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## Research Article

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**Substance P/neurokinin1 receptor is associated with the expression of cardiac stem/pluripotency-associated genes in diabetic right and left atria.**

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## Abstract

Substance P (SP) is a cardioprotective neuropeptide that interacts with the G protein-coupled neurokinin-1 receptor (NK<sub>1</sub>R). Although the expression of SP/NK<sub>1</sub>R in the right atrium (RA) of diabetic patients is known to be significantly impaired, the molecular mechanism remains unclear. LETO and OLETF rats were randomly divided into 3 groups: saline, SP injection (5 nmole/kg), and SP+RP injection (1 mg/kg RP67580, a selective non-peptide tachykinin NK<sub>1</sub>R antagonist). After 3 weeks, the left atrium (LA), RA, and left ventricle (LV) of the rats were collected. Cardiac stem/pluripotency-associated genes in the diabetic atria of each group were comprehensively examined using qRT-PCR analysis. qRT-PCR analysis demonstrated that only the RA of SP-treated OLETF rats exhibited significantly higher levels of *α-SMA*, *GATA4*, *TBX5*, and *Klf4* in the mRNA, compared to the control. RP prevented the expression of *NK<sub>1</sub>R* and four SP-associated genes in the RA of SP-treated OLETF rats. In conclusion, our findings provide novel mechanistic insights into the role of NK<sub>1</sub>R in diabetic atria.

**Keywords:** substance P; neurokinin-1 receptor; diabetic atria; cardiac stem cell/pluripotency-associated markers

## Introduction

The relative risk of cardiovascular disease (CVD) is much higher for individuals with diabetes mellitus (DM) <sup>1,2</sup>. In recent years, there has been an increased interest in validating and discovering more about known biomarkers of CVD in such individuals <sup>1-3</sup>. Prognostic biomarkers and biomarker combinations could affect processes of care and improve patient outcomes <sup>3</sup>. In general, the majority of DM research focuses on the left ventricle (LV) <sup>4,5</sup>. DM can directly affect LV mass, hypertrophy, remodeling, and function <sup>4,5</sup>. Recently, several studies have recognized relationships between DM and the atria <sup>6-8</sup>. For example, one study compared the structure and function of the right atrium (RA) of individuals with normal glucose metabolism, prediabetes, and diabetes. Using the Maastricht Study, an observational prospective population-based cohort study, two-dimensional and tissue Doppler echocardiography, and multiple linear regression analyses, the researchers found (pre)diabetes to be associated with structural RA and right ventricle (RV) changes, as well as impaired RV systolic and diastolic function <sup>6</sup>.

Advanced anatomic and pathologic imaging techniques including echocardiography, computed tomography, chest radiography, and magnetic resonance imaging have provided a better depiction of the atria, which has improved objective assessment of clinical signs and symptoms of the RA <sup>9,10</sup>. Although these techniques are not ordinarily applied to identify the molecular mechanisms underlying diabetes-induced cardiac damage, a recent study used electron microscopy paired with morphometric analysis to investigate the effects of diabetes on RA cardiomyocytes in Wistar elderly rats <sup>11</sup>. Compared to the control group, the rats with streptozotocin-induced diabetes showed increased functional activity of atrial cardiomyocytes with greater production of natriuretic peptides <sup>11</sup>. However, biomarkers and the underlying molecular mechanisms connecting DM and the atria remain elusive.

Substance P (SP), an 11-amino acid neuropeptide of the tachykinin neuropeptide family,

is an evolutionarily older neurotransmitter than acetylcholine and the catecholamines<sup>10,12,13</sup>. Neurokinin<sub>1</sub> receptor (NK<sub>1</sub>R), a G protein coupled receptor found in the central and peripheral nervous systems, is important for the biological actions of SP<sup>10,12,13</sup>. Interestingly, the expression and localization of SP and NK<sub>1</sub>R have already been described for RA of diabetic patients and rat models, shown to be significantly impaired<sup>13,14</sup>. Our recent, previous studies have demonstrated that treatment of SP enhanced the local activation of cardiac progenitor c-Kit<sup>+</sup> cells (CSCs) in LV and RA after myocardial infarction<sup>10,12</sup>. Based on these studies, we turn to unresolved key questions about how SP/NK<sub>1</sub>R relates to biomarkers and molecular mechanisms between DM and atria. The present study was performed to identify the expression of CSC-associated genes between LETO and OLETF rats with or without SP in the presence or absence of RP67580, and to assess SP's potential relevance as a novel biomarker and its role as a mechanism between atria and the diabetic heart.

