Effect of immunosuppressants on a mouse model of osteogenesis imperfecta type V harboring a heterozygous *Ifitm5* c.-14C>T mutation

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Osteogenesis imperfecta (OI) type V is an autosomal dominant disorder caused by the c.-14C>T mutation in the interferon-induced transmembrane protein 5 gene (*IFITM5*), however, its onset mechanism remains unclear. In this study, heterozygous c.-14C>T mutant mice were developed to investigate the effect of immunosuppressants (FK506 and rapamycin) on OI type V. Among the mosaic mice generated by Crispr/Cas9-based technology, mice with less than 40% mosaic ratio of c.-14C>T mutation survived, whereas those with more than 48% mosaic ratio exhibited lethal skeletal abnormalities with one exception. All heterozygous mutants obtained by mating mosaic mice with wild-type mice exhibited a perinatal lethal phenotype due to severe skeletal abnormalities. Administration of FK506, a calcineurin inhibitor, in the heterozygous fetuses improved bone mineral content (BMC) of the neonates, although it did not save the neonates from the lethal effects of the mutation, whereas rapamycin, an mTOR inhibitor, reduced BMC, suggesting that mTOR signaling is involved in the bone mineralization of heterozygous mutants. These findings could clarify certain aspects of the onset mechanism of OI type V and enable development of therapeutics for this condition.

Interferon-induced transmembrane protein 5 (IFITM5) is a type II (N-in/C-out) transmembrane protein, the amino terminus of which is located inside the cell and the carboxyl terminus is located extracellularly1. Although the function of IFITM5 was not known for a long time, it has been suggested for some time now that IFITM5 is a positive regulator of bone formation, as the *Ifitm5* gene is seen to be specifically expressed only in the mineralization stage of cultured osteoblasts2,3. Mineralization is promoted and suppressed when *Ifitm5* is overexpressed and knocked down in cultured osteoblasts, respectively2. Thus, *Ifitm5*-deficient mice were generated and their phenotype investigated to verify the role of IFITM5 in bone formation. However, studies have neither reported any major abnormalities in bone formation nor morphology in *Ifitm5*-deficient mice3,4. Although IFITM5 functions as a positive regulator of mineralization in vitro, it is considered that IFITM5 is not an essential molecule for bone formation in vivo.

Following reports stating that no major abnormality is seen in the osteogenesis of *Ifitm5*-deficient mice, many research groups have reported that patients with osteogenesis imperfecta (OI) type V have a heterozygous point mutation (c.-14C>T) in the 5' untranslated region of the *IFITM5* gene5-16. The c.-14C>T mutation shifts the start codon of *IFITM5* upstream to create a new start codon, resulting in the addition of 5 amino acids (Met-Ala-Leu-Glu-Pro, MALEP) to the amino terminus. Therefore, OI type V is thought to be caused by the mutated IFITM5 (MALEP-IFITM5) in which these 5 amino acids are added to the amino terminus of IFITM5, however, the mechanism of its onset is unknown at present.

OI is a genetic bone disease characterized by bone fragility, and at least 18 different types of OI are known till date17. OI type V is an autosomal dominant condition and is distinguished from other types of OI by features such as forearm intersosseous membrane calcification, hyperplastic callus formation, and radial head

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dislocation. However, the severity of symptoms varies widely among patients. Forearm interosseous membrane calcification is observed in essentially all patients and is associated with a limitation in forearm supination and pronation. Hyperplastic callus formation occurs mainly after bone fractures, but is not observed in all patients. Radial head dislocation is observed in many patients and causes elbow deformity. There is a remarkable difference in vulnerability to fractures, accompanied by the bone fragility caused by this condition, resulting in variable number of fractures affecting the mobility of patients ranging from being able to walk on their own to needing wheelchairs.

Presently, bisphosphonates are used as therapeutic agents for treating various types of OI including OI type V. The bisphosphonates may increase bone mass by suppressing bone resorption, but improvement of characteristic symptoms of OI type V cannot be expected. The absence of major skeletal abnormalities in Ifitm5-deficient mice leads to the possibility that OI type V may be treatable by suppressing the expression of both wild-type (WT) and c.-14C > T mutant Ifitm5, which is a similar condition to that in Ifitm5-deficient mice.

