

# Renal failure suppresses muscle irisin expression, and irisin blunts cortical bone loss in mice

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## Abstract

**Background** Chronic renal failure induces bone mineral disorders and sarcopenia. Skeletal muscle affects other tissues, including bone, by releasing myokines. However, the effects of chronic renal failure on the interactions between muscle and bone remain unclear.

**Methods** We investigated the effects of renal failure on bone, muscle, and myokines linking muscle to bone using a mouse 5/6 nephrectomy (Nx) model. Muscle mass and bone mineral density (BMD) were analysed by quantitative computed tomography 8 weeks after Nx.

**Results** Nephrectomy significantly reduced muscle mass in the whole body (12.1% reduction,  $P < 0.05$ ), grip strength (10.1% reduction,  $P < 0.05$ ), and cortical BMD at the femurs of mice (9.5% reduction,  $P < 0.01$ ) 8 weeks after surgery, but did not affect trabecular BMD at the femurs. Among the myokines linking muscle to bone, Nx reduced the expression of irisin, a proteolytic product of fibronectin type III domain-containing 5 (Fndc5), in the gastrocnemius muscles of mice (38% reduction,  $P < 0.01$ ). Nx increased myostatin mRNA levels in the gastrocnemius muscles of mice (54% increase,  $P < 0.01$ ). In simple regression analyses, cortical BMD, but not trabecular BMD, at the femurs was positively related to Fndc5 mRNA levels in the gastrocnemius muscles of mice ( $r = 0.651$ ,  $P < 0.05$ ). The weekly administration of recombinant irisin to mice ameliorated the decrease in cortical BMD, but not muscle mass or grip strength, induced by Nx (6.2% reduction in mice with Nx vs. 3.3% reduction in mice with Nx and irisin treatment,  $P < 0.05$ ).

**Conclusions** The present results demonstrated that renal failure decreases the expression of irisin in the gastrocnemius muscles of mice. Irisin may contribute to cortical bone loss induced by renal failure in mice as a myokine linking muscle to bone.

**Keywords** Renal failure; Irisin; Osteopenia; Muscle wasting

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## Introduction

Chronic kidney disease (CKD) exerts abnormalities in the mineral balance, bone turnover, bone mineralization, bone mineral density (BMD), and bone quality and is accompanied by a decrease in vitamin D and secondary hyperparathyroidism.<sup>1</sup> We previously reported an increased risk of fracture in haemodialysis patients with end-stage renal disease and postmenopausal women with mild renal dysfunction.<sup>2,3</sup>

Fibroblast growth factor (FGF) 23 has been suggested to play a key role in the progression of early-stage CKD–mineral and bone disorder (CKD-MBD).<sup>4</sup>

The prevalence of sarcopenia is high in CKD patients, particularly in the late stage.<sup>5</sup> Metabolic acidosis, systemic low-grade inflammation, increased oxidative stress, and the accumulation of advanced glycation end products (AGEs) contribute to the pathogenesis of sarcopenia and CKD-MBD with the progression of CKD.<sup>4,6</sup> However, the mechanisms by

which the CKD state affects muscle and bone have not yet been elucidated in detail.

A recent study reported that sarcopenia is highly co-morbid with disorders of bone metabolism.<sup>7</sup> Accumulating evidence suggests that skeletal muscles influence bone metabolism by secreting myokines, such as irisin, myostatin, follistatin, transforming growth factor (TGF)- $\beta$ , interleukin (IL)-6, insulin-like growth factor (IGF)-1, FGF2, and osteoglycin.<sup>7</sup> We previously reported follistatin, olfactomedin 1, and Dickkopf-related protein 2 as myokines linking muscle to bone in response to gravity changes and mechanical unloading in mice.<sup>8–10</sup> Moreover, myokine levels were shown to be regulated by various endocrinological abnormalities, such as excessive glucocorticoids and androgen deficiency.<sup>11,12</sup> However, the effects of CKD, including renal failure on myokines linking muscle to bone, remain unclear.

Irisin, a proteolytic product of fibronectin type III domain-containing 5 (Fndc5), is a peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ )-dependent myokine.<sup>13</sup> Although irisin was initially identified as a factor that induces browning in white adipose tissue (WAT) and contributes to thermogenesis regulated by exercise in mice and humans,<sup>13</sup> it also plays important roles in glucose metabolism, the cardiovascular system, and musculoskeletal system.<sup>14</sup> Previous studies showed that irisin enhanced osteoblastic bone formation and suppressed osteoclastic bone resorption in mice.<sup>15</sup> Moreover, irisin facilitated the osteogenic differentiation of osteoblastic lineage cells and inhibited osteoclast formation in mouse monocytic cells.<sup>16–18</sup> We previously demonstrated that a decrease in the expression of irisin in skeletal muscles was related to osteopenia in unloading and androgen-deficient mice.<sup>11,18</sup> Although the administration of irisin attenuated renal damage and fibrosis induced by partial nephrectomy (Nx) and folic acid in mice,<sup>19</sup> its role in renal failure remains unclear.

We therefore hypothesized that myokines linking muscle to bone may be related to muscle and bone loss in renal failure. In the present study, we aimed to examine the effects of renal failure on muscle and bone using a mouse 5/6 Nx model. We also investigated the effects of the administration of irisin on muscle and bone loss in renal failure in mice.

## Materials and methods

### *Animals and 5/6 nephrectomy*

Female C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). Eight-week-old mice were divided into two groups: sham surgery ( $n = 6$ ) and Nx ( $n = 6$ ). Nx follows a two-stage procedure as previously described.<sup>20</sup> After mice were anaesthetized with 2% isoflurane, the upper and lower poles of the left kidney were resected at the one-third

position. One week later, the right kidney was removed. In the sham surgery group, the left kidney was exposed and the right renal artery was identified. In experiments involving the administration of irisin, mice were divided into four groups: control/sham ( $n = 8$ ), irisin/sham ( $n = 8$ ), control/Nx ( $n = 8$ ), and irisin/Nx ( $n = 8$ ). Recombinant irisin (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) was dissolved in saline. Mice were intraperitoneally administered with saline or 100  $\mu\text{g}/\text{kg}$  irisin once a week for 8 weeks after the last sham surgery or Nx as previously described.<sup>11</sup> Mice were individually caged and fed food and water *ad libitum*. Food intake was measured every 3 days. Eight weeks after the last Nx or sham surgery, mice were euthanized with excess isoflurane and tissue samples were collected and weighed.

Animal experiments were performed in accordance with the guidelines of ARRIVE and the institutional rules for the use and care of laboratory animals at Kindai University. All experimental procedures on animals were approved by the Experimental Animal Welfare Committee of Kindai University (permit number: KAME-31-081).

### *Quantitative computed tomography*

Mice were scanned 8 weeks after the last sham surgery or Nx using an X-ray CT system *in vivo* (Latheta LCT-200; Hitachi Aloka Medical, Tokyo, Japan) as previously described.<sup>8,21</sup>

### *Measurement of grip strength*

Grip strength was measured using a grip strength metre (1027SM, Columbus Instruments, Columbus, OH, USA) 8 weeks after the last sham surgery or Nx as previously described.<sup>18</sup>

### *Blood chemistry*

Serum levels of blood urea nitrogen (BUN), creatinine, calcium, and phosphorus were measured using BUN Reagent L (Sysmex, Kobe, Japan), LabAssay Creatinine (Wako Pure Chemicals, Osaka, Japan), Calcium E-Test Wako (Wako Pure Chemicals), and Phospha C-Test Wako (Wako Pure Chemicals), respectively, in accordance with the manufacturers' instructions. Serum parathyroid hormone (PTH), tumour necrosis factor (TNF)- $\alpha$ , and IL-6 levels were measured using enzyme-linked immunosorbent assay kits for mouse PTH (RayBiotech, Norcross, GA, USA, Cat. No. EIAM-PTH-1), TNF- $\alpha$  (R&D Systems, Minneapolis, MN, USA, Cat. No. MTA00B), and IL-6 (RayBiotech, Cat. No. ELM-IL6-1), respectively.

