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## Successful use of bio plugs for delayed bronchial closure after pneumonectomy in experimental settings

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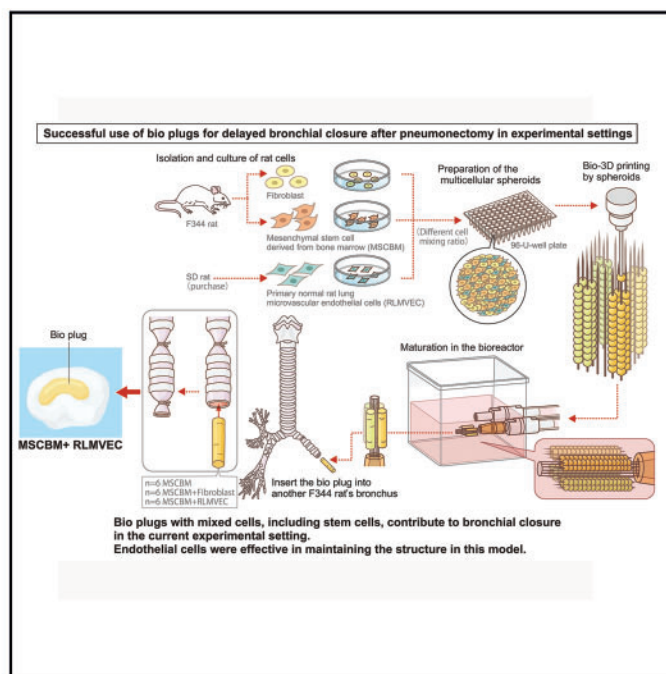
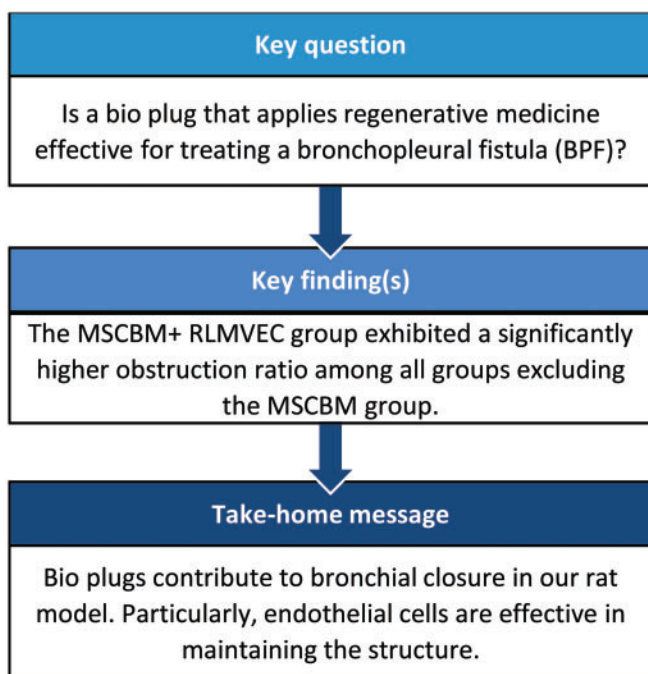
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### Abstract

**OBJECTIVES:** Cell therapies, such as stem cell suspension injection, are used to treat bronchopleural fistula. Although it is safe and effective, injected cells cannot remain within the bronchioles of the fistula due to cell leakage into the thoracic cavity. Here, we inserted a 'bio plug' into the fistula, produced using cells and a bio-3D printer, to examine the effectiveness of bio plugs for the closure of bronchopleural fistulas, the optimal cell source and the closure mechanism.

**METHODS:** Bio plugs were made with mesenchymal stem (stromal) cells derived from bone marrow (MSCBM), fibroblasts and rat lung micro-vessel endothelial cells using a bio-3D printer with different cell mixing ratios. Six groups, according to the presence or absence and the type of bio plugs, were compared. The plugs were inserted into the bronchi of F344 rats. The obstruction ratio and histological and immunohistochemical findings were evaluated.

**RESULTS:** MSCBM+ rat lung micro-vessel endothelial cell group exhibited a higher obstruction ratio among all groups excluding the MSCBM group ( $P=0.039$ ). This group had fibrosis and CD31-positive cells and fewer CD68-positive cells than MSCBM and MSCBM+ fibroblast groups.

**CONCLUSIONS:** Bio plugs with mixed cells, including stem cells, contribute to bronchial closure in the current experimental setting. Endothelial cells effectively maintain the structure in this model. Although bronchial closure for bronchopleural fistula could not be described as clinical conditions were not reproduced, we collected essential data on bronchial closure; however, further experiments are warranted.

**Keywords:** Bronchopleural fistula • Bio plug • Bio-3D printer • Mesenchymal stem cells derived from bone marrow • Endothelial cells

#### ABBREVIATIONS

BPF	Bronchopleural fistula
EWS	Endobronchial Watanabe Spigot
MSCBM	Mesenchymal stem (stromal) cells derived from bone marrow
RLMVEC	Rat lung micro-vessel endothelial cells

## INTRODUCTION

Bronchopleural fistula (BPF) and other fistulas are challenging diseases to treat; thus, they affect the quality of life of patients, and occasionally lead to death. Although the incidence of BPF in thoracic surgery has been low in the past decade, the mortality rate is still high at 12.5–71.2% [1–3].