We previously reported that IFITM5 interacts with the Fkbp11 gene product (FKBP19), a member of the FK506-binding protein (FKBP) family of peptidyl-prolyl cis/trans isomerases. FKBP19 contains a putative peptidyl-prolyl cis/trans isomerase domain with homology to FKBP12, and FK506 binds to this domain. Therefore, we performed this study to investigate the effect of FK506, an immunosuppressant, on the interaction between IFITM5 and FKBP19. However, it was not possible to investigate this because FK506 suppressed the expression of Ifitm5. Meanwhile, the result indicating that the expression of Ifitm5 was suppressed by FK506 provided us with an important hypothesis, specifically that FK506 might be able to suppress the expression of both WT and mutant Ifim5 genes, if the regulatory system for the expression of the Ifitm5 mutant is the same as that for WT Ifitm5. If FK506 could suppress the expression of both the WT and mutant Ifitm5 genes, it can be expected that bone abnormalities will not appear, as in the Ifitm5-deficient mice. Therefore, we created heterozygous Ifitm5 c.-14C > T mutant mice by Crispr/Cas9-based genome editing technology to investigate the effect of FK506 on improving bone abnormalities. This paper presents the bone abnormalities of heterozygous Ifitm5 c.-14C > T mutant mice, the partial improvement of bone abnormalities mediated by FK506, and the effect of another immunosuppressant, rapamycin, on these bone abnormalities.

**Results**

**FK506 suppresses the expression of Ifitm5 in cultured osteoblasts.** As aforementioned, there is a possibility to induce normal bone formation by suppressing the expression of both WT and mutant Ifitm5 in heterozygote mutant mice.

In osteoblasts isolated from mouse calvaria, 20 µg/ml FK506 considerably suppressed the expression of Ifitm5 (Fig. 1A). Mineralization was also suppressed depending on the concentration of FK506 (Fig. 1B). In addition, the expression of mineralization markers osteocalcin (Bglap2) and bone sialoprotein (Ibsp) were also suppressed by 20 µg/ml FK506 (Fig. 1C,D). FK506 suppressed the expression of Bglap2 and Ibsp as well as Ifitm5, however, it is unknown whether the expression of Bglap2 and Ibsp is suppressed directly by FK506. Knockdown of Ifitm5
F0-31, F0-33, and F0-43) were mosaic mice with c.-14C > T mutation, and these 6 mice had a mosaic ratio of from the tail tip of these mice and subjected to genotyping. Of the 26 surviving male mice, 6 (F0-06, F0-15, F0-18, lethal phenotype (Table S2). Of the 63 mice that survived, 26 were male mice (Table S1).

A mixture containing Cas9 protein, tracrRNA, crRNA, and ssODN was administered into fertilized eggs by electroporation. Of the mice born from these fertilized eggs, 63 survived (Table S1) and 11 exhibited a perinatal lethal phenotype. Of the mutants born by mating F0-33 and WT was 25% which reflects the mosaic ratio of germ cells in F0-33. This suggests that F0-33 is not a heterozygous mutant.

Table 2. Percentage of each sequence detected in the tail tip in 11 perinatal lethal mice. The sequences of WT, c.-14C > T, and c.-12G > A are shown in Table S4.

|         | F0-D01 | F0-D02 | F0-D03 | F0-D04 | F0-D05 | F0-D06 | F0-D07 | F0-D08 | F0-D09 | F0-D10 | F0-D11 |
|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| WT      | 6.03   | 49.10  | 32.66  | 0.19   | 0.16   | 48.48  | 18.44  | 48.37  | 0.16   | 0.16   | 48.36  |
| c.-14C > T | 89.10  | 48.26  | 64.03  | 97.21  | 97.18  | 48.89  | 62.66  | 49.06  | 97.37  | 97.20  | 48.58  |
| c.-12G > A | 0.01   | 0.06   | 0.03   | 0      | 0      | 0.04   | 16.17  | 0.03   | 0      | 0      | 0.06   |

Survival of mosaic mice depends on the mosaic ratio of the c.-14C > T mutation. In the in vitro experiment described above, FK506 suppressed the expression of Ifitm5 and also suppressed mineralization. However, as bone formation in Ifitm5-deficient mice occurs almost normally, expression of Ifitm5 is dispensable for in vivo mineralization. In addition, it has been reported that the expression level of mineralization marker genes such as Bglap is increased in Ifitm5-deficient mice. Therefore, if FK506 can suppress the expression of Ifitm5 with the c.-14C > T mutation in vivo, it is may be assumed that a similar condition as Ifitm5-deficient mice is created, wherein normal bone formation occurs. Thus, heterozygous Ifitm5 c.-14C > T mutant mice were created using Crispr/Cas9-based genome editing technology, and the effect of FK506 on the bone formation of mutant mice was investigated.