### Real-time polymerase chain reaction

A real-time polymerase chain reaction (PCR) analysis was performed using ABI PRISM 7900HT (Applied Biosystems, Foster, CA, USA) and the Fast SYBR Green Master Mix (Applied Biosystems) as previously described.<sup>9</sup> PCR primer sets are shown in Supporting Information, *Table S1*. The specific mRNA amplification of the target was normalized to 18S ribosomal RNA levels and analysed using the  $\Delta\Delta C_t$  method.

### Western blot

A western blot analysis was performed with anti-phosphorylated Akt at Ser473 (Cat. No. 4060, Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated Akt at Thr308 (Cat. No. 4056, Cell Signaling Technology), anti-Akt (Cat. No. 4691, Cell Signaling Technology), anti-phosphorylated p70 S6 kinase at Thr389 (Cat. No. 9234, Cell Signaling Technology), anti-p70 S6 kinase (Cat. No. 2708, Cell Signaling Technology), anti-irisin (Cat. No. G-067-17, Phoenix Pharmaceuticals), anti-phosphorylated focal adhesion kinase (FAK) at Tyr397 (Cat. No. 3283, Cell Signaling Technology), anti-FAK (Cat. No. 13009, Cell Signaling Technology), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Cat. No. 5174, Cell Signaling Technology) as described previously.<sup>10</sup>

### Histological analysis

Histological analyses were performed as previously described.<sup>22,23</sup> Sections of the gastrocnemius and soleus muscles were stained with haematoxylin/eosin or incubated with an anti-fast skeletal myosin heavy chain (MHC) antibody (Cat. No. ab91506, Abcam, Cambridge, UK) and anti-slow skeletal MHC antibody (Cat. No. ab234431). Sections of the femurs were stained with tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (Wako Pure Chemicals) or incubated with an anti-alkaline phosphatase (ALP) antibody (Cat. No. PAB12279, Abnova, Taipei, Taiwan). The cross-sectional areas of at least 500 myofibres were quantified using ImageJ (<https://imagej.nih.gov/ij/>) in a blinded manner. The number of ALP-positive cells and TRAP-positive multinuclear cells (MNCs) on the surface per 1 mm of the bone tissue was measured using ImageJ in a blinded manner.

### Cell culture

Mouse myoblastic C2C12 cells (ATCC, Manassas, VA, USA) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals) with 10% foetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.

Subconfluent C2C12 cells were cultured in high-glucose DMEM with 2% horse serum for 5 days to facilitate myogenic differentiation. AGE3 was prepared as previously described.<sup>24</sup>

### Statistical analysis

Each experiment was performed at least three times. Data are expressed as the mean  $\pm$  the standard error of the mean (SEM). The significance of differences was evaluated using the Mann–Whitney *U* test for two group comparisons and a one-way or two-way analysis of variance followed by Dunnett's test or the Tukey–Kramer test for multiple comparisons. A simple regression analysis was performed using Pearson's test. The significance of differences was defined as  $P < 0.05$ . Statistical analyses were performed using GraphPad Prism 7.00 software (GraphPad Software, La Jolla, CA, USA).

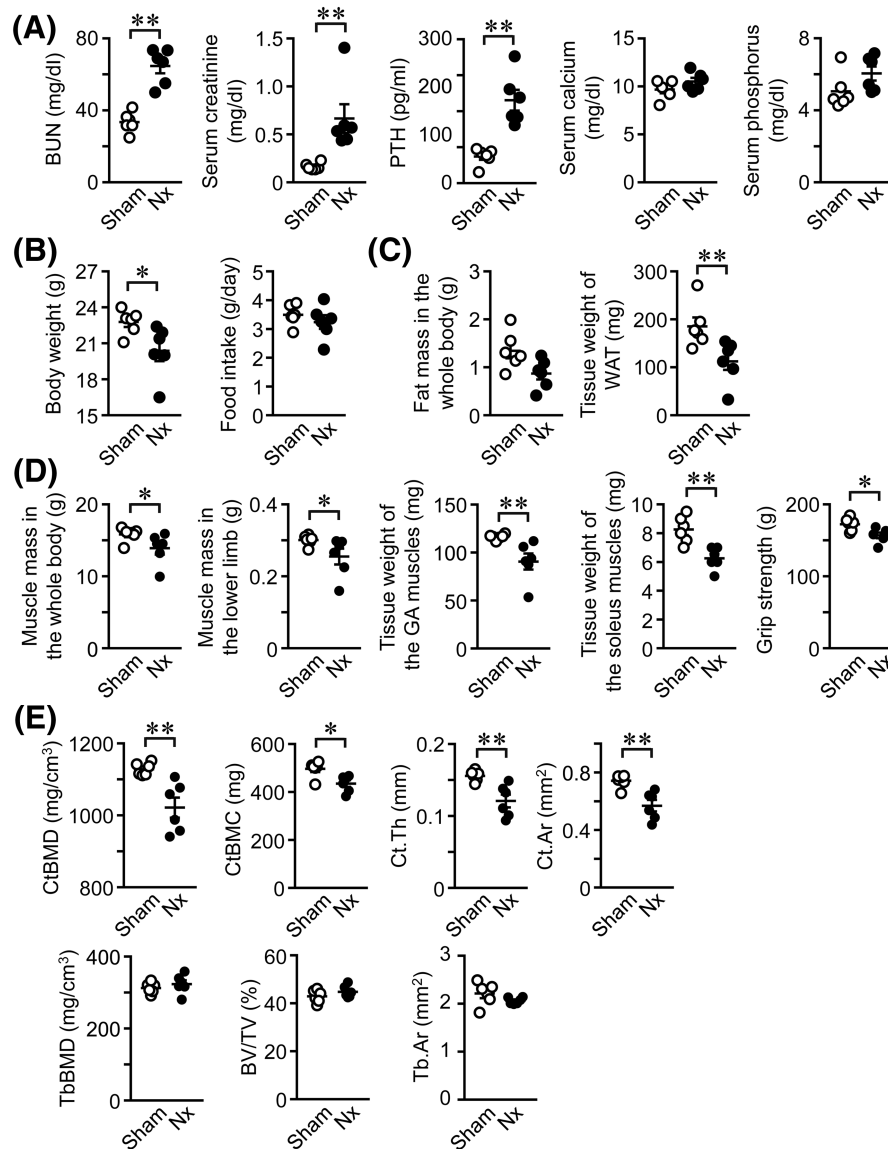
## Results

### Effects of nephrectomy on muscle and bone in mice

Nephrectomy significantly increased the serum levels of BUN, creatinine, and PTH in mice (*Figure 1A*), which indicated the successful induction of renal failure. Nx did not affect the serum levels of calcium or phosphorus (*Figure 1A*). Body weight and the tissue weight of visceral WAT were significantly lower in Nx mice than in sham mice; however, Nx did not affect food intake (*Figure 1B* and *1C*). Nx significantly decreased muscle mass in the whole body and lower limbs, the tissue weights of the gastrocnemius and soleus muscles, and grip strength in mice (*Figure 1D*). Cortical BMD, cortical bone mineral content (BMC), cortical thickness, and cortical area at the femurs were significantly lower in Nx mice than in sham mice (*Figure 1E*), although Nx did not affect trabecular BMD, trabecular bone volume fraction (BV/TV) and trabecular area at the femurs of mice (*Figure 1E*). Simple regression analyses revealed that serum PTH levels were negatively related to cortical BMD, cortical thickness, and cortical area, but not to trabecular BMD, at the femurs of sham and Nx mice (*Table S2*), suggesting that PTH contributed to Nx-induced cortical bone loss in mice.