The possible management of BPF is through surgical or endoscopic treatment [4]. Because surgical treatment is highly invasive and patients with BPF generally have poor nutrition, the success of a surgical procedure is not high (52%) [5]. Bronchoscopy is relatively less burdensome for patients compared with surgical treatment. Various treatments, such as fractionated plasma products [6] and other artificial materials, like a coil or Endobronchial Watanabe Spigot (EWS), have been reported. EWS can be temporarily placed in the bronchial fistula to promote the closure of the fistula [7]. However, these methods have some disadvantages because they cannot achieve the complete closure of BPF. The closure rate with EWS is 80% and that with the coil is 90%. Recently, several cell therapies have been applied and reported. In clinical studies, bronchoscopic injection of autologous adipose-derived stem cells into the fistula has been reported as a safe and effective procedure for the treatment of human BPF [8, 9] with a closure rate of 100%. Similarly, in goat models, bronchoscopic injection of autologous mesenchymal stem (stromal) cells derived from bone marrow (MSCBM) closed the BPF [2]. Complete BPF closure was achieved with this method. However, it has not been applied as a clinical treatment.

The clinical application of these treatments presents some disadvantages. The risk of potential infection cannot be avoided with the use of fractionated plasma products, and artificial materials, such as coil and EWS, have low biocompatibility and pose infection risks. In addition, there is a risk of these artificial materials being forced out via coughing (accounting for 14.9% of all adverse events). In the cell injection method, it is still difficult for the injected cells to stay at the bronchioles of the fistula because the cells leak into the thoracic cavity or the airway. Therefore, the efficiency of closure is unstable.

As a new method to promote bronchiole closure in the fistula, compensating for the above disadvantages, we devised a

method to insert a cylindrical cell structure, a 'bio plug', into the bronchioles. This bio plug can only be produced using cells via a bio-3D printer and retaining the cells locally. We used bio plugs of multicellular spheroids, with several types of cells, to prevent cell leakage through the airway into the thoracic cavity.

This study aimed to examine the effectiveness of bio plugs for the closure of the BPF, the optimal cell source and the closure mechanism in our experimental settings imitating BPF.

## MATERIALS AND METHODS

### Ethical statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Institutional Animal Care and Use Committee of Nagasaki University (approval number 1807261464, dated 26 July 2018). As this study was conducted on animals, obtaining informed consent was not required.

### Cell isolation and culture

We used 3-week-old male F344 rats (body weight: 50–70 g; Charles River Laboratories, Yokohama, Japan). Bone marrow cells were collected from animals as described previously [10–12]. For fibroblast isolation, the tissue piece culture method was adopted [13]. Rat lung micro-vessel endothelial cells (RLMVECs; VEC Technologies Inc., Rensselaer, NY, USA) were purchased and cultured in endothelial cell growth medium with growth supplement (Lonza, Inc., Walkersville, MD, USA).

### Preparation of the bio plug with multicellular spheroids using a bio-3D printer

Several types of bio plugs were prepared at different cell mixing ratios to confirm the best combination for transplantation. Three types of bio plugs were prepared as follows: 100% MSCBM (Group 1); 50% fibroblast and 50% MSCBM (Group 2); 50% RLMVECs and 50% MSCBM (Group 3) (Table 1). The cell suspension was adjusted to  $6.0 \times 10^6$  cells/spheroid. The mixed suspension was plated onto each well of ultra-low-attachment round 96-well plates (Sumilon PrimeSurface, Sumitomo Bakelite, Tokyo, Japan) at 100  $\mu$ l/well and then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 72 h, cells aggregated to form spheroids.

**Table 1:** Bio plug groups with different cell mixing ratios to confirm the best cell mixing combination for transplantation

Cell (%) Group	MSCBM	Fibroblast	RLMVEC
1	100	0	0
2	50	50	0
3	50	0	50

MSCBM: mesenchymal stem (stromal) cells derived from bone marrow; RLMVEC: rat lung micro-vessel endothelial cell.

We used a Regenova bio-3D printer following the Kenzan method (Cyfuse Biomedical K.K., Tokyo, Japan) [14]. Following 3D-bio printing, the printed structures were incubated with a proper flow of the appropriate medium (200 ml/h) in a bioreactor at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 2 weeks. During that period, the spheroids gradually fused and the structure was sustained. After 2 weeks, the needle array was removed, and the bio plug was prepared [15–17].

### Surgery and postoperative management

We used 8- to 12-week-old male F344 rats (body weight: 200–220 g; Charles River Laboratories, Yokohama, Japan). Animals were anaesthetized via intraperitoneal injection of ketamine at a dose of 100 mg/kg and then intubated. Anaesthesia was maintained through inhaled isoflurane and oxygen under intubation.

Left thoracotomy and pneumonectomy were performed. First, the pulmonary artery and vein were ligated with 7-0 silk and transected. The proximal side of the left bronchus was ligated with a 7-0 non-absorbable monofilament suture. The distal side of the left bronchus was opened with scissors. Bronchial lumen was scraped using a micro brush to desquamate epithelial cells, and then, for the bio plug groups, the bio plug was inserted into the lumen, and the distal side was ligated with a 7-0 non-absorbable monofilament suture. In the control (+) group, after desquamating with a brush for removing epithelial cells, promoting fibrosis induced by inflammation, and collecting blood vessels, the distal side was ligated as described above, whereas the control (-) group was not desquamated (Video 1).