Heterozygous c.-14C > T mutants exhibit a perinatal lethal phenotype. A study recently reported that heterozygous c.-14C > T mutant CD1 mice exhibited a perinatal lethal phenotype. The surviving mosaic mouse, F0-33, had a 49.08% mosaic ratio of the c.-14C > T mutation (Table 1), and therefore the possibility of it being a heterozygote could not be excluded. Thus, the possibility of obtaining viable heterozygous c.-14C > T mutant mice by mating this mosaic mouse with WT mice was explored. Of the 40 mice born by mating F0-33 and WT mice, 30 mice (75%) survived, all of which were WT (Table S5). The other 10 mice (25%) exhibited a perinatal lethal phenotype and were all heterozygous mutants (Table S5). The percentage of heterozygous mutants born by mating F0-33 and WT was 25% which reflects the mosaic ratio of germ cells in F0-33. This suggests that F0-33 is not a heterozygous mutant.

Table 1. Percentage of each sequence detected in tail tip of 6 surviving mosaic mice. The sequences of WT, c.-14C > T, 9D, 22D, and 1I are shown in Table S3.
Of the 34 mice born by mating a mosaic mouse, F0-43 with a mosaic ratio of 38.94% and WT mice, 16 mice (47.1%) survived and 18 mice (52.9%) exhibited a perinatal lethal phenotype (Table S5). In addition, of the 17 mice born by mating a mosaic mouse, F0-15 with a mosaic ratio of 35.32%, and WT mice, 14 mice (78.6%) survived and 18 mice (21.4%) exhibited a perinatal lethal phenotype (Table S5). It was also observed that of the mice born by mating the mosaic and WT mice, all the surviving mice were WT, and all those that exhibited the perinatal lethal phenotype were heterozygous mutants.

Heterozygous c.-14C > T mutants have severe skeletal anomalies. In the heterozygous c.-14C > T mutants which exhibited a perinatal lethal phenotype, malformation of long bones and ribs and a hypomineralized skull were observed (Fig. 2A). In the hindlimbs of heterozygous mutants, the tibia was severely bent at the center, and the fibula was often bent twice. (Fig. 2B and Fig. S3). A gentle bending was also observed in the femur (Fig. 2C). In addition, the radius and ulna in the forelimbs were also severely bent in one place (Fig. 2D). Ribs of the heterozygous mutants were thin and wavy, and the rib cage tended to be smaller than that of WT (Fig. 2E, Fig. S4). The characteristics of these bone anomalies were the same as those observed in the mosaic mice exhibiting perinatal lethal phenotype with a mosaic rate of 48% or higher (Fig. S1).

The bone mineral content (BMC) of the neonates born by mating mosaic and WT mice was compared. The BMC in the whole skeleton of the heterozygous mutants was 42% of that of the WT mice (Table S6), whereas it was 37% in the ribs and thoracic vertebra and 38% in the skull (Table S6). These results indicate that skeletal mineralization in heterozygous mutants is markedly reduced.

FK506 enhances mineralization in both heterozygous mutant and WT mice. The perinatal lethal phenotype exhibited by the heterozygous mutant mice prevented us from verifying the effect of FK506.
after birth. Thus, to examine whether the administration of FK506 improves the skeletal anomalies of fetuses of the heterozygous mutants and allow these neonates to survive, FK506 was subcutaneously administered to WT pregnant dams that were mated with mosaic mice once a day from day 10.5 to day 18.5 of gestation.

In spite of administration of FK506 at a dose of 0.5 mg/kg to the pregnant dams, all neonates of the heterozygous mutants exhibited a perinatal lethal phenotype. These neonates had severe bone anomalies and showed no improvement in BMC in the whole skeleton (Fig. 3A,B). Next, the dose of FK506 was increased to 1 mg/kg. Even at this dose, all heterozygous mutant neonates exhibited a perinatal lethal phenotype and the abnormalities in the skeletal morphology did not improve (Fig. 3A). However, the whole skeletal BMC of mutant neonates treated with FK506 was 1.54 times higher than that of mutant neonates without FK506 treatment (Fig. 3B).

