Deficiency of senescence marker protein 30 exacerbates angiotensin II-induced cardiac remodelling

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Aims
Ageing is an important risk factor of cardiovascular diseases including heart failure. Senescence marker protein 30 (SMP30), which was originally identified as an important ageing marker protein, is assumed to act as a novel anti-ageing factor in various organs. However, the role of SMP30 in the heart has not been previously explored. In this study, our aim was to elucidate the functional role of SMP30 on cardiac remodelling.

Methods and results
SMP30 knockout (KO) mice and wild-type (WT) mice were subjected to continuous angiotensin II (Ang II) infusion. After 14 days, the extent of cardiac hypertrophy and myocardial fibrosis was significantly higher in SMP30-KO mice than in WT mice. Echocardiography revealed that SMP30-KO mice had more severely depressed systolic and diastolic function with left ventricular dilatation compared with WT mice. Generation of reactive oxygen species related with activation of nicotinamide adenine dinucleotide phosphate-oxidase was greater in SMP30-KO mice than in WT mice. The number of deoxyribonucleotidyl transferase-mediated dUTP nick end-labelling positive nuclei was markedly increased in SMP30-KO mice with activation of caspase-3, increases in the Bax to Bcl-2 ratio and phosphorylation of c-Jun N-terminal kinase compared with WT mice. Furthermore, the number of senescence-associated β-galactosidase-positive cells was significantly increased via up-regulation of p21 gene expression in SMP30-KO mice compared with WT mice.

Conclusion
This study demonstrated the first evidence that deficiency of SMP30 exacerbates Ang II-induced cardiac hypertrophy, dysfunction, and remodelling, suggesting that SMP30 has a cardio-protective role in cardiac remodelling with anti-oxidative and anti-apoptotic effects in response to Ang II.

Keywords
Senescence marker protein 30 (SMP30) • Ageing • Remodelling • Oxidative stress • Apoptosis

1. Introduction
The prevalence and mortality rate of heart failure dramatically increase in older people, and ageing is one of the risk factors for cardiovascular events.1 With ageing, the heart shows changes in cardiac structure and function. Age-associated cardiac remodelling includes an enlargement of cardiomyocyte size, loss of myocytes due to apoptosis or necrosis, and increase of matrix connective tissue. These age-associated cardiac changes seem to be relevant to the steep increases in left ventricular hypertrophy, diastolic dysfunction, and subsequent heart failure.2

Oxidative stress is considered to be an important factor in controlling heart ageing.3 It is well known that the renin–angiotensin system (RAS) is a central component of the physiological and pathological responses of the cardiovascular system. Activation of RAS is a significant driver of oxidative stress and is involved in age-related cardiac remodelling. Angiotensin II (Ang II), the primary effector molecule of RAS, contributes not only to vasoconstriction and hypertension, but also to cardiac hypertrophy, remodelling, and heart failure. Therefore, Ang II signalling appears to play a critical role in heart ageing.
Senescence marker protein 30 (SMP30), a 34-kDa protein, was originally identified as a novel ageing marker protein in rat liver, whose expression decreases androgen-independently with age. SMP30 transcripts are detected in almost all organs, and the SMP30 gene is highly conserved among numerous animal species including humans. Intracellular localization of SMP30 is in the cytoplasm and perinuclear regions, and SMP30 exists in multiple forms under physiological conditions. It has been demonstrated that SMP30 plays multifunctional roles as Ca\(^{2+}\) regulator (named as regucalcin), anti-oxidant, and enzymatic ability to hydrolyze di-isopropyl phosphorofluoridate. Recently, SMP30 has been determined as glucurononactase, which is involved in ascorbic acid (vitamin C) biosynthesis in mammals, whereas human beings are unable to synthesize vitamin C in vivo because of mutations in L-gulonolactone oxidase.

SMP30-knockout (KO) mice have been generated and showed a shorter life span than that of the wild-type (WT) mice on a vitamin C-deficient diet. Using SMP30-KO mice, recent reports have demonstrated that SMP30 functions to protect cells from apoptosis in the liver and that SMP30 has protective effects against age-associated oxidative stress in the brain and lungs. Furthermore, SMP30-KO mice have shown accelerated senescence in the kidney and the worsening of glucose tolerance. Taken together, SMP30 is assumed to behave as an anti-ageing factor. However, the role of SMP30 in the heart has not been previously explored.