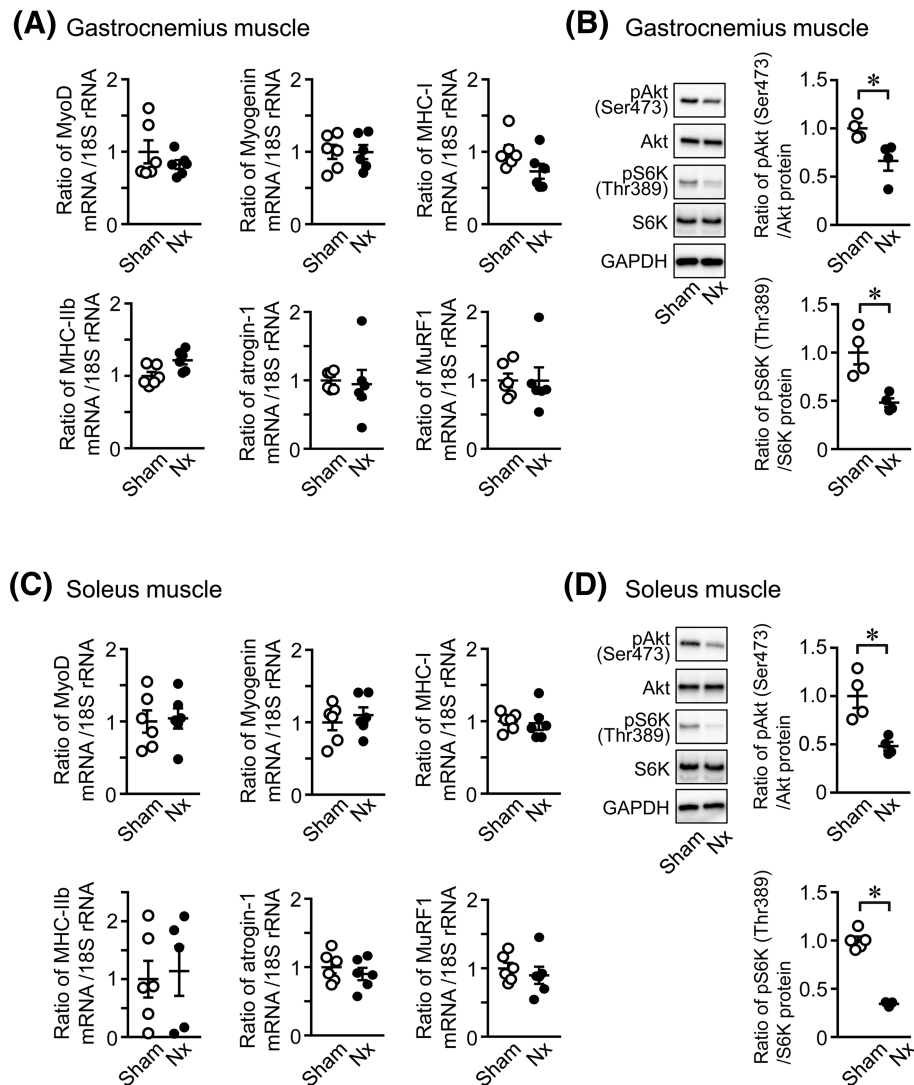
### Effects of nephrectomy on the muscle phenotype

Nephrectomy did not affect the mRNA levels of MyoD, myogenin, MHC-I or MHC-IIb, myogenic differentiation-related genes, or those of atrogen-1 or MuRF1, protein degradation-related genes, in the gastrocnemius and soleus muscles of mice (*Figure 2A* and *2C*). Nx suppressed the phosphorylation of Akt and p70 S6 kinase, protein synthesis-related mTOR sig-



**Figure 1** Effects of renal failure on muscle and bone. (A) Serum samples were collected from mice 8 weeks after the last nephrectomy (Nx) or sham surgery. Serum blood urea nitrogen (BUN), creatinine, parathyroid hormone (PTH), calcium, and phosphorus levels were analysed. (B) Data on body weight and food intake in sham and Nx mice. Body weight was measured 8 weeks after the last Nx or sham surgery. Food intake was measured for 3 days on Days 54–56 after the last Nx or sham surgery as a representative of average daily food intake. (C) Fat mass in the whole body was assessed by quantitative computed tomography (QCT) 8 weeks after the last Nx or sham surgery. After mice were anaesthetized with 2% isoflurane, computed tomography (CT) images were acquired using the following parameters: tube voltage 50 kVp, tube current 500  $\mu$ A, axial field of view 48 mm, and a voxel size of  $96 \times 192 \times 1008 \mu\text{m}$  for analyses of fat mass in the whole body. Regions of interest were defined as the whole body for the assessment of fat mass in the whole body. CT data were analysed using LaTheta software (Version 3.41). Visceral white adipose tissue (WAT) was weighed 8 weeks after the last Nx or sham surgery. (D) Muscle mass in the whole body and lower limbs was assessed by QCT 8 weeks after the last Nx or sham surgery. CT images were acquired with a voxel size of  $96 \times 192 \times 1008 \mu\text{m}$  for analyses of muscle mass in the whole body and a voxel size of  $48 \times 48 \times 192 \mu\text{m}$  for analyses of muscle mass in the lower limbs. Regions of interest were defined as the whole body for the assessment of muscle mass in the whole body and the segment from the proximal end to the distal end of the tibia for the assessment of muscle mass in the lower limbs. The gastrocnemius (GA) and soleus muscles of mice were weighed 8 weeks after the last Nx or sham surgery. The grip strength of the four limbs was measured using a grip strength metre 8 weeks after the last Nx or sham surgery. (E) Cortical bone mineral density (CtBMD), cortical bone mineral content (CtBMC), cortical thickness (Ct.Th), cortical area (Ct.Ar), trabecular bone mineral density (TbBMD), and trabecular bone volume fraction (BV/TV) at the femurs of mice were assessed by QCT 8 weeks after the last Nx or sham surgery. CT images were acquired with an isotropic voxel size of  $24 \mu\text{m}$  for analyses of the femurs. Regions of interest were defined as  $2160 \mu\text{m}$  segments of the mid-diaphysis of the femur for the assessment of CtBMD, CtBMC, Ct.Th, and Ct.Ar. Regions of interest were defined as  $1680 \mu\text{m}$  segments from  $96 \mu\text{m}$  proximal to the end of the distal growth plate towards the diaphysis of the femur for the assessment of TbBMD, bone volume, total tissue volume, and trabecular area. The trabecular bone volume fraction was calculated from bone volume and total tissue volume (BV/TV). Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). \* $P < 0.05$  and \*\* $P < 0.01$ .





**Figure 2** Effects of nephrectomy (Nx) on the expression of myogenic differentiation, protein degradation, and protein synthesis-related factors in gastrocnemius and soleus muscles of mice with or without Nx. Total RNA was extracted from the gastrocnemius (A) and soleus (C) muscles of mice 8 weeks after the last Nx or sham surgery. A real-time PCR analysis of MyoD, myogenin, MHC-I, MHC-Ib, atrogin-1, MuRF1, and 18S rRNA was performed. Data are expressed relative to the levels of 18S rRNA. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). (B,D) Total proteins were extracted from the gastrocnemius and soleus muscles of mice 8 weeks after the last Nx or sham surgery. Western blot analyses of phosphorylated Akt (pAkt) at Ser473, Akt, phosphorylated p70 S6 kinase (pS6K) at Thr389, p70 S6 kinase (S6K), and GAPDH were performed. Images represent experiments performed independently at least four times.

nals, in the gastrocnemius and soleus muscles of mice (Figure 2B and 2D).

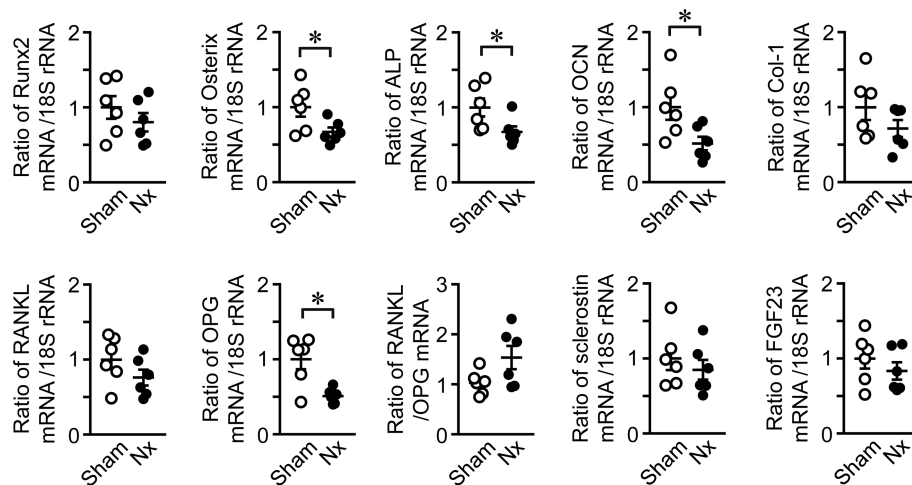
### Effects of nephrectomy on the expression of osteogenic and bone resorption-related genes in bone tissues

Nephrectomy significantly reduced the mRNA levels of osterix, ALP, and osteocalcin in the femurs of mice, but did not affect those of Runx2 or type I collagen (Figure 3). Nx significantly reduced osteoprotegerin (OPG) mRNA levels in the

femurs of mice, although it seemed to elevate the ratio of RANKL/OPG without any significant differences (Figure 3). Moreover, Nx did not affect the mRNA levels of sclerostin or FGF23 in the femurs of mice (Figure 3).

