Negative impact of recipient SPRED2 deficiency on transplanted lung in mouse model

Kohei Hashimoto[1], Masaomi Yamane[1], Seiichiro Sugimoto[1], Yutaka Hirano[1], Takeshi Kurosaki[2], Shinji Otani[2], Kentaroh Miyoshi[1], Mikio Okazaki[1], Takahiro Oto[2], Akihiro Matsukawa[3], and Shinichi Toyooka[1]

[1]Departments of General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

[2]Organ Transplant Center, Okayama University Hospital, Okayama, Japan

[3]Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Corresponding author: Masaomi Yamane, MD, PhD, Associate Professor

General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,

2-5-1, Shikata-cho, Okayama City Kita-ku, Okayama, 700-8558, JAPAN

TEL: +81-86-235-7265

19 FAX: +81-86-235-7269

20 Email: yamane-m@cc.okayama-u.ac.jp

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24 containing protein (SPRED)

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ABSTRACT

Purpose: Ischemia-reperfusion injury (IRI) after lung transplantation mainly contributes to the development of primary graft dysfunction. The Sprouty-related EVH1-domain-containing (SPRED) protein family that inhibits the mitogen activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway. Our study was aimed at examining the role of SPRED2 in IRI in mice that received orthotopic lung transplantation.

Methods: Syngeneic mouse lung transplantation was performed in wild-type C57BL/6J (WT) mice and Spred2 knockout (Spred2−/−) mice on the C57BL/6J background from the WT donor. Four hours after reperfusion, blood gas analysis was assessed, and lung grafts were then sacrificed and analyzed.

Results: By using arterial oxygen tension measurements and histological examinations, we revealed a more severe IRI in the grafts transplanted to *Spred2*$^{-/-}$ recipients, which manifested as exacerbated pulmonary edema and increased alveolar congestion with neutrophils infiltration. Intragraft ERK1/2 activation and expression levels of proinflammatory cytokines and chemokines in *Spred2*$^{-/-}$ recipients were higher than those in WT recipients.

Conclusion: SPRED2 plays an important role to protect lung from IRI in lung transplantation recipients. We suggest that focused treatments suppressing the activity of the MAPK/ERK pathway in transplantation recipients could be effective for the prevention of lung IRI.

INTRODUCTION

Primary graft dysfunction of the lung induced by ischemic reperfusion injury (IRI) after transplantation is not only a risk factor of perioperative mortality, but may also promote acute rejection or chronic allograft dysfunction [1–7]. Because the need for transplantable organs is increasing, the number of cases accepting the donation after cardiac death is also on the rise today. However, the graft of the donation after cardiac death is more likely to develop severe IRI because of the longer warm ischemic time after cardiac arrest. From the clinical standpoint, the prevention of IRI is vitally important for lung transplantation. In particular, precise algorithms of clinical manipulations in the recipient are needed, depending on the clinical strategy. The development of IRI involves multiple mechanisms that involve many signaling pathways. A direct relationship between the activity of the mitogen-activated protein kinase (MAPK) pathway and IRI has been demonstrated previously [8,9].

Sprouty-related, EVH1 domain 2 protein (SPRED2), described for the first time in 2001, is one of the inhibitors of extracellular signal-regulated kinase (ERK)1/2, which is a major signaling element of the MAPK pathway [10]. External stress stimuli to cells promote phosphorylation of various domains of several cellular receptors, causing activation of rat sarcoma virus oncogene (Ras), v-raf-leukemia viral oncogene 1 (Raf1), and MAPK/ERK kinase (MEK), which, in turn,

63 activates ERK1/2 [7,8]. Whereas ERK translocates into the nucleus, where it induces expression
64 of genes related to cell growth, differentiation, and migration, SPRED2, which is found to be
65 widely expressed in various tissues, including the lung, inhibits the MAPK/ERK pathway by
66 binding to Ras and inhibiting phosphorylation of Raf [10–15].
67 The importance of SPRED2 in counteracting IRI was revealed by our previous study, where we
68 applied hilar clamping technique to *Spred2*$^{-/-}$ mice [16]. We found that IRI of the lungs in
69 *Spred2*$^{-/-}$ mice was significantly more severe than that in wild-type (WT) mice. A positive
70 relationship between the extent of MAPK activation and lung IRI was revealed. However, the
71 selective impact on IRI of MAPK/ERK activation in the recipient has not been clarified. In this
72 study, we utilized a murine lung transplantation model to examine whether there was a
73 relationship between IRI magnitude and selected recipient factors and to explore possible clinical
74 approaches to the treatment of IRI that could be applied specifically to the recipient.
75
76 **MATERIALS AND METHODS**
77
78 **Animals**
79 WT C57BL/6J mice (8-12 weeks old) were purchased from Charles River Laboratories Japan,
80 Inc. (Yokohama, Japan). *Spred2*$^{-/-}$ mice (8-12 weeks old) backcrossed onto the C57BL/6J