We hypothesized that SMP30 has cardio-protective functions from harmful stimuli with anti-oxidative and anti-apoptotic effects. To test the hypothesis, we used SMP30-KO mice to examine the effects of SMP30 on Ang II-induced cardiac hypertrophy and remodelling in vivo.

2. Methods

For additional detailed methods, please see Supplementary material online.

2.1 Animal protocol

SMP30-KO (C57BL/6 background) mice were established as previously reported. Drinking water containing vitamin C (1.5 g/L) was provided for the SMP30-KO mice to avoid vitamin C deficiency due to their inability to synthesize vitamin C in vivo. After anaesthetizing the mice by i.p. injection of pentobarbital (50 mg/kg body weight), an osmotic minipump (ALZET micro-osmotic pump MODEL 1002, DURECT Co., Cupertino, CA, USA) was subcutaneously implanted, and Ang II (800 ng/kg/min) was continuously infused for 14 days. Controls were administered saline. The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011). Our research protocol was approved by the institutional review board, and all animal experiments were conducted in accordance with the guidelines of Fukushima Medical University Animal Research Committee.

2.2 Measurement of vitamin C

Total vitamin C levels in the heart were measured by the dinitrophenylhydrazine method according to the manufacturer’s protocol (SHIMA Laboratories Co. Ltd., Tokyo, Japan).

2.3 Measurements of blood pressure and heart rate

Mice were implanted with a radio-telemetry probe (TA11PA-C22, Data Sciences International, St Paul, MN, USA) under i.p. anaesthesia by pentobarbital (50 mg/kg body weight) as described previously. After a recovery phase of 10 days, basal arterial pressure and heart rate (HR) were started to recorded. After the measurement of control, Ang II was subcutaneously infused via an osmotic minipump, and the data were recorded.

2.4 Echocardiography

Transthoracic echocardiography was performed using Vevo 2100 High-Resolution In Vivo Imaging System (Visual Sonics, Inc., Toronto, Canada) with a high-resolution 40-MHz imaging transducer as previous reports described. Mice were lightly anaesthetized by intraperitoneal injection of isoflurane (0.5–1.5%) to achieve an HR of ~400 b.p.m., and all the measurements were obtained from three cardiac cycles.

2.5 Cardiac catheterization

The cardiac catheterization was performed as described previously. Briefly, mice were anaesthetized by i.p. injection of 2,2,2-tribromo-ethanol (250 mg/kg body weight), the right carotid artery was cannulated with the micropressure transducer (samba preclin 420 LP, Samba Sensors AB, Gothenburg, Sweden) into the left ventricle. Adequacy of anaesthesia was monitored by HR, aortic blood pressure, and respiratory rate as well as the absence of reactions of painful stimuli. The data were measured using the Labscribe 2 software (iWorx Systems, Inc., Dover, NH, USA).

2.6 Histopathological analysis

After continuous infusion of Ang II or saline for 14 days, mice were sacrificed by cervical dislocation and hearts were rapidly excised. The paraffin-embedded heart sections were stained with haematoxylin and eosin or Elastica-Masson. The cross-sectional area of cardiomyocyte and fibrosis fraction was measured using the NIH Imagej software (National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop CS2 (Adobe, San Jose, CA, USA).

In immunohistochemical analysis, the paraffin-embedded sections were incubated with anti-SMP30 antibody (SHIMA Laboratories Co. Ltd., Tokyo, Japan) with a dilution of 1:200 or negative control (normal serum). The sections were stained with horseradish peroxidase-conjugated secondary antibody (Histofine Simple Stain Mouse MAX PO (R), Nichirei Biosciences, Inc., Tokyo, Japan) and diaminobenzidine tetrahydrochloride, and counterstained with haematoxylin.

2.7 Assessment of reactive oxygen species generation

The fresh-frozen heart sections were incubated with 10 μmol/L dihydroethidium (DHE, Sigma-Aldrich Co., St Louis, MO, USA). The fluorescent images were acquired using fluorescence microscope (Olympus IX71, Olympus Optical Co., Tokyo, Japan) and the mean DHE fluorescence intensity of cardiomyocytes was quantitated with the NIH imagej software. In addition, 10 mmol/L apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, was provided in drinking water with Ang II continuous infusion, and reactive oxygen species (ROS) generation was evaluated by DHE staining.