## Results

**OETF<sup>RP+SP</sup> exhibited lower LVW/BW and RAW/BW ratios compared to both OETF<sup>saline</sup> and OETF<sup>SP</sup>, a reduction associated with the  $\alpha$ -SMA and NK<sub>1</sub>R expression**

Several studies have highlighted altered patterns of abnormality affecting the size, shape, and function of hearts in patients with DM <sup>6,7</sup>. A previous paper reported that RA of diabetic patients exhibited decreased expression of NK<sub>1</sub> <sup>14</sup>. If SP/NK<sub>1</sub>R signaling contributes to cardiac abnormality, changes in the heart size of patients with DM could be explained by a link between AF and DM. To test this, we measured the ratio of LV, RA, and LA per BW in the LETO and OETF groups. As shown in Figure 1B and 1C, compared to the OETF<sup>saline</sup> group, LVW/BW was significantly lower in the OETF<sup>SP</sup> (P<0.05) and OETF<sup>RP+SP</sup> (P<0.01) groups, while the LVW/BW ratio of the LETO<sup>SP</sup> group was not significantly different from the LETO<sup>saline</sup> group. After SP injection RAW/BW and LAW/BW both increased for the LETO<sup>SP</sup> group, while only LAW/BW of OETF<sup>SP</sup> experienced a marked increase (Fig. 2B and 2C). Unexpectedly, we found that the mean RAW/BW ratio of the OETF<sup>RP+SP</sup> group was significantly lower than for RA of the OETF<sup>saline</sup> and OETF<sup>SP</sup> groups. The weight of LA in the OETF<sup>SP</sup> group slightly increased, whereas the weight decreased in the presence of RP (Fig. 2D and 2E).

If SP/NK<sub>1</sub>R signaling correlates to change in the size of LA and RA, atrial remodeling could be caused by interstitial fibrosis, which is characterized by the myogenic properties of myofibroblasts. To test this, we further examined whether SP/NK<sub>1</sub>R signaling affects the expression of  $\alpha$ -SMA mRNA, which is used as a fibrosis marker <sup>7,11</sup> in the RA and LA of the LETO and OETF groups. As shown in Figure 3A, up-regulation of  $\alpha$ -SMA mRNA was found only in the RA of OETF<sup>SP</sup>. Inactivation of NK<sub>1</sub>R by RP inhibited the SP-mediated  $\alpha$ -SMA expression in RA of OETF<sup>SP</sup>. On the other hand, the expression of  $\alpha$ -SMA had no effect on the RA of LETO<sup>saline</sup> and LETO<sup>SP</sup>, nor on the LA of OETF<sup>saline</sup>, OETF<sup>SP</sup>, and OETF<sup>RP+SP</sup>

(Fig. 3B). The expression pattern of *NK1R* mRNA was similar to  $\alpha$ -*SMA* in RA of OLETF<sup>SP</sup> (Fig. 3C). In the LA of the LETO and OLETF groups, *NK1R* mRNA showed oppositional and low expression compared with RA in the LETO and OLETF groups (Fig. 3D). These observations indicate that SP/NK<sub>1</sub>R signaling may correlate to atrial size and fibrosis in diabetic rats.

### **SP/NK<sub>1</sub>R signaling is involved in CSC- related gene expression in OLETF models**

The number of c-Kit<sup>+</sup> cells derived from LA appendages in the atrial fibrillation (AF) heart is much lower than in a healthy heart<sup>15</sup>. In fact, c-Kit<sup>+</sup> cells are more abundant in the RA appendages<sup>10</sup>. Our previous studies have noted the local activation of c-Kit<sup>+</sup> cells by SP/NK<sub>1</sub>R signaling in RA in heart damage<sup>10</sup>. According to a recent study that constructed a rat model of right heart disease (RHD), RA can play an important role in AF maintenance with RA re-entrant activity caused by atrial fibrosis and conduction abnormalities<sup>6-8,13-15</sup>. To determine whether SP/NK<sub>1</sub>R affects CSC-related gene expression in LA and RA of LETO and OLETF groups, we selected well-studied genes of cardiac transcription factors and stem cell markers. Using qRT-PCR analysis, extremely high levels of *GATA4* and *TBX5* mRNA were observed in the RA of OLETF<sup>SP</sup> through NK<sub>1</sub>R activation (Fig. 4A and Fig. 4C), which the injection of RP prevented. On the other hand, *GATA4* and *TBX5* in LA of OLETF<sup>SP</sup> and OLETF<sup>RP+SP</sup> groups increased (Fig. 4B and Fig. 4D). Expression of c-KIT, *SCA1*, *ISL-1*, *OCT4*, and *Klf-4* mRNA in the RA of OLETF<sup>SP</sup> was impacted by SP/NK<sub>1</sub>R signaling (Fig. 5 and Fig. 6). There were no significant differences in the gene expression of *MEF2C* (LA), *NKX2.5* (RA), *NANOG* (LA), *Klf-4* (LA), and *SOX2* (LA) in the RA and/or LA of the OLETF<sup>SP</sup> group compared to the OLETF<sup>saline</sup> or OLETF<sup>RP+SP</sup> groups (Fig. 4, Fig. 5, and Fig. 6). The expression of NK<sub>1</sub>R-associated *SCA1*, *c-KIT*, *NANOG*, and *OCT4* in the LA of the OLETF<sup>SP</sup> group was

of a similarly low level as the expressions of these SP-associated genes in the RA of the OLETF<sup>RP+SP</sup> group (Fig. 5 and Fig. 6). These findings suggest that SP/NK<sub>1</sub>R signaling correlates with CSC-related gene expression in RA of OLETF groups.