background [13,17], which were bred and maintained at the Department of Animal Resources of the Okayama University (Okayama, Japan). Our experimental protocol was approved by the Animal Care Committee at Okayama University. All experimental mice received humane care in accordance with the "Principles of Laboratory Animal Care" of the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health (NIH).

Murine lung transplantation

Orthotopic transplantation of a single (left) lung was performed by using the previously described technique [18]. Two experimental groups were employed with distinct "donor → recipient" combinations: WT → WT and WT → *Spred2*$^{-/-}$ (n = 5 in both combinations). Donor mice were anesthetized by intraperitoneal injection of ketamine (0.1 mg/g bodyweight) and xylazine (0.01 mg/g bodyweight), cannulated via tracheotomy, and ventilated under the room air at a tidal volume of 0.5 mL and a respiratory rate of 120 breaths/min. Median sternotomy was performed and heparinized by an intravenous injection of 100 IU/bolus heparin. The lungs were flushed with 4 ℃ extracellular phosphate-buffered lung preservation solution (EP-TU solution), harvested, prepared for vessel anastomosis, and stored first at 4 ℃ for 120 min and then at room temperature for 30 min. Recipient mice were anesthetized by the same protocol as donor animals,

intubated, and maintained under general anesthesia with 0.5% sevoflurane admixed in 100% oxygen. The left thoracotomy was performed, and each vessel of the donor lung was anastomosed by the cuff technique. After reperfusion, the chest was closed and the recipient was kept under anesthesia for 4 h until sacrifice. After the aspiration of arterial blood, the graft was harvested and separated into four sections.

Blood gas analysis

To evaluate graft function, arterial blood gas analysis was performed. The median sternotomy was performed, the right hilum was clamped by a clip, and arterial blood was taken from the ascending aorta. Partial pressure of oxygen (PaO_2) in arterial blood was measured by a Rapid Lab 348 apparatus (Siemens Healthcare Diagnostics, Tokyo, Japan).

Histological evaluation

To evaluate the severity of graft injury after ischemic reperfusion, one of the sections of lung graft tissue was fixed in 10% formalin and stained with hematoxylin/eosin.

Flow cytometry

Lung graft tissue was used for flow cytometry analysis following a previously described protocol

[19]. In order to evaluate infiltration of neutrophils, samples were stained with fluorochrome-labeled anti-CD45, anti-CD11b, and anti-Gr-1 antibodies (BD Bio sciences, Franklin Lakes, NJ), and flow cytometry analysis was performed by using BD Accuri™ C6 (BD Bio sciences, Franklin Lakes, NJ).

Western blotting

Lung graft tissues stored at −80 ℃ were used for western blotting. A detailed protocol of this procedure has been described previously [20]. The primary antibodies were anti-p44/42 MAPK (Cell Signaling Technology, Beverly, MA, USA) to evaluate total ERK1/2 (t-ERK) level, anti-phosphorylated p44/42 MAPK (Cell Signaling Technology) to assess ERK1/2 phosphorylation (p-ERK), and anti-actin (Cell Signaling Technology) used as control. Goat anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies. The membranes were analyzed using an enhanced chemiluminescence Advanced Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA).

Real-time reverse transcription-polymerase chain reaction technique

Lung graft tissue stored in RNAlater RNA stabilization reagent (Qiagen, Venlo, Netherlands) was

used for real-time reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from the stored tissue by using an RNeasy Mini Kit (Qiagen). A high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) was used for synthesizing first strand cDNA from the respective RNA, according to the manufacture's recommendations. Quantitative real-time reverse transcription-polymerase chain reaction was performed with primers for genes encoding TNF-α, IL-1β, CCL2, CXCL2, and GAPDH, encoded by a housekeeping gene, as control. The cDNA and each primer and probe set (purchased from Thermo Fisher Scientific) were injected into TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), and mRNA expression levels were determined by an ABI StepOnePlus Real-Time PCR Instrument (Thermo Fisher Scientific) using the delta-delta-CT method.