### Effects of nephrectomy on myokine expression

Nephrectomy significantly decreased Fndc5 mRNA and irisin protein levels in the gastrocnemius muscles of mice (Figure 4A and 4B). It also significantly increased and reduced the mRNA levels of myostatin and TGF- $\beta$ , respectively, in the



**Figure 3** Effects of nephrectomy (Nx) on the expression of osteogenic and bone resorption-related genes. Total RNA was extracted from the femurs of mice 8 weeks after the last Nx or sham surgery. A real-time PCR analysis of Runx2, osterix, ALP, osteocalcin (OCN), type I collagen (Col1), RANKL, OPG, sclerostin, FGF23, and 18S rRNA was performed. Data are expressed relative to the levels of 18S rRNA. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). \* $P < 0.05$ .

gastrocnemius muscles, but did not affect the mRNA levels of follistatin, IL-6, IGF-1, FGF2, or osteoglycin (Figure 4A). Nx did not affect the expression of any of the myokines examined in the soleus muscles of mice (Figure 4C and 4D). We then investigated the expression of Fndc5 and myostatin in various tissues. Fndc5 and myostatin mRNA levels were markedly higher in the gastrocnemius muscles than in the other tissues examined, and Nx did not affect the mRNA levels of Fndc5 or myostatin in the femurs, WAT, liver, or kidneys of mice (Figure 5A). Because renal failure leads to complex pathological conditions, including the increased production of reactive oxygen species, inflammatory cytokines, PTH, and AGEs as well as a decrease in the activation of vitamin D,<sup>6,25</sup> we examined the effects of these stimuli on irisin expression in mouse C2C12 myotubes. TNF- $\alpha$  significantly reduced Fndc5 mRNA levels in C2C12 myotubes, whereas hydrogen peroxide, PTH, 1,25-dihydroxyvitamin D<sub>3</sub>, AGE3, and IL-6 did not affect them (Figure 5B and 5C). Irisin did not alter the TNF- $\alpha$ -induced reductions in Fndc5 mRNA levels in C2C12 myotubes (Figure 5D). Nx significantly increased the serum levels of TNF- $\alpha$ , but not IL-6, in mice (Figure 5E).

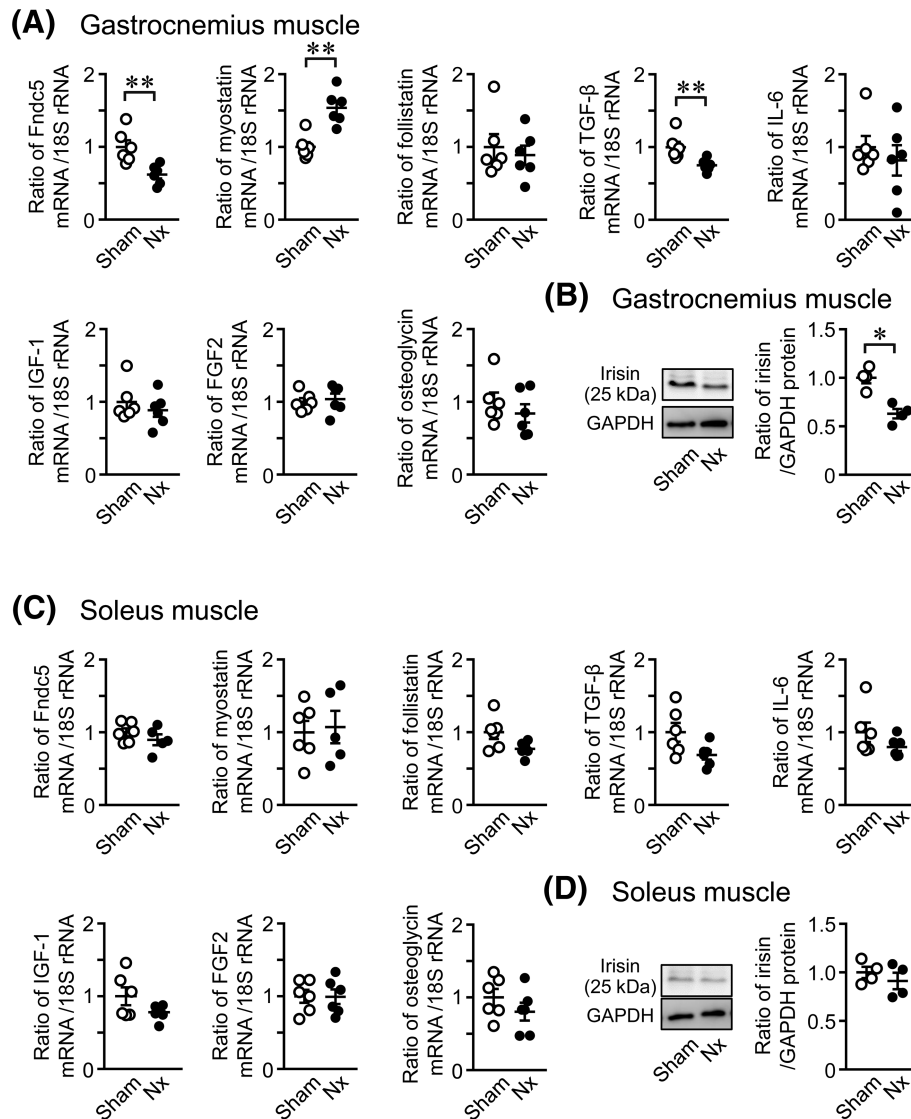
### Relationships between myokine levels in skeletal muscles and bone mineral density at the femurs of mice

We investigated the relationships between irisin and myostatin mRNA levels in the gastrocnemius muscles and bone parameters in mice using simple regression analyses. Fndc5 mRNA levels in the gastrocnemius muscles were significantly and positively related to cortical BMD and cortical thickness, but not to trabecular BMD, at the femurs of sham and Nx mice (Table S3). Myostatin mRNA levels in the gastrocnemius

muscles were significantly and negatively related to cortical BMD, cortical thickness, and cortical area, but not to trabecular BMD, at the femurs of sham and Nx mice (Table S3). We then examined the relationships between osteocalcin, sclerostin, and FGF23 levels in the femur-related and muscle-related parameters in mice. Osteocalcin mRNA levels in the femurs were significantly and positively related to muscle mass in the whole body and the tissue weights of the gastrocnemius and soleus muscles, but not grip strength, in mice (Table S4). No correlations were observed between the mRNA levels of sclerostin or FGF23 in the femur and muscle mass in the whole body, the tissue weights of the gastrocnemius and soleus muscles, or grip strength in mice (Table S4).

### Effects of the administration of irisin on muscle and bone in nephrectomy mice

The intraperitoneal administration of irisin did not affect the serum levels of BUN, creatinine, calcium, phosphorus, PTH, TNF- $\alpha$ , or IL-6 in sham or Nx mice (Figure 6A and 6B). It also did not affect body weight, fat mass in the whole body, the tissue weight of WAT, muscle mass in the whole body, the tissue weights of the gastrocnemius and soleus muscles, or grip strength in sham or Nx mice (Figure 6C–6E). Furthermore, the phosphorylation of Akt or p70 S6 kinase remained unchanged in the gastrocnemius and soleus muscles of mice (Figure 6F). Nx significantly reduced the cross-sectional areas of type II and type I myofibres in the gastrocnemius and soleus muscles of mice, respectively (Figure 7A–7C), but did not affect the ratio of type II and type I myofibres in these muscles (Figure 7D and 7E). The administration of irisin did not alter the cross-sectional area or ratio of type II and type I myofibres in the gastrocnemius or soleus muscles of mice (Figure 7A–7E).