2.8 Western blotting

Total protein was extracted from the snap-frozen left ventricle using Cell Lysis Buffer (Cell Signaling Technology, Inc., Beverly, MA, USA) with Protease Inhibitor Cocktail (BD Biosciences, San Jose, CA, USA) as previous reports described. The primary antibodies were as follows: anti-SMP30, anti-67phox, anti-Bax, anti-Bcl-2, anti-phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK, Thr183/Tyr185), anti-SAPK/JNK (Cell Signaling Technology, Inc.), anti-activated-caspase-3 (Bioworld Technology, Inc., Minneapolis, MN, USA), and mouse anti-β-actin (Santa Cruz Biotechnology, Inc.). The signals from immunoreactive bands were visualized by an Amersham ECL system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and quantified using densitometric analysis.
2.9 In vivo terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling assay

Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) method (CardioTACS In Situ Apoptosis Detection Kit, Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer’s instructions. TUNEL-positive nuclei were counted, and then expressed as a per cent of the total nuclei.28

2.10 Senescence-associated β-galactosidase activity

Senescence-associated β-galactosidase (SA-β-gal) staining was performed according to the manufacturer’s protocol (BioVision, Inc., Mountain View, CA, USA).29 SA-β-gal-positive cardiomyocytes were visualized as blue colour under light microscopy, and positive cells for SA-β-gal activity were counted.

2.11 Reverse transcription polymerase chain reaction

Total RNA was extracted from the snap-frozen left ventricle using TRIzol reagent (Invitrogen, Carlsbad, CA, USA).28 Reverse transcription polymerase chain reaction (RT–PCR) was performed using the PrimeScript RT–PCR Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer’s instructions. Primers were designed on the basis of GenBank sequences (p21, NM_001111099 and β-actin, NM_007393). The optical density of the bands was quantified using the NIH imageJ software.

3. Results

3.1 Vitamin C levels of the heart tissue in WT mice and SMP30-KO mice

First, we measured vitamin C levels of the heart tissue in basal conditions. To avoid vitamin C deficiency, drinking water containing sufficient vitamin C was supplied for SMP30-KO mice because SMP30-KO mice were unable to synthesize vitamin C due to the lack of gluconolactonase.10 The tissue concentrations of the vitamin C level were not significantly different between WT mice and SMP30-KO mice (45.7 ± 7.0 vs. 44.5 ± 10.2 μg/g tissue).

3.2 SMP30 expression in the heart tissue

Immunostaining revealed that SMP30 was expressed in cardiomyocytes, fibroblasts, and vascular endothelial cells in WT mice on the basis of cellular morphological characteristics (Figure 1A).30 We confirmed the decrease in SMP30 expression with ageing in the WT mouse heart as

Figure 1: SMP30 expression in heart tissue. (A) SMP30 is expressed in cardiomyocytes, fibroblasts, and vascular endothelial cells (left, magnification ×200; middle, ×400). Right panel indicates negative control (×400). (B) Cardiac SMP30 expression was significantly decreased by 40% at 12-month-old WT mice (old) compared with 3-month-old WT mice (young). (C) Cardiac SMP30 expression was significantly decreased by 50% after Ang II infusion for 14 days in WT mice. Results are mean ± SD from 3 mice in each group. #P < 0.05 vs. young mice or control.
Table I Gravimetric and hemodynamic data of WT and SMP30-KO mice

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<td>10 977 ± 940</td>
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Data are presented as mean ± SD from 10 to 15 mice in each group. BW, body weight; HW, heart weight; LVW, left ventricular weight; TL, tibial length; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; LVEDP, left ventricular end-diastolic pressure; max and min dp/dt, maximal and minimal rates of left ventricular pressure development, respectively; Tau, time constant of left ventricular isovolumic relaxation. *P < 0.05 and **P < 0.1 vs. control in the same strain mice; †P < 0.05 and ††P < 0.01 vs. Ang II-infused WT mice.

3.3 Effect of SMP30 deficiency on Ang II-induced cardiac hypertrophy and fibrosis

As shown in Table 1, heart weight (HW) and left ventricular weight (LVW) corrected by the tibial length (TL) were similar between control WT mice and SMP30-KO mice. After Ang II infusion, the ratios of HW to TL and LVW to TL were significantly higher in SMP30-KO mice than in WT mice (P < 0.05 and P < 0.01, respectively), although the blood pressure was similarly elevated in both Ang II-infused WT mice and SMP30-KO mice by telemetry blood pressure monitoring (Table 1).

