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# Collagen-Induced Arthritis Analysis in *Rhbdf2* Knockout Mouse

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## Abstract

Rhomboid family member 2 gene (*Rhbdf2*) is an inactive homologue lacking essential catalytic residues of rhomboid intramembrane serine proteases. The protein is necessary for maturation of tumor necrosis factor-alpha (TNF-α) converting enzyme, which is the molecule responsible for the release of TNF-α. In this study, *Rhbdf2* knockout (KO) mice were produced by CRISPR/CAS9. To see the effects of the failure of TNF-α release induced by *Rhbdf2* gene KO, collagen-induced arthritis (CIA), which is the representative TNF-α related disease, was induced in the *Rhbdf2* mutant mouse using chicken collagen type II. The severity of the CIA was measured by traditional clinical scores and histopathological analysis of hind limb joints. A rota-rod test and grip strength test were employed to evaluate the severity of CIA based on losses of physical functions. The results indicated that *Rhbdf2* mutant mice showed clear alleviation of the clinical severity of CIA as demonstrated by the significantly lower severity indexes. Moreover, a grip strength test was shown to be useful for the evaluation of physical functional losses by CIA. Overall, the results showed that the *Rhbdf2* gene has a significant effect on the induction of CIA, which is related to TNF-α.

**Key Words:** *Rhbdf2* knockout mouse, Collagen-induced arthritis, TNF-α

## INTRODUCTION

Rhomboids are a family of proteins consisting of intramembrane serine proteases and their inactive homologues (Freeman, 2014). The common ancestor of all members of the family was probably an active intramembrane protease, although the majority of existing members are not active proteases (Freeman, 2014). Rhomboid protease was initially discovered in *Drosophila* (Sturtevant *et al*., 1993; Freeman, 1994), and *Drosophila* rhomboid protease cuts epidermal growth factor receptor (EGFR) ligand Spitz and a homologue for mammalian tumor growth factor (TGF-α), triggering the secretion of the factors (Rutledge *et al*., 1992; Schweitzer *et al*., 1995). Homologs of *Drosophila* rhomboid have been identified in most prokaryotic and eukaryotic organisms (Lemberg and Freeman, 2007).

Rhomboid family members have been shown to have a common structure composed of six or seven transmembrane domains (Ha *et al*., 2013). Rhomboid proteases have conserved transmembrane segments of their polytopic rhomboid core domain. Catalytic motif is in the fourth transmembrane domain of Rhomboid proteases, and an Engelman helix dimerization motif in the sixth transmembrane domain (Urban *et al*., 2001, 2002; Lemberg *et al*., 2005; Urban and Wolfe, 2005). A tryptophan-arginine motif in loop 1 present between the first and second transmembrane domains is another invariant structure observed in rhomboid proteases.

Although the first mammalian rhomboid protease was cloned and named *RHBDL1* for rhomboid-like protein1 before *Drosophila* Rhomboid-1 was recognized as an intramembrane protease (Pascall and Brown, 1998), the function of RHBDL2 has yet to be elucidated. However, RHBDL2 has been shown to share the catalytic activity of *Drosophila* Rhomboid-1. The localizations of the five known mammalian rhomboid proteases are diversely scattered being found in the Golgi for RHBDL1, plasma membrane for RHBDL2, endosomes for RHBDL3,

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endoplasmic reticulum (ER) for RHBDL4 and mitochondrial inner membrane for PARL (Bergbold and Lemberg, 2013), suggesting that they have distinct and diverse functions. Only RHBDL2 can cleave and activate the mammalian proEGF (Adrain *et al*., 2011), and EGFR signaling is negatively modulated by RHBDL2-mediated lysosomal degradation of EGFR (Haglund and Dikic, 2012) and (or) EGFR cleavage (Liao and Carpenter, 2012). Conversely, RHBDL4 localizing to the ER can induce degradation of various substrates (Bergbold and Lemberg, 2013) as a part of the ER-associated protein degradation (ERAD) machinery (Fleig *et al*., 2012).

However, there are other subgroups of rhomboid family members, known as iRhoms, which have high sequence similarities. These rhomboid family members, iRhom1 and iRhom2 (another name of *Rhbdf2*), are inactive rhomboids that have no the key catalytic motif observed in other rhomboid proteases (Lemberg and Freeman, 2007; Ha *et al*., 2013). iRhom1 and iRhom2 lost their protease activity during their evolution but have retained key non-protease functions, which have been implicated in regulation of the epidermal growth factor (EGF) signaling pathway (Adrain *et al*., 2011) and the tumor necrosis factor-alpha (TNF-α) signaling pathway (Adrain *et al*., 2012).