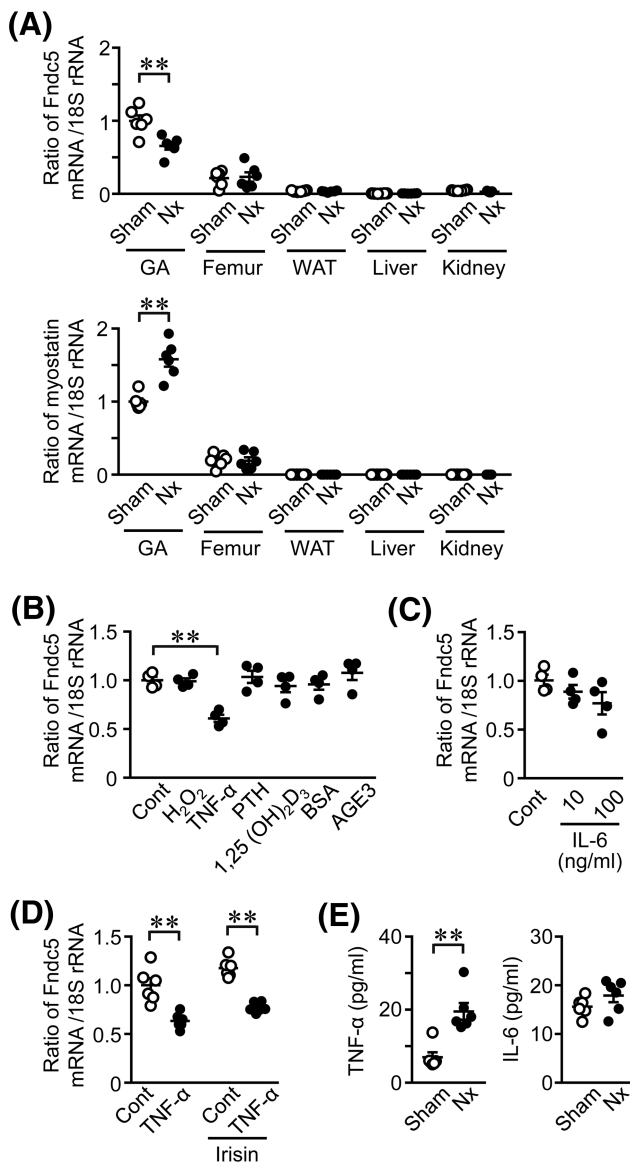
**Figure 4** Effects of nephrectomy (Nx) on the expression of myokines. Total RNA was extracted from the gastrocnemius (A) and soleus (C) muscles of mice 8 weeks after the last Nx or sham surgery. A real-time PCR analysis of *FnDC5*, *myostatin*, *follistatin*, *TGF-β*, *IL-6*, *IGF-1*, *FGF2*, *osteoglycin*, and *18S rRNA* was performed. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). \*\* $P < 0.01$ . Total proteins were extracted from the gastrocnemius (B) and soleus (D) muscles of mice 8 weeks after the last Nx or sham surgery. Western blot analyses of *irisin* and *GAPDH* were performed. Images represent experiments performed independently at least four times. Protein levels of *irisin* were quantified by densitometry and adjusted by *GAPDH* levels. Data represent the mean  $\pm$  standard error of the mean ( $n = 4$  in each group). \* $P < 0.05$ .

On the other hand, the administration of *irisin* significantly improved Nx-induced reductions in cortical BMD, cortical thickness, and cortical area as well as ALP mRNA levels in the femurs (Figure 8A and 8B). However, it did not affect trabecular BMD at the femurs of sham or Nx mice (Figure 8A). Nx and the administration of *irisin* did not alter the number of ALP-positive cells at the cortical bone surface of the femurs (Figure 8C). The administration of *irisin* significantly blunted Nx-induced increases in the number of TRAP-positive MNCs at the cortical bone surface of the femurs (Figure 8D). Although Nx suppressed the phosphorylation of Akt at Thr308 and FAK in the femurs of sham and Nx mice, the ad-

ministration of *irisin* did not affect those phosphorylation (Figure 8E).

## Discussion

Accumulating evidence indicates that *irisin* plays crucial roles in the interactions between muscle and bone.<sup>4,7,26</sup> In the present study, we showed that Nx significantly reduced the expression of *irisin* in the gastrocnemius muscles of mice, which was positively related to cortical BMD at the femurs