## DISCUSSION

Our study is the first comprehensive study analyzing the biological role of SP/NK<sub>1</sub>R on CSC-related gene expression between the RA and LA of LETO and OLETF rats. The present study highlights changes in CSC and transcription factor related gene expression underlying RA and the diabetic heart. We found that SP and/or SP/RP administration reduced the weight of LV in the OLETF group, whereas there was no difference in LV weight between the LETO<sup>saline</sup> and LETO<sup>SP</sup> group. LV dysfunction in DM generally increases LA pressures, causing atrial remodeling<sup>16,17</sup>. Accordingly, compared to the LA in the LETO<sup>saline</sup> group, the LA weight of the LETO and OLETF groups slightly increased by SP. Unexpectedly, RA of the LETO<sup>SP</sup> group seemed to have increased, while the RA weight in the OLETF<sup>SP</sup> group remained the same as the control group and RP significantly reduced in the OLETF<sup>RP+SP</sup> group. To explain these findings, we focused on the gene expression of endogenous c-Kit<sup>+</sup> CSC-related markers and a subset of activated fibrogenic cells and myofibroblasts in atrial remodeling, including fibrosis<sup>19</sup>. This focus was informed by our previous studies demonstrating the significance of SP/NK<sub>1</sub>R signaling in local activation of c-Kit<sup>+</sup> CPCs of RA and LV following heart damage<sup>10,12</sup>. Furthermore, a recent paper reported the impact of streptozotocin-induced diabetes on gene expression and DNA methylation in cardiac cells (20). Of note, cell type-specific analysis shows differentially modulate gene programs that are involved in distinct biological processes in diabetes<sup>20</sup>.

Based on these observations, we explored whether the self-renewal mechanism of CSC-related gene expression for RA remodeling is associated with SP/NK<sub>1</sub>R signaling. The findings of our current study indicate that low expression of *NK1R* in RA of OLETF<sup>SP+RP</sup>, LA of OLETF<sup>SP</sup>, and LA of OLETF<sup>SP+RP</sup> might be closely connected to the modulation of CSC-related gene expression. In particular, SP-treated RA tissues in OLETF rats increased t

he levels of  $\alpha$ -SMA, GATA4, and TBX5 mRNA. SP has similarly been found to induce pro-fibrotic changes via activation of the RhoA/ROCK pathway in an *in vitro* human corneal fibrosis model <sup>21</sup>. In addition, GATA4 and TBX5 have been shown to play a pivotal role in cardiac remodeling in ischemic heart disease <sup>22</sup>. Although we have no direct evidence, we speculate that SP-mediated CSC-related gene expression for regulating RA remodeling is not attributable to the cooperative interaction of RA NK<sub>1</sub>R-MEF2C/NKX2.5 downregulation, but rather to the RA NK<sub>1</sub>R-GATA4/TBX5 upregulation in OLETF rats under our experimental conditions. Further studies are needed to confirm this possibility.

Core cardiac transcription factors that have been usually identified as vital for heart development include the homeodomain protein Nkx2-5, GATA family zinc finger proteins GATA4, 5, and 6, MEF2 factors, SRF (MADS box proteins), T-box factors, including Tbx1, Tbx2, Tbx3, Tbx5, Tbx18, and Tbx20, and the Lim-homeodomain protein Isl-1 <sup>23-25</sup>. They function in a mutually reinforcing transcriptional network in which each of the factors modulate the expression of the others <sup>23-25</sup>. It is not remarkable that mutations in several of the genes that encode the core cardiac transcription factors are correlated with heart disease <sup>23-25</sup>. Among them, Nkx2-5, GATA4, and Tbx5 have been well established as cardiac transcription factors implicated in patients with heart disease, and all three are important for normal heart development <sup>23-25</sup>. Nkx2-5 is a key regulator of cardiac development, beginning with its role in specification and proliferation of cardiac precursors <sup>23-25</sup>. Nkx2-5 is involved in a wide range of transcriptional cascades controlling multiple cardiac genes <sup>23-25</sup>. Expression of Nkx2-5 is generally regulated by GATA factors <sup>25</sup>.

GATA4, a zinc finger transcription factor, can activate the promoters of many cardiac-specific genes such as SMADs, NKX2-5, and TBX5 <sup>26</sup>. The GATA4 sequence is highly conserved among mammals, underscoring its central role in the development and function of

the heart<sup>26</sup>. Of note, GATA4 is an important partner for Tbx5<sup>26</sup>. In addition to its coordination with Nkx2-5 and Tbx5, GATA4 partners with several other important cardiac transcription factors, including MEF2C. MEF2C physically connects with GATA factors, including GATA4 and 6 to synergistically stimulate the expression of Nppa,  $\alpha$ -MHC, a-CA, and B-type natriuretic peptide (BNP)<sup>26</sup>. GATA4 directly activates MEF2C transcription in the second heart field in combination with Is11<sup>26</sup>. Tbx5 is known to play a key role in cardiac morphogenesis and development of the conduction system. Of note, embryonic mice that lack Tbx5 have abnormal heart tube formation with hypoplastic atria<sup>27</sup>. Therefore, the molecular mechanisms of SP/NK<sub>1</sub>R appear to be associated with core cardiac transcription factors, and may indicate a CSC-mediated relationship between DM and atrial remodeling. Overall, our findings suggest a potential mechanism underlying modulation of core cardiac transcription factors via SP/NK<sub>1</sub>R signaling, including the local activation of RA CSCs and structural change of RA in diabetic atria.