As more distant rhomboid family members, many other genes without the key catalytic motif, such as derins, UBAC2, RHBDDs and TMEM115, have also been annotated as rhomboid-like proteins by bioinformatics searches based on their sequence similarities (Koonin *et al*., 2003; Lemberg and Freeman, 2007; Finn *et al*., 2010). However, the structural relationships among these proteins remain to be investigated because of their limited overall sequence conservation (Bergbold and Lemberg, 2013).

Currently, there are 14 rhomboid family members, five rhomboid proteases and nine catalytically inactive homologues (Bergbold and Lemberg, 2013). Among these rhomboids, iRhoms comprise a unique family, not only possessing the key catalytic motif and highly conserved sequences between species, but also the unique iRhom homology domain and cytosolic N-terminal cytosolic domain, suggesting that these proteins have an important biological role, despite their lack of protease activity (Koonin *et al*., 2003; Lemberg and Freeman, 2007; Freeman, 2014). Rhomboid is a family member 2 protein in humans that is encoded by the *RHBDF2* gene (Puente *et al*., 2003). The alternative name iRhom2 has been proposed to clarify that it is a catalytically inactive member of the rhomboid family of intramembrane serine proteases (Lemberg and Freeman, 2007) .

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by synovitis and joint damage. The etiology of RA is multi-factorial, including various genetic and environmental factors, and its pathogenesis is complex, involving synovial tissue proliferation, pannus formation, and cartilage and bone destruction.

In this study, *Rhbdf2* knockout (KO) mouse was produced using the CRISPR/CAS9 system. This type of genome editing is a useful tool for generating mutant animals that introduces mutations in genes of interest using artificial DNA nucleases such as the CRISPR/Cas9 systems in living cells (Hara *et al*., 2015). It is well known that *Rhbdf2* inhibits the release of TNF-α by blocking the maturity of the TNF-α converting enzyme (TACE) (McIlwain *et al*., 2012; Siggs *et al*., 2012). Collagen-induced arthritis (CIA), which is used for animal models of RA, was developed as it is expected to be affected by these types of features. Accordingly, it was predicted that the induction of arthritis would be impaired in this mutant mouse because TNF-α is not released in the *Rhbdf2* KO mouse.

## MATERIALS AND METHODS

### *Rhbdf2* mutant mouse

A *Rhbdf2* KO founder mouse with the C57BL/6J background was produced at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) using CRISPR/Cas9, and the mutation was confirmed by a T7E1 assay. For the mutant lineage establishment, the founder mouse was crossed with C57BL/6J mice to maintain the pure C57BL/6J background.

### Mutant allele analysis of *Rhbdf2* mutant mouse

To identify the exact mutated sequences, the tail DNA from F1 mutants was extracted and used as a template for PCR amplification with the primer pairs F1 (5'-TTCCATAAACACGAGCACCA-3') and R1 (5'-CTCTGGCTACTCCCATCTGC-3'), which span the target area for the guide RNA for CRISPR/Cas9. The PCR amplicon was TA cloned for sequence determination using a TA cloning kit TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA). The PCR primer pairs for genotyping the mutant allele were then designed based on the results of the wild and mutant allele sequences.

To confirm the null mutation of *Rhbdf2* gene, Western blot analysis for *Rhbdf2* was performed with mouse tissues. Briefly, lysates from mouse tissues were subjected to 8% SDS-PAGE, and the resolved proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h at room temperature in 5% skim milk, and then incubated with anti-*Rhbdf2* (MyBioSource, San Diego, CA, USA). The bands were visualized with enhanced chemiluminescence reagents (Ab Frontier, Seoul, Korea). Furthermore, to confirm the functional impairment in TNF-α secretion in the mutant mouse, TNF-α secretion assay was perfromed with bone marrow derived macrophages (BMDM). Briefly, bone marrow cells were collected from femurs and tibias of mice, and red blood cells (RBCs) were lysed with ACK lysing buffer (CMABREX Bio Science, Walkersville, MD, USA). The bone marrow cells were cultured with M-CSF (20 ng/ml) for 6 days to generate BMDM. BMDMs ($1x10^5$ cells/well) were cultured in a 96-well plate with RPMI1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 50 nM 2-mercaptoethanol (Life Technologies, Gaithersburg, MD, USA), 100 μg/mL streptomycin, 100 U/mL penicillin and with or without 1 μg/ml of lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The concentrations of TNF-α secreted from BMDM was measured using commercially available ELISA kits (Quantikine Mouse ELISA kit, R&D Systems, Minneapolis, MN, USA), according to the manufacturers' protocol.

### Collagen-induced arthritis induction protocol

Ten week old *Rhbdf2*$^{-/-}$ and *Rhbdf2*$^{+/+}$ male mice were obtained from KRIBB and raised in a SPF environment with free access to standard diet and water. Collagen-induced arthritis was induced using 14 *Rhbdf2*$^{-/-}$ and 14 *Rhbdf2*$^{+/+}$ mice and referred to as RA-homo and RA-wild groups, respectively. In addition, RA was induced in 14 *Rhbdf2*$^{+/+}$ mice by injection with vehicle without collagen and they were used as the negative

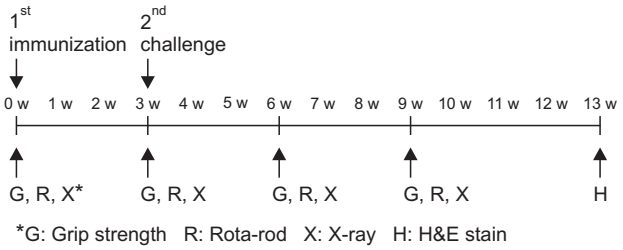


**Fig. 1.** Diagram describing collagen-induced arthritis induction in mice. The experiment was carried out for 13 weeks. Boosting was 3 weeks after the first immunization. Grip strength and rota-rod tests were carried out 3, 6 and 9 weeks after the first immunization. At the end of the experiment, all the mice were autopsied, and histological assessment for the severity of CIA was performed (n=14 for each group).

controls (referred to as the vehicle-wild group). Commercial complete Freund's adjuvant (CFA) including 5 mg/ml heat-killed *M. tuberculosis* (Chondrex, Inc., Redmond, WA, USA) was used for RA induction. Briefly, 2 mg/ml chick collagen type II (CII; Sigma Chemical Company, St. Louis, MO, USA) was dissolved in 10 mM acetic acid (Sigma Chemical Company) at 4°C by overnight incubation, then emulsified with an equal volume of CFA. For the vehicle-wild group, the emulsion was prepared without chick collagen type II. The mice in the RA-homo and RA-wild groups received intradermal injection with a 100 μl emulsion on day 0 of the experiment, and all animals were boosted again with the same type of emulsions 3 weeks later. The schedule of CIA induction and assessment is shown in Fig. 1.

### Grip strength test

The grip strength test was designed to enable measurement of the grip strength of two forelimbs and combined forelimbs and hind limbs (Dunnett *et al*., 1998). This test measures the maximum muscle strength (g) using a Chatillon Force Gauge (Chatillon DFE2, C.S.C Force Measurement, San Diego, CA, USA). At 3, 6 and 9 weeks after the immunization, the same measurements were repeated with the same mice. Ten week old *Rhbdf2* CIA induction male mice were then allowed to grip the grid either with the forelimbs or with the combined forelimbs and hind limbs, after which they were pulled backwards until they released the grid and the maximum strength of the mouse before releasing the grid was recorded. Three consecutive trials separated by 1 min intervals were performed for each mouse.

### Rota-rod test

The rota-rod test was used to assess motor coordination, balance and motor learning. At 3, 6 and 9 weeks after the immunization, the same measurements were repeated with the same mice. The 10 week old CIA induction male mice were evaluated using a rota-rod apparatus (RotaRod 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). The test was performed by placing the mice on rotating drums (3 cm diameter) that rotated under continuous acceleration from 4 to 40 rpm over 300 s, and the latency (the time until the mouse falls off the rod) was measured. This experiment consisted of three trials separated by 15 min intervals.

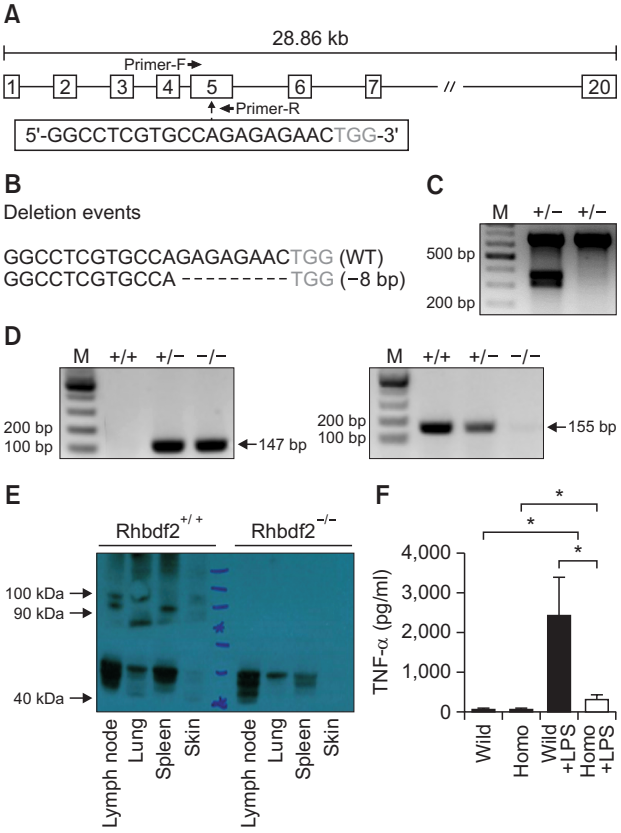


**Fig. 2.** Confirmation of *Rhbdf2* mutant allele in mutant mice. (A) Schematic representation of the target site on exon 5 in the *Rhbdf2* gene. The target sequences and PAM sequences (gray letters) are indicated. (B) The mutated sequences were shown to be 8 bp deletions by sequencing analysis. (C) In the T7E1 cleavage assay, *Rhbdf2* heterozygous mice showed two smaller bands that were cut by the enzyme and did not appear in homozygous mice. (D) PCR genotyping was performed with newly designed PCR primers to distinguish the wild allele (155 bp) from the mutant allele (147 bp). bp, base pair; M, DNA size maker; +/+, wild; +/-, heterozygous; -/-, homozygous. (E) Abolishment of full-length *Rhbdf2* proteins production in *Rhbdf2*$^{-/-}$ mouse was confirmed by Western blot analysis. (F) TNF-α secretion assay was perfromed with macrophages derived from bone marrow cells. The TNF-α concentration in the culture medium of LPS-stimulated cells was determined by ELSIA. **p*<0.5.

### X-ray analysis

X-ray analysis was used to assess the morphology of hind limbs swelling. At 3, 6 and 9 weeks after the immunization, the same measurements were repeated with the same mice. After the 10-week-old CIA induction male mice were anesthetized by intraperitoneal injection of 1.2% avertin solution (0.02 ml/g body weight), the x-ray images were obtained using a Faxitron (MX20, Faxitron, Tucson, AZ, USA).

### Hematoxylin and eosin staining

After completing the experiment, mice were sacrificed and hind limbs were fixed in 10% neutral buffered formalin, then decalcified with 10% EDTA at pH 7.4 for 2 days. The knee joints of the tissues were then embedded in paraffin and cut into 5-μm thick sections. The deparaffinized sections were subsequently stained with hematoxylin and eosin and ob-

**Table 1.** Severity scores for collagen-induced arthritis in mice

| Severity score | The degree of rheumatoid arthritis |
|---|---|
| 0 | No evidence of erythema and swelling |
| 1 | Erythema and mild swelling confined to the tars or ankle joint |
| 2 | Erythema and mild swelling extending from the ankle to the tarsals |
| 3 | Erythema and moderate swelling extending from the ankle to metatarsal joints |
| 4 | Erythema and severe swelling encompass the ankle, foot and digits, or ankyloses of the limb |

served under a light microscope.

### Statistical analysis

All data were expressed as the means ± standard deviations (SD). Student's *t*-tests were conducted to compare the two groups using the Graph pad Prism software (San Diego, CA, USA). The *p*-values<0.05 were considered statistically significant.

## RESULTS

### Confirmation of *Rhbdf2* deficiency in mutant mice

*Rhbdf2* mutant mice were produced using the CRISPR/Cas9 system. To determine the exact mutated sequences, the DNA region including the sgRNA targeting site was amplified by PCR as shown in Fig. 2A. The PCR product was subjected to TA cloning, and the sequence was determined Fig. 2B. *Rhbdf2* mutant mouse was shown to have an eight base pair deletion within the targeting sequences, resulting in the frame shift mutation. Although the mutation could be detected by T7E1 assay as in Fig. 2C, an easier way for genotyping of the mutation is required for massive production of the mutant mouse. Therefore, through sequence analysis for the wild and mutant genomic DNA regions, new PCR primer pairs were selected as followings; for the wild allele, wild-F1 (5'-CGTG CCAGA GAGCT GCCCA-3') and common-R1 (5'-GAACA ATGTT GCCCA GACCC-3') and for the mutant allele, mutant-F1 (5'-CGTGC CAGAG AGCTG CCCA-3') and common-R1 (5'-GAACA ATGTT GCCCA GACCC-3'). The PCR primers could be used successfully to determine the exact genotyping as shown in Fig. 2D.

Western blot analysis for *Rhbdf2* demonstrated that the full-length of *Rhbdf2* proteins were disappeared in the *Rhbdf2* mutant mouse (Fig. 2E). We also confirmed that the macrophages derived bone marrow of the mutant mouse could not secrete TNF-$\alpha$ when stimulated with LPS (Fig. 2F).

### Collagen-induced arthritis analysis

*Rhbdf2* is known to be related to TACE maturation, which is essential for TNF-$\alpha$ release. Although the causes of RA development are not known, it is well known that TNF-$\alpha$ is one of the accelerating agents for RA (Issuree *et al*., 2013; Lee *et al*., 2016). Therefore, *Rhbdf2* deletion may alleviate the RA symptoms in mice. To confirm this assumption, two groups of mice (wild type and *Rhbdf2* homozygous mutant) were used for the induction of CIA and designated as RA-wild and RA-homo, respectively, as described in the Materials and Methods. One additional group of wild type mice was used for CIA induction without collagen and designated as vehicle-wild. The severity of arthritis during the CIA induction period was scored as described in Table 1. From 4th week after first immunization, the severity score increased to 1.75 ± 0.17 and 1.2 ± 0.18 in the RA-homo and RA-wild group, respectively, but not in the vehicle-wild group. However, the severity score in the RA-homo group did not rise after that point, whereas the score in the RA-wild group rose continuously. Therefore, the severity score was reversed between the two groups from 5 weeks, and the gap in the scores between the groups became bigger with time (Fig. 3).

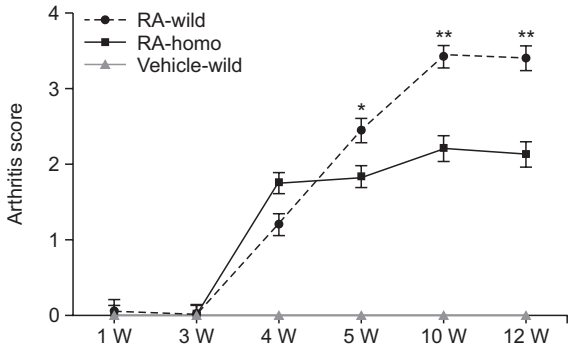


**Fig. 3.** Collagen-induced arthritis in *Rhbdf2* mice. Mice were immunized with type II collagen in complete Freund's adjuvant, and paw diameter and clinical score were measured for 12 weeks (n=14 arthritic mice per group). Paw swelling peaked in RA-wild mice at week 10. In contrast, the mice in the RA-homo group did not show an increase in paw swelling from 4 weeks after the immunization through the end of the experiment. n=14 for each group. **p*<0.05; ***p*<0.01.

### Evaluations of CIA by histopathology in *Rhbdf2* deficient mice

To evaluate the pathological severity of the CIA, the knee joints of the mice were stained with hematoxylin and eosin after sectioning the decalcified tissues at the 13th week as shown in Table 2. As shown in Fig. 4, the knee joints of mice in the RA-wild group showed irregular erosion in the articular cartilage surface (Fig. 4B). Conversely, the knee joints of mice in the vehicle-wild or RA-homo group had cartilage surfaces that were almost completely intact (Fig. 4A, 4C). The histopathological severity scores for CIA in the knee joints were allocated as described in Table 2 and expressed in the bar graph (Fig. 4D). The results clearly demonstrated that the severity of CIA in *Rhbdf2* deficient mice was significantly lower than in wild type mice.

### Evaluation of CIA effects on physical functions

The grip strength test and rota-rod test were employed to

**Table 2.** Histopathological severity scores for collagen-induced arthritis in mice

| Severity score | The degree of CIA lesions |
|---|---|
| 0 | No more than any cartilage |
| 1 | Wxeak and damaged articular cartilage softens |
| 2 | The cartilage destruction and severe damage to the cartilage |
| 3 | The severe erosion of cartilage and bone destruction |

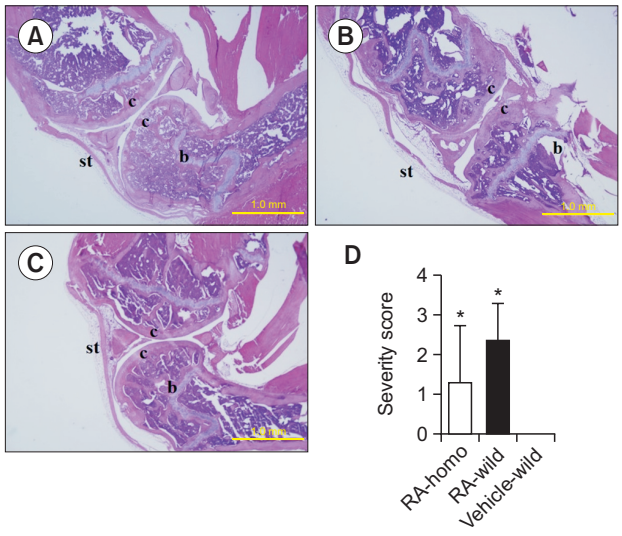


**Fig. 4.** The representative histopathologic observations in synovial membranes of *Rhbdf2* mice. Representative H&E stained sagittal sections of the knee joints from vehicle-wild (A), RA-wild (B) and RA-homo (C) mice are shown. (D) The histopathological severity scores for RA were determined from the affected joints and expressed as the means ± SD (males: n=14 for each group). **p*<0.05. The letters b, c and st indicate bone, cartilage, and synovial tissue, respectively.

evaluate the CIA effect on physical functions during the CIA induction process. At 0 weeks after first the immunization, the grip strength test and rota-rod test were conducted in all individual mice as shown in Fig. 1, and the measured values were used as a basal level (severity score=0) for the severity score as indicated in Table 3 for the grip strength test and Table 4 for the rota-rod test. On the 3rd, 6th and 9th week after the immunization, the same measurements were repeated for the same mice. The CIA severity score tables for the grip strength measurement with combined forelimbs and hind limbs and for the rota-rod measurement are shown in Table 3 and Table 4, respectively. The severity scores converted from measurements in the grip strength test and rota-rod test are expressed as a graph in Fig. 5. The severity score of the grip strength measurement for the RA-wild group was observed to increase abruptly at the 6th week after the first immunization (Fig. 5A). Conversely, the RA-homo group did not show a conspicuous increase until the 9th week after the first immunization, and the score was significantly lower than that of the RA-wild group. On the other hand, irregular patterns were depicted with severity scores from the rota-rod measurements. As shown in Fig. 5B, although the severity scores in the RA-wild group increased as early as at 1st week after the first immunization, the other two groups also showed the same level of severity scores.

**Table 3.** Severity scores for collagen-induced arthritis of grip strength test in mice

| Severity score | Fore limb and hind limb grip strength (g) |
|---|---|
| 0 | 160≤ |
| 1 | 150-159 |
| 2 | 140-149 |
| 3 | 130-139 |
| 4 | 120-129 |
| 5 | ≤110 |

**Table 4.** Severity scores for rota-rod test in mice

| Severity score | Latency time in roar-rod test (s) |
|---|---|
| 0 | 130≤ |
| 1 | ≥120-<129 |
| 2 | ≥110-<119 |
| 3 | ≥100-<109 |
| 4 | ≥90-<99 |
| 5 | 0-<80 |

### Paw thickness comparison by X-ray analysis

X-ray analysis was used to assess the morphology of paw thickness. The results revealed that at the 3rd, 6th and 9th week after immunization, the hind paw of the RA-wild mouse was relatively thicker than that of the RA-homo mouse after CIA induction, as indicated by an arrow (Fig. 6A). It was considered that the thickness of mice in the RA-homo increased due to inflammation, which was comparatively weaker than that of RA-wild mice (Fig. 6B). Overall, the results indicate that thickening was due to inhibition of TNF-$\alpha$ expression in *Rhbdf2* KO mice.

## DISCUSSION

In this study, we tried to reveal the function of the *Rhbdf2* gene by conducting an arthritis study using *Rhbdf2* KO mice. RA is an autoimmune disease that leads to chronic inflammation in the joints, destruction of cartilage, and erosion of the bone in the affected joint. One inactive homologue lacking essential catalytic residues of rhomboid intramembrane serine proteases is *Rhbdf2*. This protein is known to help the maturation of TACE in the RA induction of mice. Rhomboid protease was first discovered in *Drosophila* (Ha *et al*., 2013). *Drosophila* rhomboid protease cuts epidermal growth factor receptor (EGFR) ligand Spitz and a homologue for mammalian tumor growth factor (TGF-$\alpha$), triggering secretion of the factors (Rutledge *et al*., 1992; Schweitzer *et al*., 1995). Homologs of fly

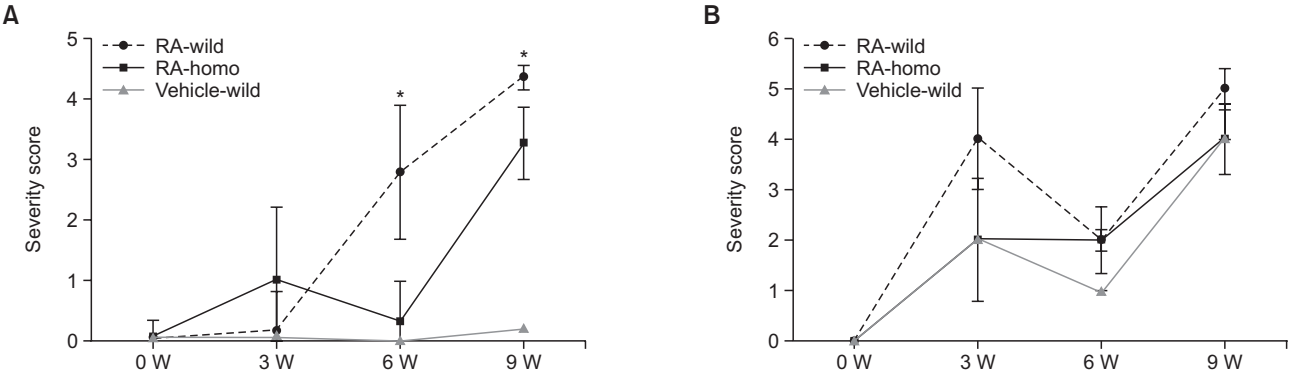


**Fig. 5.** Severity score determined using the results of the grip strength test with *Rhbdf2*$^{-/-}$ mice. (A) The grip strength was measured for mice in the RA-homo and RA-wild groups. The measurements were converted to the severity scores as described in Table 3. (B) The rotarod test was measured for mice in RA-homo and RA-wild groups. The measurements were converted to the severity scores as described in Table 4. The severity scores were expressed as the means ± SD for each group. n=14 for each group. **p*<0.05.

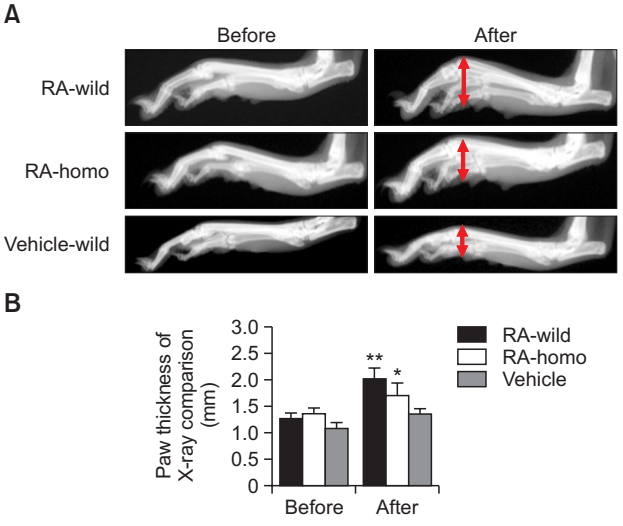


**Fig. 6.** X-ray test in CIA mice. The skeletons of *RA-homo* mice and *RA-wild* mice at each week of age were analyzed by X-Ray system. Radiological examination was conducted 3, 6 and 9 weeks after immunization, and the same measurements were repeated with the same mice. The results are expressed as the means ± SD for 14 mice for each genotype (males: *RA-homo*, n=14; *RA-wild*, n=14). **p*<0.5.

rhomboid proteases have been identified in most prokaryotic and eukaryotic organisms (Basso *et al*., 2007). Additionally, rhomboid proteases comprise a superfamily of proteins consisting of intra-membrane serine proteases and their inactive homologs (Freeman, 2014). The common ancestor of all members of the family is probably an active intra-membrane protease, although the majority of existing members are not active proteases (Freeman, 2014).

TACE, also known as ADAM17, is a membrane-anchored metalloproteinase that controls two major pathways, the EGFR pathway and proinflammatory TNF-α pathway, which play important roles in development and disease (Black *et al*., 1997; Peschon *et al*., 1998; Sahin *et al*., 2004). TACE is essential to the release of EGFR ligands (Sahin *et al*., 2004; Sahin and Blobel, 2007) and TNF-α (Black *et al*., 1997; Issuree *et al*., 2013), which is the primary trigger of inflammation (Adrain *et al*., 2012; Lim *et al*., 2016). TACE and its regulator, iRhom2, can be rapidly activated by small amounts of cytokines, growth factors, and pro-inflammatory mediators present in the blood (Hall and Blobel, 2012). iRhoms, which are co-expressed with TACE, are essential to the specific regulation of TACE activity (Christova *et al*., 2013). Considering these points, we were able to speculate about the functions of various *Rhbdf2* genes. Previously known mutation in *Rhbdf2* gene increased the protein stability of EGFR and over-activated it by secretion of amphiregulin (Hosur *et al*., 2014).

The known mechanism of *Rhbdf2* gene is as follows. TNF-α is secreted from the cell, after which TACE present on the cell membrane cleaves the membrane-bound TNF-α and releases the TNF-α molecule. It is known that the TACE protein acting here functions only when it is matured from the ER to the cell membrane through the Golgi, at which time the molecule that facilitates the maturation of TACE in the cell is *Rhbdf2* (Adrain *et al*., 2012). In this study, *Rhbdf2* KO mice were generated by the CRISPR/CAS9 system. This gene editing technique has been used in living cells to create animals by mutating the gene of interest using artificial DNA nucleases such as those present in the CRISPR/CAS9 system (Hara *et al*., 2015). Therefore, in this study, *Rhbdf2* gene deficient mice were isolated, and the *Rhbdf2* mutant was identified through the T7E1 assay and TA cloning sequencing, which confirmed that 8 bp was deleted from the target site. Evaluation of the expression level of TNF-α in these mice by ELISA and Western blotting confirmed that TNF-α in the *Rhbdf2*$^{-/-}$ mouse tissues was expressed at lower levels than in *Rhbdf2*$^{+/+}$ mouse, and that TNF-α production was functionally blocked in bone marrow derived macrophages stimulated with LPS. In this study, CIA-related experiments were performed using *Rhbdf2* deficient mice. The deficiency of the *Rhbdf2* gene restricted the maturation of TACE, and the expression of TNF-α was thought to be inhibited. The CIA model is commonly used to analyze the relationship between the occurrence of CIA and the existence of the *Rhbdf2* gene. When we numerically evaluated the results of the CIA induced experiment, erythema and edema of the front and hind paws of RA-wild and RA-homo mice increased from the 5th and 7th week, and the severity score of RA-homo mice was significantly reduced. The results of the histological evaluation of arthritis-induced knee joints showed that the synovial membrane of the vehicle-wild group was smooth and looked

normal, while there was severe erosion of bones and tissues in the RA-wild group. But only weak mild erosion of cartilage in RA-homo mice was observed in their knee joints. Recent studies showed that TACE is controlled by iRhom1 in most of cells except for myeloid cells and microglia where iRhom2 have the role. Actually, in other paper, it has been shown that iRhom2 KO mouse has normal appearance and no spontaneous pathological phenotypes (McIlwain *et al*., 2012; Issuree *et al*., 2013) and that myeloid cells lacking iRhom2 release very little TNF-α in response to LPS stimulation (McIlwain *et al*., 2012). These results coincide with our present results. On the other hand, iRhom1/2 double KO mice die perinatally with open eyes, misshapen heart valves, and growth plate defects (Li *et al*., 2015). Therefore, in other than myeloid cells, it is considered that iRhom1 can compensate the iRhom2 deficiency *in vivo* of iRhom2 KO mouse. Accordingly, the milder severity of RA in RA-homo group is considered due to the low level of TNF-α production from myeloid cells.

In this experiment, a new method was used to evaluate the biological functions caused by CIA induction. The CIA score determined from the grip strength progress for the RA-homo group was unclear at 3th and 6th week, but was high from the 9th week, while the degree of arthritis was low when compared to the RA-wild group. The rota-rod test revealed that the strength of the RA-wild mice was high at week 3 and the degree of arthritis was similar to that of 9th week. Moreover, from the 6th week to the 9th week the CIA gradually increased in the RA-homo mice in this study. Based on these experimental results, the rota-rod test was more sensitive to CIA measurements than the grip strength test. This was likely because the rota-rod test was correlated with the motility of arthritis by measuring latency time on it. Overall, the results of this experiment revealed no abnormal phenotypes in the *Rhbdf2* KO mouse; however, arthritis induction showed that RA was weakly induced in the RA-homo mice. Furthermore, the induction of arthritis was shown to be caused by the deficiency of TNF-α, which can be used as an important resource in investigation of the function of the *Rhbdf2* gene and as an indicator of disease status.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with respect to publication of these results.

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