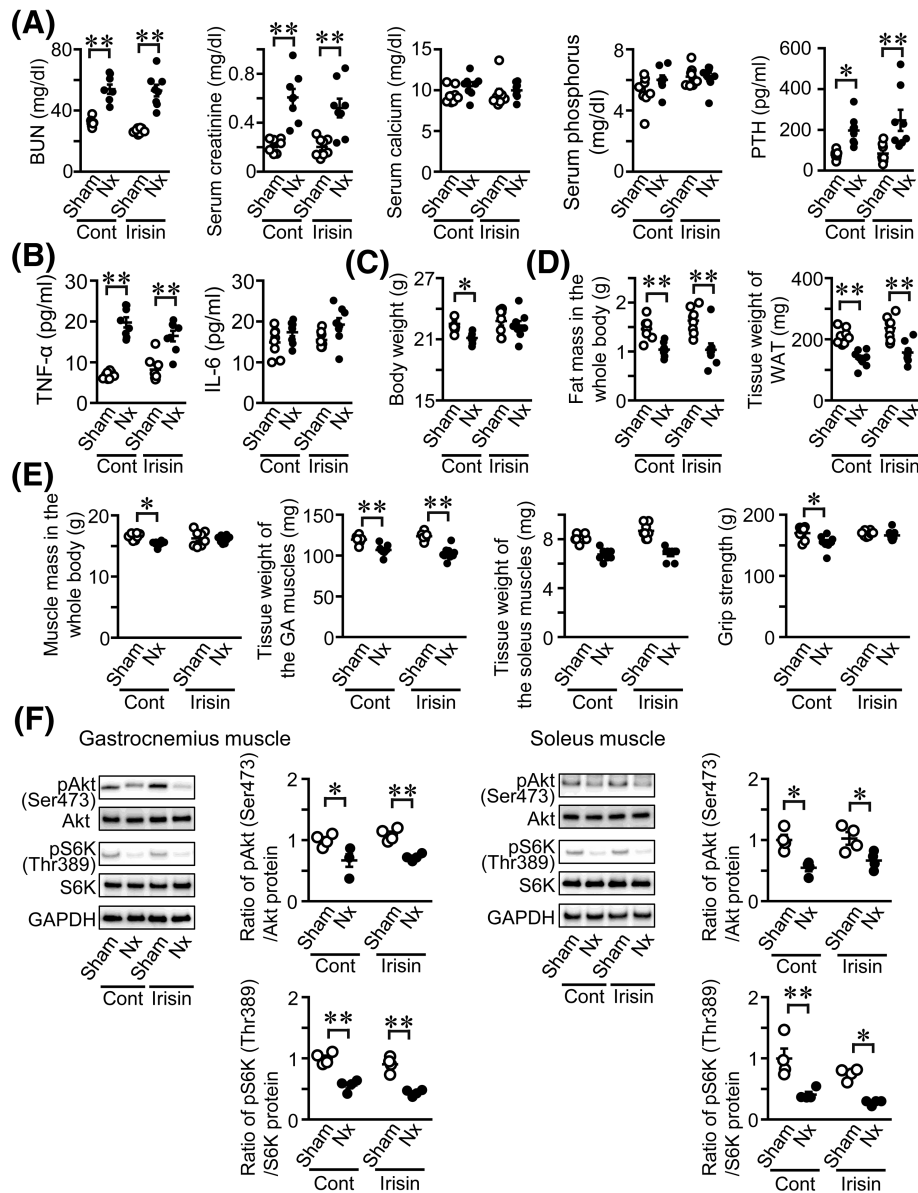


**Figure 5** Effects of nephrectomy (Nx) on the expression of Fndc5 and myostatin in mouse tissues. (A) Total RNA was extracted from the gastrocnemius muscles (GA), femurs, visceral white adipose tissue (WAT), liver, and kidneys of mice 8 weeks after the last Nx or sham surgery. A real-time PCR analysis of Fndc5, myostatin, and 18S rRNA was performed. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). (B,C) Total RNA was extracted from C2C12 myotubes 24 h after exposure to  $10^{-4}$  M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 20 ng/mL TNF- $\alpha$ ,  $10^{-8}$  M PTH 1-34 (PTH),  $10^{-8}$  M 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), 200  $\mu$ g/mL BSA, 200  $\mu$ g/mL AGE3, or 10 and 100 ng/mL IL-6, and real-time PCR analysis of Fndc5 and 18S rRNA was performed. Data represent the mean  $\pm$  standard error of the mean ( $n = 4$  sample in each group). Cont, control. \*\* $P < 0.01$ . (D) Total RNA was extracted from C2C12 myotubes 24 h after the 20 ng/mL TNF- $\alpha$  treatment with or without 100 nM irisin, and real-time PCR analysis of Fndc5 and 18S rRNA was performed. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  sample in each group). (E) Serum samples were collected from mice 8 weeks after the last Nx or sham surgery. Serum TNF- $\alpha$  and IL-6 levels were analysed. Data represent the mean  $\pm$  standard error of the mean ( $n = 6$  mice in each group). \* $P < 0.05$  and \*\* $P < 0.01$ .

in simple regression analyses of mouse samples employed in our study. Moreover, we revealed that the administration of irisin ameliorated Nx-induced reductions in cortical bone mass-related parameters and ALP expression at the femurs of mice. Previous studies showed that irisin facilitated the proliferation and osteogenic differentiation of mouse bone marrow stromal cells and mouse osteoblasts, but suppressed osteoclast differentiation from mouse monocytic cells.<sup>16–18</sup> Moreover, the administration of irisin increased cortical BMD and protected against unloading-induced bone loss in mice.<sup>15,27</sup> We previously reported that a decrease in the expression of irisin in skeletal muscles is related to bone loss by mechanical unloading and androgen deficiency in mice.<sup>11,18</sup> We also showed that irisin is involved in the improvement by chronic exercise of bone loss in oestrogen-deficient mice.<sup>21</sup> Collectively, these findings suggest that a decrease in the expression of irisin in skeletal muscles contributes to bone loss by Nx in mice. Irisin may be a crucial myokine linking muscle to bone in the pathophysiology of renal failure. Zhu *et al.* reported that irisin deficiency in osteogenic lineage cells decreased bone formation in mice, suggesting that osteoblast-derived irisin plays some roles in bone metabolism.<sup>28</sup> However, Nx did not affect irisin expression in the femurs of mice in the present study. We therefore speculate that skeletal muscles rather than bone tissues are responsible for bone loss induced by Nx in mice. We were unable to examine blood irisin levels in Nx mice due to the poor reliability of the enzyme-linked immunoassay kits available.<sup>29</sup> The development of reliable quantification methods is needed to examine blood irisin levels in Nx mice.

Chronic kidney disease has been shown to increase the production of reactive oxygen species and inflammatory cytokines as well as the incidence of cardiovascular diseases,<sup>30</sup> which may influence the effects of the pathophysiology of renal insufficiency on interactions between muscle and bone. We showed that TNF- $\alpha$ , but not hydrogen peroxide, decreased the expression of irisin in C2C12 myotubes. Based on this result, we speculate that the chronic inflammatory state induced by renal insufficiency suppresses irisin expression in the skeletal muscles of mice. Remels *et al.* revealed that TNF- $\alpha$  reduced PGC-1 $\alpha$  levels through the activation of nuclear factor- $\kappa$ B signalling in mouse myotubes.<sup>31</sup> Because PGC-1 $\alpha$  is a crucial transcription factor for the induction of irisin,<sup>13</sup> TNF- $\alpha$  may reduce the expression of irisin through PGC-1 $\alpha$  in muscle cells.

Secondary hyperparathyroidism is commonly observed in patients with renal failure.<sup>1</sup> Anastasilakis *et al.* reported that circulating irisin levels in postmenopausal women with osteoporosis were not influenced by a treatment with teriparatide for 3 months,<sup>32</sup> and Palermo *et al.* did not find any correlations between circulating irisin and PTH levels in postmenopausal women with primary hyperparathyroidism.<sup>33</sup> In the present study, we showed that neither PTH nor 1,25-dihydroxyvitamin D<sub>3</sub> affected the expression of irisin in C2C12