Although the mutant neonates showed an increase in BMC when the pregnant dams were administered with FK506 at a dose of 1.0 mg/kg, the abnormalities in skeletal morphology did not improve, implying that the c.-14C > T mutation containing Ifitm5 continued to be expressed in the fetuses of the heterozygous mutants. Administration of FK506 directly into the peritoneal cavity of E14.5 fetuses through the uterus of WT pregnant dams mated with mosaic mice at an increased dose of 1 mg/kg increased the efficiency of FK506. However, even when FK506 was administered directly to the fetuses, all the neonates of the heterozygous mutants exhibited a perinatal lethal phenotype and the abnormalities in skeletal morphology did not improve (Fig. 3A). Conversely, the whole skeletal BMC in the mutant neonates treated with FK506 was 1.69 times that of the untreated mutant neonates (Fig. 4B). The BMC, particularly in the ribs and thoracic vertebrae of the mutant neonates treated with FK506 increased 1.86 times that of the untreated mutant neonates (Fig. 4C, Fig. S5). The BMC in the skull of the mutant neonates treated with FK506 was also seen to be increased 1.66 times compared to that of untreated mutant neonates (Fig. 4D and Fig. S6).

Furthermore, an increase in BMC was also observed in WT neonates. The BMC in whole skeleton, ribs and thoracic vertebrae, and skull in WT neonates treated with FK506 was 1.32, 1.26, and 1.43 times that of untreated WT neonates, respectively (Fig. 4A–C, Fig. S5, S6). These results indicate that FK506 increases the BMC in both heterozygous mutants and WT mice.

The fact that direct administration of FK506 to the fetus failed to improve abnormalities of skeletal morphology suggests that FK506 did not suppress the expression of c.-14C > T mutation containing Ifitm5. This inference led us to assess the expression level of Ifitm5 in the hind limb bones of the fetuses directly administered with FK506. Real-time quantitative polymerase chain reaction (qPCR) assay suggested that the expression of Ifitm5
was not suppressed by FK506 administration (Fig. S7), although this result does not formally exclude the possibility of the suppression of mutant allele, because the qPCR assay did not discriminate between WT and mutant allele. Also, the expression levels of *Bglap2*, *Sp7*, and *Runx2* were not significantly altered (Fig. S7).

**Rapamycin reduces BMC in heterozygous mutants but not in WT mice.** The effect of rapamycin, an immunosuppressant with a mode of action different from FK506, administered directly to the E14.5 fetuses was investigated to verify increased BMC in the neonates. Heterozygous neonates treated with rapamycin also exhibited a perinatal lethal phenotype and had severe skeletal anomalies (Fig. 5A, Fig. S8). This observation suggested that rapamycin does not suppress the expression of *Ifitm5* with the c.-14C > T mutation. The whole skeletal BMC in heterozygous neonates treated with rapamycin was reduced to 54% of that in untreated heterozygous neonates (Fig. 5B, Fig. S8). In contrast, the BMC of WT neonates treated with rapamycin did not differ from that of untreated WT neonates (Fig. S5B, Fig. S8). The BMC of ribs and thoracic vertebrae was reduced to 47% and that of the skull reduced to 46% in the mutant neonates treated with rapamycin as compared to the BMC in untreated mutant neonates; no significant difference was observed in the BMC of ribs and thoracic vertebrae between the rapamycin treated and untreated mutant neonates (Fig. 5C,D, Fig. S9, S10). The BMC of ribs, thoracic vertebrae, and skull in WT neonates treated with rapamycin was not significantly different from that of untreated WT neonates (Fig. 5C,D, Fig. S9, S10). These results suggested the possibility that the sensitivity to rapamycin in the heterozygous mutants is different from that in WT mice at least during embryogenesis.

**Discussion**

Among the mosaic mice generated by Crispr/Cas9-based genome editing technology, mice with a mosaic ratio of 48% or more at the tail tip, with one exception (F0-33 with a mosaic ratio of 49.08%), exhibited a perinatal lethal phenotype and severe bone abnormalities were observed. Mice with a mosaic ratio of 40% or less survived, and no skeletal abnormality was observed even after growth. These results suggest a relationship between mosaic rates and bone abnormalities. However, the relationship between the mosaic rate and bone abnormalities was not continuous, and it seemed that bone abnormalities appear above a certain threshold of mosaic rate. The
mosaic rate was examined at the tail tip; although the mosaic rate of the tail tip does not reflect the mosaic rate of osteoblasts, it is proposed that abnormal bone formation is likely to occur in mosaic mice when the mosaic rate of osteoblasts exceeds a certain threshold.