Rats with an RLMVEC bio plug were injected with FK506 (0.5 mg/kg/24 h) after surgery to suppress the immune response until the day before euthanasia. In this experiment, RLMVECs were not derived from F344 rats, and therefore, immunosuppression was required because cell plugs containing RLMVECs were allogeneic rather than autologous. The rats had access to food and water *ad libitum* and were weighed daily under inhaled isoflurane anaesthesia during the observation period. After the observation period, the rats were euthanized, and the left bronchus was removed for evaluation. The observation period was 2 weeks, and there were 6 samples in the bio plug (Groups 1, 2 and 3) and control groups (+, -). Group 3 was observed for 6 and 12 weeks (Fig. 1).

### Histology and immunohistochemistry

After overnight fixation in 10% formalin (Japan Tanner Corporation, Osaka, Japan), structures were embedded in paraffin

and sectioned at a thickness of 5 µm. They were stained with haematoxylin and eosin (HE) for general histological evaluation. Masson's trichrome and Elastica van Gieson staining was used for evaluating fibrosis. Immunohistochemistry was performed with the following primary antibodies: anti-CD31 (1:400; rabbit polyclonal; bs-195R; Bioss, Boston, MA, USA) to evaluate endothelial cell distribution, and anti-CD68 (1:800; mouse monoclonal; ab31630; Abcam, Cambridge, UK) to evaluate the expression of general macrophages.

### Measuring the bronchus obstruction ratio

The proportion of cell structure in the bronchial lumen with HE staining after the observation period was measured. Isolated bronchi following euthanasia were all sectioned and stained with HE every 7 slices. We adopted the highest proportion of cells in the bronchial lumen to measure the bronchus obstruction ratio. Visual analysis was performed using Image J (version 1.51, U.S. National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>, 1997–2018).

### Statistical analysis

Data are represented as the mean (standard deviation). All statistical analyses were performed using SPSS statistics (ver 24.0; IBM Japan, Ltd., Tokyo, Japan). For the statistical tests, due to the small sample size, the non-parametric method was used instead of normality determination. Comparisons were performed using Mann–Whitney *U*-test and Bonferroni correction. The statistical significance level ( $\alpha$ ) was set at 0.05, and the significance probability (*P*-value) was calculated using the Bonferroni method and displayed.

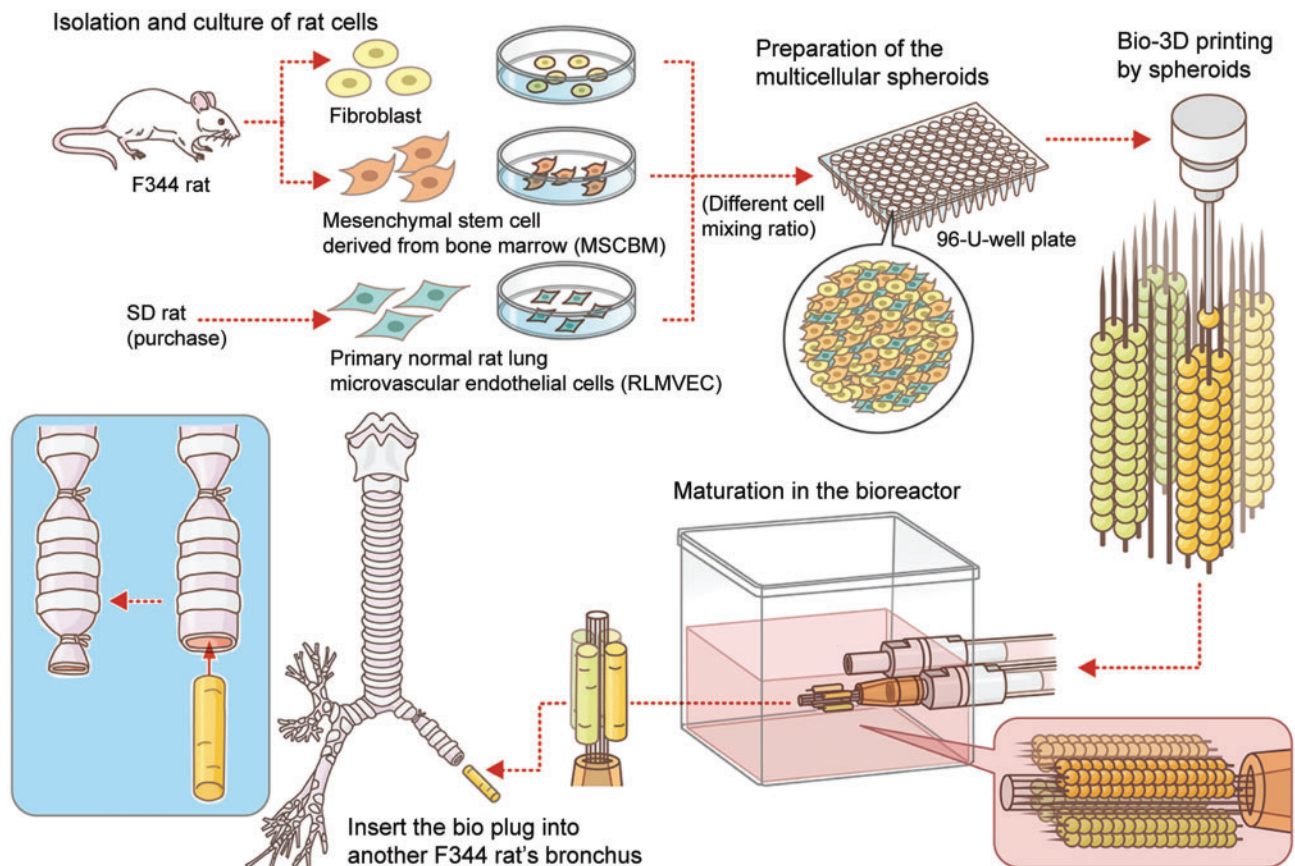
## RESULTS

### Macroscopic and microscopic findings (haematoxylin and eosin staining) (2-week model)

Macroscopically, no obstruction of the bronchus was found in the control groups. Furthermore, there was no difference between the 2 controls [(+) and (-)]. In contrast, in all bio plug groups, almost the entire lumen was sealed up with the bio plug 2 weeks after insertion. Macroscopically, the bio plug had a yellowish colour. Microscopic findings with HE staining showed different trends in bio plug groups. In Group 3, the bronchus lumen was almost filled with the bio plug, similar to the macroscopic findings. However, in Group 1 and Group 2, there were some spaces in the lumen between the bio plug and the epithelial layer of the bronchus (Fig. 2).

### Obstruction ratio of the bronchus (2-week model)

The mean obstruction ratio was 0.08 (0.06) and 0.08 (0.06) for control groups [(-) and (+), respectively]. The ratio was 0.34 (0.20), 0.27 (0.15) and 0.69(0.11) for Groups 1, 2 and 3, respectively. There was no significant difference between the (-) and (+) control groups. Group 3 showed a significantly higher obstruction ratio among all groups, excluding Group 2 (Table 2).



**Figure 1:** Study overview. Cells: MSCBM (isolated from F344 rat), fibroblasts (isolated from F344 rat) or RLMVECs (isolated from SD rat, purchased) were cultured. Multicellular spheroids were created using mixed cell suspensions, and the bio plug was then constructed via bio-3D printing using these spheroids. The plugs were matured in a bioreactor for a total of 2 weeks before transplantation. MSCBM: mesenchymal stem (stromal) cells derived from bone marrow; RLMVEC: rat lung micro-vessel endothelial cell.

## Histological findings (2-week model)

Masson's trichrome staining showed that fibrosis was present in a part of the plug, connected with bronchial fibrotic tissues in Group 3. In contrast, in other groups, the bio plug cells were damaged, and as such, there were no fibrotic changes in the plug. Elastica van Gieson staining showed slight fibrosis in Group 3, similar to the results observed with Masson's trichrome staining (Fig. 3).

## Immunohistochemistry findings (2-week model)

CD31-positive cells were observed only in Group 3, particularly around the connective tissue between the bio plug and the bronchial tissue. Furthermore, there were some small capillary structures with CD31-positive cells (Fig. 4A–C). Many CD68-positive cells were found in the plug of Groups 1 and 2. Only a few CD68-positive cells were found in the plugs and the bronchi in Group 3 (Fig. 4D–F).

## Findings in the long-term models of Group 3 (6 and 12 weeks)

Macroscopic findings showed that the bronchioles in 6- (Fig. 5A) and 12-week (Fig. 5E) models were sealed up with the bio plug. HE staining showed that in the bronchial lumen, cells and tissues

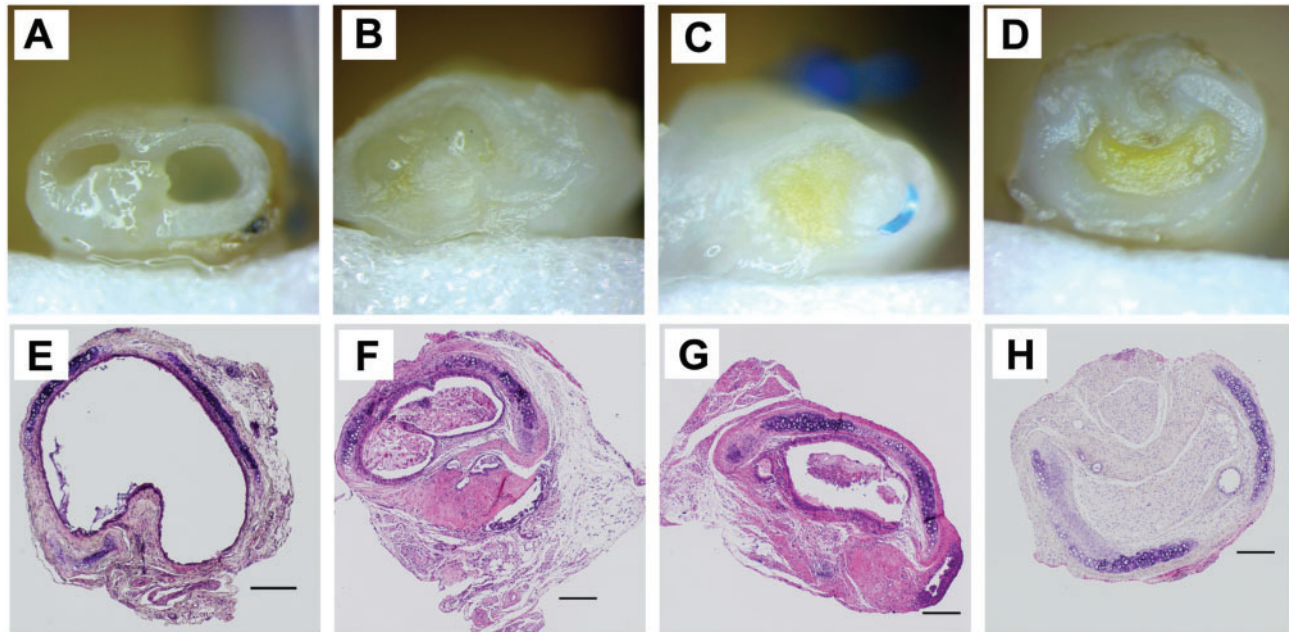
were still present in the plug, which connected with the bronchial tissues in the long-term models (Fig. 5B and F). There were spaces in the bronchial lumen between the plug and bronchial tissues; however, bronchial spaces were smaller than those in a normal bronchus. Some fibrotic tissues were also present in the bronchus lumen; however, some tissues were necrotic (Fig. 5C, D, G, and H).

## DISCUSSION

We devised a novel strategy to close the bronchial lumen and treat BPF using bio plugs constructed from several types of cells. Bio plugs entirely sealed the bronchial lumen, in both short- and long-term models. Bio plugs that consisted of MSCBM+ RLMVEC showed the best microscopical closure of the bronchial lumen in the short term and could narrow the lumen via fibrotic tissues in the long term. Bio plugs consisting of patient-derived cells show the potential to treat a severe BPF.

Although empyema with BPF is associated with high mortality, the recovery rate can be improved with BPF closure. For empyema without BPF, methods and devices, such as vacuum-assisted closer, have been developed [18, 19]. Thus, one of the goals of treating empyema with fistulas is BPF closure.

Cell therapies have recently been applied for the bronchoscopic management of BPF, and the complete repair of BPF has been reported. Aho *et al.* [20] first described the use of an



**Figure 2:** Macroscopic and microscopic findings (haematoxylin and eosin staining). Control (-) group: No bio plugs and without desquamation using a brush; control (+) group: No bio plugs and with desquamation using a brush; Group 1: 100% mesenchymal stem (stromal) cells derived from bone marrow; Group 2: 50% fibroblasts and 50% mesenchymal stem (stromal) cells derived from bone marrow; Group 3: 50% rat lung micro-vessel endothelial cell and 50% mesenchymal stem (stromal) cells derived from bone marrow. **(A–D)** Macroscopic findings; **(A)** Control (-), **(B)** Group 1, **(C)** Group 2, **(D)** Group 3, **(E–H)** Microscopic findings; **(E)** Control (+), **(F)** Group 1, **(G)** Group 2, **(H)** Group 3. Scale bar: 200  $\mu$ m. Macroscopically and microscopically, no obstruction of the bronchus was found in the control groups, which had no bio plug. In macroscopic findings, all bio plug groups showed that almost the entire lumen was sealed with the bio plug. However, microscopic findings showed different trends in the bio plug groups. The bronchial lumen in Group 3 appeared to be the most obstructed with the bio plug.

**Table 2:** Obstruction ratio of the bronchus

	Mean (SD)	P-value (Bonferroni correction)			
		Control +	Group 1	Group 2	Group 3
Control -	0.08 (0.06)	>0.999	0.065	0.250	<b>0.039</b>
Control +	0.08 (0.06)		<b>0.039</b>	0.374	<b>0.039</b>
Group 1:MSCBM	0.34 (0.20)			>0.999	0.250
Group 2:MSCBM+ Fibroblast	0.27 (0.15)				<b>0.039</b>
Group 3:MSCBM+ RLMVEC	0.69 (0.11)				

Control (-) group: No bio plug and without desquamation using a brush. Control (+) group: No bio plug and with desquamation using a brush. Group 1: 100% MSCBM, Group 2: 50% fibroblasts and 50% MSCBM, Group 3: 50% RLMVEC and 50% MSCBM. P-value: Mann-Whitney U-test with Bonferroni correction. The statistical significance level is set to 0.05, and the numbers in bold are those that meet that level.

MSCBM: mesenchymal stem (stromal) cells derived from bone marrow; RLMVEC: rat lung micro-vessel endothelial cell; SD: standard deviation.

autologous MSC-seeded matrix graft for repairing post-pneumectomy BPF and reported complete BPF closure. In multiple case reports in humans and large animal experiments, a 100% success rate has been reported [2, 8, 9]. In almost all cases, injection methods were adapted and a scaffold, fibrin glue or a matrix of synthetic bio-absorbable poly (glycolide: trimethylene carbonate) copolymer was used. Although the side effects of these materials are not apparent, there are potential risks of foreign body reaction, infection and outflow owing to their liquid state.

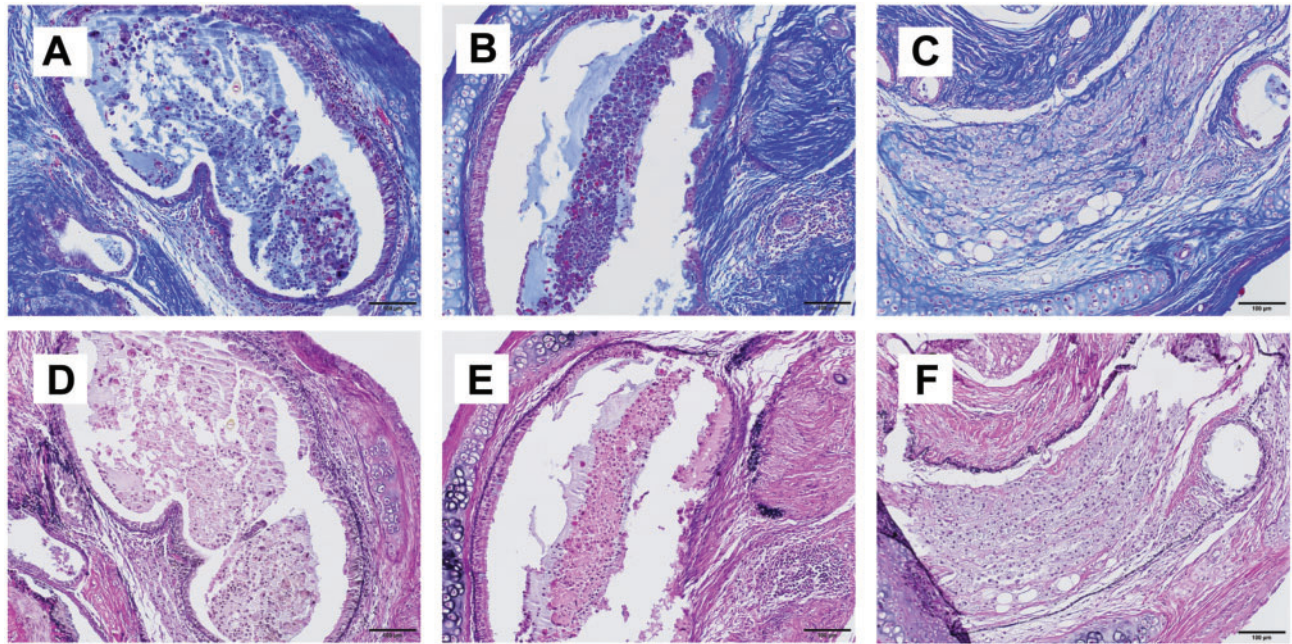
Our method was developed to overcome these disadvantages. As autologous, scaffold-free and solid cell structures are ideal for curative treatment, we devised a cylindrical cell structure, a 'bio

plug' without scaffolds, using a bio-3D printing system, Regenova. We have developed human organs, trachea and oesophagus, using this technology [12, 15, 16]. Bio-3D printing technology has allowed the construction of any 3D structure using cells alone, without scaffold materials [21]. In this study, we investigated whether we could attain a histological obstruction of the bronchi in a rat model. Bio plugs have advantages in the long term because cell plugs have a possibility of histological occlusion and an ability to keep the cells *in situ* for a longer time.

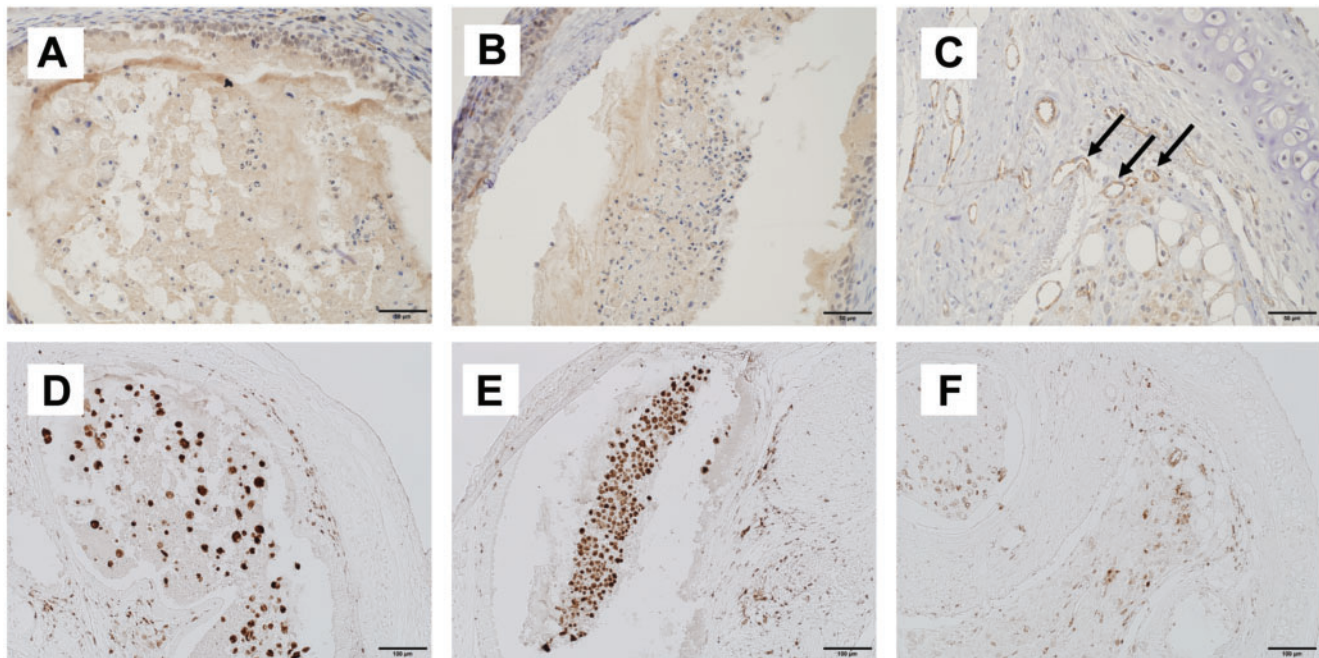
The efficiency of bronchial closure using bio plugs depends on the type of the cell. For a bio plug that consisted of only fibroblasts or stem cells, macrophage phagocytosis started within a short period; thus, the obstruction efficiency decreased. Even though there was no difference in macroscopic findings, there were notable microscopic differences in the bronchial obstruction ratio of the residual cells and tissues. Phagocytosis of macrophages indicated that the cells had died. However, the addition of endothelial cells into the bio plug led to tissue engraftment and persistence, which might be due to connections with the surrounding tissues and blood vessel formation.

Furthermore, the fibrous tissue was observed, possibly due to the blood flow into the plug by endothelial cells and the interaction of stem cells with endothelial cells [22]. The National Clinical Database of Japan shows that bronchoplasty, hilar nodal dissection, induction radiotherapy or chemoradiotherapy are high-risk factors for BPF [23]. The decrease in blood flow to the bronchial stumps, caused by these risk factors, may have been involved in BPF development, which might be related to the success of our experiment as the addition of endothelial cells to the bio plug caused angiogenesis.

The obstruction ratio was reduced in the long-term model compared with that in the 2-week model, and complete occlusion was



**Figure 3:** Histological findings, and Masson's trichrome and Elastica van Gieson staining. Group 1: 100% mesenchymal stem (stromal) cells derived from bone marrow; Group 2: 50% fibroblasts and 50% mesenchymal stem (stromal) cells derived from bone marrow; Group 3: 50% rat lung micro-vessel endothelial cell and 50% mesenchymal stem (stromal) cells derived from bone marrow. (A–C) Masson's trichrome staining; (A) Group 1, (B) Group 2, (C) Group 3, (D–F) Elastica van Gieson; (D) Group 1, (E) Group 2, (F) Group 3. Scale bar: 100  $\mu$ m. In Group 3, Masson's trichrome and Elastica van Gieson staining showed that fibrosis was found in the part of the bio plug that was connected with bronchial fibrotic tissues. In other groups, there were no fibrotic changes in the bio plug.