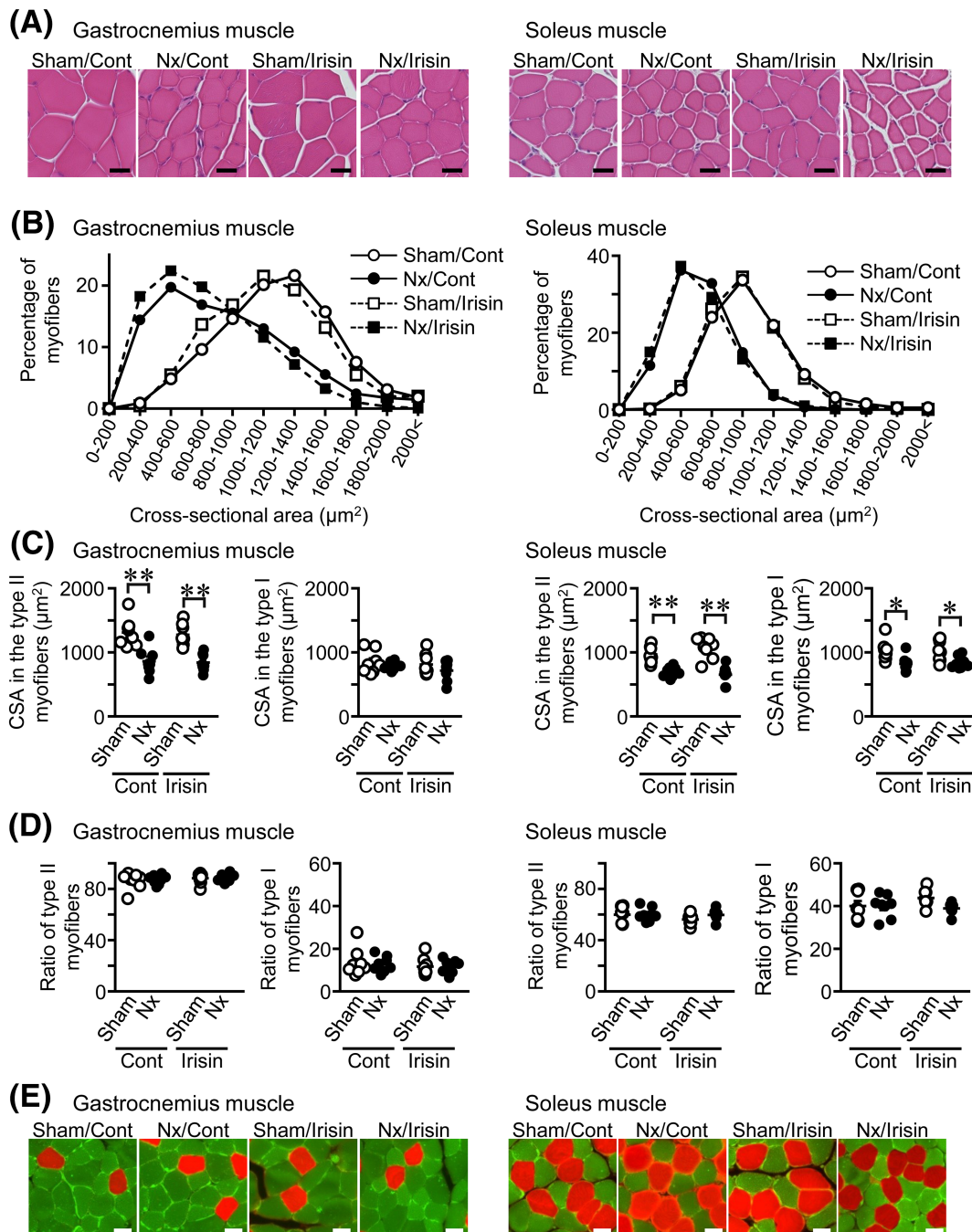


**Figure 6** Effects of the irisin treatment on muscle and body weights in sham and nephrectomy (Nx) mice. (A,B) Serum samples were collected from mice 8 weeks after the last Nx or sham surgery. Serum blood urea nitrogen (BUN), creatinine, calcium, phosphorus, parathyroid hormone (PTH), TNF- $\alpha$ , and IL-6 levels were analysed. (C,D) Body weight was measured 8 weeks after the last Nx or sham surgery. Fat mass in the whole body was assessed by QCT 8 weeks after the last Nx or sham surgery. Visceral white adipose tissue (WAT) was weighed 8 weeks after the last Nx or sham surgery. (E) Muscle mass in the whole body was assessed by QCT 8 weeks after the last Nx or sham surgery. The gastrocnemius (GA) and soleus muscles of mice were weighed 8 weeks after the last Nx or sham surgery. The grip strength of the four limbs was measured using a grip strength metre in mice 8 weeks after the last Nx or sham surgery. (F) Total proteins were extracted from the gastrocnemius and soleus muscles of mice 8 weeks after the last Nx or sham surgery. Western blot analyses of phosphorylated Akt (pAkt) at Ser473, Akt, phosphorylated p70 S6 kinase (pS6K) at Thr389, p70 S6 kinase (S6K), and GAPDH were performed. Images represent experiments performed independently at least four times. Data represent the mean  $\pm$  standard error of the mean.  $n = 8$  mice (A–E) and 4 mice (F) in each group. Cont, control. \* $P < 0.05$  and \*\* $P < 0.01$ .

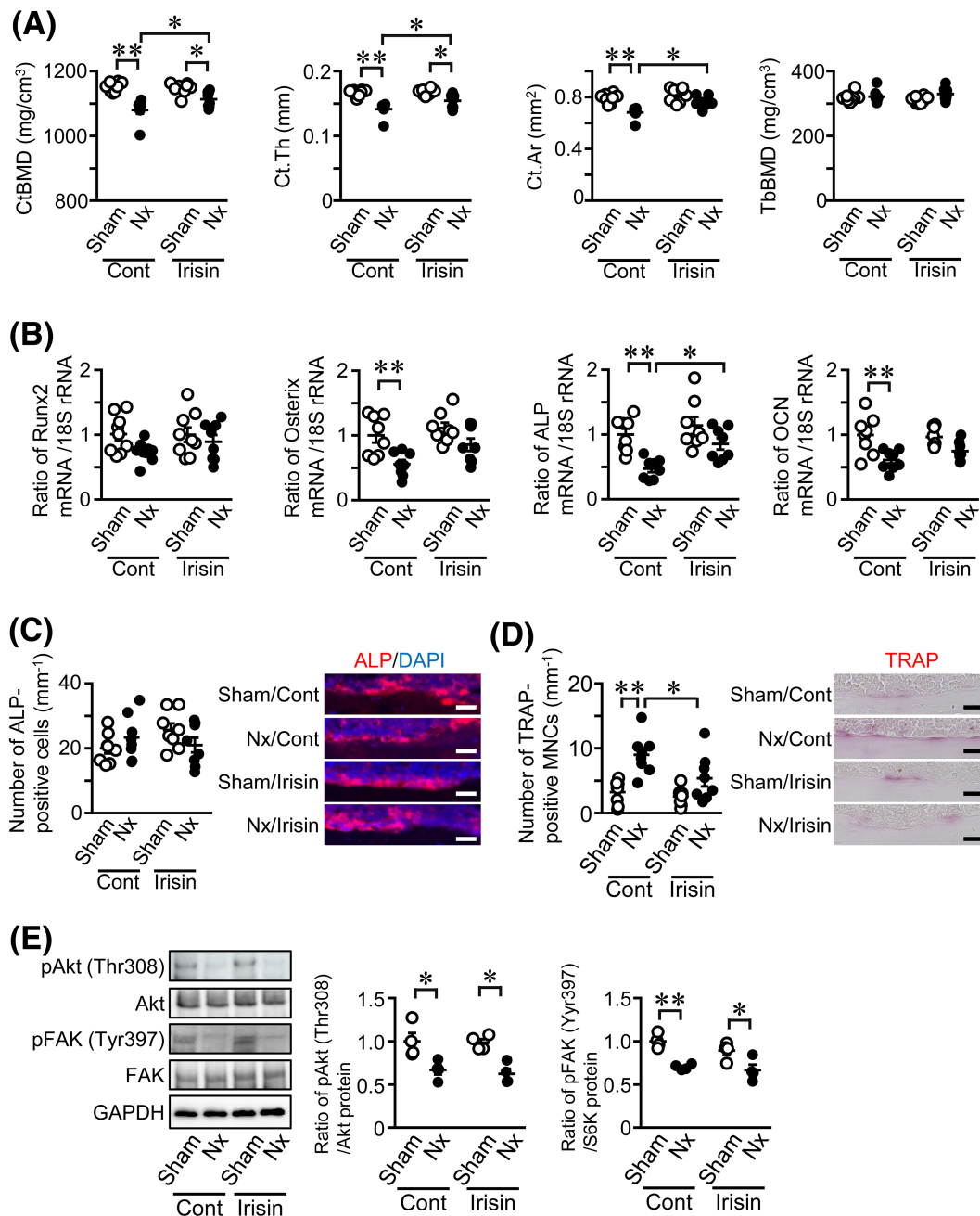
myotubes. These results suggest that the contribution of PTH or 1,25-dihydroxyvitamin D is not responsible for the effects of renal failure on irisin expression in the skeletal muscles of mice. Glycolaldehyde-derived toxic AGE3, which is toxic, has been implicated in the pathogenesis of renal failure and arteriosclerosis in diabetic patients.<sup>34</sup> However, AGE3 did

not affect irisin expression in C2C12 myotubes in the present study, suggesting that AGEs are not responsible for the effects of renal failure on irisin expression in the skeletal muscles of mice. Further studies using other AGEs, such as methylglyoxal-derived hydroimidazolone and  $N^{\epsilon}$ -fructosyllysine-derived  $N^{\epsilon}$ -carboxymethyllysine, are needed to clarify





**Figure 7** Effects of irisin on the cross-sectional area (CSA) and myofibre type of the gastrocnemius and soleus muscles of mice. (A) Haematoxylin and eosin staining was performed on the gastrocnemius and soleus muscles of mice 8 weeks after the last nephrectomy (Nx) or sham surgery. Scale bars indicate 25  $\mu\text{m}$ . (B) The CSA of individual myofibers was measured in haematoxylin and eosin-stained sections of the gastrocnemius and soleus muscles of mice 8 weeks after the last Nx or sham surgery. Distribution of myofibre sizes in the gastrocnemius and soleus muscles of mice. (C,D) Immunohistochemistry of type II and type I myofibers was performed on the gastrocnemius and soleus muscles of mice 8 weeks after the last Nx or sham surgery. The CSA of individual type II and type I myofibers was measured (C). The ratio of type II and type I myofibers was analysed (D). (E) The representative images of immunohistochemistry of type II and type I myofibers. Scale bars indicate 25  $\mu\text{m}$ . Green and red colours indicate type II and type I myofibers, respectively. Data represent the mean  $\pm$  standard error of the mean.  $n = 8$  mice in each group. Cont, control. \* $P < 0.05$  and \*\* $P < 0.01$ .



**Figure 8** Effects of the administration of irisin on bone in nephrectomy (Nx) mice. (A) Cortical bone mineral density (CtBMD), cortical thickness (Ct.Th), cortical area (Ct.Ar), and trabecular bone mineral density (TbBMD) at the femur of mice were assessed by QCT 8 weeks after the last Nx or sham surgery. (B) Total RNA was extracted from the femurs of mice 8 weeks after the last Nx or sham surgery. A real-time PCR analysis of Runx2, osterix, ALP, osteocalcin (OCN), and 18S rRNA was performed. Data are expressed relative to the levels of 18S rRNA. (C) Immunohistochemistry of ALP in the femurs of mice 8 weeks after the last Nx or sham surgery. The number of ALP-positive cells per 1 mm<sup>2</sup> of the cortical bone surface was counted. Red colour indicates ALP-positive signals. Scale bars indicate 25 μm. (D) TRAP staining was performed on the femurs of mice 8 weeks after the last Nx or sham surgery. The number of TRAP-positive multinucleated cells (MNCs) per 1 mm<sup>2</sup> of the cortical bone surface was counted. Scale bars indicate 25 μm. (E) Total proteins were extracted from the femurs of mice 8 weeks after the last Nx or sham surgery. Western blot analyses of phosphorylated Akt (pAkt) at Thr308, Akt, phosphorylated focal adhesion kinase (FAK) at Thr389, FAK, and GAPDH were performed. Images represent experiments performed independently at least four times. Data represent the mean ± standard error of the mean. *n* = 8 mice (A–D) and 4 mice (E) in each group. Cont, control. \**P* < 0.05 and \*\**P* < 0.01.

the effects of AGEs on irisin expression in skeletal muscles with renal failure.

Previous studies suggested the involvement of myostatin in the muscle/bone relationship as a circulating myokine<sup>7</sup>; however, myostatin regulates muscle mass as a local factor in a pathological state, such as immobilization.<sup>35</sup> Clinical studies showed that the expression of myostatin was up-regulated in the skeletal muscles of patients with CKD Stage 5.<sup>36</sup> The present results showed that Nx increased the expression of myostatin in the gastrocnemius muscles of mice. Moreover, myostatin levels in the gastrocnemius muscles were negatively related to cortical BMD at the femurs of mice in the simple regression analysis. Therefore, we speculate that an elevation in the expression of myostatin in skeletal muscles in mice is involved in muscle wasting induced by renal failure. Zhang *et al.* reported that pharmacological inhibition of myostatin suppressed muscle atrophy in Nx mice.<sup>37</sup> Moreover, myostatin may play a role in cortical osteopenia induced by renal failure in mice as a circulating myokine. However, the contribution of myostatin as a myokine to bone loss induced by renal failure remains unclear because we could not show the data with the specific inhibition of myostatin signalling *in vivo* for the universal physiological effects of myostatin and related Smad2/3 signalling.

Chronic kidney disease-related muscle wasting is associated with multiple factors, such as chronic low-grade inflammation, metabolic acidosis, insulin resistance, and vitamin D deficiency, which lead to a negative protein balance.<sup>5</sup> In the present study, Nx reduced the phosphorylation of Akt and p70 S6 kinase in the gastrocnemius and soleus muscles of mice, but did not affect myogenic differentiation or the expression of muscle protein degradation factors, such as atrogin-1 and MuRF1. These results suggest that Nx induces muscle wasting partly through the suppression of protein synthesis. However, the proportional decrease in the phosphorylation of Akt seemed somewhat higher in the slow-twitch myofibre-dominant soleus muscles than in the fast-twitch myofibre-dominant gastrocnemius muscles. We therefore speculate that other protein degradation pathways, such as the autophagy-lysosome system, may be involved in the atrophy of fast-twitch myofibre-dominant muscle induced by renal failure in mice. Carlson *et al.* reported that myostatin was abundantly expressed in the gastrocnemius muscles, but not the soleus muscles of mice.<sup>38</sup> In the present study, basal myostatin mRNA levels were 26.7-fold higher in the gastrocnemius muscles than in the soleus muscles (data not shown). We therefore speculate that the regulation of myostatin may be crucial for fast-twitch myofibre-dominant muscle atrophy induced by renal failure. Rodriguez *et al.* showed that irisin facilitated myogenic differentiation in mouse muscle cells.<sup>39</sup> Furthermore, it induced skeletal muscle hypertrophy and rescued denervation-induced muscle atrophy in mice when injected three times at a dose of 2500 µg/kg/week.<sup>40</sup> However, we demonstrated that a weekly irisin treatment at a

dose of 100 µg/kg, a 75-fold lower dose than that used to induce muscle hypertrophy in mice, did not affect muscle wasting induced by renal failure in mice in the present study; however, it blunted Nx-induced cortical bone loss at the femurs, which is consistent with our previous findings from androgen-deficient mice.<sup>11</sup> Therefore, irisin appears to influence bone mass at a lower dose than that to induce muscle hypertrophy in mice. The administration of irisin seemed to prevent body weight loss in Nx mice without statistical significant differences in the present study. Although irisin did not affect muscle or fat mass or the tissue weights of muscles and WAT, it attenuated Nx-induced cortical bone loss in mice. We therefore speculate that the irisin-induced putative recovery of body weight loss may be partly due to the improvement of cortical bone loss in Nx mice.

In the present study, Nx reduced cortical, but not trabecular, bone parameters at the femurs of mice, which is consistent with clinical evidence showing that CKD-induced osteoporosis mainly affects cortical bone.<sup>1</sup> Hyperparathyroidism plays a crucial role in the development of cortical porosity in the CKD state.<sup>1</sup> Indeed, we showed that serum PTH levels were negatively related to cortical bone parameters, but not trabecular BMD, in simple regression analyses of mice. These findings suggest that hyperparathyroidism is partly responsible for cortical bone loss induced by renal failure. In addition, a previous study suggested the cortical bone-dominant effects of irisin on bone mass in mice.<sup>15</sup> Therefore, a decrease in the secretion of irisin from skeletal muscles may be crucial for renal failure-induced cortical bone loss.

Previous studies indicated that bone-derived humoral factors, osteokines, affect other tissues.<sup>41</sup> In the present study, the levels of osteocalcin, but not sclerostin or FGF23, were decreased in the femurs of Nx mice and related to muscle mass as well as the tissue weights of the gastrocnemius and soleus muscles in mice, implying that osteocalcin is related to muscle wasting induced by renal insufficiency in mice. However, because the roles of osteocalcin as an osteokine are controversial,<sup>42</sup> further studies are needed to clarify its roles in the linkage from bone to muscle in renal failure.

Irisin has been suggested to play important roles in the muscle and bone relationships regulated by exercise and mechanical stress in order to prevent the progression of osteoporosis.<sup>21,26</sup> We revealed that the expression of irisin in the gastrocnemius muscles was reduced by renal failure in mice. Moreover, the administration of irisin ameliorated cortical bone loss induced by renal failure in mice. These results raised the possibility that irisin could be a treatment tool for CKD-MBD.

There are several limitations in the present study that need to be addressed. The administration of irisin at 100 µg/kg was previously used to increase cortical bone mass in mice and recover bone loss and muscle atrophy in hindlimb-unloading mice.<sup>15,27</sup> The administration of irisin at 100 µg/kg blunts muscle loss and increases glycogen storage

and GLUT4 levels in mice.<sup>27,43</sup> It also blunted bone mass reductions caused by androgen deficiency in mice in our previous study.<sup>11</sup> We therefore administered 100 µg/kg of irisin in the present study. However, we cannot rule out the possibility that a higher dose of irisin may rescue muscle wasting by Nx. Furthermore, renal failure may alter various humoral factors, including the inflammatory cytokines, PTH and FGF23, which directly influence bone independently of skeletal muscles, because Nx increased the serum levels of PTH and TNF-α in the present study. Therefore, we were unable to precisely distinguish between the direct and myokine-induced effects of renal failure on bone. Another limitation is that Nx did not significantly affect the ratio of RANKL/OPG in femoral bone tissues. However, an elevated ratio of RANKL/OPG in bone tissues may be expected with renal failure because bone resorption is generally promoted in renal failure.<sup>1</sup> This may be attributed to the low sensitivity to detect changes in the ratio of RANKL/OPG in our experiments using femoral bone tissue samples, including whole bone and bone marrow cells as well as connective tissue cells. In addition, we could not investigate the precise mechanisms by which muscle-derived irisin contributes to osteopenia in Nx mice or how TNF-α affects irisin expression in skeletal muscles *in vivo*. Therefore, further studies using tissue-specific gene-deficient mice are warranted to clarify these issues.

In conclusion, we herein demonstrated that renal failure induces a decrease in irisin expression and an increase in myostatin expression in the skeletal muscles of mice. The present findings suggest that a decrease in irisin expression in skeletal muscle is related to cortical bone loss induced by renal failure in mice, possibly as a myokine linking muscle to bone.

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## Ethics statement

The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.

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## Conflict of interest

None declared.

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## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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