## **Methods**

### **Materials**

SP (6.7  $\mu\text{g}/\text{kg}/0.1 \text{ ml}$ ) was purchased from Sigma (St. Louis, MO, USA). RP-67580 (RP is a selective non-peptide tachykinin NK<sub>1</sub>R antagonist; 1 mg/kg) was obtained from R&D Systems Inc. (Minneapolis, MN, USA). AccuPower®RocketScript™ Cycle RT PreMix (dN12) and AccuPower®ProFi Taq PCR PreMix were purchased from Bioneer (DaeJeon, Korea). SYBR®Green Mix was obtained from Applied Biosystems (Lincoln, CA, USA).

### **Experimental animals**

All procedures for animal models in this study were approved by the Institutional Animal Care and Use Committee of Kyung Hee Medical Center (KHMC-IACUC:2015-028). All methods were carried out in accordance with relevant guidelines and regulations. Based on the ARRIVE guidelines derived from Institutional Animal Care and Use Committee of Kyung Hee Medical Center standardized pain protocol, all LETO and OLETF rats continually was monitored for signs of distress. The LETO and OLETF rats were housed in the same pathogen-free facility under a 12 h light and dark cycle with ad libitum feeding. No more than two animals were housed per cage<sup>10,12</sup>.

### **Experimental design, sample collection, and measurement of LA, RA, LV, and body weight ratio**

As shown in Fig. 1A, the rats were randomly divided into 5 groups (n=4 each); LETO (LETO<sup>saline</sup>), LETO with 5 nmole/kg SP injection (LETO<sup>SP</sup>), OLETF<sup>saline</sup>, OLETF with 5 nmole/kg SP injection (OLETF<sup>SP</sup>), and OLETF<sup>SP</sup> with a 1 mg/kg injection of RP67580 (OLETF<sup>RP+SP</sup>). SP and RP were injected twice into the tail vein of the OLETF<sup>SP</sup> at 27 and 28 weeks. At 29 weeks, the rats were anaesthetized by using isoflurane (Hana Pharm Co.,Ltd.,

Seoul, Korea), and then were euthanized. The hearts were gently grasped with blunt forceps, then punctured with a 21 G needle to aspirate blood from LV. The hearts were washed with PBS and then LA, RA, and LV tissue samples were collected (Fig. 2A). LA mass, RA mass, LV mass, and body weight were measured using an electronic balance (model GR-200 and CUX220H). The percentage of each weight ratio was calculated as follows: the ratio of LA, RA, or LV = (LA, RA, or LV weight/body weight) x %. The weight for every sample was immediately measured and then frozen in liquid nitrogen and stored at -80°C.

### **Quantitative reverse-transcription PCR (qRT-PCR)**

cDNA was synthesized using AccuPower®RocketScript™ Cycle RT PreMix (dN12) (Bioneer, DaeJeon, Korea). To correlate the targeted gene expression profiles, qRT-PCR assays were used with SYBR®Green Mix and the appropriate primers (Applied Biosystems), and were performed on a StepOnePlus real-time PCR system (Applied Biosystems). The relative gene expression profiles from all data were evaluated using the  $\Delta$  Ct method with normalization versus *RPL-32* as previously described<sup>10,12</sup>. The primers used are as follows: *c-KIT* (Forward-AGACGTACAGATCCAGAATG, Reverse-TGCTCTTTGCTGTTACCTT); *SCA-1* (Forward-TGCCCAACGAGTCCTCAAAC, Reverse-TGCTGCGTTGCAAAGATCTG); *ISL-1* (Forward-AGTCCGGAGAGACATGATGG, Reverse-TGCAAGGCGAAGTCACTCAG); *Klf-4* (Forward-GATGGGGTCTGAGACTGGAT, Reverse-AACTTCCAGTCACCCCTTGG); *NANOG* (Forward-CTCTCTACCATTCTGAACCTGAGC, Reverse-TCAGGCCGTTGCTAGTCTTC); *OCT4* (Forward-GCCCCATTTACCACACT, Reverse-CCAGAGCAGTGACAGGAACA); *SOX2* (Forward-GACAGCTACGCGCACATGAA, Reverse-CGAGCTGGTCATGGAGTTGT); *GATA4* (Forward-ACCCTGCGAGACACCCAAT, Reverse-GTAGAGGCCACAGGCGTTGC); *MEF2C* (Forward-CGAGATACCCACAACACACG, Reverse-GGAGTGGAATTCGTTCCGGT);

*NKX2.5* (Forward-AGCGGCGCTTCAAGCAAC, Reverse-ACCAGATCTTGACCTGCGTG); *TBX5* (Forward-TGAGAACAACCCCTTCGCC, Reverse-CTGTGGTTGGCCACTTTGT); *NK1R* (Forward-TACTGTGGGCCAGTGAGATC, Reverse- GGTACACACAACCACGATCATCA); and *RPL-32* (Forward- TGTC AAGGAGCTGGAAGTGC, Reverse-AGGCACACAAGCCATCTATTCA).

### Statistical analysis

A one-way analysis of variance (ANOVA) (for comparisons between three or more groups) or student's *t*-tests (for comparisons of two groups) or followed by Tukey post hoc tests were used for the statistical analyses. GraphPad Prism software (GraphPad Software Inc) and SPSS software ver. 17.0 (SPSS, Chicago, IL) were used to determine the statistical analysis. A value of  $P < 0.05$  was considered significant. Data are expressed as means  $\pm$  standard error (SD). \* $P < 0.05 - 0.01$ , \*\* $P < 0.01 - 0.001$ , and \*\*\* $P < 0.001$  vs. corresponding LETO<sup>saline</sup> and/or OLET<sup>saline</sup>. All error bars mean the standard deviation of three or more biological replicates of indicated data.