Statistical analysis

All data were analyzed by using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). PaO_2 levels, numbers of cells sorted by flow cytometry, and levels of proinflammatory molecules are expressed as the mean ± SEM. Statistical analysis was performed by using the Student's *t* test. All statistical analyses were conducted with a significance level of $\alpha = 0.05$ ($P < 0.05$).

RESULTS

Pulmonary graft function

PaO_2 level in *Spred2*$^{-/-}$ group was significantly lower than that in WT group (WT: 235.2 ± 9.7; *Spred2*$^{-/-}$: 152.5 ± 11.7 mmHg; P = 0.0006; Fig.1-A). Observations of the transplanted lung hematoxylin-eosin stained sections revealed stronger proinflammatory changes, such as interstitial edema, hemorrhagic congestion, septal thickening, and neutrophils infiltration, in *Spred2*$^{-/-}$ mice compared to those in WT animals (Fig. 1-B).

Number of infiltrated neutrophils in the transplanted lung graft

Gr-1$^+$, CD11b$^+$, and CD11c$^-$ characteristics were used as markers of neutrophils. Gr-1$^+$CD11b$^+$cells and Gr-1$^+$CD11c$^-$cells were sorted under CD45$^+$ gating (Fig. 2) by flow cytometry. Both cell subtypes were more abundant in *Spred2*$^{-/-}$ group than in WT group (Gr-1$^+$CD11b$^+$ cells: WT, 378.6 ± 57.7; *Spred2*$^{-/-}$, 856.5 ± 149.6; P = 0.018; Gr-1$^+$CD11c$^-$ cells: WT, 353.3 ± 46.5; *Spred2*$^{-/-}$, 857.4 ± 152.8; P = 0.014). These findings strongly suggested that more activated neutrophils infiltrated graft lungs in *Spred2*$^{-/-}$ than in WT recipients.

Intragraft expression status of proinflammatory molecules and ERK1/2

We examined mRNA expression levels of cytokines and chemokines involved in the regulation of neutrophil migration and infiltration in transplanted lung grafts. We found that mRNAs

171 encoding CCL2, CXCL2, IL-1β, and TNF-α were expressed at a significantly higher level in
172 *Spred2*$^{-/-}$ group than in WT animals: CCL2, $P = 0.035$; CXCL2, $P = 0.012$; IL-1β, $P = 0.046$;
173 TNF-α, $P = 0.028$. (Fig.3-A). In addition, to examine the activation of ERK1/2 of the MAPK
174 pathway, western blotting for p-ERK1/2 was performed. The level of p-ERK1/2 was higher in
175 *Spred2*$^{-/-}$ group than in WT mice, whereas t-ERK1/2 expression was equivalent in both groups
176 (Fig. 3-B).
177
178 **DISCUSSION**
179 This study investigated the role of SPRED2, an inhibitor of the MAPK/ERK signaling pathway,
180 in the development of lung IRI in recipient mice that underwent orthotopic lung transplantation.
181 We found that *Spred2*$^{-/-}$ recipient mice showed more severe IRI even if the transplanted lung was
182 supposed to be a normal graft, which indicated that SPRED2 plays an important role in recipients
183 by inhibiting ERK1/2 activation and attenuating IRI development.
184 It has been shown that several complex signaling pathways affect the emergence of lung IRI.
185 Under ischemic circumstances, vascular endothelial cells and alveolar macrophages in the lung
186 tissue produce various pro-inflammatory molecules. Once reperfusion occurs, pro-inflammatory
187 molecules stimulate migration and infiltration of the inflammatory cells, typified by the
188 neutrophils, and induce further stress response and activation of signaling cascades, including the

189 MAPK pathway. These physiological reactions lead to the increased microvascular permeability,
190 pulmonary vascular resistance, pulmonary edema, impaired oxygenation, and pulmonary
191 hypertension. Collectively, these processes ultimately cause primary graft dysfunction after lung
192 transplantation [2,8,9,21].