Histological examination showed that Ang II-infused SMP30-KO mice had substantial left ventricular hypertrophy with left ventricular dilatation compared with Ang II-infused WT mice, which suggested eccentric hypertrophy in Ang II-infused SMP30-KO mice compared with concentric hypertrophy in Ang II-infused WT mice (Figure 1B, top). The cardiomyocyte cross-sectional area was significantly larger in Ang II-infused SMP30-KO mice than in Ang II-infused WT mice (399 ± 17 vs. 372 ± 11 μm², P < 0.01, Figure 2A, middle and 8). Additionally, ex vivo analysis demonstrated that cell width, length, and surface area of isolated cardiomyocytes were significantly greater in SMP30-KO mice than in WT mice after Ang II stimulation (Supplementary material online, Figure S1). The degree of cardiac fibrosis was significantly higher in Ang II-infused SMP30-KO mice than in Ang II-infused WT mice (6.4 ± 0.8 vs. 7.5 ± 0.7%, P < 0.01, Figure 2A, bottom and C). These data revealed that the deficiency of SMP30 exacerbates Ang II-induced cardiac hypertrophy and fibrosis, independently of blood pressure.

3.4 Effect of SMP30 deficiency on Ang II-induced cardiac dysfunction

As shown in Table 1, there were no differences in cardiac function between WT mice and SMP30-KO mice under basal conditions. Echocardiography showed that left ventricular end-diastolic and end-systolic dimensions were enlarged and fractional shortening was reduced in SMP30-KO mice compared with WT mice at 14 days after Ang II infusion (P < 0.01, Table 1 and Supplementary material online, Table S1 and Figure S2, top). The left ventricular mass was greater in Ang II-infused SMP30-KO mice than in Ang II-infused WT mice, which was concordant with the histological findings (P < 0.01). Ang II-infused SMP30-KO mice had significantly higher peak E velocity, E/A, and E/E′ compared with Ang II-infused WT mice (Supplementary material online, Table S1 and Figure S2, middle and bottom). The mitral inflow showed the restrictive pattern in Ang II-infused SMP30-KO mice in contrast to the relaxation abnormality pattern or the pseudo-normalization pattern in Ang II-infused WT mice. These echocardiographic data revealed that left ventricular systolic and diastolic functions were remarkably depressed in SMP30-KO mice compared with WT mice after Ang II infusion.

Haemodynamic assessment by cardiac catheterization showed that max dp/dt and min dp/dt were significantly lower in SMP30-KO mice than in WT mice after Ang II infusion (P < 0.01 and P < 0.05,
respectively), supporting that cardiac systolic and diastolic functions were more severely depressed in SMP30-KO mice (Table 1).

### 3.5 Effect of SMP30 deficiency on Ang II-induced myocardial oxidative stress

We examined myocardial oxidative stress by DHE staining which indicates the $O_2^-$ levels of living cells because oxidative stress is considered to be one of the important mechanisms of heart failure and cardiac remodelling. Although Ang II infusion dramatically increased the ROS generation in both WT mice and SMP30-KO mice, the ROS generation in Ang II-infused SMP30-KO mice was significantly greater than in Ang II-infused WT mice ($P < 0.01$, Figure 3A). In addition, we found that the level of superoxide generation was significantly decreased in Ang II-infused WT mice with apocynin treatment, compared with that of Ang II-infused WT mice without apocynin treatment ($P < 0.01$, Figure 3A). As well as WT mice, SMP30-KO mice revealed that Ang II-induced superoxide generation was significantly down-regulated by apocynin treatment ($P < 0.01$, Figure 3A).

To investigate the involvement of NADPH oxidase in Ang II-induced ROS generation, we examined the expression of p67phox subunit of NADPH oxidase by western blotting. The expression levels of p67phox were significantly elevated in Ang II-infused SMP30-KO mice compared with Ang II-infused WT mice ($P < 0.01$, Figure 3B). These data suggested that the deficiency of SMP30 increased Ang II-induced myocardial oxidative stress via up-regulation of NADPH oxidase.

### 3.6 Effect of SMP30 deficiency on Ang II-induced apoptosis

As previously demonstrated, SMP30 has anti-apoptotic effects in other organs. We, therefore, checked apoptosis using TUNEL staining.
After Ang II infusion, the numbers of TUNEL-positive nuclei including cardiomyocytes and non-cardiomyocytes were increased in both WT and SMP30-KO mice. The numbers of TUNEL-positive nuclei in Ang II-infused SMP30-KO mice were remarkably greater than in Ang II-infused WT mice, as shown in Figure 4A ($P < 0.01$).

