Circulation Research

ORIGINAL RESEARCH

Brown Adipocyte ADRB3 Mediates Cardioprotection via Suppressing Exosomal iNOS

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BACKGROUND: The ADRB3 (β3-adrenergic receptors), which is predominantly expressed in brown adipose tissue (BAT), can activate BAT and improve metabolic health. Previous studies indicate that the endocrine function of BAT is associated with cardiac homeostasis and diseases. Here, we investigate the role of ADRB3 activation-mediated BAT function in cardiac remodeling.

METHODS: BKO (brown adipocyte-specific ADRB3 knockout) and littermate control mice were subjected to Ang II (angiotensin II) for 28 days. Exosomes from ADRB3 antagonist SR59230A (SR-exo) or agonist mirabegron (MR-exo) treated brown adipocytes were intravenously injected to Ang II–infused mice.

RESULTS: BKO markedly accelerated cardiac hypertrophy and fibrosis compared with control mice after Ang II infusion. In vitro, ADRB3 KO rather than control brown adipocytes aggravated expression of fibrotic genes in cardiac fibroblasts, and this difference was not detected after exosome inhibitor treatment. Consistently, BKO brown adipocyte-derived exosomes accelerated Ang II–induced cardiac fibroblast dysfunction compared with control exosomes. Furthermore, SR-exo significantly aggravated Ang II–induced cardiac remodeling, whereas MR-exo attenuated cardiac dysfunction. Mechanistically, ADRB3 KO or SR59230A treatment in brown adipocytes resulted an increase of iNOS (inducible nitric oxide synthase) in exosomes. Knockdown of iNOS in brown adipocytes reversed SR-exo-aggravated cardiac remodeling.

CONCLUSIONS: Our data illustrated a new endocrine pattern of BAT in regulating cardiac remodeling, suggesting that activation of ADRB3 in brown adipocytes offers cardiac protection through suppressing exosomal iNOS.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: brown adipocytes ■ cardiovascular disease ■ fibroblast ■ fibrosis ■ homeostasis

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The β-adrenoceptors have 3 subtypes β1-, β2-, and β3- adrenoceptors (ADRB3 [β3-adrenergic receptors]) and play an important role in the regulation of heart function.[1] Chronic activation or heterologous high-level expression of β1-, β2- adrenoceptors leads to cardiac damage.[2–4] Beta-adrenoceptors antagonists targeting β1, and β2 adrenoceptors are extensively used to improve cardiac remodeling. In contrast, ADRB3 activation has recently been implicated in cardiac protection in response to hemodynamic stress and ischemia-reperfusion.[5,6] It is worth noted that ADRB3 is abundantly expressed in adipose tissue especially in brown adipose tissue (BAT), rather than the heart and blood vessels. ADRB3 activation stimulates energy expenditure, nutrient consumption, lipolysis, and thermogenesis in the BAT.[7,8] However, whether ADRB3 activation-mediated BAT function is involved in regulating cardiac remodeling remain unknown.

BAT is now emerged as a dynamic endocrine organ, secreting several bioactive products, including adipokines, gaseous messengers, and microvesicles, that can target distant tissues such as white adipose tissue, liver,

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ORIGINAL RESEARCH

Novelty and Significance

What Is Known?

- Brown adipose tissue (BAT) protects against pathological cardiac remodeling via secreting adipokines.
- The ADRB3 (β3-adrenergic receptors) is predominantly expressed in BAT and regulates thermogenesis.
- ADRB3 activation is implicated in cardiac protection in response to hemodynamic stress and ischemia reperfusion.

What New Information Does This Article Contribute?

- Exosomal bioactive enzyme iNOS (inducible NOS) from BAT promotes Ang II–induced cardiac remodeling.
- ADRB3 in BAT negatively regulates exosomal iNOS and pathological cardiac remodeling.

The β adrenoceptors play an important role in the regulation of heart function. Previous studies have demonstrated that cardiac resident ADRB3 activation protects against cardiac damage. In fact, ADRB3 expression is much higher in BAT than heart. We herein provided a new organ-to-organ crosstalk pattern between BAT and cardiac function, in which ADRB3 knockout brown adipocyte derived-exosomes mediate the pathological communication between BAT and heart. Mechanically, ADRB3 deletion in BAT leads to increased release of iNOS-containing exosomes, which directly aggravated cardiac remodeling.

Nonstandard Abbreviations and Acronyms

ADRB3	β3-adrenergic receptors
Ang II	angiotensin II
BAT	brown adipose tissue
BKO	brown adipocyte-specific ADRB3 knockout
BKO-exo	exosomes from BKO brown adipocytes
CFs	cardiac fibroblasts
Col1a1	collagen type I alpha 1
eNOS	endothelial nitric oxide synthase
iBAT	intrascapular BAT
iNOS	inducible nitric oxide synthase
Lv-shiNOS	lentiviral vectors to silence iNOS
MR-exo	exosomes from mirabegron brown adipocytes
NMCMs	neonatal mouse cardiomyocytes
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
SR&LvshiNOS-exo	exosomes from SR59230A and Lv-shiNOS treated brown adipocytes
SR-exo	exosomes from SR59230A treated brown adipocytes
Tgfβ	transforming growth factor β1
Ucp1	uncoupling protein 1
α-Sma	α-smooth muscle actin

pancreas, heart, and bone.[9–11] Experimental BAT transplantation and activation provide metabolic improvements and cardiac protection through releasing endocrine factors, such as insulin-like growth factor I, interleukin-6, and fibroblast growth factor-21.[12–17] However, it is still unclear whether the other organ-to-organ crosstalk patterns are involved in the interaction between BAT and the heart. Exosomes are membrane-enclosed spherical structures engaged in the transport of various bioactive molecules from parental to distant target cells. The exosome-associated cellular content includes proteins, lipids, multi-molecular complexes, and nucleic acids, which have been shown to modulate the signaling pathways in target cells.[18] Accumulating evidence have demonstrated the important regulatory effects of exosomes in cardiovascular diseases.[18,19] Exosomes derived from cardiomyocytes, endothelial cells, fibroblasts, and stem cells are involved in pathophysiological processes of cardiovascular diseases, such as cardiac hypertrophy, cardiomyocyte survival and apoptosis, cardiac fibrosis, and angiogenesis.[20–22]

In the current study, we show that brown adipocyte-specific deletion of ADRB3 aggravates Ang II (angiotensin II)–induced cardiac remodeling in an exosome-dependent manner. Exosomes from ADRB3 antagonist SR59230A-treated brown adipocytes accelerate Ang II–induced cardiac remodeling, whereas exosomes from ADRB3 agonist mirabegron-treated brown adipocytes attenuate Ang II–induced cardiac remodeling. Further, we uncover that ADRB3-inhibition results in the release of iNOS (inducible nitric oxide synthase)-containing exosomes from brown adipocytes, which contributes to aggravated cardiac remodeling after Ang II infusion. Collectively, these findings suggest a new BAT-to-heart crosstalk pattern in regulating cardiac remodeling.

METHODS

Data Availability

A detailed description of materials and methods is available in the Supplemental Material. The data that support the findings

of this study are available from the corresponding author upon reasonable request.

