SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture
HEK293 (WTβ1AR-Flag, PKAβ1AR-Flag and GRKβ1AR-Flag) and H9c2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 200 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were then transfected with 1 μg of cDNA encoding for S1PR1 coniugated with GFP (S1PR1-GFP) while H9c9 cells were also transfected with 1 μg of cDNA encoding for WTβ1AR-Flag. All transfections were performed using FuGENE6 reagent according to the manufacturer’s instructions (Roche Applied Science). Following transfection cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, 10 mM HEPES (pH 7.4), and 1% penicillin prior to stimulation. Under serum starvation conditions, cells were stimulated as described in the figure legends.

Immunoblotting
Cells and left ventricular (LV) samples were lysed in a RIPA buffer with protease and phosphatase inhibitors cocktail (Roche). Protein concentrations in all lysates were measured using a dye-binding protein assay kit (Bio-Rad) and a spectrophotometer reader (Biorad) at a wavelength of 750 nm. Phosphorylation of ERK 1/2 was detected by protein immunoblotting using a 1:1000 anti-mouse IgG (Cell Signaling). β1AR was detected by using 1:1000 anti-rabbit IgG (Thermo Scientific). GRK2 was detected by using 1:1000 anti-mouse IgG (Santa Cruz). S1PR1 was detected by using 1:1000 anti-mouse IgG (abm). Secondary antibodies were
purchased from Amersham Life Sciences Inc. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Life Sciences Inc.) according to the manufacturer’s instructions, and were quantified using densitometry (Chemidoc, Biorad, USA). Each experiment and densitometric quantification was separately repeated at least three times.

_**Plasma membrane proteins fractionation and S1PR1 levels analysis**_

Membrane protein purification was performed using a membrane protein extraction kit (Biovision) according to manufacturer instructions. Briefly, 100 mg of LV section were lysed in 1 mL of Homogenization Buffer. Then centrifuged at 700 g for 10 minutes at 4°C. The supernatant (cytosol fraction) was stored at -80°C while pellet (total cellular membrane protein) was further processed in order to separate organelles membrane from plasma membranes. Plasma membrane proteins were dissolved in RIPA buffer and used to evaluate the levels of S1PR1 by immunoblot. Na⁺/K⁺ ATPase (Upstate) antibody (1:1000 anti-mouse IgG) and an anti-Actin antibody (Santa Cruz) were respectively used as plasma membrane purification and loading controls.

_**Measurement of hypertrophic growth in H9c2 cardiomyoblasts**_

H9C2 cells were grown on glass coverslip. Following co-transfection, the cells were serum starved and treated with ISO or S1P and pre-treated also with selective β1AR blocker metoprolol tartrate (MET, 1 μM; MP biomedical) or S1PR1 antagonist W146 (10 μM; Sigma-Aldrich) as described in figure legends.

_**TUNEL staining**_
Tunel staining was performed with the use of an ApopTag Fluorescein Direct in Situ Apoptosis Detection kit (Chemicon, UK) according to the manufacturer’s instructions. Images were visualized by specific green fluorescence and nuclei by 4’-6-diamidino-2-phenylindole (DAPI) (nuclear counterstain). The TUNEL positives cells were examined with a microscope (Nikon TE-2000 U) and images were acquired with a digital camera (Nikon).

**Rat MI model**

30 Wistar Kyoto male rats (300 gr.) entered the study and underwent surgically induced myocardial infarction (MI) by permanent ligation of the left anterior descending coronary artery (LAD). Mortality rate was ~25% at 8 weeks post-MI and raised to ~30% the end of the study period.

**Myocardial in vivo gene delivery**

8-weeks post-MI (HF) a total of 4X10^{11} total particles of rAAV6-S1PR1 or rAAV6-GFP in a total volume of 500 μL were injected using a 32½ G needle in five different area of the LV free wall (two injections into the anterior wall, two into the posterior and one into the lateral wall immediately above the infarct scar).

8 weeks following rAAV6-GFP (n=5 for each delivery technique) in vivo gene delivery to HF rats using an Olympus IX 71 microscope, a mercury arc light and suitable filters. Moreover, transfection efficiency of *in vivo* gene transfer was assessed by GFP fluorescence (510 nm) in sectioned hearts (10 μm) using an Olympus IX81 confocal microscope.

**In vivo Gene Therapy Efficiency**

The percentage (%) of GFP-stained isolated myocytes was assessed as previously described (23) and briefly reported in the online supplemental methods.

**Immunohistochemistry**
LV specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and re-hydratation, 4 μm-thick sections were prepared and mounted on glass slides. Sections were deparaffinized, rinsed in xylene, and rehydrated. Subsequently they were quenched with 0.3% hydrogen peroxide, washed in water, treated with 2% bovine albumin in PBS and incubated with the primary antibodies at 4°C overnight. After being washed in PBS the primary antibody was detected with biotinylated anti-mouse IgG for 1 hour at room temperature. Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin (1:5000; Dako), and developed with 3,3-diaminobenzidine. Negative controls (Blank) were prepared by substitution of the primary antibody with an irrelevant antibody. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**Capillary density**

To determine capillary density, LV sections were incubated with a biotinilated lectin from Bandeiraea simplicifolia (Sigma) and amplified by a Tyramide Signal Amplification (TSA) Biotin System kit (Perkin Elmer Life Sciences, MA). Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin (1:5000; Dako), and developed with 3,3-diaminobenzidine. Negative controls (Blank) were prepared by substitution of the primary antibody with an irrelevant antibody. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**Echocardiography**

Echocardiography was performed on conscious mice (following 7 days of ISO treatment) and on rats (8-weeks post-MI and 12-weeks following gene therapy) respectively, with a Vevo770 (VisualSonics) or an HDI 5000 (Philips) echocardiograph.
Catheter-based in vivo hemodynamic measurements

Cardiac function was measured 12 weeks following gene therapy (20 weeks after MI) in anesthetized rats (2% isofluorane; v/v) using 2 F pressure catheter (SPR-320; Millar instruments; Houston, TX). The pressure transducer was placed into the LV cavity through the right carotid artery and the right external jugular vein was cannulated with a P-10 catheter (Becton-Dickinson, Sparks, MD) that was used for ISO administration (333 ng/Kg BW).

Measurement of infarct size

Briefly, hearts were frozen in liquid nitrogen and sectioned from apex to base into 2-mm slices. To delineate the infarct size, sections were incubated in 1% (wt/vol) triphenyltetrazolium chloride (TTC,Sigma) in PBS (pH 7.4) at room temperature for 15 min. For each section, the infarct size of the LV was calculated from enlarged digital photos using SigmaScan 5.0 software.

Co-Immunoprecipitation (Co-IP) assay

WTβ1AR-Flag cells were transfected with cDNA encoding for S1PR1-GFP. Following stimulation with ISO (1µM) or S1P (250 nM) for 30 minutes the cells were lysed and subjected to Co-IP assay, using a commercial kit (Pierce), according to manufacturer instructions. IP of S1PR1 was performed using an Anti-GFP antibody (Upstate). Total lysates and Co-IP elutions were then subjected to immunoblot. For β1AR immunoblot an anti-Flag antibody (Sigma) was used.

Treatment protocol for mice

As previously described (1), C57BL/6 mice (n=5) were intra-peritoneal injected, with SEW2871, dissolved in DMSO Tween 20, at the total rate of 1 mg/kg/d over a period of 7 days. Control mice (SHAM, n=5) were injected with vehicle (0.002% ascorbic acid). At sacrifice, after heart
weight (HW) and body weight (BW) ratio calculation, the hearts were removed and cardiac chambers dissected.

*SphK1 and Akt immunoblots*

SphK1 was detected by using 1:1000 anti-mouse IgG (Santacruz). Phosphorylation of Akt was detected by protein immunoblotting using a 1:1000 anti-mouse IgG (Cell Signaling). Total Akt was detected by protein immunoblotting using a 1:1000 anti-rabbit IgG (Cell Signaling).

*ELISA assay*

Total blood serum S1P levels were measured using a commercial kit (Echelon), according to manufacturer instructions. 1 mL of blood was collected from rat groups (n=10 of each group) at the end of the study period prior to heart explantation. Then the blood was centrifuged to 2000 rpm a 15°C for 15 minutes and 25 μL of serum were used for the ELISA assay.