Then, we examined signalling pathways of Ang II-induced apoptosis in the heart. Caspase-3 is a key mediator of apoptosis, and activation of caspase-3 leads to DNA injury and subsequently apoptotic cell death. The activation of caspase-3 was induced by Ang II infusion in both WT and SMP30-KO mice, and the activation of caspase-3 in Ang II-infused SMP30-KO mice was significantly greater than in Ang II-infused WT mice ($P < 0.01$, Figure 4B).

After Ang II infusion, Bax expression which functions as pro-apoptotic protein was increased, whereas the expression of anti-apoptotic protein Bcl-2 was decreased in both genotypes of mice. The ratio of Bax to Bcl-2 was significantly higher in Ang II-infused SMP30-KO mice than in Ang II-infused WT mice ($P < 0.01$, Figure 4C).

Furthermore, we examined the involvement of SAPK/JNK which has a crucial role in cell apoptosis as one main subgroup of the mitogen-activated protein kinase family. Phosphorylation activity of SAPK/JNK in Ang II-infused SMP30-KO mice was significantly increased compared with Ang II-infused WT mice ($P < 0.01$, Figure 4D).

These findings demonstrated that SMP30 deficiency exacerbates Ang II-induced apoptosis through these signalling pathways.

### 3.7 Expression of senescence markers in SMP30-KO mice after Ang II infusion

Senescent cells can be identified by the expression of enzymatic SA-β-gal activity in left ventricular tissues (Figure 5A). SA-β-gal activity was induced by Ang II stimulation. The numbers of SA-β-gal-positive cells were significantly greater in Ang II-infused SMP30-KO mice than in Ang II-infused WT mice (1.7 ± 0.8 vs. 0.6 ± 0.5/mm², $P < 0.01$) as demonstrated in Figure 5A.

To evaluate the gene expression of cell cycle inhibitor to confirm senescence of cardiomyocytes, we analysed mRNA expression of p21 gene by RT–PCR (Figure 5B). Following Ang II infusion, the expression levels of p21 mRNA were increased in both WT mice and SMP30-KO mice. Compared with Ang II-infused WT mice, Ang II-infused SMP30-KO mice showed a significant increase in p21 expression ($P < 0.01$). These results indicate that deficiency of SMP30 induced cellular senescence after Ang II infusion by the p21-dependent pathway.

### 4. Discussion

Previous studies have shown that SMP30 acts as an anti-ageing factor, and SMP30 prevents oxidative stress and apoptosis in the liver, lungs, and brain. However, the role of SMP30 in the heart has not...
Figure 4 Apoptosis and apoptotic signalling pathways in WT and SMP30-KO mice after Ang II infusion. (A) Upper panels show representative images of TUNEL staining of left ventricular tissue sections. Lower bar graph shows the per cent of TUNEL-positive nuclei. (B) Activation of caspase-3 was examined by western blotting with anti-activated-caspase-3 antibody using myocardial samples. Expression levels of activated-caspase-3 were normalized by β-actin. (C) Expressions of Bax and Bcl-2 were analysed by western blotting. The Bax to Bcl-2 ratio was calculated and presented in the bar graph. (D) Phosphorylation activity of SAPK/JNK. Expressions of phosphorylated and total SAPK/JNK were analysed by western blotting. Relative expression levels of phosphorylated SAPK/JNK (P-SAPK/JNK) were expressed in relation to those of SAPK/JNK. Results are mean ± SD from 6 to 10 mice in each group. *P < 0.01 vs. control in the same strain mice; †P < 0.01 vs. Ang II-infused WT mice.
been investigated. In this study, we demonstrated the first evidence that deficiency of SMP30 exacerbates Ang II-induced cardiac hypertrophy, dysfunction, and adverse remodelling. Our results revealed that SMP30 has a cardio-protective role with anti-oxidative and anti-apoptotic effects in response to Ang II.