RESULTS

BKO Aggravates Ang II–Induced Cardiac Remodeling

We detected the expression level of ADRB3 in different organs and tissues and found that ADRB3 was highly expressed in intrascapular BAT (iBAT) compared with other adipose tissues and organs. We then generated brown adipocyte-specific *Adrb3*-knockout mice (BKO). *Adrb3*$^{flox/+}$ mice were bred with *Ucp1*-Cre (for brown adipocyte knockout), the *Ucp1*-Cre+; *Adrb3*$^{flox/flox}$ (BKO) and littermates *Ucp1*-Cre-; *Adrb3*$^{flox/flox}$ (control) mice were used for subsequent experiments. BKO blocked ADRB3 expression in iBAT but not the other adipose tissues and heart (Figure S1A). Ang II–induced cardiac contractile dysfunction as reflected by decreased left ventricular ejection fraction and fractional shortening and increased left ventricular mass compared with saline-treated mice (Figure 1A and 1B). Hematoxylin-eosin and wheat germ agglutinin staining showed that Ang II treatment increased cardiomyocyte size (Figure 1C). Masson trichrome staining, collagen I, and α-smooth muscle actin (α-SMA) immunohistochemical staining revealed that Ang II treatment induced cardiac fibrosis (Figure 1D and 1E). Although, ADRB3 KO did not affect blood pressure with Ang II infusion (Figure S1B), these hypertrophic and fibrotic effects were more serious in BKO mice than control mice after Ang II infusion (Figure 1A through 1E). mRNA expression of hypertrophic genes, including *Anp* (atrial natriuretic polypeptide), *Bnp* (brain natriuretic peptide), *β-Mhc* (myosin heavy chain β), and fibrotic genes, including *α-Sma* (alpha-smooth muscle actin), *Col1a1* (collagen type I alpha 1 chain), and *Tgfβ* (transforming growth factor β1) were elevated in BKO mice treated with Ang II (Figure 1F). These results indicate that brown adipocytes ADRB3 knockout aggravates Ang II–induced cardiac remodeling.

To determine the role of ADRB3 on BAT function, we further detected the adipocyte browning and thermogenic gene expression of BAT. Ang II treatment significantly promoted iBAT activity as shown by smaller-sized adipocyte and increased UCP1 (uncoupling protein 1) staining, but this effect was absent in BKO mice (Figure S1C). Consistently, the expression of thermogenic genes, including *Ucp1*, *Pparγ* (peroxisome proliferator-activated receptor γ), *Prdm16* (PRD1-BF1-RIZ1 homologous domain containing 16), and *Cidea* (cell death-inducing DNA fragmentation factor-like effector A) were increased in the iBAT of WT but not BKO mice treated with Ang II (Figure S1D). These results suggest that Ang II regulates BAT function through ADRB3 activation. To characterize how Ang II activates ADRB3, we examined the effect of Ang II on adipocyte browning in cultured adipocytes. The result showed that Ang II did not directly promote adipocyte browning, inconsistent with the in vivo Ang II infusion in mice. It is known that the renin-angiotensin-aldosterone system modulates sympathetic response. To determine whether sympathetic activation is involved in regulating iBAT activity, we detected the level of dopamine, adrenaline, and noradrenaline in iBAT in response to Ang II. We found that Ang II significantly increased the level of noradrenaline, but not dopamine, adrenaline in iBAT (Figure S2A). Furthermore, we found that the noradrenaline increased the lipid droplet sizes and the expression level of thermogenic genes, including *Ucp1*, *Pparγ*, *Prdm16*, and *Cidea*, while Ang II treatment had no such effects. The effects of noradrenaline on morphological changes of white to brown-like adipocytes as well as upregulation of browning markers were blocked with ADRB3-specific inhibitor SR59230A treatment (Figure S2B through S2D). Thus, our findings suggest that Ang II–induced sympathetic response, in particularly noradrenaline, is important for the function of Ang II in ADRB3-mediated adipocyte browning in vivo.

Characterization of Brown Adipocyte-Derived Exosomes and Their Incorporation Into Fibroblasts

Next, a coculture assay system was used to study the crosstalk between brown adipocytes and cardiac fibroblasts (CFs). We found that brown adipocytes from BKO mice increased the mRNA and protein levels of α-SMA, Col1a1 (collagen type I alpha 1), and TGFβ (transforming growth factor β1) in Ang II–stimulated CFs (Figure 2A and 2B). To determine whether the profibrotic effect of ADRB3 knockout is associated with brown adipocyte-derived exosomes, we used GW4869 (10 μmol/L), a widely used blocker of exosome biogenesis/release. GW4869 abrogated the increased protein levels of α-SMA, Col1a1, and TGFβ in CFs, indicating that exosomes released from BKO-brown adipocytes are necessary for activating CFs (Figure 2B).

We then isolated exosomes from primary brown adipocyte-conditioned medium using 2 different methods. After a shared clearing procedure to remove sedimented live cells, dead cells and debris, exosomes were isolated by ultracentrifugation or using an affinity column (exoEasy Maxi Kit from QIAGEN) as previously described[27] (Figure 2C). Isolated exosomes were identified using transmission electron microscopy and Delta Nano C particle analyzer. The 2 methods yielded a similar population of exosomes that appeared round, cup-shaped morphology and about 100 nm in diameter (Figure 2D). Exosome-specific markers CD63 and TSG101 were expressed in brown adipocyte-derived exosomes from both control and BKO mice (Figure 2E).

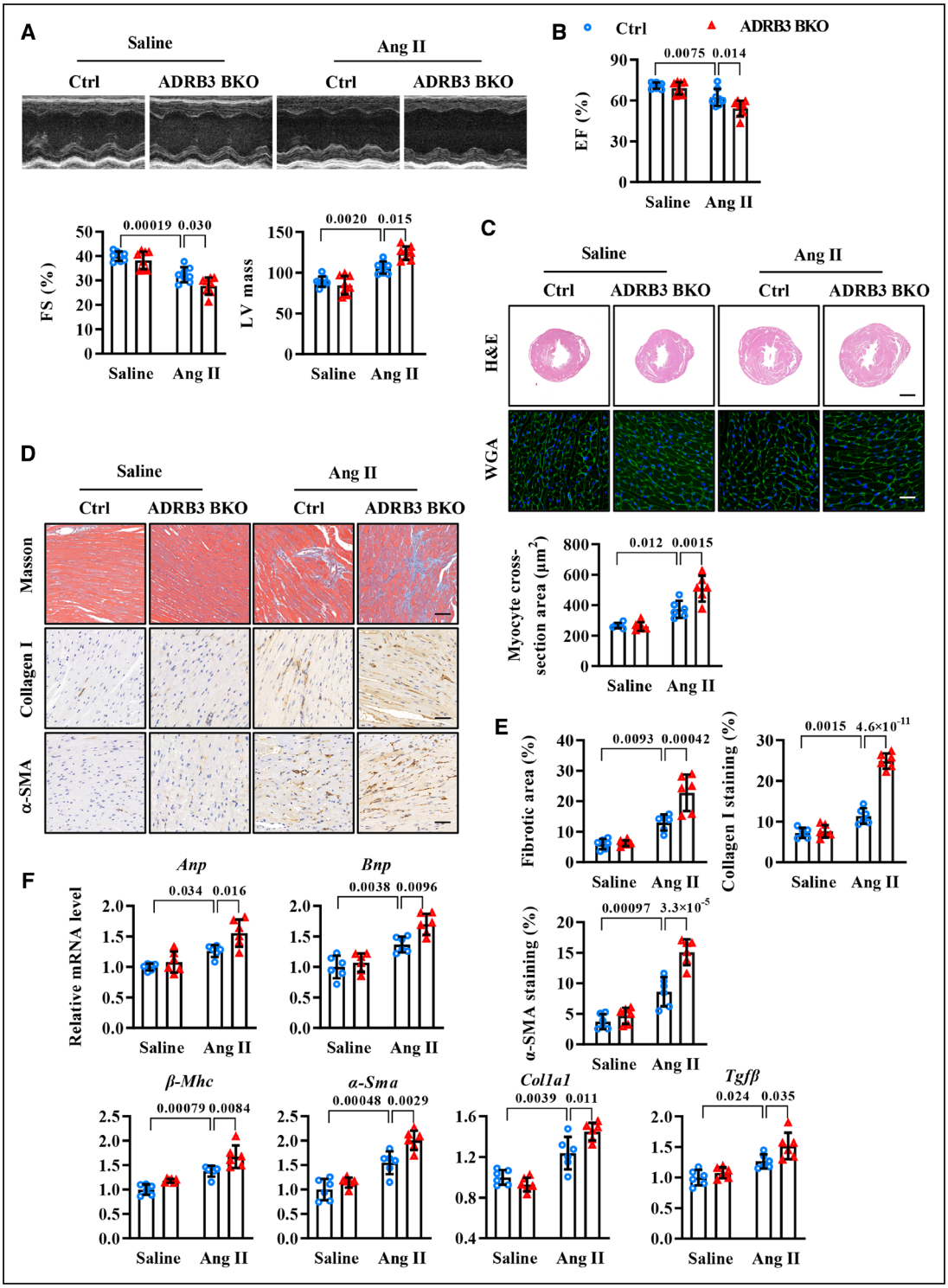

Figure 1. Brown adipocyte-specific ADRB3 (β3-adrenergic receptor) knockout (BKO) exacerbates Ang II (angiotensin II)–induced heart failure.

A, Representative M-mode echocardiography of brown adipocyte-specific ADRB3 knockout (BKO) and littermate control mice (Ctrl) treated with saline or Ang II. **B**, Ejection fraction (EF), fractional shortening (FS), and left ventricular (LV) mass of BKO and Ctrl mice treated with saline or Ang II. **C**, Hematoxylin-eosin (H&E) and wheat germ agglutinin (WGA) staining and quantitative analysis of cardiomyocyte size in heart tissue of BKO and Ctrl mice treated with saline or Ang II. Scale bar: 500 and 30 μm. **D**, Masson trichrome staining, collagen I and α-smooth muscle actin (α-SMA) immunohistochemical staining in heart tissue of BKO and Ctrl mice treated with saline or Ang II. Scale bar: 100 μm. **E**, Quantification of cardiac fibrosis in heart tissue of BKO and Ctrl mice treated with saline or Ang II. **F**, Quantitative real-time PCR (qRT-PCR) was performed to analyze the mRNA levels of hypertrophic (*Anp*, *Bnp,* and *β-Mhc*,) and fibrotic (*α-Sma*, *Col1a1,* and *Tgfβ*) genes. *Anp* indicates atrial natriuretic peptide; *Bnp*, brain natriuretic peptide; β-*Mhc*, myosin heavy chain β; *α-Sma*, alpha smooth muscle actin*; Col1a1*, alpha-1 type I collagen; and *Tgfβ*, transforming growth factor β1. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey multiple comparisons test (n=6).

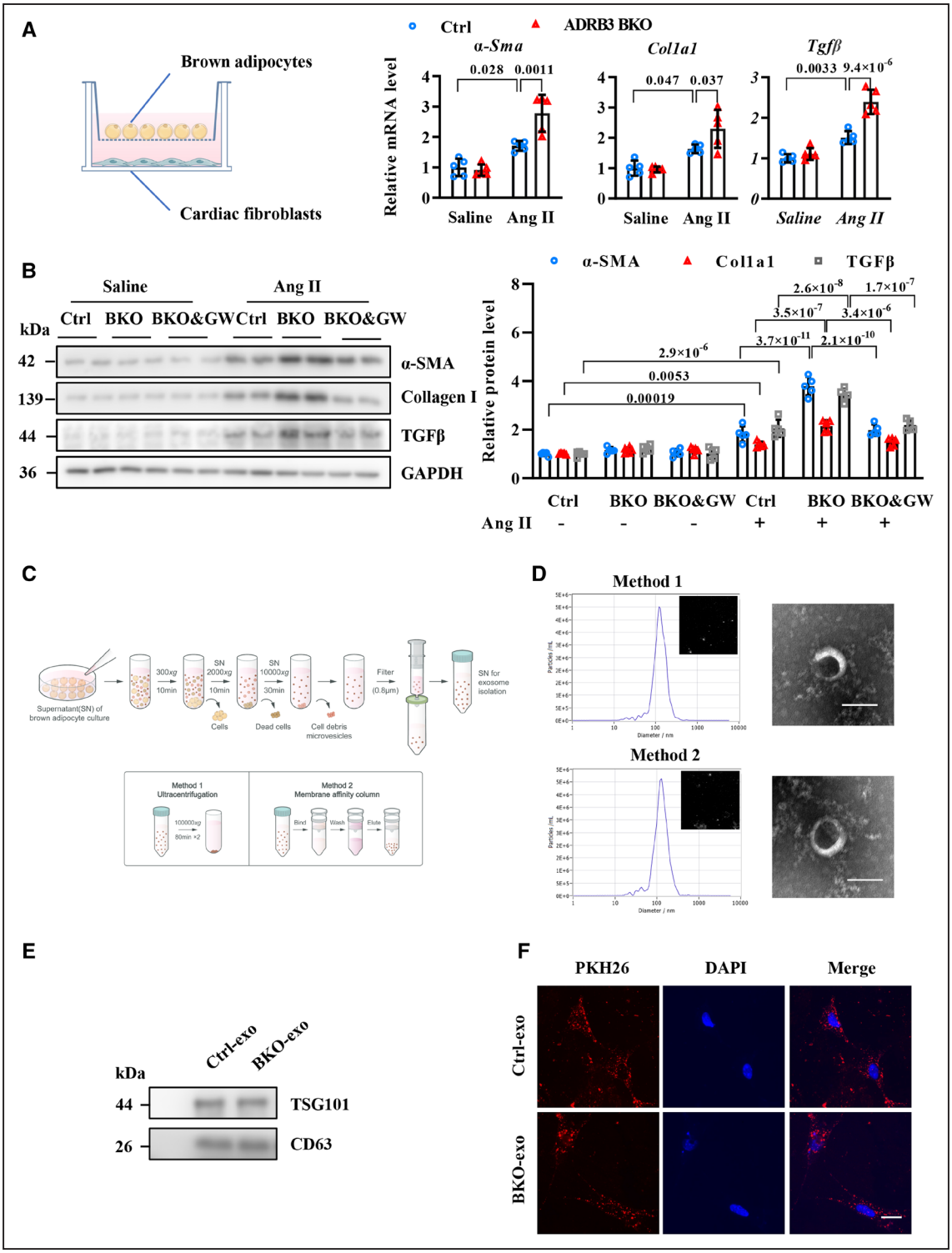

Figure 2. Characterization of brown adipocyte-derived exosomes.

A, Quantification of *α-Sma*, *Col1a1*, and *Tgfβ* mRNA levels in cardiac fibroblasts (CFs) cocultured with brown adipocytes from brown adipocyte-specific ADRB3 knockout (BKO) and control (Ctrl) mice treated with saline or Ang II (angiotensin II). **B**, Western blot and quantitative analysis of α-SMA (α-smooth muscle actin), Col1a1 (collagen type I alpha 1), and TGFβ (transforming growth factor β1) in CFs cocultured with brown adipocytes from BKO and Ctrl mice treated with saline, Ang II or GW4869 (GW). **C**, Schematic representation of the exosome isolation methods. **D**, Concentration, size distribution and representative electron microscopic photographs of exosomes isolated by the 2 different methods. Scale bar: 100 nm. **E**, Western blot analysis of TSG101 and CD63 of brown adipocyte-derived exosomes of BKO and Ctrl mice. **F**, Representative images of brown adipocyte-derived exosomes staining of BKO and Ctrl mice in CFs. Nuclei stained by DAPI in blue and exosomes stained by PKH26 in red. Scale bar: 30 μm. Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=5).

To test whether brown adipocyte-derived exosomes could be taken up by CFs, we labeled exosomes with the red fluorescent membrane dye PKH26 and then incubated them with CFs. Twenty-four hours later, we observed that the majority of CFs acquired the red dye-labeled exosomes (Figure 2F), revealing that CFs can efficiently assimilate brown adipocyte-derived exosomes.

To identify whether myocytes are also involved in the BKO-induced cardiac damage, brown adipocytes and neonatal mouse cardiomyocytes (NMCMs) coculture assay was performed. Brown adipocytes from BKO mice increased the mRNA levels of hypertrophic genes in NMCMs. Moreover, labeled exosomes were incorporated into the NMCMs (Figure S3).

Brown Adipocyte-Derived Exosomes Induce CF Activation

To determine the direct effects of brown adipocyte-derived exosomes on CFs and NMCMs, we incubated cells with exosomes for 24 hours. Exosomes derived from BKO brown adipocytes (BKO-exo) by the 2 methods equally increased the mRNA levels of *α-Sma, Col1a1, Tgfβ, Anp, Bnp,* and *β-Mhc* in Ang II–stimulated CFs and NMCMs compared with the control mice (Figure 3A and Figure S4A and S4B). The 2 methods tested here yielded a similar population of exosomes from brown adipocytes. Subsequent studies were conducted using exosomes isolated with method 2. BKO-exo also increased the protein levels of α-SMA, Col1a1, and TGFβ (Figure 3B). We then performed EdU staining to measure CF proliferation and found that BKO-exo visibly increased the proliferation of CFs (Figure 3C). Consistently, exosomes from ADRB3 inhibitor SR59230A-treated brown adipocytes (SR-exo) showed similar effects on CFs. In contrast, exosomes from ADRB3 agonist mirabegron-treated brown adipocytes (MR-exo) attenuated Ang II–stimulated CF proliferation and fibrotic gene expression. (Figure 3D and 3E and Figure S4D).

To further identify the role of brown adipocyte-derived exosomes on cardiac myocytes, we cultured NMCMs with isolated exosomes. Quantitative PCR assay and α-actinin staining showed that BKO-exo and SR-exo incubation markedly increased the cross-section area of myocytes and hypertrophic gene expression, while MR-exo inhibited Ang II–induced morphological hypertrophy (Figure S4C through S4E).

ADRB3-Activated Brown Adipocyte-Derived Exosomes Protect Against Ang II–Induced Cardiac Remodeling

To determine whether brown adipocyte-derived exosomes are necessary for ADRB3 intervention on cardiac remodeling, SR-exo and MR-exo were intravenously injected (4 mg/kg body weight) to the Ang II–infused mice every 3 days. After 1 month, cardiac contractile function was measured by echocardiography, showing deterioration in SR-exo mice and improvement in MR-exo mice compared with the control group (Figure 4A and 4B). Hematoxylin-eosin and wheat germ agglutinin staining showed that SR-exo aggravated Ang II–mediated increases in cardiomyocyte size and MR-exo ameliorated the Ang II–induced cardiac hypertrophy (Figure 4C). Masson trichrome staining, collagen I, and α-SMA immunohistochemical staining revealed that Ang II–induced fibrotic features were enhanced in the heart of SR-exo mice and improved in MR-exo mice (Figure 4D and 4E). Consistently, the quantitative PCR results showed that these abnormal pathological phenotypes were accompanied by the regulation of hypertrophic and fibrotic genes (Figure S5). These data show that ADRB3 activation in brown adipocytes protects against Ang II–induced cardiac remodeling via exosome transportation.

Exosomes Transport iNOS From Brown Adipocytes to CFs

Exosome-mediated organ-to-organ crosstalk is mainly dependent on the nucleic acids, proteins, and metabolites contained within the exosome. Recently, several studies revealed that exosome-derived proteins are involved in the regulation of cardiovascular disease.[22,30] Previous studies have showed that ADRB3 regulates NOS (nitric oxide synthase) in the heart and vasculature under pathological conditions or pharmacological stimulation.[31–33] So we first detected the level of nNOS (neuronal NOS), iNOS, and eNOS (endothelial NOS) in brown adipocyte-derived exosomes and found that only iNOS was significantly increased in the exosomes from BKO mice under Ang II treatment (Figure 5A and Figure S6A).

Then, we isolated the serum exosomes and detected the level of iNOS, and found that iNOS was significantly increased in serum exosomes and the heart of BKO mice (Figure S6B and S6C). Consistently, we found that the level of iNOS was remarkably increased in SR-exo compared with control-exo (Figure 5B). The iNOS protein level was upregulated in the heart after SR-exo injection under Ang II treatment (Figure 5C), whereas iNOS mRNA level was not changed (Figure S6D), suggesting that the increased iNOS protein level in the heart might be derived directly from exosomes.

To examine whether iNOS could be transferred by brown adipocyte-derived exosomes in vivo, BKO-exo were labeled with the near-infrared dye DiR and injected intravenously into the mice at different times. We observed the presence of exosomes in the CFs within 1 and 48 hours after injection in vivo (Figure 5D). The iNOS levels significantly reached a peak at 6 hours and then declined at 12, 24, and 48 hours postinjection (Figure S6E).

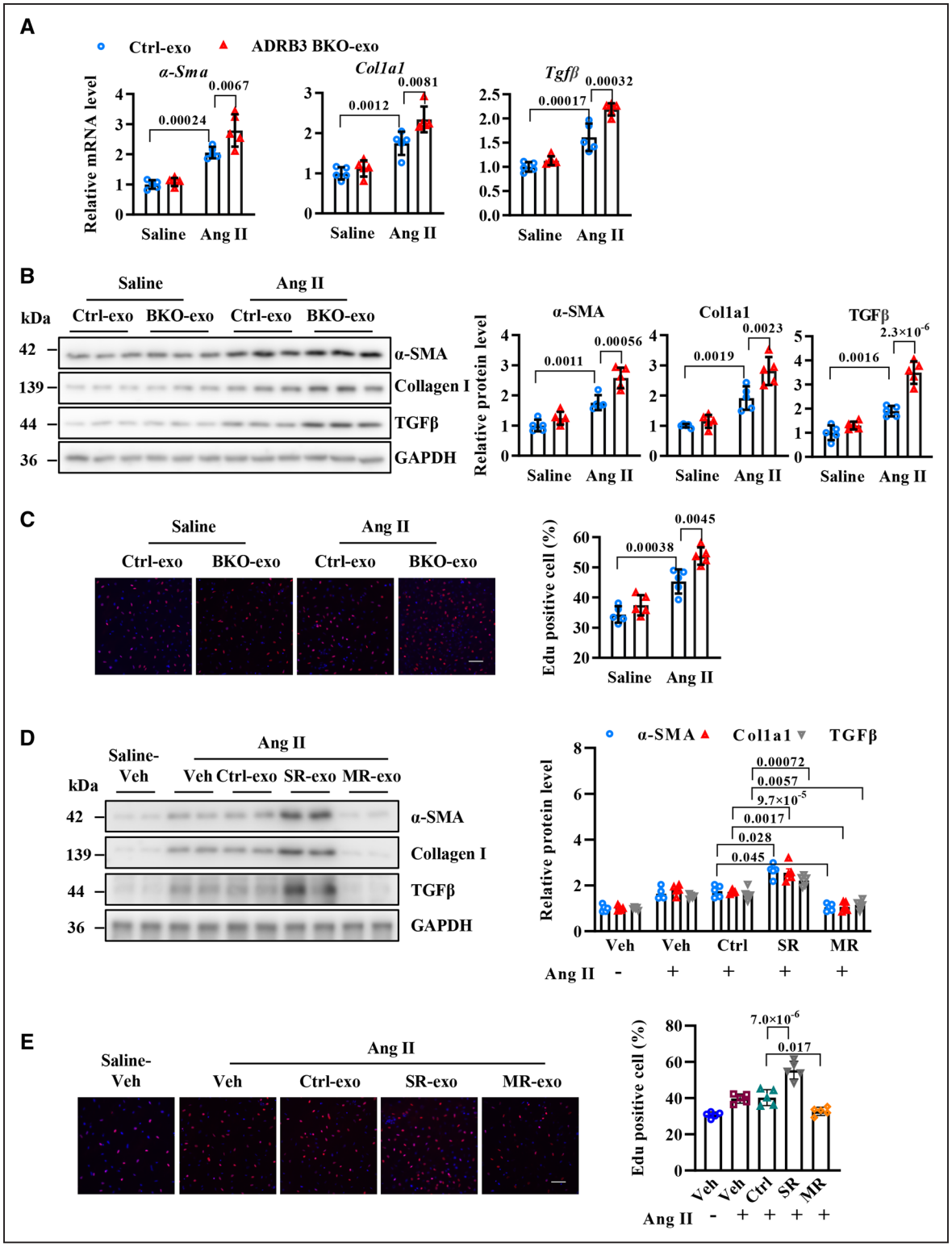

Figure 3. Brown adipocyte-derived exosomes induces cardiac fibroblasts (CFs) activation.
A, Quantification of *α-Sma*, *Col1a1*, and *Tgfβ* mRNA levels in CFs cultured with brown adipocyte-derived exosomes from BKO (BKO-exo) and control (Ctrl-exo) mice treated with saline or Ang II (angiotensin II). **B**, Western blot and quantitative analysis of α-SMA (α-smooth muscle actin), Col1a1 (collagen type I alpha 1), and TGFβ (transforming growth factor β1) in CFs cultured with BKO-exo and Ctrl-exo treated with saline or Ang II. **C**, Representative image and quantitative analysis of BrdU incorporation in CFs cultured with BKO-exo and Ctrl-exo treated with saline or Ang II. Scale bar: 100 μm. **D**, Western blot and quantitative analysis of α-SMA, Col1a1, and TGFβ protein level in CFs cultured with exosomes isolated from brown adipocytes treated with control (Ctrl-exo), SR59230A (SR-exo) or mirabegron (MR-exo) with saline or Ang II. **E**, Representative image and quantitative analysis of BrdU incorporation in CFs cultured with Ctrl-exo, SR-exo, or MR-exo with saline or Ang II. Scale bar: 100 μm. Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=5).

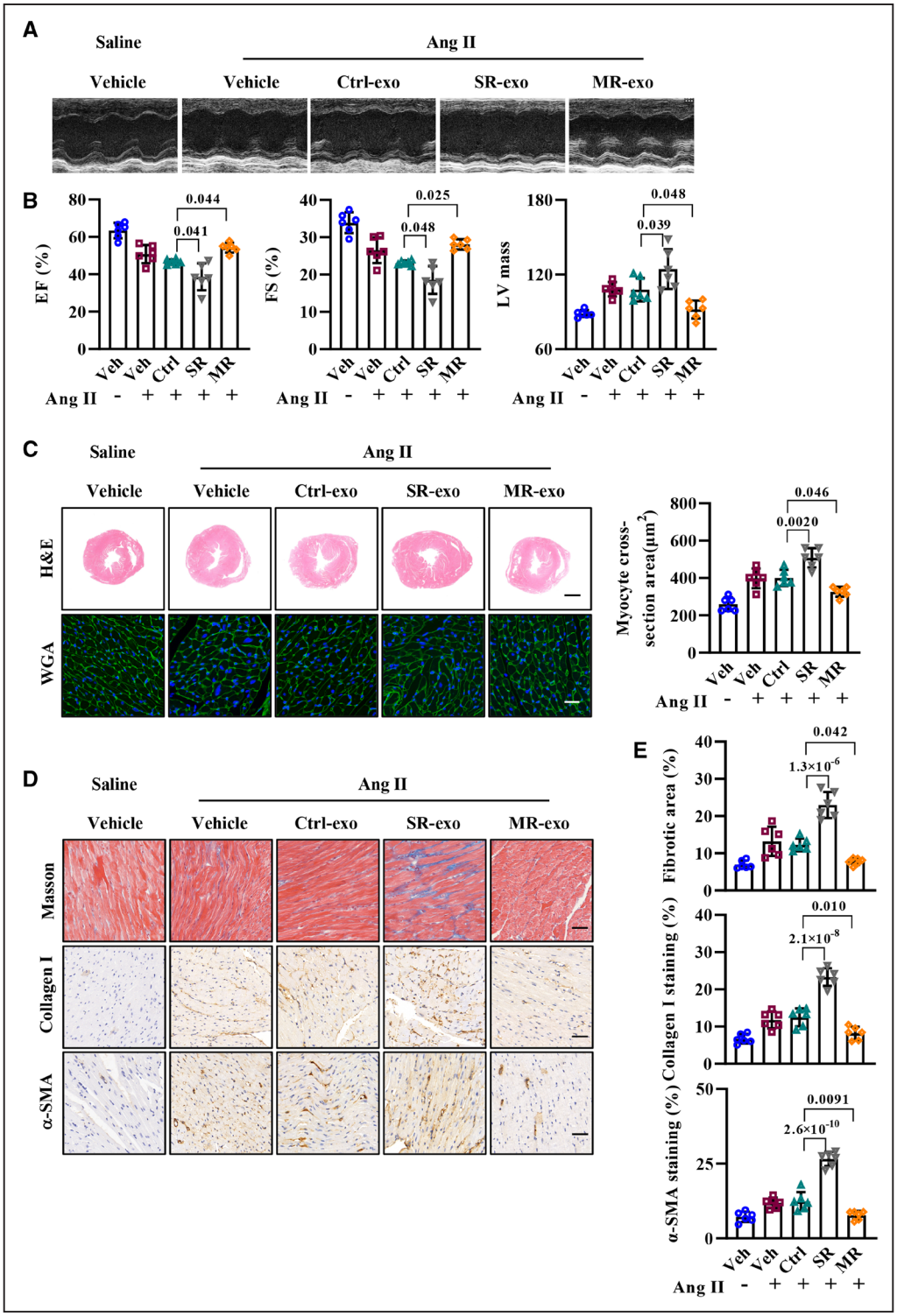

Figure 4. Brown adipocyte ADRB3 (β3-adrenergic receptor) activation protects against cardiac remodeling through exosome injection.

A, Representative M-mode echocardiography of wild type (WT) mice intravenously injected (4 mg/kg body weight) with Ctrl-exo, SR-exo or MR-exo treated with saline or Ang II (angiotensin II). **B**, Ejection fraction (EF), fractional shortening (FS), and left ventricular (LV) mass of wild-type (WT) mice injected with Ctrl-exo, SR-exo, or MR-exo treated with saline or Ang II. **C**, Hematoxylin-eosin (H&E) and wheat germ agglutinin (WGA) staining and quantitative analysis of cardiomyocyte size in heart tissue of WT mice injected with Ctrl-exo, SR-exo, or MR-exo treated with saline or Ang II. Scale bar: 500 and 30 μm. **D**, Masson trichrome staining, collagen I, and α-smooth muscle actin (α-SMA) immunohistochemical staining in heart tissue of WT mice injected with Ctrl-exo, SR-exo or MR-exo treated with saline or Ang II. Scale bar: 100 μm. **E**, Quantification of cardiac fibrosis in heart tissue of WT mice injected with Ctrl-exo, SR-exo, or MR-exo treated with saline or Ang II. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey multiple comparisons test (n=6).

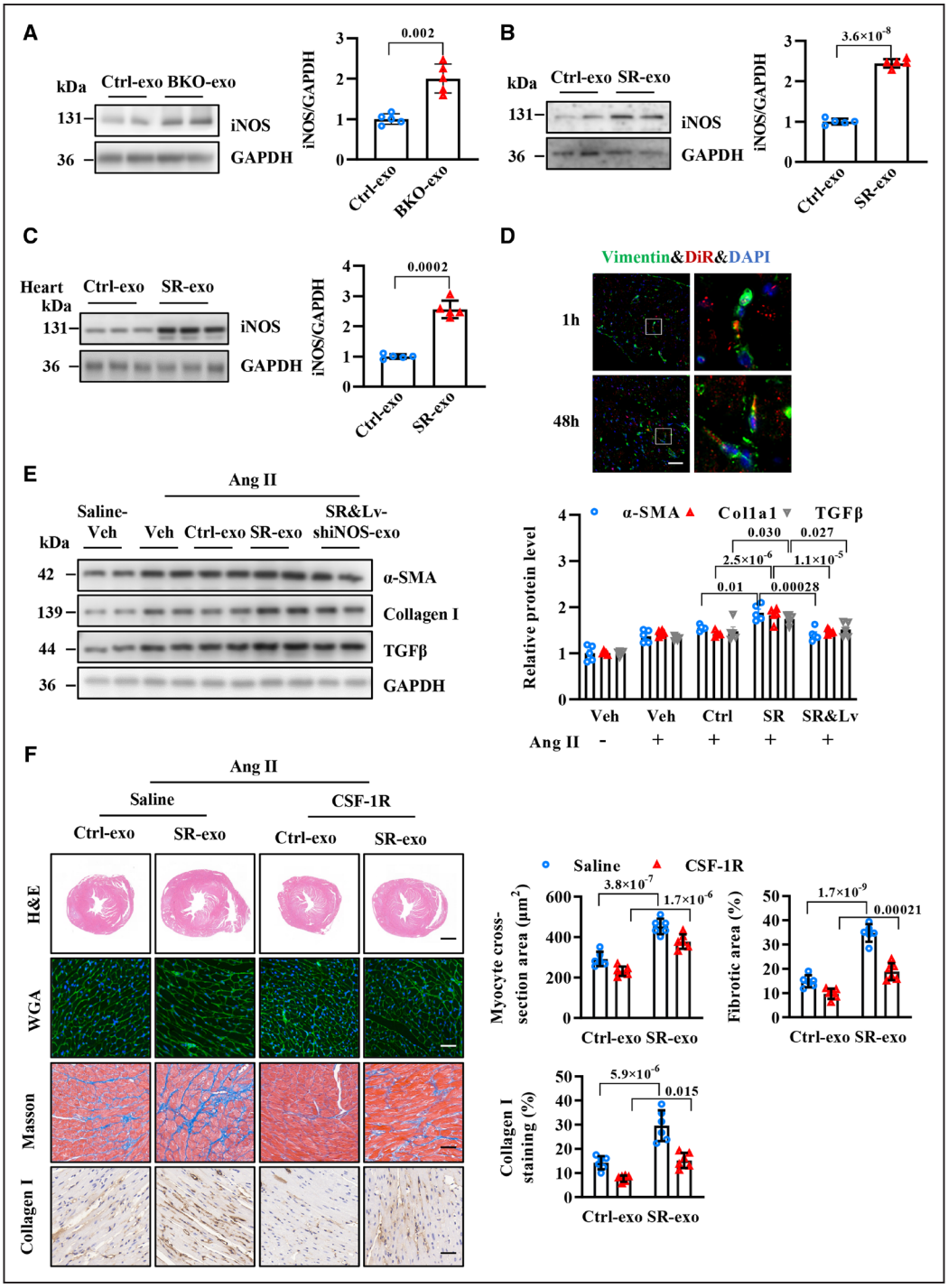

Figure 5. Exosomes transport iNOS (inducible nitric oxide synthase) from brown adipocytes to fibroblasts.
A, Western blot and quantitative analysis of iNOS in brown adipocyte-derived exosomes of brown adipocyte-specific ADRB3 knockout (BKO) and control (Ctrl) mice treated with Ang II (angiotensin II). Significant differences were examined by unpaired 2-tailed Mann-Whitney *U* test (n=5). **B**, Western blot and quantitative analysis of iNOS in Ctrl-exo and SR-exo. Significant differences were examined by unpaired 2-tailed Mann-Whitney *U* test (n=5). **C**, Western blot and quantitative analysis of iNOS in heart tissue of wild-type (WT) mice injected with Ctrl-exo or SR-exo under Ang II treatment. Significant differences were examined by unpaired 2-tailed Mann-Whitney *U* test (n=5). **D**, Immunofluorescence of DiR-labeled (red) brown adipocyte-derived exosomes in heart tissue (Vimentin, green) 1 and 48 h after the tail-vein injection in vivo. **E**, Western blot and quantitative analysis of α-smooth muscle actin (α-SMA), Col1a1 (collagen type I alpha 1), and TGFβ (transforming growth factor β1) in cardiac fibroblasts (CFs) cultured with SR-exo or SR&Lv-shiNOS-exo (SR&Lv; exosomes isolated from primary brown adipocytes treated with iNOS-silencing Lentivirus virus construct and SR). Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=5). **F**, Hematoxylin-eosin (H&E), wheat germ agglutinin (WGA), Masson trichrome staining, and collagen I immunohistochemical staining in heart tissue of mice injected with saline or anti-CSF-1R antibody during Ang II infusion. Scale bar: 100 μm. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey multiple comparisons test (n=6).

To further demonstrate the role of iNOS-containing exosomes in cardiac function, we prepared the Lv-shiNOS (lentiviral vectors to silence iNOS). Primary brown adipocytes were treated with SR and Lv-shiNOS and the CFs were incubated with the isolated exosomes (SR&LvshiNOS-exo). SR-exo-aggravated CFs proliferation, myocyte hypertrophy, hypertrophic and fibrotic gene expression were reversed by SR&LvshiNOS-exo (Figure 5E and Figure S6F through S6H).

Previous studies indicated that heart and macrophage-derived iNOS contributes to cardiac remodeling after Ang II infusion.[34,35] To further determine the role of macrophage in cardiac remodeling after exosome injection, we used neutralizing antibody CSF-1R to deplete macrophages in vivo (Figure S7A). Although SR-exo injection increase the protein level of iNOS, macrophages depletion significantly decreased iNOS protein level (Figure S7B). Echocardiographic examination and histological analyses showed that macrophage depletion remarkably improved cardiac remodeling in both control-exo and SR-exo treated mice with Ang II infusion. However, SR-exo accelerated Ang II–induced cardiac remodeling regardless of the presence or absence of macrophage depletion (Figure S7C and Figure 5F).

To further determine the important role of iBAT-derived iNOS in cardiac remodeling, Cre-dependent adeno-associated virus-DIO-shRNA against iNOS were injected into iBAT of *Ucp1*-cre mice to acquire BAT specific iNOS knockdown mice (Figure S8A). Western blot analysis confirmed the significant decrease expression of iNOS in iBAT and heart (Figure S8B). Consistent with the macrophage depletion, echocardiographic examination and histological analyses showed that BAT specific iNOS knockdown remarkably improved cardiac remodeling with Ang II infusion (Figure S8C and S8D).

These results suggest that ADRB3 inhibition-mediated iNOS production in brown adipocytes contributes to cardiac remodeling after Ang II infusion.

ADRB3/iNOS Pathway in Brown Adipocytes Impair Cardiac Function

Previous studies showed that ERK, p38, and JNK are involved in the regulation of iNOS expression.[36] We found that ADRB3 knockout inhibited ERK phosphorylation in iBAT of Ang II–infused mice. In contrast, p38 and JNK phosphorylation did not change in ADRB3 knockout iBAT after Ang II infusion (Figure 6A).

We then used Ang II, mirabegron, and ERK inhibitor PD98059 to pretreat brown adipocytes in vitro. Mirabegron-inhibited iNOS expression and exosomal iNOS level was blocked after PD98059 treatment accompanied with downregulation of ERK phosphorylation (Figure 6B). These data suggest that ADRB3-mediated iNOS expression suppression is most likely mediated through promoting ERK phosphorylation in brown adipocytes.

iNOS Silencing in Brown Adipocytes Protects Against Cardiac Remodeling

To further investigate the role of iNOS-containing exosomes in cardiac function in vivo, SR-exo, and SR&LvshiNOS-exo were injected into the Ang II treated mice as described earlier. SR-exo-induced decreases in ejection fraction, fractional shortening, and increases in left ventricular mass were significantly diminished in SR&LvshiNOS-exo mice (Figure 7A and 7B). Similarly, SR&LvshiNOS-exo improved cardiac hypertrophy and fibrosis, as evidenced by decreases in myocyte area, fibrotic area, α-SMA, and Col1a1 expression and levels of hypertrophic and fibrotic genes (Figure 7C through 7F). Collectively, these results demonstrate that iNOS silencing in brown adipocytes elicits cardiac protective effects.

DISCUSSION

Here, we reveal an endocrine crosstalk between BAT and the heart that is mediated by exosomal delivery of iNOS. We report that ADRB3-BKO attenuates BAT thermogenic activity and accelerates Ang II–induced cardiac remodeling. ADRB3 deletion in BAT leads to the expression and release of iNOS in exosomes into the circulatory system and subsequently, iNOS-containing exosomes target CFs and induce cardiac fibrosis. Besides, intravenous injection of SR-exo significantly aggravates Ang II–mediated cardiac hypertrophy, while MR-exo protects against the Ang II–induced cardiac dysfunction (Figure 8). Importantly, the silencing of iNOS in brown adipocytes blocks the effects of SR-exo in aggravating cardiac remodeling. Our study highlights the role of BAT ADRB3-mediated exosome production in regulating cardiac function and pinpoints iNOS as a critical exosomal component that participates in CF dysfunction in hypertensive mice.

Prolonged activation of β1- and β2-adrenoceptors leads to cardiovascular damage and β-adrenergic blockers were used to prevent or treat heart failure.[37] Accumulating evidence has demonstrated that unlike the classical β1-and β2-ARs, ADRB3 activation may result in inhibition of myocardial contractility in response to hemodynamic stress.[38,39] Besides the heart, we found that ADRB3 is present in many types of tissues and abundantly expressed in iBAT. We previously found that ADRB3 was significantly increased in the perivascular adipose tissue of the Doca-salt hypertensive model and ADRB3 antagonist SR59230A treatment aggravated the vascular damage.[40] However, the role of BAT ADRB3 in the cardiovascular system is not clear. BAT is an important endocrine organ, and healthy BAT plays a cardiovascular protective role.[41,42] We detected the blood pressure in the studied group and found that ADRB3 KO in BAT did not change blood pressure in Ang II–infused animals,

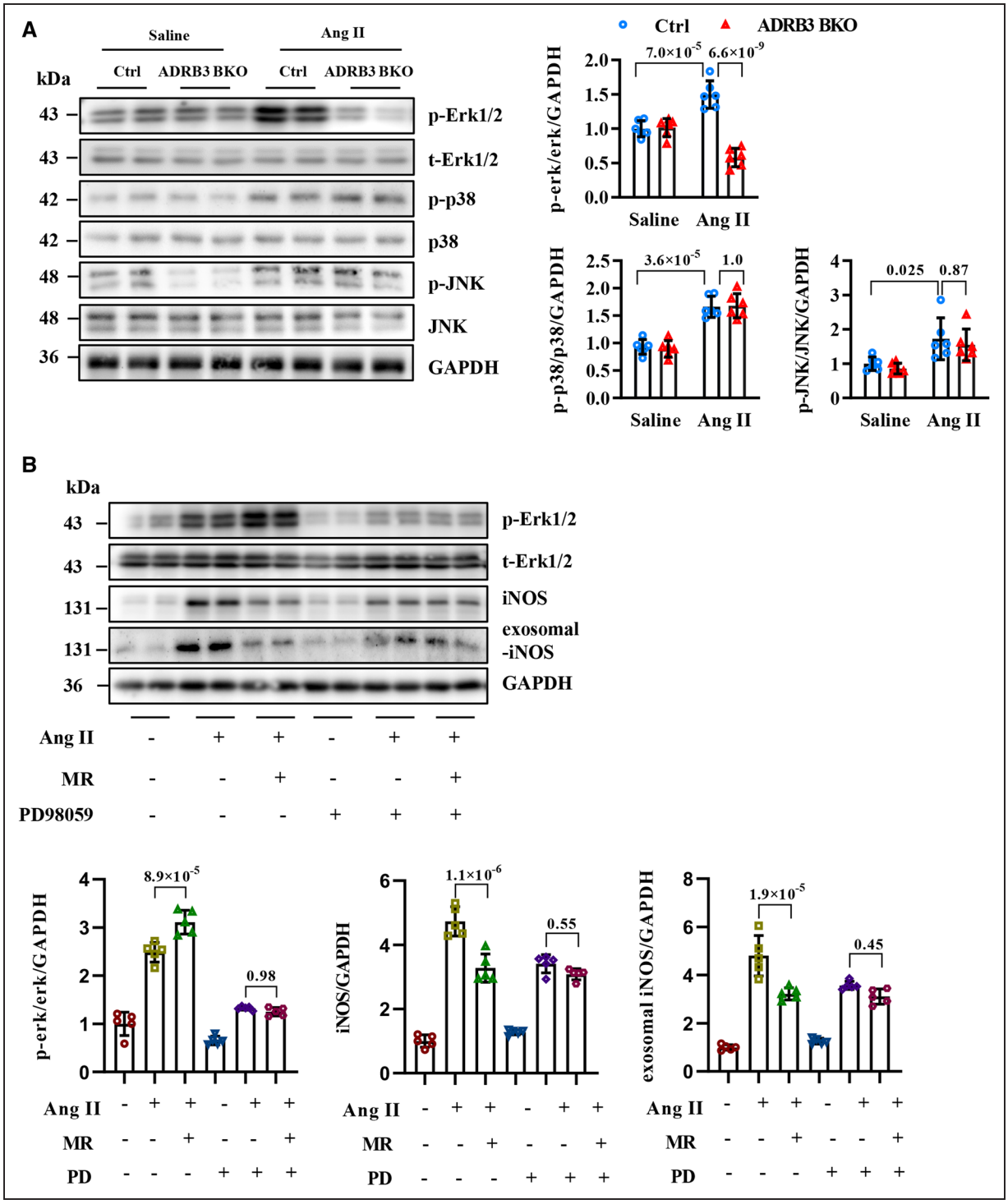

Figure 6. ADRB3 (β3-adrenergic receptor)/iNOS (inducible nitric oxide synthase) pathway in brown adipocytes impair cardiac function.

A, Western blot and quantitative analysis of ERK, p38, JNK phosphorylation in brown adipose tissue from BKO and Ctrl mice treated with saline or Ang II (angiotensin II). Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by Tukey multiple comparisons test (n=6). **B**, Western blot and quantitative analysis of phosphorylation, iNOS, exosomal iNOS in brown adipocytes treated with MR or PD98059 with or without Ang II. Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=5).

suggesting that ADRB3 KO-induced cardiac damage is blood pressure-independent. We demonstrated that ADRB3-BKO decreased BAT activity and aggravated Ang II–induced cardiac hypertrophy and fibrosis. Ang II, the major bioactive component of the renin-angiotensin system, is involved in regulating adipose functions and

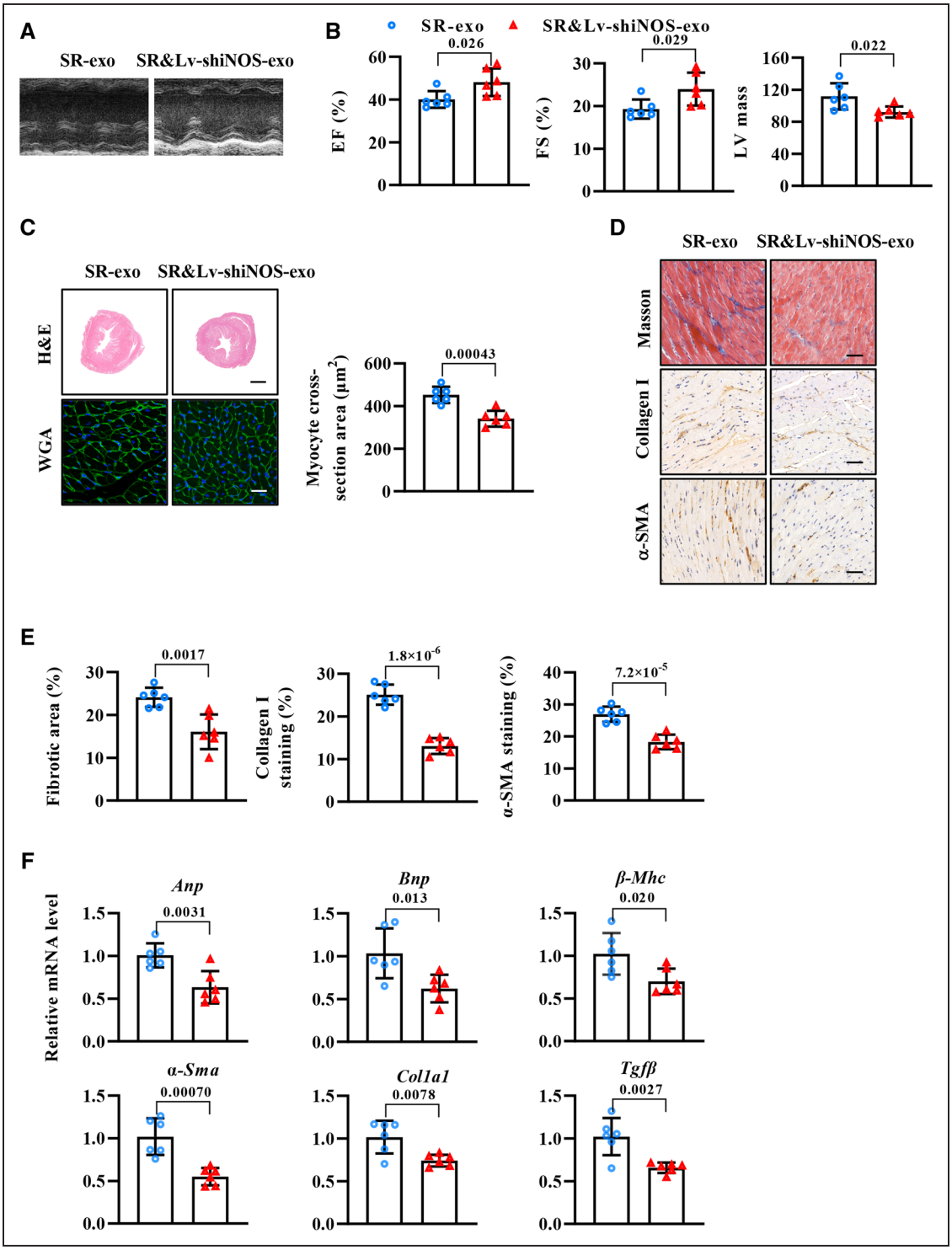

Figure 7. iNOS (inducible nitric oxide synthase) silencing in brown adipocytes protects against cardiac remodeling.
A, Representative M-mode echocardiography of wild-type (WT) mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II (angiotensin II) treatment. **B**, Ejection fraction (EF), fractional shortening (FS), and left ventricular (LV) mass of WT mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II treatment. **C**, Hematoxylin-eosin (H&E) and wheat germ agglutinin (WGA) staining and quantitative analysis of cardiomyocyte size in heart tissue of WT mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II treatment. Scale bar: 500 and 30 μm. **D**, Masson trichrome staining, collagen I, and α-smooth muscle actin (α-SMA) immunohistochemical staining in heart tissue of WT mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II treatment. Scale bar: 100 μm. **E**, Quantification of cardiac fibrosis in heart tissue of WT mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II treatment. **F**, qRT-PCR of *Anp*, *Bnp*, *β-Mhc*, *α-Sma*, *Col1a1*, and *Tgfβ* genes in heart tissue of WT mice injected with SR-exo or SR&Lv-shiNOS-exo under Ang II treatment. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired *t* test (n=6).

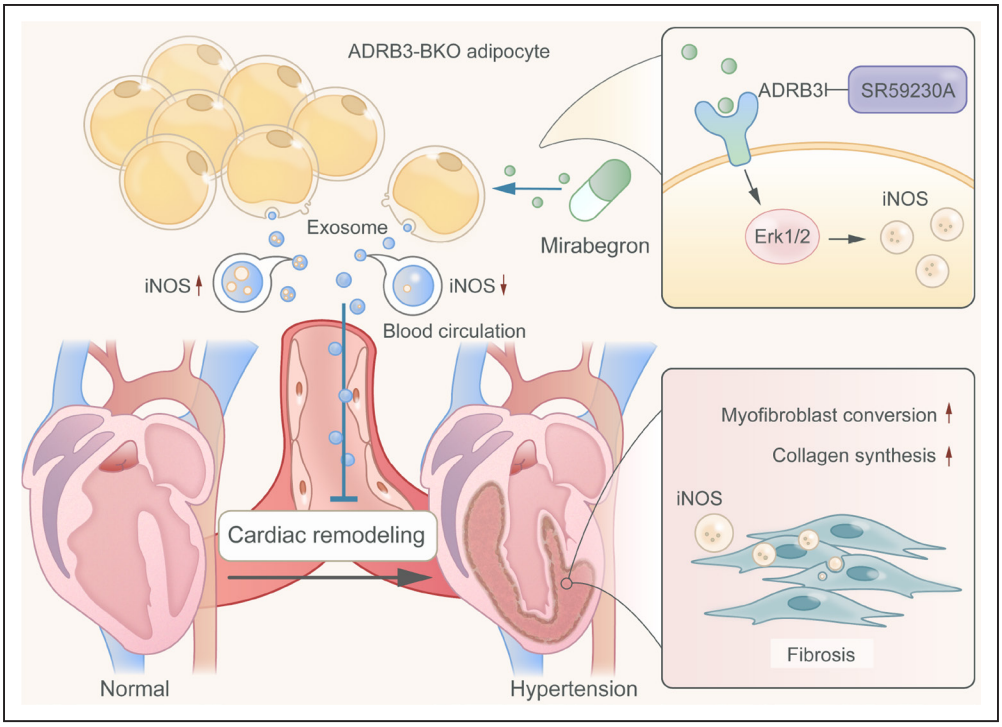

Figure 8. Schematic illustration of ADRB3 (β3-adrenergic receptor)-brown adipocyte exosomes-iNOS (inducible nitric oxide synthase) pathway in cardiac remodeling.

remodeling. Recent studies indicate that dysfunctional adipose tissue contributes to increased activity of the renin-angiotensin-aldosterone system, which is thought to play a crucial role in the pathogenesis of cardiovascular diseases.[43] Here, we revealed that Ang II promoted the iBAT browning and these effects were absent in ADRB3 BKO mice. These findings indicate that ADRB3 could represent an undervalued mechanism for a vicious circle between Ang II and BAT remodeling, which further contributes to cardiac damage.

Exosome, small heterogeneous microvesicle, has recently garnered great clinical interest as they carry diverse cargoes including proteins, lipids, and nucleic acids, and influence recipient cell function. Exosomes have been shown to play an important role in maintaining cardiovascular homeostasis.[18,44] Cardiomyocytes mediate antiangiogenesis through the exosomal transfer of mir-320 into endothelial cells in type 2 diabetic rats.[45] Mesenchymal stromal cell-derived exosomes prevent pulmonary inflammation and the development of pulmonary hypertension in the murine model.[46] In the present study, we provide in vitro and vivo evidence that BAT-derived exosomes were taken up by CFs and the heart. Furthermore, we reveal that ADRB3 knockout in BAT leads to the expression and release of iNOS in exosomes and eventually cardiac dysfunction. Our results showed that BAT-derived exosomes play a key role in distant communications between the BAT and heart. Importantly, special attention should be paid to the role of BAT-derived exosomes in other tissues in future studies.

NO is involved in the vascular contractility and regulation of cardiovascular tissue remodeling and metabolism. NO is synthesized by 3 NOS isoforms: nNOS, iNOS, and eNOS.[47] iNOS-derived NO has been associated with the pathogenesis and progression of cardiovascular diseases.[48,49] iNOS deficiency lowers plasma lipid peroxides and reduces atherosclerosis in apolipoprotein E-knockout mice.[50] We found that only the expression of iNOS was significantly increased in BAT-delivery exosomes of BKO mice. iNOS silencing using lentivirus blocks the adverse effects of SR-exo in cardiac tissues and CFs. Our data imply that iNOS might be secreted from BAT and transported in exosomes that may target cardiac tissue and contribute to cardiac remodeling. Previous studies indicated that Ang II stimulated ERK phosphorylation in cardiomyocytes and VSMCs without noradrenaline[51,52] and increased iNOS level in aorta and macrophages.[53,54] In this study, we showed that Ang II activates ERK and increase iNOS in adipocytes, which maybe through other signaling pathway that is ADRB3 independent. Nevertheless, additional research is needed to further delineate the detail mechanism that involved.

ADRB3 recently gained much attention and the current clinical practice of mirabegron is being tested in patients with structural cardiac disease, highlighting ADRB3's great potential as a novel therapeutic target.[55] To elucidate role of brown adipocyte-derived iNOS in mirabegron cardioprotection, we injected the adeno-associated virus-DIO-iNOS into the iBAT of *Ucp1*-cre mice to acquire BAT specific iNOS overexpression mice.

Echocardiographic examination and histological analyses showed that BAT specific iNOS overexpression significantly reversed a large part of cardioprotective effects after mirabegron treatment (Figure S9).

In summary, here, we report that exosomes from ADRB3 knockout-brown adipocytes promote cardiac remodeling by iNOS delivery. Our data illustrated an important role of ADRB3 in endocrine crosstalk between BAT and the heart via exosomal delivery, suggesting ADRB3-engineered exosomes might be a novel therapeutic agent for cardiac remodeling.

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Disclosures

None.

Supplemental Materials

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