193 The reaction of MAPK pathways proceeds rapidly, from several minutes to just a few hours
194 from the activation, resulting in the stimulation of ERK. Translocation of ERK into the nucleus
195 promotes synthesis of several cytokines and chemokines that facilitate migration of inflammatory
196 cells, including neutrophils, edema, and other proinflammatory manifestations [22–24]. These
197 observations suggested that the severity of IRI almost entirely depend on the intrinsic characteristics
198 of the lung graft itself due to rapid MAPK pathway-dependent responses and subsequent activation of
199 IRI signaling pathways. Indeed, the majority of studies on IRI after transplantation have focused
200 on the donor rather than recipient factors. However, activated inflammatory cells or molecules of
201 the recipient play an important role in the promotion of additional inflammatory phenomena.
202 Several studies reported that a number of recipient factors correlated with the extent of lung IRI
203 [25, 26]. Liu *et al*. reported that recipient monocyte migration caused by ischemia reperfusion
204 triggered IRI in a mouse model of lung transplantation [25]. Additionally, other researchers
205 demonstrated that the severity of inflammation in lung transplantation recipients influenced
206 overall lung IRI in a clinical study [26]. They focused on several inflammatory cytokines and

chemokines, including CCL2, in their experiment and showed that preoperative levels of those molecules in the recipient blood samples correlated with the extent of IRI development. From these findings, it follows that inflammatory cells and proinflammatory signaling pathway molecules of the recipient also have key roles in IRI development.

In this study, WT mice were utilized as normal recipients, whereas *Spred2*$^{-/-}$ mice mimicked recipients having inflammation potential. The lung grafts, which were supposed to retain their function, caused severe IRI at 4 h after reperfusion when transplanted into recipients with the deletion of *Spred2*. Thus, the recipient with potential inflammatory hyperactivation of the MAPK pathway can suffer from IRI irrespective of the lung graft condition. Moreover, our data suggest that anti-inflammatory treatment of the recipient in the perioperative period can prevent lung IRI. In our previous study of lung IRI, in which we utilized the hilar clamping technique, it was revealed that an ERK inhibitor ameliorated IRI that developed in *Spred2*$^{-/-}$ mice [16].

In conclusion, activation of the MAPK/ERK pathway in the recipient affects IRI severity after lung transplantation. Furthermore, anti-inflammatory treatment for the recipient may be effective in cases of the lung transplantation characterized by augmented inflammatory potential due to MAPK pathway hyperactivation.

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295 **FIGURE LEGENDS**

296

297 **[Fig. 1] Histological evaluation and pulmonary function of the graft after ischemia-reperfusion**

298 (A) Comparison of the hematoxylin and eosin-stained pulmonary parenchyma sections from WT and

299 *Spred2*$^{-/-}$ mice 4 h after the reperfusion (40× magnification). The section from a *Spred2*$^{-/-}$ animal

300 showed more severe inflammatory changes, such as interstitial edema, hemorrhagic congestion, septal

301 thickening, and neutrophil infiltration, than the section from a WT mouse. (B) Pulmonary graft

302 function 4 h after the reperfusion was evaluated by PaO_2 levels after ventilation with 100% oxygen.

303 Arterial blood sample was obtained from ascending aorta after right hilar clamping in order to evaluate

304 the graft function independently. PaO_2 level was significantly lower in *Spred2*$^{-/-}$ group than in WT

305 group ($^{*}P < 0.01$).

306

307 **[Fig. 2] Flow cytometry analysis of lung neutrophil infiltration** Flow cytometry was performed to

308 sort Gr-1^{+}/CD11b^{+} and Gr-1^{+}/CD11c^{-} cells under CD45^{+} gating, as they were deemed to be

309 neutrophils. Both types of cells were significantly more abundant in *Spred2*$^{-/-}$ lung grafts (*$P < 0.05$).

310

311 **[Fig. 3] Expression levels of ERK1/2 and mRNAs encoding proinflammatory molecules.** (A) Total

312 ERK1/2 (t-ERK) and phosphorylated ERK (p-ERK) expression levels were examined by western

313 blotting to evaluate the extent of MAPK pathway activation. (B) Relative expression levels of mRNAs

314 encoding proinflammatory cytokines and chemokines involved in neutrophil activation were

315 examined by real time PCR. mRNAs encoding CCL2, CXCL2, IL-1 β , and TNF- α were

316 significantly more highly expressed in *Spred2*$^{-/-}$ group than in WT animals (*$P < 0.05$).

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