It has been well known that Ang II plays an important role in the development of pathological cardiac hypertrophy, remodelling, and subsequent heart failure. Subcutaneous chronic infusion of Ang II induces cardiac hypertrophy and fibrosis with hypertension. Ang II also stimulates NADPH oxidase to produce ROS, and consequent myocardial oxidative stress is associated with the development of left ventricular remodelling and heart failure. Furthermore, it has been considered that apoptosis plays an adverse role in cardiac remodelling and contributes to progressive myocardial dysfunction and that Ang II exaggerates apoptotic responses in cardiomyocytes. Interestingly, we observed that deficiency of SMP30 exacerbates Ang II-induced cardiac hypertrophy and fibrosis in SMP30-KO mice (Table 1, Figure 2, and Supplementary material online, Figure S1). Moreover, we found that Ang II-infused SMP30-KO mice showed left ventricular dilatation and depressed systolic function in addition to more severely impaired diastolic function compared with Ang II-infused WT mice, suggesting that the absence of SMP30 caused more progressive cardiac dysfunction and remodelling (Table 1 and Supplementary material online, Table S1 and Figure S2). These remarkable changes were independent of Ang II-induced hypertension because increased systemic blood pressure of SMP30-KO mice was similar to that of WT mice (Table 1). SMP30-KO mice had much more elevated NADPH oxidase-generated ROS by Ang II stimulation (Figure 3). In addition, SMP30-KO mice were more susceptible to Ang II-induced apoptosis associated with activation of caspase-3, increase in Bax, decrease in Bcl-2, and phosphorylation of SAPK/JNK (Figure 4). Although we were unable to show the direct observation of TUNEL-positive cardiomyocyte nuclei, apoptosis of non-cardiomyocytes plays an important role in Ang II-induced cardiac remodelling and dysfunction as previously reported. These data indicate that SMP30 has a protective role against Ang II-associated cardiac hypertrophy, dysfunction, and remodelling by inhibiting oxidative stress and apoptosis.

SMP30 has been proposed as an important ageing marker, and the lack of SMP30 causes various dysfunctions of organs during ageing process. Concerning the vitamin C biosynthesis pathway, similar to humans, SMP30-KO mice cannot synthesize vitamin C and SMP30-KO mice may mimic the human physiology closer than other rodents. The potent anti-ageing and anti-oxidative actions of a low-calorie diet effectively suppressed the age-related down-regulation of SMP30, indicating that SMP30 expression was influenced by oxidative stress. These previous reports suggest that SMP30 expression accounts for the age-associated deterioration of cellular function and the enhanced susceptibility to harmful stimuli in aged tissues. On the other hand, very few reports demonstrated cellular senescence of cardiomyocytes in vivo. We demonstrated that Ang II could increase senescent cells detected by SA β-gal activity in vivo. Importantly, Ang II-induced cellular senescence was accompanied with markedly elevated p21 gene expression. SMP30-KO mice showed significantly increased SA β-gal-positive cells with elevated expression of p21 gene by Ang II stimulation, indicating that SMP30 inhibits premature cellular senescence through the signalling pathway of p21 in response to Ang II (Figure 5).

Figure 5 Senescence markers in hearts of WT and SMP30-KO mice after Ang II infusion. (A) Senescent cells were detected by SA β-gal staining of left ventricular tissue sections, and the numbers of SA β-gal-positive cells were counted. (B) The mRNA expression levels of p21 gene were analysed by RT–PCR. Expression levels of p21 gene were normalized by β-actin. Results are mean ± SD from 6 to 8 mice in each group. *P < 0.01 vs. control in the same strain mice; †P < 0.01 vs. Ang II-infused WT mice.
There were no differences between WT mice and SMP30-KO mice under basal conditions at 12- to 16-week-old, but we observed that 12-month-old SMP30-KO mice showed exaggerated left ventricular hypertrophy, diastolic dysfunction, and myocardial fibrosis compared with 12-month-old WT mice (Supplementary material online, Table S2). One central role of SMP30 in the heart is considered to be the suppressive effect of ROS generation by inhibiting NADPH oxidase activation, as demonstrated in the present study (Figure 3). The suppressive role of SMP30 in oxidative stress contributes to the reduction of senescent marker expressions, suggesting that SMP30 prevents myocardial dysfunction from various stresses such as Ang II stimulation and ageing (Supplementary material online, Figure S3). Since detailed mechanisms have not been fully clarified, we should evaluate the cellular compartment specific effects of SMP30 in the future study.

5. Conclusions
In summary, deficiency of SMP30 adversely modifies Ang II-induced cardiac hypertrophy and remodelling through increase in oxidative stress and progression of apoptosis. These data provide that SMP30 has a protective role in cardiac remodelling and up-regulation of SMP30 could be a therapeutic target for treatment of heart failure.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest:
none declared.

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References


