, the luminal area at eight weeks after treatment was the largest in the SgHi group. The maximum thickness of the tracheal cartilage was not markedly different among the groups, but histologically, the process of tracheal cartilage regeneration was advanced in the SgHi group. In this study, the reproducibility of the accelerated regenerative process was experimentally confirmed using this combination therapy.

5. Conclusions

A combination of locally controlled release of SDF-1α using gelatin hydrogels and systemic administration of HMGB1 peptide promotes the regeneration of tracheal cartilage by recruiting Pα cells. Combination therapy is useful strategy for TBM treatment.

Author's contribution

K.S. conceived and designed the study; performed experiments; collected, analyzed, and interpreted the data; and wrote the manuscript. T.O. interpreted the data and helped write the manuscript. Y.T. interpreted the data, helped write the manuscript and approved the final draft. K.T. and E.H. supervised this study. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of this work.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.06.017.

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