*Hematoxylin and Eosin staining*

Left ventricular specimens were fixed in 4% formaldehyde and embedded in paraffin. After de-paraffinization and re-hydratation, 4 μm-thick sections were prepared, mounted on glass slides and were stained with hematoxylin for 5 min and with eosin for 2 min. Then the slides were washed for 30 sec with tap water and then rapidly dehydrated with 100% ethanol and mounted. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

**References**

Supplementary Figure legends

Supplementary Figure 1

β1AR and S1PR1 reciprocal interaction

A. Representative panels of Co-IP assay in total lysates from HEK293 cells stably expressing WTβ1AR-Flag-S1PR1-GFP. Immunoprecipitated proteins (IP) for GFP (S1PR1) were blotted with an antibody anti-Flag (β1AR).

Supplementary Figure 2

The lack of GRKs-phosphorylation sites on S1PR1 inhibits the cross-talk between β1AR and S1PR1

A. HEK293 cells stably expressing WTβ1AR-Flag transfected with cDNA encoding for S1PR1-GFP or S1PR1-Δ32-GFP were pre-treated with β2AR antagonist ICI-118,551-HCl (ICI, 10 μM), then were stimulated with (-)-isoproterenol bitartrate (ISO) (1 μM) or sphingosine 1-phosphate (S1P) (250 nM) for 30 min and compared with unstimulated (NS). Representative panels of S1PR1-GFP, S1PR1-Δ32-GFP and β1AR-Flag immunofluorescence images, showing cumulative data of multiple independent experiments. Arrows indicate receptor internalization; Representative immunoblots showing ERK1/2 activation following 5 min of stimulation with ISO (1 μM) or S1P (250 nM) in WTβ1AR-Flag. GAPDH was used as loading control.

Supplementary Figure 3
In vivo chronic S1PR1 agonism (7 days) resulted in a GRK2 upregulation and a β1AR plasma membrane downregulation.

A. Bar graphs showing the heart weight/body weight (HW/BW) ratio in SHAM and SEW2871-7d;

B. Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments to evaluate GRK2 levels in SEW2871-7d groups compared to SHAM (GRK2 levels Fold over SHAM);

C. Representative immunoblots (upper panels) and densitometric analysis (lower panel) of multiple independent experiments to evaluate β1AR plasma membrane levels in crude LV membrane preparations from SHAM and SEW2871-7d mice (βAR Membrane levels Fold over SHAM). ACTIN was used as loading control.

Data are expressed as means ± SEM. Statistical significance between groups was determined by Mann-Whitney exact test. N= 5 for each group. *p<0.05 vs SHAM.

Supplementary Figure 4

S1PR1 natural agonist levels are decreased during HF

A. Bar graphs showing S1P levels (µM) in blood serum collection from SHAM, HF+ rAVV6-GFP and HF+rAVV6-S1PR1 groups.

B. Representative immunoblots (upper panels) and densitometric analysis (lower panel) evaluating Sphingosine kinase 1 (SphK1) levels in HF+rAVV6-GFP and HF+rAVV6-S1PR1 groups compared to SHAM (SphK1 levels Fold over SHAM). GAPDH was used as loading control.
Data are presented as mean ± SEM. Statistical significance between groups was determined by one-way ANOVA with Bonferroni post-hoc correction. N= 10 for each group. *p<0.01 vs SHAM; †p<0.05 vs HF.

**Supplementary Figure 5**

**Reduced immune cells infiltration following S1PR1 gene therapy**

A. Representative Hematoxylin/Eosin panels of remote and infarcted region from cardiac sections of HF, HF+rAVV6-S1PR1 and HF+rAAV6-GFP rats, performed at the end of the study period;

**Supplementary Figure 6**

**S1PR1 gene-delivery preserve Akt activation**

A. Representative immunoblots (upper panels) and densitometric analysis (lower panel) evaluating pAkt levels in HF+rAVV6-GFP and HF+rAVV6-S1PR1 groups compared to SHAM (pAkt levels Fold over SHAM). tAkt was used as loading control.

Data are presented as mean ± SEM. Statistical significance between groups was determined by one-way ANOVA with Bonferroni post-hoc correction. N= 10 for each group. *p<0.01 vs SHAM; †p<0.05 vs HF.
Supplementary Figure 1

A

IP: Anti-SIPR1

Total Lysates

IB: β1AR

KS WT ISO WT SIP

KS WT ISO WT SIP

β1AR
Supplementary Figure 5

A

SHAM      HF      HF+AAV6-S1PR1

Remote

Infarct