## FIGURE LEGENDS

**Figure 1. The effect of SP and RP67580 on LVW/BW of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFRP+SP hearts.** (A) Schematic of *in vivo* rat study design. (B and C) Bar graphs depicting the percentages of the LV mass to body weight ratio levels of each group. \**P* <0.05 and \*\**P* <0.01 vs. corresponding controls using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

**Figure 2. The effect of SP and RP67580 on RAW/BW and LAW/BW of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFRP+SP hearts.** (A) A simple illustration of RA and LA in a rat heart. (B - D) Bar graph indicating the percentages of the RA or LA mass to body weight ratio levels of each group. \*\**P* <0.01 vs. corresponding controls using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

**Figure 3. Expression levels of  $\alpha$ -SMA and *NK1R* mRNA in RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFRP+SP hearts.** (A - D) qRT-PCR analysis of relative mRNA levels of  $\alpha$ -SMA and *NK1R* in the RA and LA of each group, respectively. \**P* < 0.05, \*\**P* <0.01, and \*\*\**P* <0.01 vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

**Figure 4. *GATA4* and *TBX4* are overexpressed in the RA of OLETFS<sup>SP</sup> hearts via SP/NK1R signaling.** (A-H) Graphs of qRT-PCR analyses of targeted cardiac transcriptional gene expression in the RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFRP+SP hearts. \**P* <0.05 and \*\**P* <0.01 vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

**Figure 5. SP affects the gene expression of CSC markers.** (A-F) Graphs of qRT-PCR analyses of targeted gene expression in the RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFS<sup>RP+SP</sup> hearts. \* $P < 0.05$  and \*\*\* $P < 0.001$  vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

**Figure 6. SP impacts on expression of *Klf-4* in OLETFS<sup>SP</sup> hearts.** (A-H) Graphs of qRT-PCR analyses of targeted gene expression in the RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFS<sup>saline</sup>, OLETFS<sup>SP</sup>, and OLETFS<sup>RP+SP</sup> hearts. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc tests*.

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### **Authors' contributions**

Y.M.J and W.K., wrote the manuscript, handled the work for the manuscript, conceived and designed the research. Y.M.J. performed statistical analysis of data and all experiments. W.K. handled funding and supervision. S.R.L. was responsible for animal experiments. All authors reviewed and approved the final manuscript.

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## **Data Availability**

(1) All data used to support the observations of present study are included within the article, provided a statement to confirm that all methods were carried out in accordance with relevant guidelines and regulations and compliance with ARRIVE guidelines {animal research of Institutional Animal Care and Use Committee of Kyung Hee Medical Center (KHMC-IACUC:2015-028)}. (2) All data used to support the findings of this study are available from the corresponding author upon request.

## **Ethics declarations**

## **Competing interests**

The authors declare that they have no competing interests.

