EXPERIMENTAL STUDY

# Catecholamine-Induced Senescence of Endothelial Cells and Bone Marrow Cells Promotes Cardiac Dysfunction in Mice

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**Summary**

Previous studies have suggested that cellular senescence plays a central role in the progression of pathologic changes in the failing heart. It is well known that the sympathetic nervous system is activated in patients with heart failure, and this change is associated with poor clinical outcomes. Sympathetic activation increases the levels of various catecholamines, such as epinephrine and norepinephrine, but the contribution of these catecholamines to cellular senescence associated with heart failure remains to be determined. We found that catecholamine infusion induced senescence of endothelial cells and bone marrow cells, and promoted cardiac dysfunction in mice. In C57BL/6NCr mice, the continuous infusion of isoproterenol-induced cardiac inflammation and cardiac dysfunction. Expression of p53, a master regulator of cellular senescence, was increased in the cardiac tissue and bone marrow cells of these mice. Suppression of cellular senescence by genetic deletion of p53 in endothelial cells or bone marrow cells led to improvement of isoproterenol-induced cardiac dysfunction. In vitro studies showed that adrenergic signaling increased the expression of p53 and adhesion molecules by endothelial cells and macrophages. Our results indicate that catecholamine-induced senescence of endothelial cells and bone marrow cells plays a pivotal role in the progression of heart failure. Suppression of catecholamine-p53 signaling is crucial for inhibition of remodeling in the failing heart.

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**Key words:** Chronic inflammation, p53, Cellular senescence

Heart failure has become one of the most important healthcare issues, causing a huge social and economic burden.[1)] Aging is associated with deterioration of organ function that is characterized by cellular senescence, altered intercellular communication, mitochondrial dysfunction, and deregulation of nutrient sensing.[2)] Extensive analysis of the role of cellular senescence has indicated that it underlies the progression of pathology in age-related disorders.[3,4)] Accumulation of senescent cells with an altered genetic profile disturbs organ homeostasis.[3)] Elimination of senescent cells via senolytic agents or genetic manipulation leads to rejuvenation of organs and contributes to prolongation of the lifespan in mice.[5,6)] p53 protein has been called a guardian of the genome and it contributes to maintenance of genomic stability, thereby inhibiting development of cancer.[7)] However, p53 is also known to induce cellular senescence and has a detrimental role in diseases associated with aging, such as obesity, diabetes, and heart failure.[8-10)] Aging promotes a shift from the compensated to decompensated phase of heart failure in response to various stresses, including mechanical and metabolic stresses,[11,12)] and studies have indicated that p53-induced cellular senescence is critically involved in this process. In a murine model of left ventricular (LV) pres-

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sure overload, increased p53 signaling was shown to suppress angiogenesis and promote hypoxia in cardiac tissues, with these changes leading to systolic dysfunction.[13] It was recently reported that p53-induced senescence of endothelial cells and bone marrow cells induces chronic sterile inflammation (characterized by infiltration of macrophages) and cardiac remodeling in response to LV pressure overload.[14] Another study showed that suppression of p53 signaling in endothelial cells contributed to re-activation of angiogenesis in the heart and ameliorated cardiac dysfunction.[15] The pathogenesis of heart failure is multifactorial and complex, so this condition is not dependent on a single molecule or a simple mechanism.[16-20] However, accumulating evidence indicates a central role of cellular senescence in the process of heart failure.[9,13,14] It is well known that circulating catecholamine levels are increased in patients with heart failure and that such elevation predicts a poor prognosis,[21] but the role of catecholamines in cellular senescence is yet to be defined. Accordingly, we investigated the pathological role of catecholamine-induced cellular senescence in heart failure.

## Methods

**Animal models:** These animal experiments were conducted in compliance with the study protocol, which was reviewed by the Institutional Animal Care and Use Committee of Niigata University and approved by the President of Niigata University. C57BL/6 mice were purchased from SLC Japan (Shizuoka, Japan). Isoproterenol (30 mg/kg/day, I6504, Sigma-Aldrich) or phosphate-buffered saline (PBS) was administered by an infusion pump (DURECT Corporation) for 4 or 6 weeks, as described previously[22] Mice expressing Cre recombinase in Tie2-positive cells (Tie2-Cre) were purchased from Jackson Laboratories. We crossed Tie2-Cre mice (with a C57BL/6 background) and mice carrying floxed Trp53 alleles (with a C57BL/6 background) to generate mice that showed endothelial cell-specific knockout of p53 (Tie2-Cre$^{+\ Trp53flox/flox}$). The genotype of the littermate controls was Tie2-Cre$^{-\ Trp53flox/flox}$. In addition, p53-deficient mice (with a C57BL/6 background) were purchased from Jackson Laboratories.