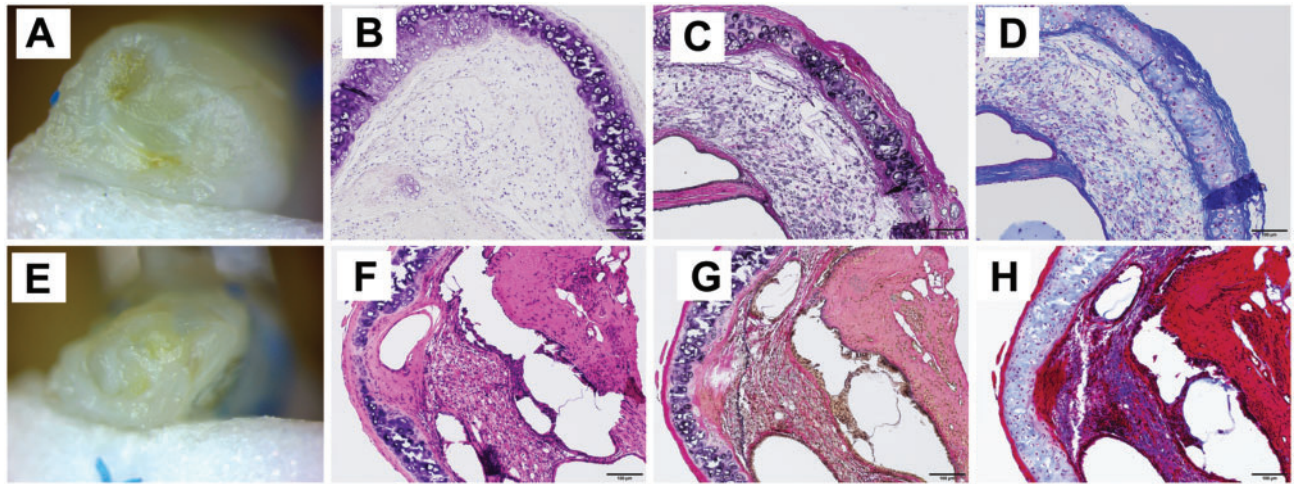


**Figure 4:** Immunohistochemistry findings. Group 1: 100% mesenchymal stem (stromal) cells derived from bone marrow; Group 2: 50% fibroblasts and 50% mesenchymal stem (stromal) cells derived from bone marrow; Group 3: 50% rat lung micro-vessel endothelial cell and 50% mesenchymal stem (stromal) cells derived from bone marrow. (A–C) CD31; (A) Group 1, (B) Group 2, (C) Group 3, (D–F) CD68; (D) Group 1, (E) Group 2, (F) Group 3. Scale bar: 100  $\mu$ m. CD31-positive cells were only observed in Group 3. Arrows show CD31-positive cells. Numerous CD68-positive cells were found in the bio plug of Groups 1 and 2. Group 3 had only a few CD68-positive cells.

not observed. For clinical applications, this is a viable problem, which could be solved by multiple insertions of bio plugs.

We could not unravel the mechanism of complete BPF closure, but the paracrine signalling by MSCBM may play a role [24].

MSCBM secretes paracrine factors, such as growth factors and cytokines, which enhances local angiogenesis and tissue repair [25]. The plug groups containing MSCBM had a higher tendency to close the lumen than the control groups. Angiogenesis and



**Figure 5:** Long-term model of Group 3. (A–D) Six-week models; (A) macroscopic, (B) haematoxylin and eosin, (C) Elastica van Gieson, (D) Masson's trichrome. (E–H) Twelve-week models; (E) macroscopic, (F) HE, (G) EVG, (H) Masson's trichrome. Scale bar: 100  $\mu$ m. There are some spaces in the bronchial lumen between the bio plug and bronchial tissues; however, the bronchial spaces are smaller than those of the normal bronchus.



**Video 1:** Left thoracotomy and pneumonectomy were performed. First, the left main bronchus, pulmonary artery and vein were exposed. Pulmonary artery and vein were ligated with 7-0 silk. The proximal side of the left bronchus was ligated with a 7-0 non-absorbable monofilament suture. The distal side of the left bronchus was opened with scissors. Bronchial lumen was scrapped using a micro brush to desquamate epithelial cells, and then, for the bio plug groups, the bio plug was inserted into the lumen, and the distal side was ligated with a 7-0 non-absorbable monofilament suture. In the control (+) group, after desquamating with a brush, the distal side was ligated as described above, and the control (-) group was not desquamated.

tissue healing potential may contribute to the closure of the BPF via MSCBM paracrine effects.

Herein, we used a rat model to collect essential data. Therefore, unlike the actual bronchial fistula model, because the central bronchus communicates with the trachea and the peripheral bronchi communicates with the thoracic cavity in BPF, the bronchial

closure potential for BPF cannot yet be described. However, it is vital to prove that bio plugs remain in the bronchus due to endothelial cell-mediated angiogenesis and contribute to the bronchus narrowing. Further verification using large animals is needed in the future. However, in the bronchial fistula model in large animals, the effects of positive and negative pressure in the respiratory tract due to respiration cannot be ignored. These may prevent the plug from staying in place. It is necessary to devise further methods and prove their effects. Although high efficiency is required, narrowing the lumen by autologous cell plugs could be a treatment option for intractable bronchial fistulas.

Our rat model did not reproduce clinical conditions. Proximal and distal bronchi were ligated to prevent deviation of the bio plug. In clinical BPF, the bio plug can be changed according to the size of the fistula with a bio-3D printer and, therefore, avoiding bio plug leakage into the thoracic cavity. We plan to use a new insertion device and procedure for bio plugs in large animal BPF models. In this procedure, a new silicone device will be placed on the proximal side to prevent the bio plug from invading the airway. Because patients with BPF are susceptible to sepsis, we plan to collect and store autologous cells from patients at high risk of developing BPF to produce bio plugs before the initial surgery.

## CONCLUSION

We show that a bio plug with scaffold-free cells contributes to delayed bronchial closure in the current experimental setting. Endothelial cells are effective in maintaining the structure. Further experiments, including large-scale animal experiments, are needed. The establishment of the BPF model in large animals, the examination of a cell source that is easier to isolate and cultivate in humans, developing a new device for inserting cell plugs and preventing displacement of the cell plugs by coughing, evaluation of the effectiveness of bio plugs, and a new device to be used in large animal models are the next steps of this study.

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**Conflict of interest:** K.N. is a co-founder and shareholder of Cyfuse Biomedical K.K. He only provides the method for producing 3D cell structures and is not involved in analysis and data extraction. The remaining authors declare no competing interests.

## Data Availability Statement

All relevant data are within the manuscript and its supporting information files.

## Author contributions

**Masaaki Moriyama:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing—original draft; Writing—review & editing. **Keitaro Matsumoto:** Conceptualization; Data curation; Investigation; Methodology; Project administration; Supervision; Writing—review & editing. **Daisuke Taniguchi:** Data curation; Investigation. **Ryusuke Machino:** Data curation; Investigation. **Tomoshi Tsuchiya:** Conceptualization; Data curation; Investigation. **Koichi Nakayama:** Conceptualization; Supervision. **Takeshi Nagayasu:** Conceptualization; Supervision.

## Reviewer information

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