## **Additional Information**

## **Supplementary information**

NA

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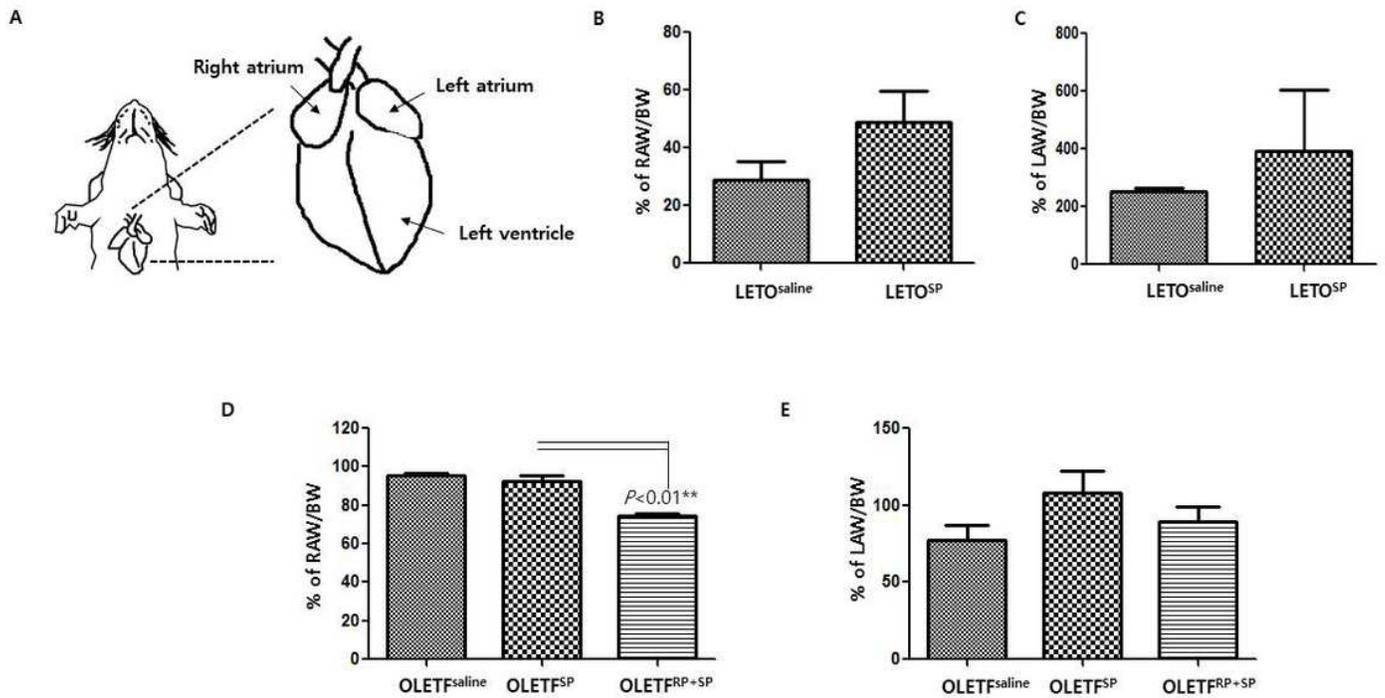
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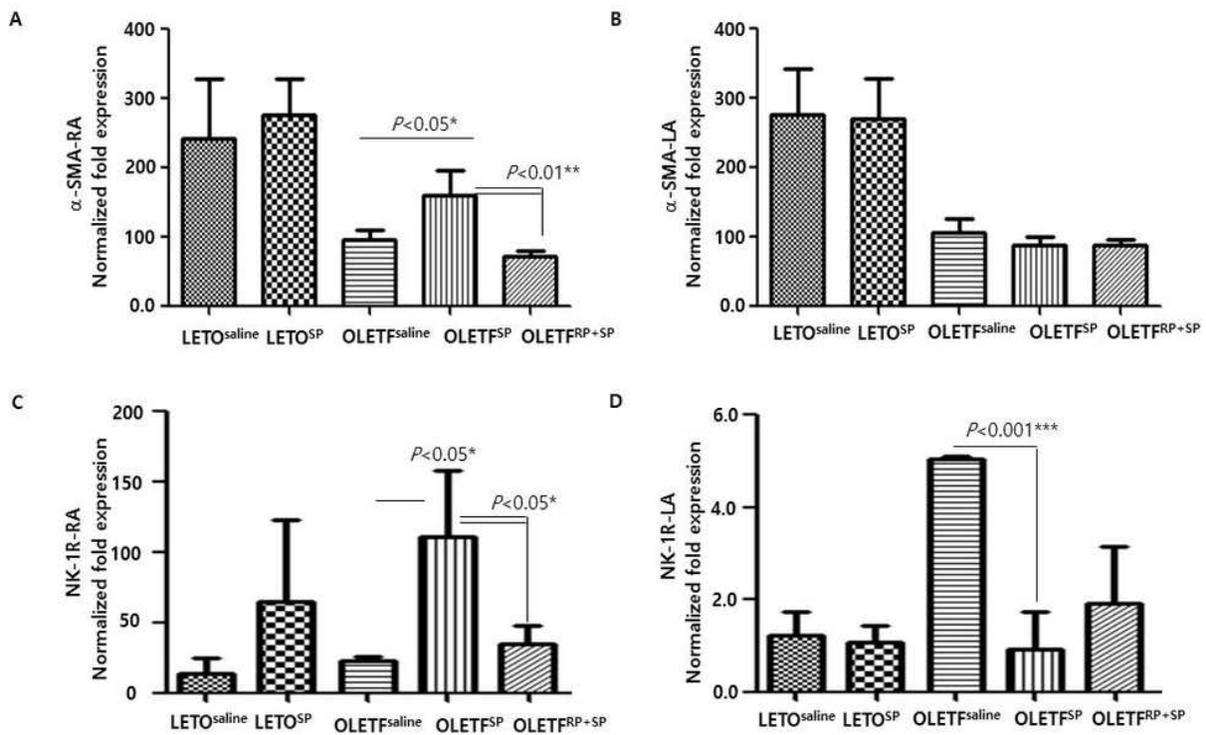
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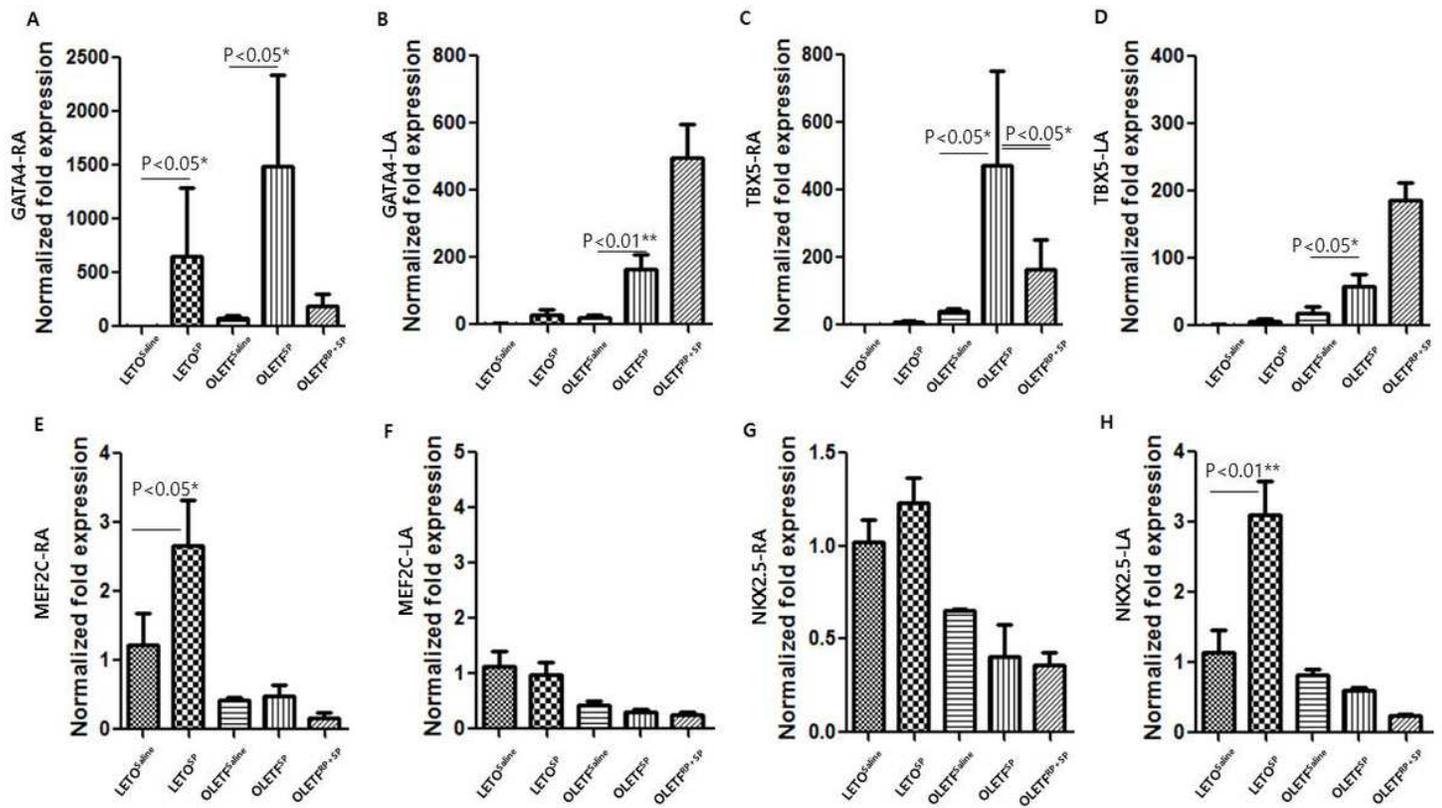
**Figure 2**