Administration of FK506 suppressed the expression of *Ifitm5* in osteoblasts in vitro and at the same time suppressed mineralization. This result complied with the function of *Ifitm5* as a positive regulator of mineralization in vitro and also suggests the possibility of reduced mineralization in vivo. However, the absence of any significant abnormality in the skeleton of *Ifitm5*-deficient mice led to the possibility that mineralization would not be reduced if FK506 could completely suppress *Ifitm5* expression in vivo. In addition, it was considered that a complete suppression of both WT and mutated *Ifitm5* by FK506 may improve severe skeletal abnormalities in heterozygous c.-14C>T mutants. Based on this hypothesis, FK506 administration experiments were carried out. Neonates of heterozygous mutants obtained by direct administration of FK506 at a dose of 1 mg/kg to the fetuses showed improved BMC, but contrary to expectations, morphological bone abnormality did not improve suggesting that the expression of mutated *Ifitm5* was not suppressed. Assessment of *Ifitm5* expression in fetuses directly administered with FK506, revealed no suppression of *Ifitm5* expression. Therefore, the improvement in BMC seen after FK506 administration in heterozygous neonates is not due to suppression of *Ifitm5* expression. Although FK506 suppressed the expression of *Ifitm5* in vitro, it did not suppress the expression of the gene in vivo. This indicates that the sensitivity of osteoblasts to FK506 is different in vitro and in vivo.

Direct administration of FK506 to the fetuses increased BMC not only in the heterozygous mutants but also in the WT mice, which indicates that FK506 has the same effect on heterozygous mutants and WT mice. In addition, an increase in BMC was observed in neonates of mutant mice even when FK506 was administered to the pregnant dams at a dose of 1.0 mg/kg. This result implies that FK506 administration to a pregnant dam also results in increased fetal BMC. FK506 forms a complex with the FK506-binding protein 12 (FKBP12), inhibiting the activation of the nuclear factor of activated T cells (NFAT), which is dependent on calcineurin. NFAT functions as a co-factor for Sp1, Sp3, and Osterix, and activates these transcription factors. Sp1, Sp3,
Bio Co., Ltd.) at a density of 2.53 × 10⁴ cells/ml, and 500 μl of growth medium containing the dispersed cells from Cosmo Bio Co., Ltd. (Tokyo, Japan). The osteoblasts were dispersed in growth medium (OBCM, Cosmo specific or undifferentiated stem cell-specific BMP1 signaling, resulting in aberrant chondrogenesis and subsequent induction of ectopic ossification. The mechanism of the interosseous membrane calcification and hyperplastic callus formation, which are characteristics of OI type V, are presently unknown. Although the initial phase of hyperplastic callus formation resembles an inflammatory reaction, the effectiveness of indomethacin, an anti-inflammatory agent, has not been observed in OI type V patients as reported in a previous study. The result of this study indicating that the BMC of heterozygous mutants was reduced by rapamycin, which is involved in mTOR signaling may provide some clue to elucidate the mechanisms of interosseous membrane calcification and hyperplastic callus formation in OI type V.

In recent years, it has been reported that IFITM2 and IFITM3 that are potent inhibitors of virus-cell fusion and are degraded by rapamycin. It has been found that the PPxY site (NEDD4 motif) is involved in this degradation, and IFITM1 without a PPxY site is not degraded by rapamycin. It is expected that IFITM5 is not degraded by rapamycin, similar to because IFITM1, as IFITM5 does not have a PPxY site.

The perinatal lethal phenotype exhibited by the heterozygous c.-14C > T mutant mice led to an ambiguity as to whether the mutants exhibit characteristic symptoms of OI type V after birth. The skeletal phenotype of the heterozygous neonates, however, does not accurately reflect that of OI type V. This study has indicated that mineralization of heterozygous neonates may be partially dependent on mTOR signaling, and FK506 improves their BMC. As the skeletal phenotype caused by heterozygous mutation in IFITM5 differs between mouse and human, it is difficult to extrapolate the effects of FK506 or rapamycin on human OI type V. However, the findings obtained in this study are expected to enable elucidation of the mechanism by which MALEP-IFITM5 causes skeletal anomalies and provide essential information for development of therapeutic strategies.

Materials and methods

Osteoblast culture. Mouse skull-derived osteoblasts (# OBC11: Lot No. SBL C, OBM) were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). The osteoblasts were dispersed in growth medium (OBGM, Cosmo Biotech, Ltd.) at a density of 2.53×10⁴ cells/ml, and 500 μl of growth medium containing the dispersed cells was seeded in each well of a 24-well plate. After 5 days, the medium was changed to differentiation medium (OGCMO, Lot. SAI-DM-O, Cosmo Bio Co. Ltd) containing FK506 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved in dimethyl sulfoxide (DMSO) (FUJIFILM Wako Pure Chemical Corporation). The differentiation medium containing FK506 was replaced every 3 days. Detection of mineralized nodules was performed using Alizarin red S (FUJIFILM Wako Pure Chemical Corporation) after fixation of cultures by 70% ethanol.

Generation of mosaic mice expressing Ifitm5 containing c.-14C>T mutation. C57BL/6J mice were purchased from CREA Japan Inc. (Tokyo, Japan). The differentiation medium containing FK506 was replaced every 3 days. Detection of mineralized nodules was performed using Alizarin red S (FUJIFILM Wako Pure Chemical Corporation) after fixation of cultures by 70% ethanol.

Superovulation was induced by intraperitoneal administration of CARD HyperOva (Kyudo Co., Ltd., Saga, Japan) to 4-week-old C57BL/6J female mice. 48 h after which hCG (Asuka Pharmaceutical Co., Ltd., Tokyo, Japan) was intraperitoneally administered at 5 units/mouse. On the following day, the ova were transferred to human tubal fluid (HTF) medium (ARK Resource Co., Ltd., Kumamoto, Japan). Spermatozoa were collected from the epididymal tail of a 10-week-old
male mouse and incubated with in FERTIUP (mouse sperm pre-culture solution, Kyudo Co., Ltd.) at 37 °C for 1 h. In vitro fertilization was carried out by mixing the incubated spermatozoa with ovum in HTF medium. Five hours after in vitro fertilization, the mixture of Cas9 protein (final concentration of 100 ng/μl), tracrRNA (final concentration of 100 ng/μl), cRNA (final concentration of 200 ng/μl), and ssODN (final concentration of 200 ng/μl) was introduced into the embryos with male and female pronucleus by NEPA21 Tyepell electroporator (voltage: 225 V, pulse width: 0.5 and 1.0 ms, pulse interval: 50 ms, number of times: 4 times; NEPAGENE Co., Ltd., Chiba, Japan). The electroporated embryos were transferred to KSOM medium (ARK Resources Co., Ltd.) and cultured for 16 h.

Transplantation of electroporated fertilized ova was done under anesthesia. Two-cell staged embryos after electroporation were transplanted into pseudo-pregnant mice on the day of confirmation of vaginal plugs. Birth of neonates was confirmed on the 19th day after embryo transplantation. DNA was recovered from the tail tip and the nucleotide sequence of the Ifitm5 region was analyzed using the next-generation sequencer to investigate the mosaic ratio of the neonates. The mosaic mice described above were created at KAC Co., Ltd (Kyoto, Japan) with approval from the Animal Care and Use Committee of the Company.

Generation of heterozygous Ifitm5 c.-14C>T mutant mice. Male mosaic mice carrying the c.-14C>T mutation were mated with female wild-type (WT) C57BL/6j mice to obtain heterozygous mutants. Pregnant dams were bred by three methods until delivery. In the first method, pregnant dams were bred normally until delivery. In the second method, FK506 was administered subcutaneously to pregnant dams once a day from the gestation day 10.5 to day 18.5. In the third method, pregnant dams were anesthetized with isoflurane and surgically opened for direct administration of FK506 or rapamycin (LC Laboratories, MA, USA) from the uterus into the peritoneal cavity of E14.5 fetuses using a 33-gauge syringe. FK506 was administered to pregnant dams via subcutaneous injection (1.0 mg/ml in Soybean oil) at varying volumes depending on the concentration used (12.5 μl (0.5 mg/kg) or 25 μl (1.0 mg/kg)). FK506 was administered to E14.5 fetuses [10 μl (1.0 mg/kg)] via intra-peritoneal injection at a concentration of 0.03 mg/ml in phosphate buffered saline (PBS). Rapamycin, (0.5 mg/ml) was diluted in a solution containing 5% PEG400 and 5% Tween80 to a final concentration of 0.03 mg/ml with PBS and was administered 10 μl (1.0 mg/kg) to E14.5 fetuses via in-traperitoneal injection.

Generation of heterozygous mutant mice described above were conducted at the Center for Promotion of Platform for Research on Biofunctional Molecules, Hokkaido University with approval from the Hokkaido University Animal Experiment Committee and were performed according to the guidelines for animal experimentation of the Hokkaido University.

Determination of DNA sequence. The nucleotide sequence of the Ifitm5 region in the mosaic mice was analyzed by a next generation sequencer. The tail tip of mice cut at 5 mm in length was placed in 300 μl of 25 mmol/l NaCl solution and incubated at 98 °C for 30 min to extract the DNA. The extracted DNA was purified with ethanol and then subjected to 1st PCR using ExTaq HS (Takara Bio Inc., Shiga, Japan). After purifying this PCR product with Agencourt AMPure XP (Beckman Coulter Inc., CA, USA), a second PCR was performed. The primer sequences used for the first and second PCR are given in Table S8. The obtained PCR product was purified with Agencourt AMPure XP and then the base sequence was analyzed with MiSeq (Illumina Inc., CA, USA) using MiSeq Reagent Kit v3 (illumina Inc.).

The nucleotide sequence of the Ifitm5 region in the heterozygous mice was analyzed by the Sanger sequencing method. The DNA extracted from the tail tip was amplified by PCR using forward (5′-CTGTTGGGTTGCTC AAGGCCACTGC-3′) and reverse (5′-GGCGCAACGATTCGAGTGATCG-3′) primers. The base sequence of the amplified DNA was analyzed using Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific, MA, USA) with Applied Biosystems Big Dye Termination V3.1 (Thermo Fisher Scientific).

X-ray micro-computed tomography (μCT) scanning and data analysis. An X-ray μCT apparatus (SMX-160CTS, Shimazu Corporation, Kyoto, Japan) was used for skeletal analysis. The whole body of the mouse was scanned. It was confirmed that the CT value was not saturated in any of the samples. The X-ray μCT measurement of the standard substance (FANTOM No.06-U5D1mmH for bone mineral density, Ratoc System Engineering Co., Ltd., Chiba, Japan). The X-ray μCT measurement of the bone samples was performed under the same measurement conditions as the bone sample measurement conditions.

Gene expression analysis. In the extraction of nucleic acids from cultured osteoblasts, the cells were washed twice with PBS, then mixed with 800 μl of ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan), and were homogenized by pipetting. Nucleic acid extraction from the bones, the femur,ibia, and fibula was done using RNAlater (Thermo Fisher Scientific) after cutting the hindlimbs. These bones were placed in a disposable homogenizer (AS ONE, Osaka, Japan) containing ISOGEN and homogenized.
Chloroform was added to the homogenized cells and bones, followed by centrifugation at 12,000 × g for 15 min, and the supernatant was collected. An equal volume of 2-propanol was added to the supernatant, followed by centrifugation at 15,000 × g for 10 min to obtain nucleic acid pellet. The pellet was washed with 70% ethanol, dried, and dissolved in DEPC-treated water. DNA in DEPC-treated water was degraded with DNase I (Takara Bio Inc.), and then RNA was purified using Agencourt RNAClean XP (Beckman Coulter Inc., CA, USA) according to the manufacturer’s instruction. Purified RNA was reverse-transcribed with PrimeScript RT reagent Kit (Takara Bio Inc.) to obtain complementary DNA (cDNA). The light Cycler 480 SYBR Green I Master (Roche Life Science, IN, USA) was added to the synthesized cDNA, and the gene expression level was analyzed using qPCR (LightCycler 480, Roche Life Science). The primer sequences for qPCR of each gene are given in Table S9. The expression level of each gene was normalized with the expression level of Gapdh.

Statistical analysis. All analyses were replicated with two to ten samples. Multiple comparisons were facilitated by assessment of significance using analysis of variance followed by Ryan’s method. A p-value < 0.05 was considered statistically significant.

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Author contributions
N.H. and T.K. conceived and designed the study. T.T., K.K., and T.K. carried out the experiments. N.H. and T.T. analyzed the data. N.H. drafted manuscript, and all authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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