**Bone marrow transplantation model:** The bone marrow transplantation model was generated as described previously[14] with slight modifications. Bone marrow cell suspensions were isolated by flushing marrow from the femurs and tibias of 8- or 9-week-old wild-type or p53-deficient male mice. After 7-week-old wild-type male mice were subjected to irradiation, bone marrow cells ($1.5 \times 10^7$ cells) were injected via the tail vein within 3 h. Two weeks after bone marrow transplantation, the mice were subjected to isoproterenol treatment as described above.

**Cell culture:** Human umbilical vein endothelial cells (HUVECs) and the specified medium for these cells (CC-3162) were purchased from Lonza and the cells were cultured according to the manufacturer's instructions. RAW 264.7 cells were cultured in DMEM with 10% FBS. In some experiments, isoproterenol (Sigma-Aldrich, I6504, 100 nM) was added to HUVEC or RAW264.7 cells. The cells were incubated with isoproterenol for 30 minutes before DHE staining and incubated with isoproterenol for 1 or 3 hours before western blotting or immunofluorescence studies. Adenovirus encoding p53 (Adeno-p53) was introduced into RAW264.7 (20MOI for 6hours), and Adeno-Mock was used as a control.

**Echocardiography:** Echocardiography was performed with a Vevo 770 High Resolution Imaging System (Visual Sonics Inc.). To minimize data variation, cardiac function was assessed when the heart rate was within the range of 550-650 /minute.

**Histological analysis:** Cardiac tissue samples were harvested and fixed overnight in 10% formalin, followed by embedding in paraffin and sectioning (Leica Biosystems) for Hematoxylin-eosin (HE) staining or immunofluorescence studies. For histological examination of HUVECs, the cells were dispersed on glass-bottomed dishes before being studied. After treatment with isoproterenol, HUVECs were fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then were subjected to staining. Reactive oxygen species (ROS) were evaluated by dihydroethidium (DHE) staining with Hoechst (Thermofisher, H21491, 1:1000). The following antibodies were used for detection of p53: anti-p53 antibody (Abcam, ab 31333, 1:50), anti-Mac3 (BD Pharmingen, 550292, 1:50), Wheat Germ Agglutinin Alexa Fluor™ 488 Conjugate (Thermofischer, W11261, 1:50) and anti-rabbit Cy5 conjugated (Thermofischer, A10523, 1:50). Five fields per section were randomly selected for quantification with Image-J software. For evaluation of MAC3-positive cells, five fields were randomly selected in each section and percentage of MAC3-positive cells to nuclear number per field at a magnification of x400 (5 mice per l group) was calculated.

**Western blotting:** Whole-cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl, 5 mM EDTA, 0.025% NaN3, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg $mL^{-1}$ leupeptin, 2 μg $mL^{-1}$ aprotinin, 50 mM NaF, and 1 mM $Na_2VO_3$). Then the lysates (10-50 μg) were subjected to SDS-PAGE and proteins were transferred to a PVDF membrane (Millipore), which was incubated with the primary antibody followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin-G (Jackson). Specific proteins were detected by enhanced chemiluminescence (RPN2106 or RPN2232, GE Healthcare). The primary antibodies for western blotting were anti-p53 antibody (#2524, Cell Signaling), anti-actin antibody (#4967, Cell Signaling), and anti-GAPDH antibody (sc-20357, Santa Cruz). For studies of HUVECs, anti-p53 antibody (DO1) (sc-126, Santa Cruz) was used. The primary antibodies were used at a dilution of 1:1000, except for anti-actin antibody and anti-GAPDH antibody (both 1:5000). The secondary antibody for anti-p53 antibody (#2524) was peroxidase-conjugated AffiniPure goat anti-mouse IgG (light chain-specific) (Jackson Immunoresearch, 115-035-174), while peroxidase-conjugated AffiniPure goat anti-mouse IgG(H + L) (Jackson Immunoresearch, 115-035-003) was used for anti p53antibody (DO1) (sc-126), peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) (Jackson Immunoresearch, 111-035-003) was employed for anti-actin antibody (#4967),

and peroxidase-conjugated AffiniPure donkey anti-goat IgG (H + L) (Jackson Immunoresearch,705-035-147) was used to detect anti-GAPDH antibody (sc-20357). Secondary antibodies were used at dilutions ranging from 1: 5000 to 1:20,000.

**RNA analysis:** Total RNA (1 μg) was isolated from tissue samples with RNA-Bee (Tel-Test Inc.). Real-time quantitative PCR (qPCR) was performed using a Light Cycler 480 (Roche) with the Universal Probe Library and Light Cycler 480 ProbesMaster (Roche) according to the manufacturer's instructions. *Rplp0* (for mouse primers) or *RNA 18S5* (for human primers) was used as the internal control. The primers and their sequences were as follows:

Mouse primers:

*Adgre1*(*Emr1*); 5'-CCTGGACGAATCCTGTGAAG-3', 5'-GGTGGGACCACAGAGAGTTG-3'

*Tnf*; 5'-TCTTCTCATTCCTGCTTGTGG-3', 5'-GGTCTGGGCCATAGAACTGA-3'

*Ccl2*; 5'-CATCCACGTGTTGGCTCA-3', 5'-GATCATCTTGCTGGTGAATGAGT-3'

*Icam1*; 5'-CCCACGCTACCTCTGCTC-3', 5'-GATGGATACCTGAGCATCACC-3'

*Cdkn1a*; 5'-TCCACAGCGATATCCAGACA-3', 5'-GGACATCACCAGGATTGGAC-3'

*Itgal*; 5'-CCCCAGACTTTTGCTACTGG-3', 5'-CGTGTGTCCAGGTTGTAGCTC-3'

*Rplp0*; 5'-GATGCCCAGGGAAGACAG-3', 5'-ACAATGAAGCATTTTGGATAATCA-3'.

Human primers:

*CDKN1A*; 5'-TCACTGTCTTGTACCCTTGTGC-3', 5'-GGCGTTTGGAGTGGTAGAAA-3'

*ICAM1*; 5'-CCTTCCTCACCGTGTACTGG-3', 5'-AGCGTAGGGTAAGGTTCTTGC-3'

*RNA18S5*; 5'-GGAGAGGGAGCCTGAGAAAC-3', 5'-TCGGGAGTGGGTAATTTGC-3'

**Statistical analysis:** Results are shown as the mean ± 2 SEM. Statistical analysis was performed with SPSS version 22.0.0.1. Differences between groups were examined by Student's t-test or analysis of variance, followed by Tukey's multiple comparison test or the non-parametric Kruskal-Wallis test for comparisons among more than two groups. In all analyses, $P < 0.05$ was considered to indicate statistical significance.

## Results

**Isoproterenol infusion increases p53 in cardiac tissue and bone marrow cells:** To investigate whether catecholamine infusion induced cellular senescence, we administered isoproterenol to mice via an infusion pump, leading to the development of cardiac hypertrophy associated with cardiac systolic dysfunction (Figure 1A-D). Under these conditions, there was an increase in p53 and the transcript of its target molecule, p21 (*Cdkn1a*), in cardiac tissues (Figure 1E, F). We previously detected p53-induced senescence of endothelial cells and bone marrow cells in a murine model of LV pressure overload.[14] These findings led us to analyze p53 expression in bone marrow cells under hyperadrenergic conditions in the present study. After continuous infusion of isoproterenol, there was a marked increase of p53 and its transcript p21 (*Cdkn1a*) in bone marrow cells. These results suggested that the hyperadrenergic state associated with heart failure promotes p53-induced cellular senescence in cardiac tissue and in the bone marrow.

**Isoproterenol induces cardiac inflammation:** Chronic sterile inflammation develops in patients with heart failure and promotes pathologic changes.[23-25] Activation of p53-mediated signaling increases the expression of adhesion molecules by endothelial cells and bone marrow cells, and also induces cardiac inflammation in a murine model of LV pressure overload.[14] In agreement with this report, we found that isoproterenol infusion increased the infiltration of mononuclear cells and Mac3 positive cells into the left ventricle (Figure 2A, B), and was associated with increased LV expression of transcripts for inflammatory markers such as *Adgre1* (*Emr1*), *Tnf*, *Ccl2*, and *Icam1* (Figure 2C). Isoproterenol infusion also increased transcripts for integrin alpha-L (*Itgal*) in bone marrow cells (Figure 2D). These results suggested that activation of adrenergic signaling *per se* contributes to elevation of integrin expression by bone marrow cells and to increased cardiac tissue expression of intercellular adhesion molecule-1 (ICAM-1), which may promote sterile inflammation and remodeling in the failing heart.

**Suppression of p53 expression in endothelial cells or bone marrow cells ameliorates isoproterenol-induced cardiac dysfunction:** Our data indicated that the activation of adrenergic signaling resulted in up-regulation of p53 in both cardiac tissue and bone marrow (Figure 1E, G). In addition, transcripts of *Icam1* and *Itgal* were respectively increased in cardiac tissue and bone marrow by a hyperadrenergic state (Figure 2C, D). We previously reported that p53 positively regulates the expression of these adhesion molecules at the transcriptional level, inducing cardiac inflammation and remodeling in a murine model of LV pressure overload.[14] To obtain further direct evidence that p53 expression in endothelial cells and/or bone marrow cells contributes to progression of catecholamine-induced heart failure, we performed isoproterenol infusion in endothelial cell (EC) - or bone marrow cell (BM)-p53 knockout (KO) mice. EC-p53 KO mice were generated by crossing Tie-2 Cre mice with $Trp53^{flox/flox}$ mice, and Tie2-$Cre^{+}$ $Trp53^{flox/flox}$ (EC-p53 KO) mice and their littermate controls ($Trp53^{flox/flox}$ mice) were analyzed. Infusion of isoproterenol led to cardiac dysfunction and dilatation in control mice, while these changes were ameliorated in EC-p53 KO mice (Figure 3A). We generated a BM-p53 KO model by transplanting bone marrow cells from systemic p53 KO mice into wild-type mice after irradiation, while control mice were generated by transplanting bone marrow cells from wild-type mice into irradiated wild-type mice. Isoproterenol infusion caused systolic dysfunction along with cardiac dilatation in the control groups, while such changes were ameliorated in BM-p53 KO mice (Figure 3B). These results suggested that up-regulation of p53 signaling in ECs and BM cells by a hyperadrenergic state induce cardiac dysfunction. Interestingly, we found that intraventricular septal thickness (IVSTd) becomes thicker in EC-p53 KO group compared to WT group under isoproterenol treatment (Figure 3A). In BM-p53 KO group, IVSTd showed a trend to increase

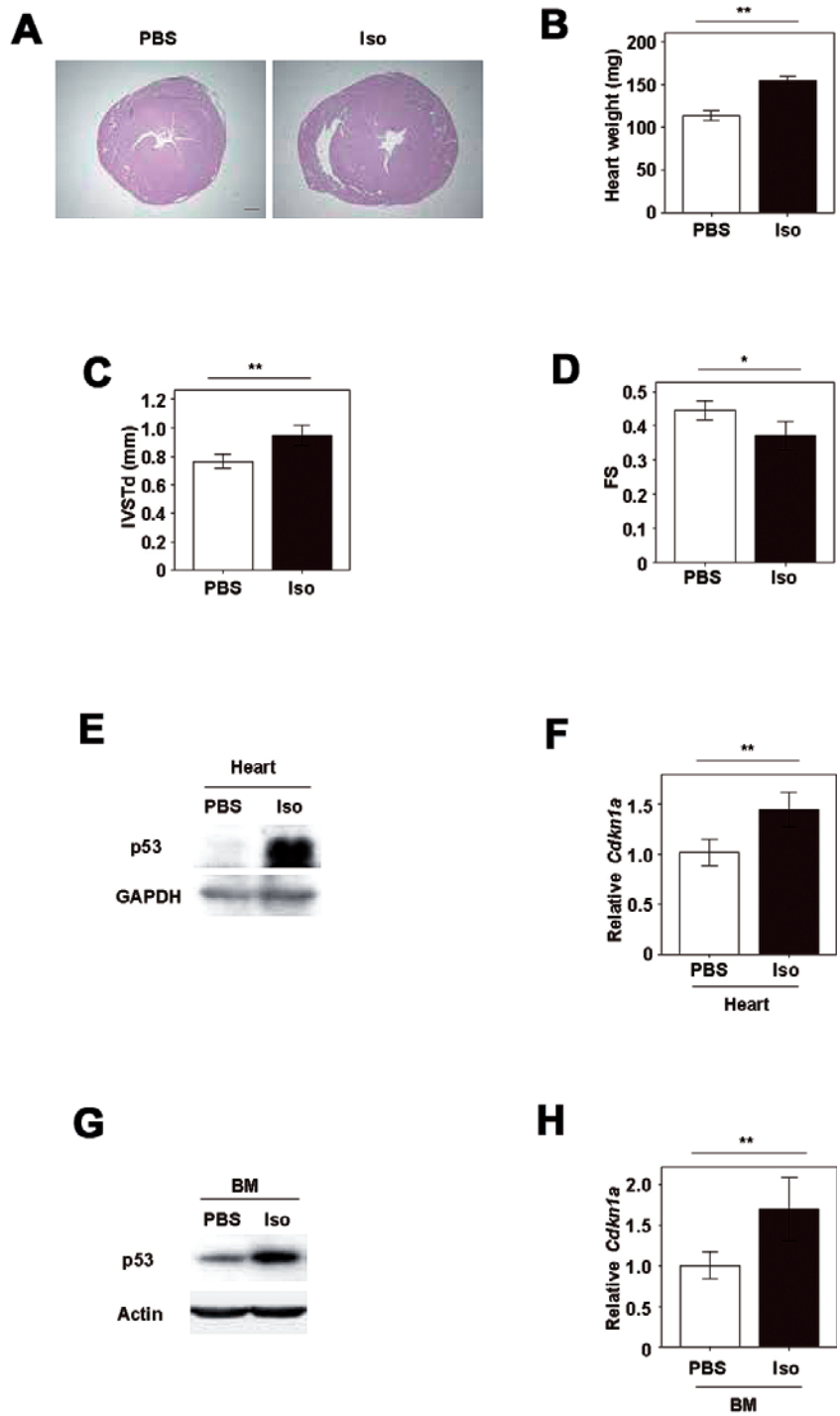


**Figure 1.** Isoproterenol up-regulates p53 in cardiac tissue and bone marrow. **A:** Hematoxylin-eosin (HE) staining of cardiac tissue after infusion of PBS or isoproterenol (Iso). Scale bar = 500 μm. **B:** Heart weight was measured after 4 weeks of PBS or isoproterenol (Iso) infusion ($n$ = 4, 4). **C, D:** The end-diastolic interventricular septal thickness (IVSTd) (**C**) and fractional shortening (FS) (**D**) were measured by echocardiography after 4 weeks of PBS or isoproterenol (Iso) infusion ($n$ = 4, 4). **E:** Western blot analysis of p53 expression in cardiac tissue after 6 weeks of PBS or isoproterenol (Iso) infusion. **F:** qPCR analysis of *Cdkn1a* in cardiac tissue after 4 weeks of PBS or isoproterenol (Iso) infusion ($n$ = 8, 11). **G, H:** Western blot analysis of p53 expression (**G**) and qPCR analysis of *Cdkn1a* expression in bone marrow cells (BM) ($n$ = 3, 5) (**H**) after 6 weeks of PBS or isoproterenol (Iso) infusion. Data were analyzed by the two-tailed Student's $t$-test. $^*P < 0.05$, $^{**}P < 0.01$. Values represent the mean ± 2 SEM. NS indicates not significant.

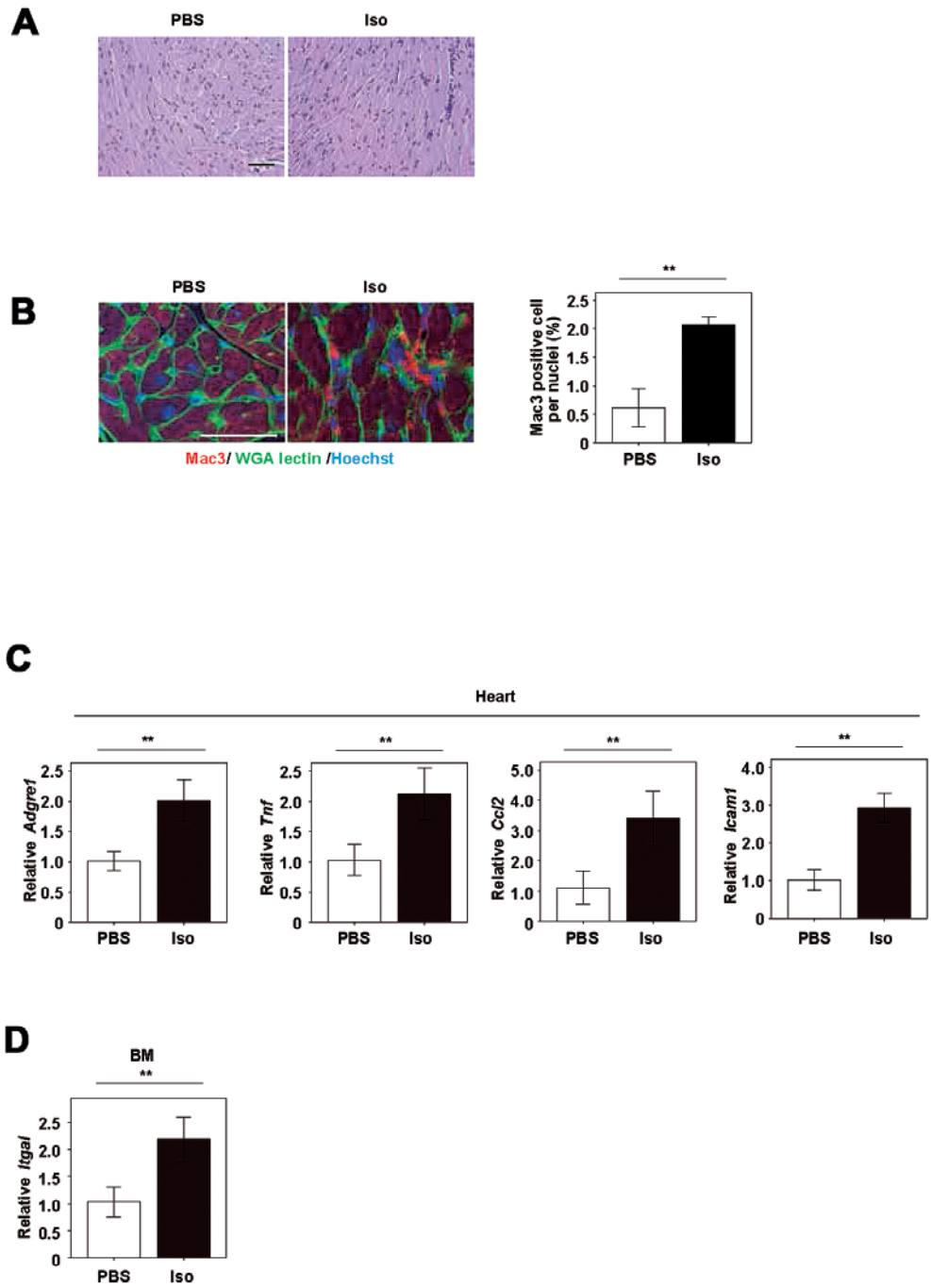


**Figure 2.** Isoproterenol induces cardiac inflammation. **A:** Hematoxylin-eosin (HE) staining of left ventricle after PBS or isoproterenol (Iso) infusion. Scale bar = 50 μm. **B:** Immunofluorescence study detecting Mac3 positive cells in left ventricle after PBS or isoproterenol (Iso) infusion. Scale bar = 50 μm. Right panel indicates quantification ($n$ = 4, 5). **C:** qPCR analysis of *Adgre1* (*Emr1*), *Tnf*, *Ccl2*, and *Icam1*, expression in cardiac tissue after 4 weeks of PBS or isoproterenol (Iso) infusion ($n$ = 4, 6). **D:** qPCR analysis of *Itgal* expression in bone marrow cells (BM) after 6 weeks of PBS or isoproterenol (Iso) infusion ($n$ = 3, 5). Data were analyzed by the two-tailed Student's $t$-test. *$P < 0.05$, **$P < 0.01$. Values represent the mean ± 2 SEM. NS indicates not significant.

in the KO group compared to WT group under isoproterenol treatment (Figure 3B), and further studies are needed to test whether the suppression of endothelial and/or BM cell-p53 contributes to promote protective cardiac hypertrophy under stressed condition.

**Isoproterenol up-regulates p53 in endothelial cells and macrophages:** Next, we investigated whether isoproterenol caused up-regulation of p53 in cultured cells. When HUVECs were cultured with isoproterenol, there was a significant increase of ROS (Figure 4A), and this was as-

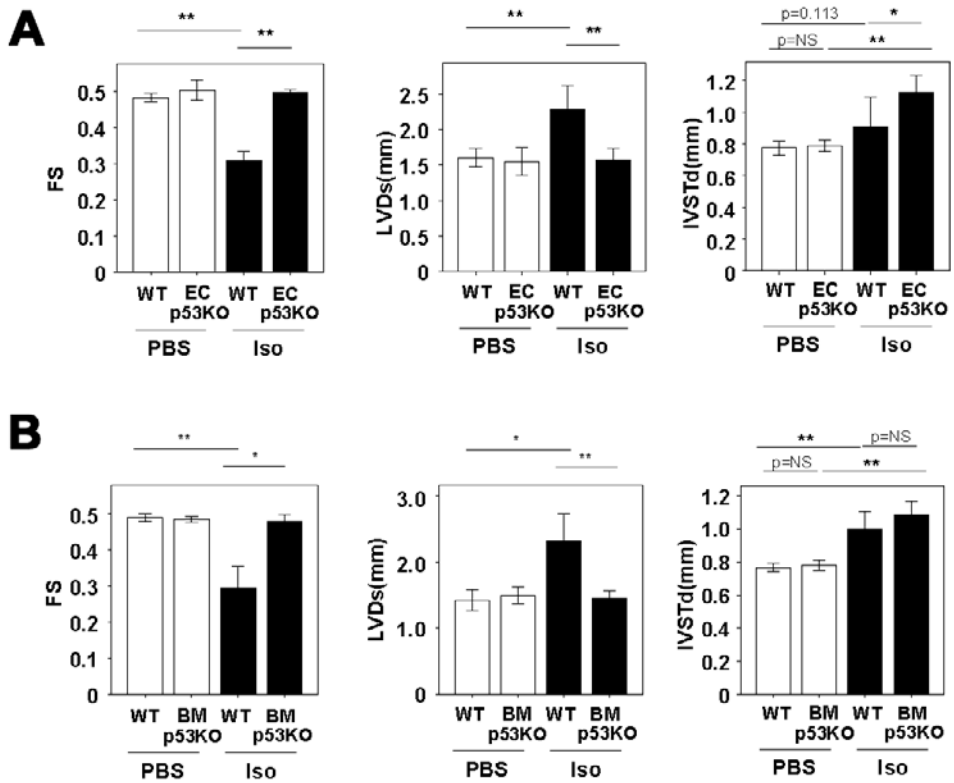


**Figure 3.** Suppression of p53 in endothelial cells or bone marrow cells ameliorates isoproterenol-induced cardiac dysfunction. **A:** Echocardiography shows fractional shortening (FS), left ventricular end-systolic diameter (LVDs) and intraventricular septal thickness (IVSTd) in endothelial cell-specific p53 knockout (EC-p53KO) mice and littermate controls (WT) after PBS or isoproterenol (Iso) infusion for 6 weeks ($n$ = 7, 9, 3, 4). **B:** Echocardiography shows FS, LVDs and IVSTd in bone marrow p53-depleted (BM-p53KO) mice and wild-type control mice (WT) after PBS or isoproterenol (Iso) infusion for 6 weeks ($n$ = 3, 4, 5, 5). Data were analyzed by 2-way ANOVA followed by Tukey's multiple comparison test (**A**) or by the non-parametric Kruskal-Wallis test (**B**). *$P$ < 0.05, **$P$ < 0.01. Values represent the mean ± 2 SEM. NS indicates not significant.

sociated with increased p53 expression (Figure 4B, C). Transcripts of p53 target molecules, p21 (*CDKN1A*) and *ICAM1*, were also increased in HUVECs by incubation with isoproterenol (Figure 4D). Some subsets of macrophages were reported to promote cardiac remodeling in the failing heart.[26] Our study indicated that Mac3 positive cells would increase in cardiac tissue by isoproterenol treatment (Figure 2B). This evidence promoted us to test the role of isoproterenol in macrophage like (RAW264.7) cells. Incubation of RAW264.7 macrophages with isoproterenol increased ROS and p53 levels (Figure 4E, F), along with an increase of transcripts including p21 (*Cdkn1a*) and *Itgal* (Figure 4G). Introducing p53 with adenovirus encoding p53 in these cells also led to an increase of *Cdkn1a* and *Itgal* expression (Figure 4H). Taken together, our results indicated that a hyperadrenergic state induces p53-ICAM1 signaling in ECs and p53-integrin signaling in inflammatory cells, contributing to the progression of pathological changes in the failing heart (Figure 4I).

## Discussion

In this study, we showed that catecholamine-induced senescence of ECs and BM cells had a pivotal role in the progression of pathological changes associated with heart failure. In a murine model of isoproterenol-induced heart failure, the infusion of isoproterenol led to an increase of p53 expression in both cardiac tissue and BM, while genetic suppression of p53 in these organs ameliorated cardiac dysfunction. In addition, we demonstrated that isoproterenol up-regulates ROS production in HUVECs and RAW264.7 macrophages. Because high ROS levels have been reported to induce genomic instability, it is possible that such instability resulted in the up-regulation of p53 in these cells. Long-term exposure to catecholamines is reported to induce desensitization of β-adrenergic receptors in cardiomyocytes, with β1-adrenergic receptors generally thought to predominantly undergo down-regulation.[27] In contrast, β2-adrenergic receptor expression is elevated in the failing heart,[28] and chronic β2-adrenergic receptor activation increases ROS production by up-regulation of NADPH oxidase activity.[29] We previously reported that β2-adrenergic receptor-mediated signaling induces cardiac inflammation in a murine model of LV pressure overload.[14] Taken together, these findings indicate that chronic exposure to catecholamines may promote pathologic changes, possibly via catecholamine/β2-adrenergic recep-

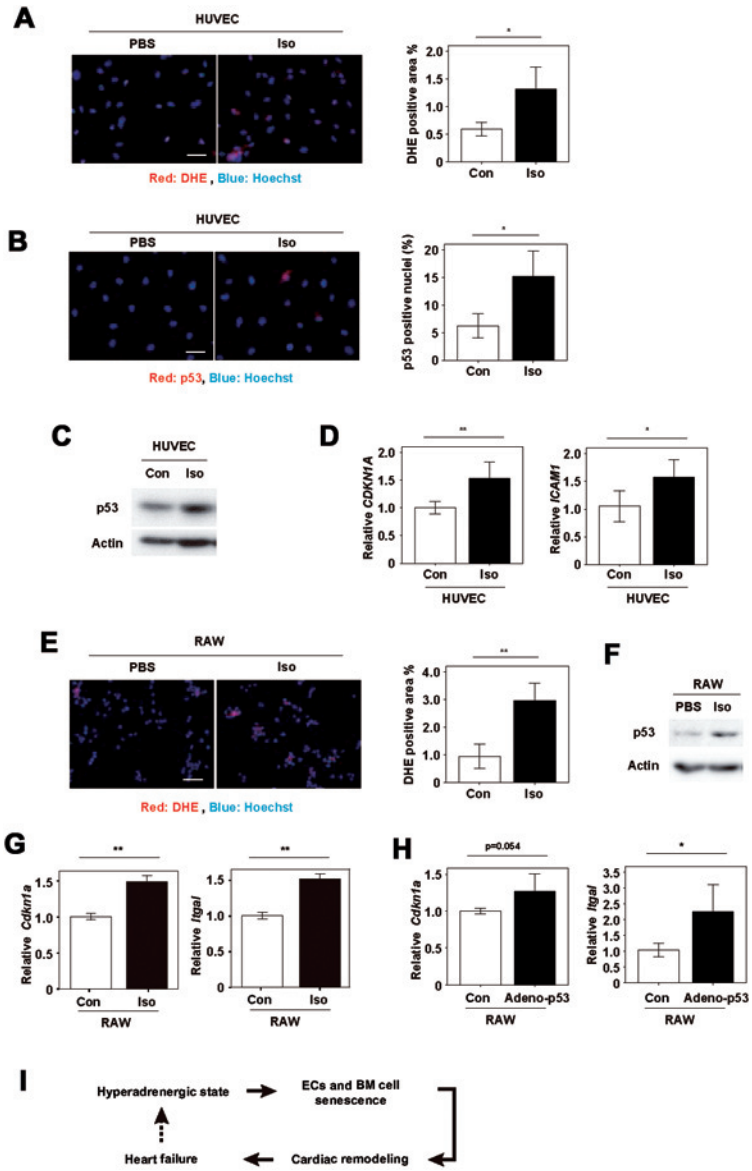


**Figure 4.** Isoproterenol up-regulates ROS/p53 signaling and increases adhesion molecule expression. **A:** Dihydroethidium (DHE) staining for reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVECs). Cells were incubated with isoproterenol (Iso) (100 nM) for 30 minutes. Scale bar = 50 μm. The right panel shows quantification of the DHE-positive area ($n$ = 3, 3). **B:** Immunofluorescent staining for p53 in HUVECs treated with isoproterenol (Iso) (100 nM) or PBS for 3 hrs. Scale bar = 50 μm. The right panel indicates quantification of p53-positive nuclei per field (%) ($n$ = 3, 3). **C:** Western blot analysis of p53 expression in HUVECs treated with isoproterenol (Iso) (100 nM) for 3 hours. **D:** qPCR analysis of *CDKN1A* and *ICAM1* expression in HUVECs treated with isoproterenol (Iso) (100 nM) for 6 hours ($n$ = 6, 6). **E:** Dihydroethidium (DHE) staining of ROS in RAW264.7 cells incubated with isoproterenol (Iso) (100 nM) for 20 minutes. The right panel shows quantification of the DHE-positive area ($n$ = 3, 3). Scale bar = 50 μm. **F:** Western blot analysis of p53 expression in RAW264.7 cells treated with isoproterenol (Iso) (100 nM) for 1 hour. **G:** qPCR analysis of *Cdkn1a* and *Itgal* expression in RAW264.7 cells treated with isoproterenol (Iso) (100 nM) for 3 hours ($n$ = 4, 6). **H:** qPCR analysis of *Cdkn1a* and *Itgal* expression in RAW264.7 cells treated with adeno-p53 (20MOI) or Adeno-Mock (Con) for 6 hours ($n$ = 9, 10). **I:** Scheme showing the role of catecholamine-induced cellular senescence in heart failure. A hyperadrenergic state develops with the onset of heart failure. Catecholamines induce senescence of endothelial cells (ECs) and bone marrow (BM) cells, and these changes promote cardiac remodeling. Data were analyzed by the two-tailed Student's $t$-test. $^*P < 0.05$, $^{**}P < 0.01$. Values represent the mean ± 2 SEM. NS indicates not significant.

tor/ROS/p53 signaling, but further investigation is needed to clarify the β-adrenergic receptor type involved. Whether catecholamine desensitization develops in ECs or inflammatory cells (such as macrophages) in patients with heart failure is another topic to be explored. While β-blockers were initially contraindicated for patients with heart failure, these drugs have since become first-line therapy for this critical condition. Carvedilol is a non-selective β-blocker with lower affinity for the β1-adrenergic receptor and higher affinity for the β2-adrenergic receptor. Among various β-blockers (including selective and non-selective drugs), carvedilol is recognized as first-line therapy for heart failure.[30,31] Our findings suggest that β-blockers like carvedilol may have a beneficial effect via suppression of β2-adrenergic receptor signaling and prevention of cellular senescence in the hyperadrenergic state associated with heart failure.

## Conclusion

Inhibition of catecholamine-induced cellular senescence is important for suppression of pathologic changes in the failing heart.

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## Disclosures

**Conflicts of interest:** The authors disclose no conflict of interest.

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