The effect of SP and RP67580 on RAW/BW and LAW/BW of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLET<sup>saline</sup>, OLET<sup>SP</sup>, and OLET<sup>RP+SP</sup> hearts. (A) A simple illustration of RA and LA in a rat heart. (B - D) Bar graph indicating the percentages of the RA or LA mass to body weight ratio levels of each group.  $^{**}P < 0.01$  vs. corresponding controls using the Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.



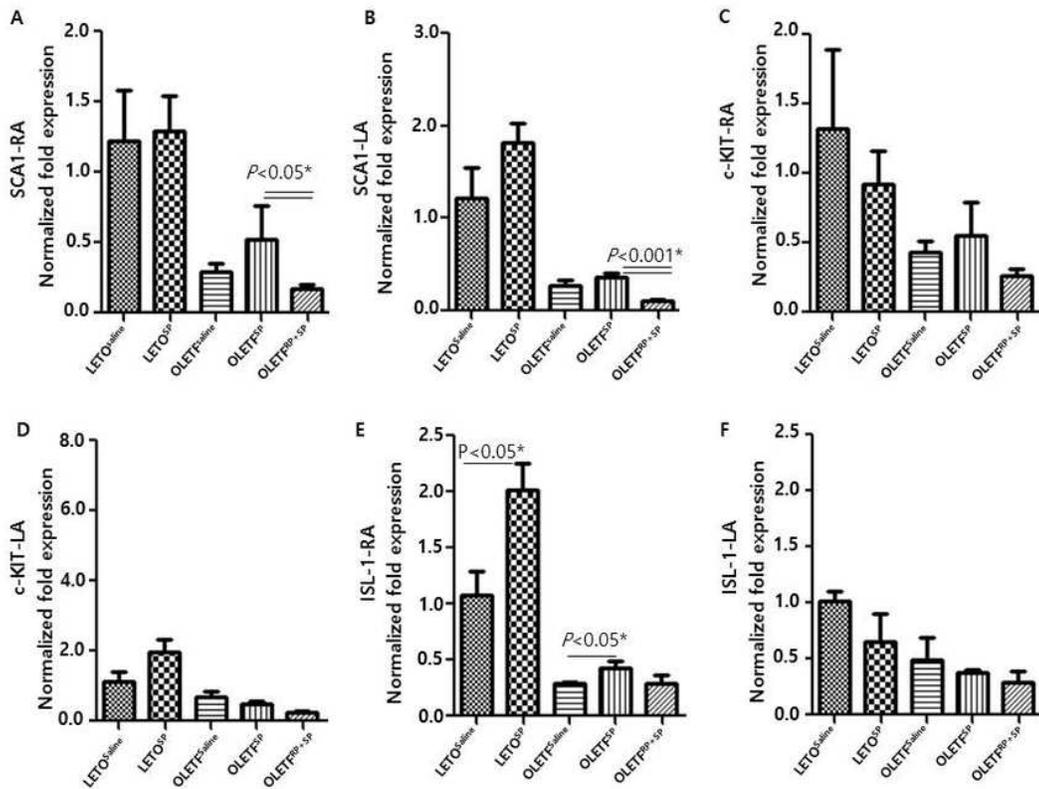
**Figure 3**

Expression levels of  $\alpha$ -SMA and NK1R mRNA in RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETF<sup>saline</sup>, OLETF<sup>SP</sup>, and OLETF<sup>RP+SP</sup> hearts. (A - D) qRT-PCR analysis of relative mRNA levels of  $\alpha$ -SMA and NK1R in the RA and LA of each group, respectively. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.01$  vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.



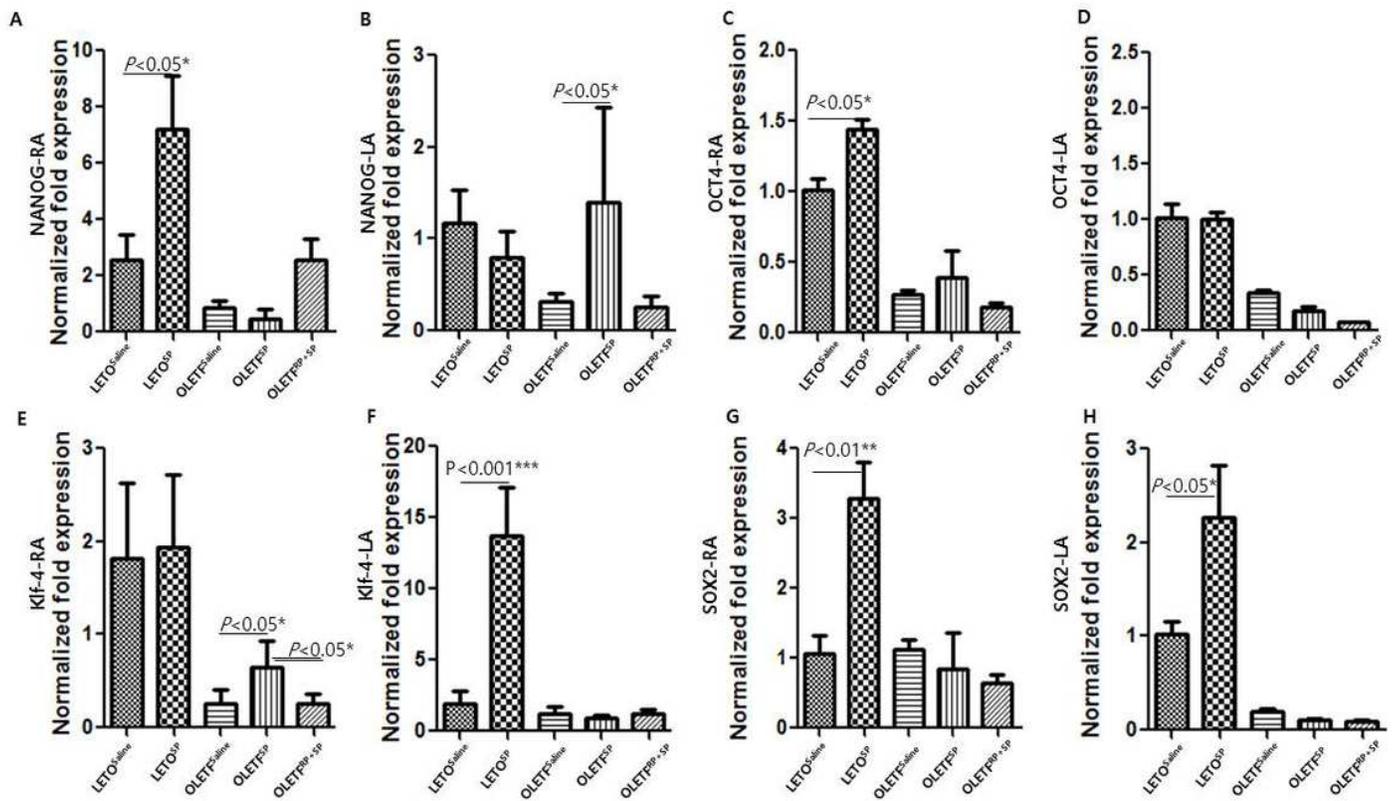
**Figure 4**

GATA4 and TBX4 are overexpressed in the RA of OLETFRP hearts via SP/NK1R signaling. (A-H) Graphs of qRT-PCR analyses of targeted cardiac transcriptional gene expression in the RA and LA of LETO<sup>saline</sup>, LETO<sup>SP</sup>, OLETFSaline, OLETFSP, and OLETFRP+SP hearts. \*P < 0.05 and \*\*P < 0.01 vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.



**Figure 5**

SP affects the gene expression of CSC markers. (A-F) Graphs of qRT-PCR analyses of targeted gene expression in the RA and LA of LETOsaline, LETOSP, OLETFSaline, OLETFSP, and OLETFRP+SP hearts. \* $P < 0.05$  and \*\*\* $P < 0.001$  vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.



**Figure 6**

SP impacts on expression of Klf-4 in OLETFSP hearts. (A-H) Graphs of qRT-PCR analyses of targeted gene expression in the RA and LA of LETOsaline, LETOSP, OLETFSaline, OLETFSP, and OLETFRP+SP hearts. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  vs. corresponding controls using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests.