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## **β1-Adrenergic Receptor and Sphingosine-1-Phosphate Receptor 1 Reciprocal Down-Regulation Influences Cardiac Hypertrophic Response and Progression Toward Heart Failure: Protective Role of S1PR1 Cardiac Gene Therapy**

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### **Abstract**

**Background**—The Sphingosine-1-phosphate receptor 1 (S1PR1) and β1-adrenergic receptor (β1AR) are G protein-coupled receptors (GPCRs) expressed in the heart. These two GPCRs have opposing actions on adenylyl cyclase due to differential G protein-coupling. Importantly, both of these receptors can be regulated by the actions of GPCR kinase-2 (GRK2), which triggers desensitization and down-regulation processes. Although, classical signaling paradigms suggest that simultaneous activation of β1ARs and S1PR1s in a myocyte would simply be opposing action on cAMP production, in this report we have uncovered a direct interaction between these two receptors with a regulatory involvement of GRK2.

**Methods and Results**—In HEK293 cells overexpressing both β1AR and S1PR1, we demonstrate that β1AR down-regulation can occur after sphingosine 1-phosphate (S1PR1 agonist) stimulation while S1PR1 down-regulation can be triggered by isoproterenol (βAR agonist) treatment. This cross-talk between these two distinct GPCRs appears to have physiological significance since they interact and show reciprocal regulation in mouse hearts undergoing chronic βAR stimulation and also in a rat model of post-ischemic heart failure (HF).

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**Conclusions**—We demonstrate that restoring cardiac plasma membrane levels of S1PR1 produce beneficial effects counterbalancing deleterious  $\beta$ 1AR overstimulation in HF.

### Keywords

gene therapy; heart failure; hypertrophy; receptors; adrenergic; beta; signal transduction

### Introduction

G protein-coupled receptors (GPCRs) transduce cell signals via heterotrimeric G proteins from neurohormones, ions, and sensory stimuli to regulate every aspect of mammalian physiology. GPCRs are regulated by GPCR kinases (GRKs) that trigger termination of signaling, a process known as desensitization. Phosphorylation of agonist-occupied receptors by GRKs induces recruitment and binding of  $\beta$ -arrestins that displace bound G proteins, therefore uncoupling receptors from their downstream signaling effectors. This process continues through  $\beta$ -arrestin-dependent internalization of receptors, that lead either to their degradation and down-regulation or recycling (resensitization) to the membrane. Moreover,  $\beta$ -arrestin recruitment to GRK-phosphorylated receptors has been shown to lead to novel intracellular signaling, a process called G protein-independent signaling<sup>1</sup>. In the heart, an important GRK-mediated  $\beta$ -arrestin-dependent signal has been shown to be ERK MAP kinase activation<sup>2</sup>. Given the central role in cardiac pathophysiology, GPCRs are critical therapeutic targets in cardiac diseases. This is especially true in heart failure (HF) where  $\beta$ -adrenergic receptor ( $\beta$ AR) antagonists and angiotensin II receptor blockers are standard of care for human HF patients.

$\beta$ AR and angiotensin II receptor blockade are warranted as a consequence of the sympathetic nervous system (SNS) and renin-angiotensin system (RAS) hyperactivity that induces their overstimulation. This phenomenon represents the molecular basis for  $\beta$ AR down-regulation in the failing human myocardium. Since GPCRs are dynamically regulated in disease processes a better understanding of down-stream signaling is imperative. In particular, GPCR dimerization<sup>3</sup> and interaction between different GPCR signaling pathways<sup>4,5</sup> have become the cornerstone of current cardiovascular research in order to better clarify molecular alterations underlying cardiovascular diseases and to identify novel potential therapeutic targets. In cardiac physiology and pathophysiology, the  $\beta$ 1AR is the predominant  $\beta$ AR that regulates inotropic and chronotropic responses of SNS catecholamines through Gs-dependent activation of adenylyl cyclase (AC)<sup>6,7</sup>. Chronic  $\beta$ 1AR hyperstimulation results in their down-regulation and consequently there is a marked reduction of the inotropic reserve of the failing heart<sup>8,9</sup>. The sphingosine-1-phosphate receptor 1 (S1PR1), which mediates the effect of the lysophospholipid sphingosine-1-phosphate (S1P, a natural agonist) is also expressed on cardiomyocytes<sup>10</sup> and its signaling opposes  $\beta$ 1AR-mediated AC activation through its coupling to the AC-inhibitory G protein, Gi<sup>11</sup>. Thus, in the heart, S1PR1 is able to antagonize the effects mediated by isoproterenol (ISO) and other  $\beta$ AR agonists<sup>11,12</sup>. Furthermore, S1PR1 and  $\beta$ 1AR undergo GRK-mediated regulation through phosphorylation<sup>13,14,15</sup>. Although GRK2, the primary GRK isoform expressed in myocytes, can regulate both receptors, the  $\beta$ 1AR is also regulated by phosphorylation by protein kinase A (PKA)<sup>13,14</sup>, while the S1PR1 can be regulated by protein kinase C (PKC)<sup>15</sup>. Importantly, in respect to novel non-canonical signaling both receptors have been implicated in ERK activation that can lead to protective signaling<sup>2</sup>.

Recently, a functional interaction between  $\beta$ AR and S1PR1 signaling has been reported *in vivo*. In fact, ISO administration in mice induces cardiac hypertrophy via engagement of the S1PR1 signaling pathway<sup>12</sup>. However, there is no proof of direct cross-talk at the receptor level between  $\beta$ 1ARs and S1PR1s. In the present study, we provide biochemical and

functional evidence of a direct connection between these two highly expressed GPCRs in the heart, demonstrating their reciprocal regulation via an important regulator, GRK2, which has potential significance for cardiac pathophysiology.

## Methods

### Cell Culture

Human embryonic kidney (HEK) 293 cells overexpressing the mouse wild type  $\beta$ 1AR carrying a Flag epitope (WT $\beta$ 1AR-Flag) or 2 mutants lacking, respectively: the putative PKA phosphorylation sites (PKA $\beta$ 1AR-Flag) and the putative GRK phosphorylation sites (GRK $\beta$ 1AR-Flag) obtained as previously described<sup>14</sup> and cardiomyoblasts H9c2 obtained from American Type Culture Collection (ATCC), were cultured and transfected as briefly described in online methods.

### Confocal microscopy

S1PR1-GFP and  $\beta$ 1AR-Flag internalization was visualized by confocal laser scanning microscopy (CLSM). Following (–)-isoproterenol bitartrate (ISO, 1  $\mu$ M; Sigma-Aldrich) or sphingosine-1 phosphate (S1P, 250 nM; Sigma-Aldrich) stimulation cells were fixed and visualized as previously described (16). After fixation cells were incubated with an anti-Flag Cy3 conjugated mouse IgG (Sigma-Aldrich) using a dilution 1:100 in PBS containing 0.5% BSA for 1h at room temperature. CLSM was performed at 488 nm (GFP) or 568 nm (Cy3). The fluorescent data sets were analyzed by LSM 510 software. The cells treated with ISO were pre-treated with selective  $\beta$ 2AR-antagonist ICI-118,551-HCl (ICI, 10  $\mu$ M; Sigma-Aldrich). Each experiment was separately repeated at least three times.

### Immunoblotting

Immunoblotting on cells and left ventricular (LV) samples were performed as previously described<sup>16</sup> and briefly reported in the online supplemental methods.

### Treatment protocol for mice

As previously described<sup>2,17,18</sup>, C57BL/6 mice (n=5) were subcutaneously injected, twice a day, with ISO, dissolved in 0.002% ascorbic acid, at the total rate of 3 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.

### Measurement of hypertrophic growth in H9c2 cardiomyoblasts

Hypertrophy was assessed by measurement of relative cell surface area of H9c2 cells as previously described<sup>19,20,21</sup> (please see online supplemental methods).

### TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on H9C2 cells (please see online supplemental methods).

### Rat MI model

Myocardial infarction (MI) in rats was performed as previously described<sup>22</sup> and briefly reported in the online supplemental methods.

### **Myocardial in vivo gene delivery**

Myocardial gene transfer in rats was achieved by direct intra-myocardial injection 8 weeks post-MI as previously described<sup>23</sup> and briefly reported in the online supplemental methods.

### **Echocardiography**

Echocardiography was performed as previously described<sup>22,24</sup> and briefly reported in the online supplemental methods.

### **Catheter-based in vivo hemodynamic measurements**

Cardiac function was measured 12 weeks following gene therapy as previously described<sup>23</sup>.

### **Myocardial perfusion studies**

Myocardial perfusion was determined using 15  $\mu\text{m}$  fluorescent microspheres (Triton Inc.). Cardiac and blood samples were processed for microspheres determination. Myocardial blood flow was measured basal and after maximal vasodilation with dipyridamole (6 mg  $\text{kg}^{-1} \text{min}^{-1}$  i.v.).

### **Measurement of infarct size**

Infarct size was examined in all experimental rats at the end of the study period, as previously described<sup>23</sup> and briefly reported in the online supplemental methods.

### **Histology**

Left ventricular paraffin embedded specimens were immunohistochemically stained for S1PR1 (anti-S1PR1 mouse monoclonal, 1:100; abm) and GFP (anti-GFP mouse monoclonal, 1:200; Upstate) or stained for capillary density determination as previously described<sup>16</sup> and briefly reported in online methods.

### **RT-PCR**

Total RNA was isolated from cardiac samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed to generate cDNA. To evaluate the expression of recombinant human S1PR1-GFP and GFP was performed a PCR using specific primers (hS1PR1-For 5'-CAGCAAATCGGACAATTCCT-3', hS1PR1-Rev 5'-GAACTTCAGGGTCAGCTTGC-3' GFP-For 5'-GACGTAAACGGCCACAAGTT-3' GFP-Rev 5'-AAGTCGTGCTGCTTCATGTG-3') with respectively amplified products of 250 bp and 180 bp.

### **$\beta$ -Adrenergic Receptor Radioligand Binding**

Receptor binding with 20  $\mu\text{g}$  of protein from plasma membrane was performed using [<sup>125</sup>I]cyanopindolol (350 pM) as previously described<sup>24</sup>. Receptor density (fmol) was normalized to milligrams of membrane protein.

### **Statistical analysis**

Normally distributed, continuous variables with sample size greater than 10 are expressed as mean  $\pm$  SEM and compared by one-way or repeated measures ANOVA test followed by Bonferroni post hoc correction, as appropriate. When sample size was less than 10, exact tests (Mann-Whitney or Kruskal-Wallis as required), followed by Bonferroni post hoc correction, were performed and data represented with median and dot plots. Normality was tested using Shapiro-Wilk test (<50 values) or Kolmogorov-Smirnov test ( $\geq$  50 values), as appropriate. All analyzed data showed no significant departure from normal distribution.

Statistical Analysis was performed using SPSS software (SPSS Inc., Chicago, Illinois). Level of statistical significance was set to  $p < 0.05$ .

## Results

### *In vitro* reciprocal downregulation of $\beta$ 1AR and S1PR1

In order to establish a functional correlation between  $\beta$ 1AR and S1PR1 signaling, HEK293 cells stably expressing the mouse wild-type  $\beta$ 1AR (WT $\beta$ 1AR) or 2 mutants lacking, respectively: the putative PKA phosphorylation sites (PKA $\beta$ 1AR) or the putative GRK phosphorylation sites (GRK $\beta$ 1AR) were transfected with S1PR1 cDNA. By confocal microscopy experiments we evaluated  $\beta$ 1AR and S1PR1 internalization following ISO and S1P stimulation. As shown in Figure 1A, ISO and S1P stimulations resulted in a marked loss of both WT $\beta$ 1AR and S1PR1 from the cell surface and surprisingly, in a co-localization of the two receptors in the cytosol peaking after 30 min of treatment. The absence of PKA phosphorylation sites (PKA $\beta$ 1AR) did not affect the reciprocal internalization and cytosolic co-localization following ISO or S1P stimulation (Figure 1B). In contrast, as shown in Figure 1C, in GRK $\beta$ 1AR cells, ISO stimulation only induced  $\beta$ 1AR but not S1PR1 internalization. On the other hand, S1P stimulation induced S1PR1 downregulation with no effect on  $\beta$ 1AR localization. Interestingly, ISO or S1P induced a similar significant increase in pERK levels in all cell subtypes (Figure 1A–B–C). To better determine whether  $\beta$ 1AR and S1PR1 form stable complexes,  $\beta$ 1AR and S1PR1 were expressed at same level in HEK293 cells and then a co-immunoprecipitation (Co-IP) assay was performed. Notably, as shown in Supplemental Figure 1, IP of S1PR1 resulted in a Co-IP of  $\beta$ 1AR in the absence (NS) or in presence of ISO or S1P, confirming our hypothesis of a direct receptor-receptor interaction.

To further investigate how GRK-dependent phosphorylation was involved in the cross-talk between  $\beta$ 1AR and S1PR1, we also used a mutated form of S1PR1 (S1PR1- $\Delta$ 32) lacking GRK2 phosphorylation sites<sup>15</sup>. As shown in supplementary Figure 2A, S1PR1- $\Delta$ 32 lost the ability to be co-internalized with  $\beta$ 1AR following agonists stimulation. Furthermore, ERK1/2 phosphorylation resulted increased following ISO stimulation but at a lower extent following S1P stimulation (Supplemental Figure 2A).

### $\beta$ 1AR- and S1PR1-dependent GRK2 upregulation

Since it is known that both  $\beta$ 1AR and S1PR1 are substrates of GRK2 phosphorylation<sup>13,14,15</sup>, and our data suggest a crucial role of this kinase in the co-dependence of internalization of these two receptors, we further explored the potential role of GRK2. A 2-fold increase in GRK2 levels in WT $\beta$ 1AR cells stimulated for 12 hrs either with ISO or S1P, was observed (Figure 2A). In PKA $\beta$ 1AR cells, ISO or S1P stimulation resulted in blunted, yet significant, GRK2 upregulation (Figure 2B). Importantly, GRK $\beta$ 1AR cells showed enhanced GRK2 expression only following S1P administration (Fig. 2C).

### *In vitro* protective role of S1PR1 on deleterious $\beta$ 1AR overstimulation

It is known that both  $\beta$ 1AR and S1P are able to induce cardiomyocyte hypertrophy<sup>24,25</sup>. Therefore, we tested the effects of chronic ISO and S1P administration *in vitro* in H9c2 cells stimulated for 48 hrs in absence or presence of the  $\beta$ 1AR antagonist metoprolol or the S1PR1 selective antagonist W146. Treatment with both agonists resulted in a consistent increase of cell surface area indicating a hypertrophic response that was blocked with the respective antagonist (metoprolol or W146) (Figure 3A). Notably, only ISO induced an increase in cardiomyocytes apoptosis (Figure 3B). This apoptotic response in ISO stimulated cells was prevented by metoprolol pre-treatment (Figure 3B). Interestingly, S1P induced a

robust hypertrophic response and blunted apoptotic reaction in metoprolol pre-treated cells (Figure 3A–B). Reciprocally, ISO stimulation in the presence of S1PR1 antagonist did not induce an hypertrophic response but increased apoptosis (Figure 3A–B).

### In vivo reciprocal $\beta$ 1AR and S1PR1 downregulation

As widely demonstrated<sup>2,17,18</sup>, chronic *in vivo* ISO administration induces cardiac hypertrophy, pathology and strong cardiac  $\beta$ 1AR plasma membrane downregulation. Thus, to determine whether our *in vitro* findings could be translated *in vivo*, we analysed S1PR1 density in crude myocardial membrane preparations from mice after 7 days of ISO administration. As expected, after chronic ISO stimulation mice exhibited a significant increase in Heart to Body Weight (HW/BW) ratio (Figure 4A), LV-septum thickness (Figure 4B) and a robust cardiac GRK2 upregulation compared to untreated mice (Figure 4C). Notably, 7 days of ISO administration resulted in a S1PR1 downregulation at the plasma membrane level (Figure 4D).

In addition, in order to evaluate whether S1PR1-selective agonist was able to induce a similar  $\beta$ 1AR downregulation *in vivo*, we treated mice for 7 days (via daily intraperitoneal injections) with the S1PR1 agonist, SEW2871 (Supplemental Figure 3). Interestingly, SEW-treatment resulted in a consistent increase in HW/BW ratio (Supplemental Figure 3A) and in a strong increase in GRK2 protein levels (Supplemental Figure 3B) compared to untreated mice. Consistently, with our *in vitro* observations, this S1PR1 selective agonist resulted in a robust decrease in  $\beta$ 1AR cardiac plasma membrane levels compared to untreated group (Supplemental Figure 3C).

### Cardiac S1PR1 membrane downregulation during heart failure

Since our data show that S1PR1 internalization occurs *in vitro* as well as *in vivo* following chronic ISO stimulation, we studied potential S1PR1 dysregulation in a clinically relevant experimental model of HF, a pathological condition characterized by a sustained elevation of circulating catecholamines. Accordingly, plasma membranes were extracted from LV lysates of 8 week post-myocardial infarction (post-MI) rats. Importantly, we found a significant down-regulation of S1PR1 in HF rats compared to control (Figure 5A). Accordingly, in order to assess whether S1PR1 down-regulation during HF was induced by an increase in S1P levels we performed an ELISA assay for S1P on blood serum and an immunoblots on LV lysates to assess the expression of Sphingosine kinase 1 (SphK1) in our HF and SHAM groups (Supplemental Figure 4A–B). Notably, both S1P circulating levels and SphK1 expression were robustly reduced in HF group compared to SHAM, proving that S1PR1 behaves similarly to  $\beta$ 1AR in a significant pathophysiological setting such as HF<sup>22</sup>. In contrast, S1PR1 gene therapy was able to restore SphK1 expression at the levels observed in sham and to increase S1P circulating levels compared to HF control group.

### In vivo S1PR1 gene therapy

Since we have demonstrated that S1PR1 signaling is beneficial in cardiomyocytes *in vitro*, and that this receptor is down-regulated in our animal model of post-MI HF, we next explored the effects of long-term S1PR1 receptor overexpression during HF. Adult male Sprague-Dawley rats underwent MI (n=30) or SHAM operation (n=10). 8 weeks post-surgery HF rats were randomly assigned to one of the following group: 1) HF Saline (HF, n=10); 2) HF Adeno-associated type 6-GreenFluorescent Protein (HF-rAAV6-GFP, n=10); 3) HF rAAV6-S1PR1-GFP (HF-rAVV6-S1PR1, n=10). A baseline echocardiogram was performed in all groups one day before treatment onset, to confirm the presence of similar levels of LV dysfunction before gene delivery. All groups were then studied over the course of 12 additional weeks (20 weeks after MI) (Figure 5B), and all assays in the HF groups were compared with a control SHAM-operated group that received neither MI nor gene



transfer. At 12 weeks after gene delivery, both transgenes (S1PR1 and GFP) were robustly expressed in the LV, as assessed by immunohistochemistry (Figure 5C). Consistently, RT-PCR analysis (Figure 5D) and immunoblots (Figure 5E) confirmed the expression of the human S1PR1 in the heart of rAAV6-S1PR1 group and of GFP in both hearts of rAAV6-S1PR1 and -GFP groups. As shown in Figure 5F, 20 weeks following MI, cardiac rAAV6-S1PR1 gene therapy resulted in restoration of plasma membrane S1PR1 at the level observed in SHAM animals.

### Effects of S1PR1 overexpression on in vivo cardiac function at 12 weeks after gene delivery

Eight weeks after experimental MI, LV ejection fraction (EF) was dramatically decreased and end-diastolic diameter was increased as expected (Figure 6A–B). Treatment with GFP or saline had no impact on cardiac performance with a further deterioration of cardiac function 12 weeks later. On the other hand, S1PR1 overexpression ameliorated LV contractility. In fact, EF was significantly increased in S1PR1 infected rats compared to HF controls (Figure 6B). Adverse LV remodeling as measured by ventricular dilatation also progressed further in saline and GFP groups, and this was prevented by S1PR1 gene delivery. As expected no differences in infarct size were observed among all HF groups since gene therapy was performed 8 weeks post-MI when the infarct scar was completely established (data not shown). Of note, S1PR1 gene delivery affected the immune response as observed by Hematoxylin/Eosin staining of cardiac sections. In fact, S1PR1 overexpression resulted in a reduced infiltration of immune cells compared to HF control group (Supplemental Figure 5A). Interestingly, S1PR1 overexpression in the LV induced a consistent decrease in heart rate. LV invasive hemodynamic analysis in rats performed at the end of the study period (12 weeks after gene delivery) showed significant decreases in  $+dP/dt$  and  $-dP/dt$  in all HF groups compared to SHAM, proving HF-related reduction in LV contractility and relaxation (Table 1 and Figure 6C). LV systolic pressure was significantly reduced, whereas LV end-diastolic pressure was significantly increased in all HF groups compared to SHAM (Table 1). Cardiac S1PR1 overexpression significantly improved LV contractility and relaxation 12 weeks after treatment (Table 1 and Figure 6C). Furthermore, S1PR1 gene delivery increased LV systolic pressure and decreased end-diastolic pressure compared to HF control groups (Table 1). Notably, total plasma membrane  $\beta$ AR density was completely restored in rAAV6-S1PR1 hearts compared with HF controls (Figure 6D). Accordingly, S1PR1 infected rats showed significantly improved LV  $+dP/dt$  and LV  $-dP/dt$  after maximal  $\beta$ AR stimulation by ISO (Table 1 and Figure 6C).

### Effect of S1PR1 on cardiac remodeling and angiogenesis

The beneficial effect of S1PR1 overexpression on cardiac function was accompanied by a hypertrophic response of the failing heart. This observation was evident at echocardiographic evaluation where we found increased anterior wall diastolic thickness (AWd) and posterior wall diastolic thickness (PWd) in S1PR1 rats compared to HF controls (Table 1). Consistently, HW/BW ratio was significantly higher in hearts treated with S1PR1 compared to GFP and saline treated groups (Table 1). Importantly, the S1PR1-dependent increase of LV mass was accompanied by significant decrease of LV systolic and diastolic internal diameter compared to rAAV6-GFP HF hearts, thus indicating that S1PR1 gene therapy induced a compensatory hypertrophic response able to contrast LV dilatation. In accordance with previous observations reported by us and others<sup>25,26</sup>, this adaptive LV remodeling was associated with a significant growth of cardiac capillary network. In fact, S1PR1 gene delivery resulted in a significant increase in capillary density compared to HF groups and a complete recovery of myocardial blood flow that was indistinguishable from SHAM (Figure 7A–B). At the molecular level, we investigated the effects on Akt activation since this kinase is one of the major pro-angiogenic molecules involved in S1PR1

signaling<sup>27,28</sup>. Notably, S1PR1 down-regulation was associated with a robust reduction in Akt activation compared to SHAM (Supplemental Figure 6A). Of note, S1PR1 gene therapy was able to enhance Akt activation (Supplemental Figure 6A).

## Discussion

Heart failure is a major and growing public health problem affecting 1–6% of the US population<sup>29</sup>. It is a disease characterized by LV dysfunction associated with a complex of symptoms that relate to inadequate perfusion of tissues and pulmonary congestion. One of the consequences is the activation of the SNS, which plays a crucial role in adapting circulatory homeostasis to changes in environment. Further, circulating levels of catecholamines are increased in HF in proportion to the severity of the disease<sup>30</sup>. However, sympathetic hyperactivity can also initiate or accelerate cardiac dysfunction and provoke major cardiovascular events, thus justifying why HF patients with higher plasma levels of norepinephrine have the most unfavorable prognosis<sup>31</sup>. These observations have led to the hypotheses that sympathetic activation may play an important role in HF progression<sup>8,32,33</sup> and that pharmacological interference with this system can produce hemodynamic and clinical benefits<sup>34</sup>.

Currently, some of the most effective treatments for HF target  $\beta$ 1AR and  $\beta$ 2AR and angiotensin II type IA receptor, which are both GPCRs<sup>4</sup>. Several experimental evidences have demonstrated that GPCRs can actually interact and can be reciprocally regulated. For instance, it has been shown that the  $\beta$ 1AR is able to transactivate EGFR, conferring a  $\beta$ -arrestin dependent cardioprotective effect<sup>2</sup>. These data have led to hypothesize the development of new therapies for HF that would be able not only to antagonize harmful cardiac signaling, but also potentiate the beneficial pathways<sup>35</sup>. In that regard, a deeper understanding of the signaling mechanisms underlying the development and progression of heart failure is absolutely needed.

Our data show, for the first time, a direct interaction between  $\beta$ 1AR and S1PR1, representing dynamic regulation present between two important and predominant GPCRs in the heart that appears to have significant physiological effects. Using HEK293 cells overexpressing the WT $\beta$ 1AR, and transiently transfected with S1PR1, we demonstrated *in vitro* that either ISO or S1P stimulation induce a dual-internalization with a cytosolic co-localization of both  $\beta$ 1AR and S1PR1. As a functional consequence, ERK activation occurs. Importantly, we also demonstrated that both  $\beta$ 1AR and S1PR1 are able to form a stable complex and that the reciprocal downregulation of the two receptors occurs only in presence of agonist-dependent GRK2 activation. In fact, we examined the molecular mechanisms of such interaction, and interestingly found that the absence of GRK-phosphorylation sites but not the absence of PKA-phosphorylation sites on the  $\beta$ 1AR abolished the cross talk between the two receptors. Similarly, the lack of GRK2-phosphorylation sites on S1PR1 inhibited the reciprocal down-regulation between  $\beta$ 1AR and S1PR1 following agonists stimulation. Therefore, GRK2 seems to be a nodal regulator of the cellular response to both catecholamines and S1P and it is able to modulate the phosphorylation and the reciprocal internalization of both  $\beta$ 1AR and S1PR1.

It is known that  $\beta$ 1AR is a very important regulator of cardiac function both in physiological and pathophysiological settings<sup>36,37</sup> and its action can become deleterious over time. An important role has also been recently shown for the S1PR1. In fact, this receptor can mediate an hypertrophic response in neonatal rat cardiomyocytes<sup>38</sup> and it is cardioprotective for adult cardiomyocytes under hypoxia<sup>10</sup> and in the intact heart exposed to myocardial infarction<sup>39,40,41,42</sup>. Our data confirm S1PR1 ability to mediate hypertrophy in cultured cells as well as the  $\beta$ 1AR. However, while  $\beta$ 1AR is also able to induce significant apoptosis, this



is not the case of S1PR1. More interestingly, the apoptotic response to ISO stimulation increases if the S1PR1 receptor is blocked. Our data demonstrate that S1PR1 signaling results to be cardioprotective, in particular during a sustained catecholamine stimulation which reproduces pathological condition such as HF. In fact, our demonstration that S1PR1 is downregulated in the pathological cardiac hypertrophy induced by ISO injection in mice, which is known to predispose to cardiac dysfunction, confirms that S1PR1 inactivation could have a role in the progression toward HF. More interestingly, we show that in a rat model of post-ischemic HF, S1PR1 plasma membrane levels are significantly downregulated. Further, our results showed that the observed S1PR1 down-regulation was not correlated to an S1P increase. In fact, this molecule was even strongly reduced in blood serum of post-MI HF mice compared to SHAM, strengthening our hypothesis of an active role of catecholamine overstimulation *in vivo* on reciprocal down-regulation between  $\beta$ 1AR and S1PR1.

Of note, rAAV6 gene therapy, as previously described<sup>43</sup>, allowed us to obtain the long-term and stable myocardial gene expression of rAAV6-S1PR1 cardiac gene transfer. For the first time, we directly investigated the therapeutic effects of 12 weeks expression of S1PR1 in the failing heart. We were able to demonstrate that rAAV6-S1PR1 gene-delivery restored S1PR1 plasma membrane levels and its signaling, observed by a consistent increase in SphK1 expression in the heart and S1P active secretion in blood serum, and exerts an important functional and structural cardiac recovery, improving cardiac function and blocking the negative remodelling in our post-MI rat HF model. Three months after gene delivery we found significantly increased LV EF, dP/dt and systolic blood pressure whereas LV-end diastolic diameter and pressure were decreased in comparison to the control HF animals.

The beneficial effects of S1PR1 gene therapy were also evident on post-MI  $\beta$ AR dysfunction. In fact, maximal inotropic responses during  $\beta$ AR stimulation were almost completely restored after S1PR1 gene delivery. It is reasonable to hypothesize that the favorable action of S1PR1 overexpression in the post-MI heart is probably due to either a direct cardioprotective effect and to an increase of the angiogenic response that promotes an adaptive, angiogenesis-dependent LV hypertrophy instead of transitioning to a maladaptive state. Consistent with this, S1PR1 gene delivery induced the activation of Akt signaling, preventing capillary rarefaction and completely restoring myocardial blood flow in our experimental groups. Taken together, our data show that S1PR1 downregulation, probably induced by an excessive catecholamine stimulation during HF, can be responsible for the progression toward a further decrease of cardiac performance. Importantly, in the present study we focused on S1PR1 that represents the S1PR subtype with highest expression in the heart and further studies will be needed to uncover potential relevant roles of S1PR2 and S1PR3 in HF. In conclusion, the ability of S1PR1 gene delivery to prevent LV failure in a setting of established myocardial damage, confers to this molecule the potential to be a candidate for HF treatment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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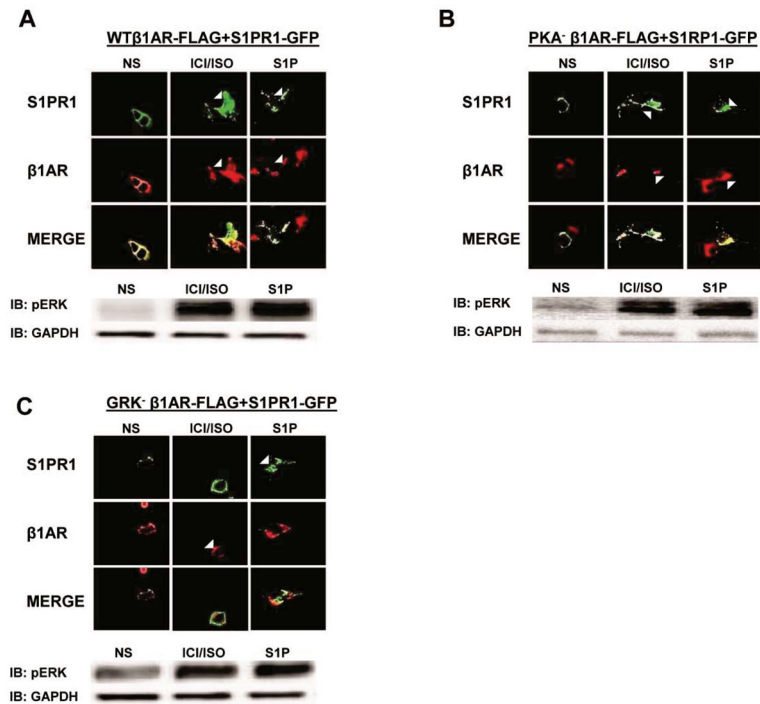
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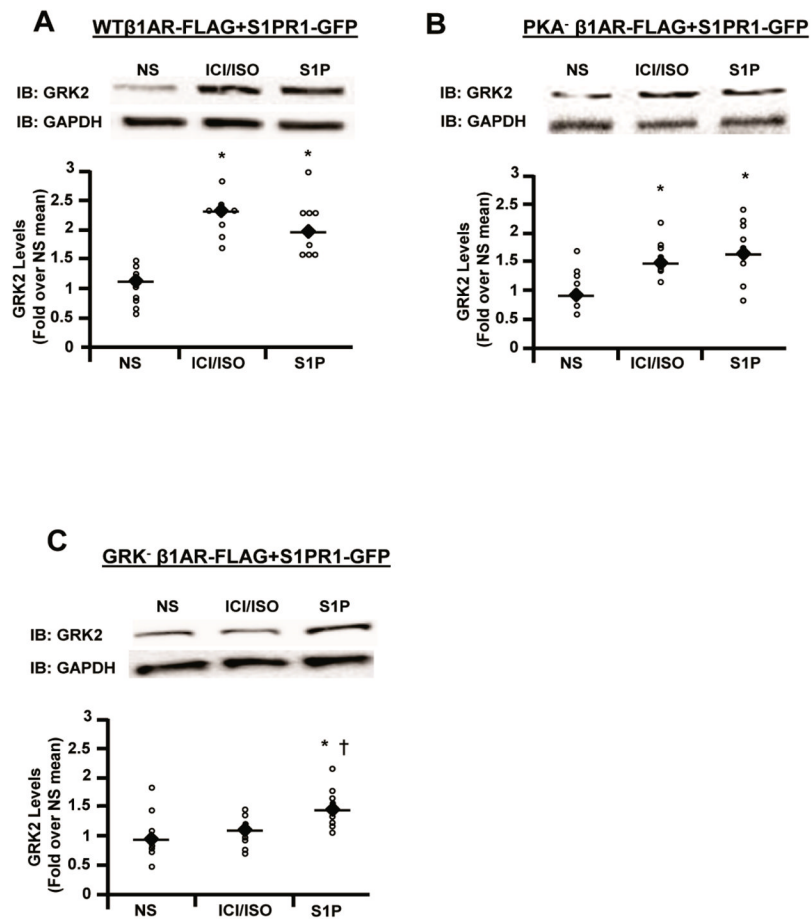
### Clinical Perspective

In chronic heart failure (HF), sympathetic nervous system overdrive induces the up-regulation of G protein-coupled receptor kinase 2 (GRK2) with a consequent  $\beta$ -adrenergic receptor ( $\beta$ AR) down-regulation/desensitization. Importantly in failing myocardium  $\beta$ AR dysregulation is clinically seen by loss of inotropic reserve. Currently,  $\beta$ -blockers represent a solid “pillar” in HF therapy which at molecular level efficiently, counteract both  $\beta$ AR down-regulation and GRK2 up-regulation. Noteworthy, GRK2 inhibition represent a new promising strategy to rescue the failing heart and we have recently recently demonstrated that in some animal models could be used as substitution or in conjunction with  $\beta$ -blockers. GRK2 inhibition may be effective in HF interfering with other intracellular processes. The present study provides the first evidence of a direct and GRK2-dependent interaction between  $\beta$ 1AR and S1PR1. We show that the reciprocal down-regulation between these two predominant GPCRs in the heart, appears to have significant physiological effects on cardiac hypertrophy, apoptosis and remodeling. Clinically, HF-related S1PR1 down-regulation worsens left ventricular dysfunction in a setting of established myocardial damage. Noteworthy, we show that the increase of S1PR1 density, through gene therapy, in failing cardiomyocytes may represent a novel therapeutic strategy for HF. This study clearly opens a new chapter in the understanding of the molecular mechanisms involved in HF, demonstrating that beside the well documented therapeutic effects of  $\beta$ AR signalling “resensitization” in HF via GRK2 inhibition or beta-blockade, a new field of research could be the reconstitution of S1PR1 cardioprotective signaling as a new therapeutic target along with other unexplored signaling pathways.

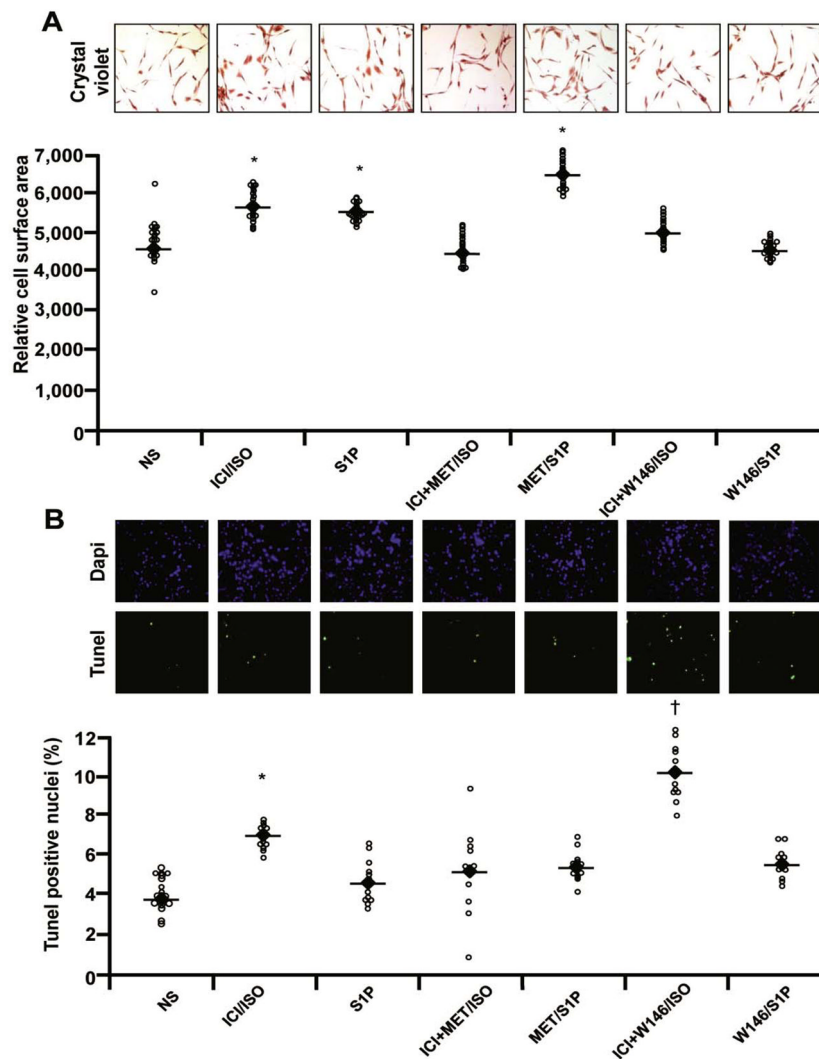


**Figure 1.**

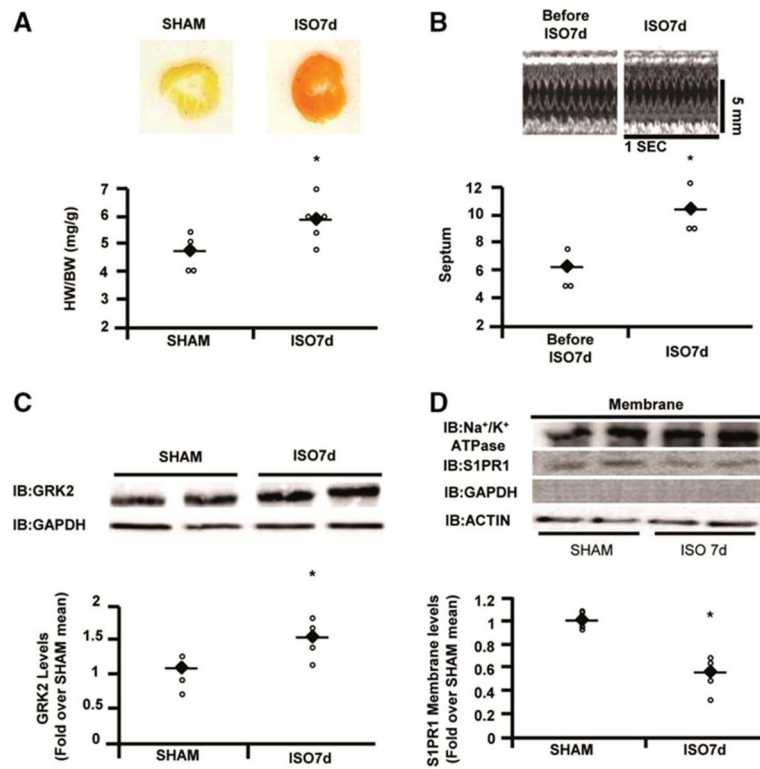
GRKs-phosphorylation sites removal inhibits the cross-talk between  $\beta$ 1AR and S1PR1. HEK293 cells stably expressing WT $\beta$ 1AR-Flag (**A**), PKA $\beta$ 1AR-Flag (**B**), GRK $\beta$ 1AR-Flag (**C**) and transfected with S1PR1-GFP, were pre-treated with  $\beta$ 2AR antagonist ICI-118,551-HCl (ICI, 10  $\mu$ M), then were stimulated with (-)-isoproterenol bitartrate (ISO) (1  $\mu$ M) or sphingosine 1-phosphate (S1P) (250 nM) for 30 min and compared with unstimulated (NS). Representative panels of S1PR1-GFP and  $\beta$ 1AR-Flag immunofluorescence images, showing cumulative data of multiple independent experiments in WT $\beta$ 1AR-Flag+S1PR1-GFP (**A**), the PKA $\beta$ 1AR-Flag+S1PR1-GFP (**B**) or the GRK $\beta$ 1AR-Flag+S1PR1-GFP (**C**). Arrows indicate receptor internalization; Representative immunoblots showing ERK1/2 activation following 5 min of stimulation with ISO (1  $\mu$ M) or S1P (250 nM) in WT $\beta$ 1AR-Flag (**A**), PKA $\beta$ 1AR-Flag (**B**), GRK $\beta$ 1AR-Flag (**C**). GAPDH was used as loading control.



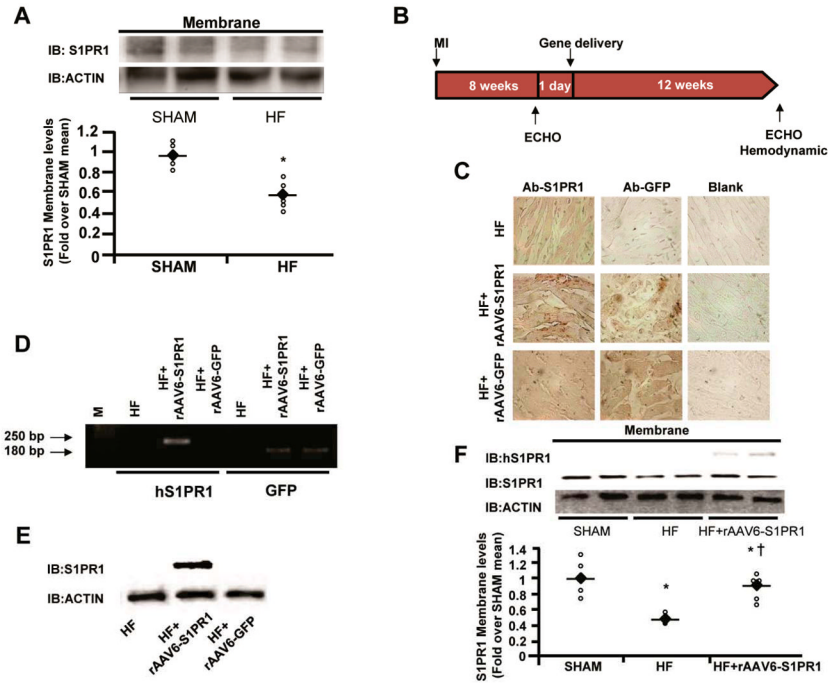
**Figure 2.** Reduced effect on GRK2 upregulation following GRKs-phosphorylation sites removal in  $\beta$ 1AR. Representative immunoblots (upper panels) and dot plots (lower panels) showing GRK2 levels following 12 hours of stimulation with ISO ( $1 \mu\text{M}$ ) or S1P ( $250 \text{ nM}$ ) in WT $\beta$ 1AR-Flag (A), PKA- $\beta$ 1AR-Flag (B), GRK- $\beta$ 1AR-Flag (C). GAPDH was used as loading control (GRK2 levels Fold over NS mean); Statistical significance between groups was determined by exact tests (Mann-Whitney and Kruskal-Wallis) followed by Bonferroni post-hoc correction. Barred diamond represents the median.  $N=9$  for each group. \* $p<0.05$  vs. NS; † $p<0.05$  vs. ICI/ISO.



**Figure 3.** Physiological role for  $\beta$ 1AR and S1PR1 interaction. Representative images (upper panels) and dot plots (lower panels) showing (A) hypertrophic (relative cell surface area) and (B) apoptotic (TUNEL positive nuclei) response in H9c2 cells transfected with cDNA encoding for S1PR1-GFP and the mouse WT $\beta$ 1AR-Flag. Cells were pre-treated with ICI (10  $\mu$ M) and stimulated with ISO (10  $\mu$ M) or S1P (250 nM); cells pre-treated with  $\beta$ 1AR antagonist Metoprolol tartrate (MET, 1  $\mu$ M) or S1PR1 selective antagonist W146 (10  $\mu$ M) were then treated with ISO (10  $\mu$ M) or S1P (250 nM) for 48 h. Each experiment was independently repeated 3 (panel A) and 4 (panel B) times. Statistical significance between groups was determined by one-way ANOVA followed by Bonferroni post-hoc correction. Barred diamond represents the mean. \* $p < 0.05$  vs NS; †  $p < 0.05$  vs. all.



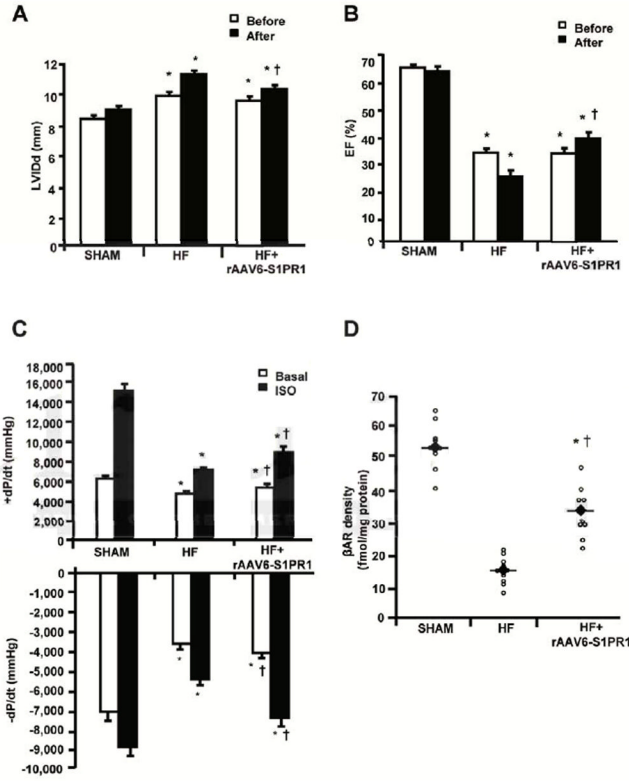
**Figure 4.** *In vivo* chronic ISO treatment (7 days) resulted in a GRK2 upregulation and an S1PR1 plasma membrane downregulation. **A.** Representative images (upper panel) of sirius red staining of mouse cardiac sections from SHAM and ISO7d groups (25X) and dot plots (lower panel) showing the heart weight/body weight (HW/BW) ratio in SHAM and ISO 7d; **B.** Representative serial M-mode echocardiographic tracings (upper panel) before ISO and following ISO7d and dot plots (lower panel) showing Septum thickness; **C.** Representative immunoblots (upper panels) and dot plots (lower panel) of GRK2 levels in ISO7d groups compared to SHAM (GRK2 levels Fold over SHAM mean). **D.** Representative immunoblots (upper panels) and dot plots (lower panel) of S1PR1 membrane levels in crude LV membrane preparations from SHAM and ISO7d mice. ACTIN was used as loading control. GAPDH and Na<sup>+</sup>/K<sup>+</sup>-ATPase were used as membrane purification control (S1PR1 levels Fold over SHAM mean). Statistical significance between groups was determined by Mann-Whitney exact test. N= 5 for each group. Barred diamond represents the median. \*p<0.05 vs SHAM.



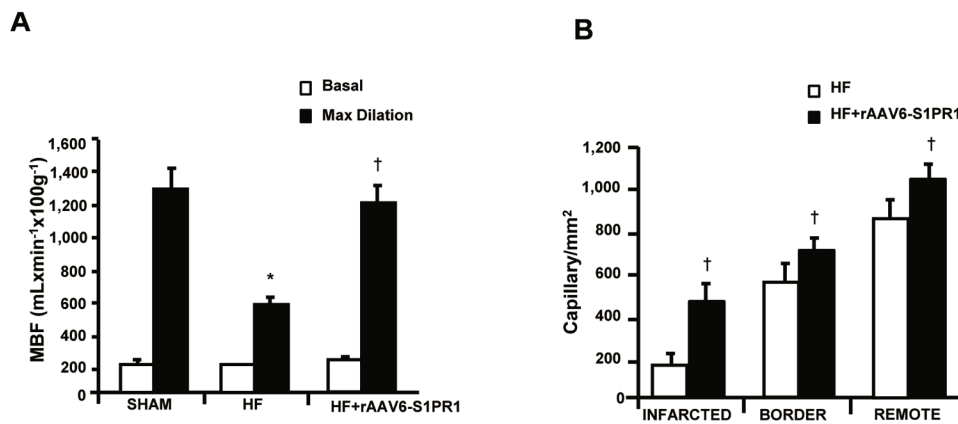
**Figure 5.**

The overexpression of S1PR1 restores the receptor plasma membrane levels impaired during heart failure. **A**. Representative immunoblots (upper panel) and dot plots (lower panel) showing S1PR1 membrane density in crude LV membrane preparations from SHAM and HF rats. ACTIN was used as loading control (S1PR1 levels Fold over SHAM mean). Statistical significance between groups was determined by Mann-Whitney exact test.  $N=5$  for each group. Barred diamond represents the median.  $*p<0.05$  vs SHAM; **B**. Overall design of 20-week study of S1PR1 gene delivery in HF rats; **C**. Representative images of S1PR1 and GFP immunohistochemistry stainings on cardiac sections from HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats, performed at the end of the study period; **D**. Representative images (upper panel) of RT-PCR on cardiac total RNA lysates from HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats, showing the overexpression of transgenic human S1PR1 (hS1PR1) and GFP; **E**. Representative immunoblots (lower panel) showing the transgenic expression of human S1PR1 (hS1PR1) and GFP in HF, HF+rAAV6-S1PR1 and HF+rAAV6-GFP rats; **F**. Representative immunoblots (upper panels) and dot plots (lower panel) of S1PR1 membrane density in crude LV membrane preparations from SHAM, HF and HF+rAAV6-S1PR1 rats. ACTIN was used as loading control (S1PR1 Fold over SHAM mean). Statistical significance between groups was determined by exact tests (Mann-Whitney and Kruskal-Wallis) followed by Bonferroni post-hoc correction.  $N=5$  for each group, except  $N=7$  for HF+rAAV6-S1PR1 group. Barred diamond represents the median.  $*p<0.05$  vs SHAM ;  $\dagger p<0.05$  vs HF.



**Figure 6.**

rAAV6-S1PR1 gene therapy at 12 weeks after MI ameliorates cardiac function. Bar graphs showing cardiac parameters measurement at 12 weeks after MI and/or after 12 weeks of treatment. **A.** Data are presented as mean  $\pm$  SEM; Statistical significance between groups was determined LV internal diameter at diastole (LVIDd) measured by echocardiography 12 weeks after MI and after 12 weeks of treatment; **B.** Ejection Fraction (EF) as measured by echocardiography 12 weeks after MI (A) and 12 weeks after *in vivo* gene delivery; **C.** Average LV +dP/dt and LV -dP/dt values evaluated under basal conditions and after maximal isoproterenol stimulation; **D.**  $\beta$ 1AR density in cardiac homogenates purified from hearts of all experimental groups performed at the end of the study period. Statistical significance between groups was determined by repeated measure ANOVA with Bonferroni post-hoc correction (panels A, B and C) and, by one-way ANOVA with Bonferroni post-hoc correction (panel D; barred diamond represents the mean). N= 10 for each group. \*p<0.05 vs SHAM; †p<0.05 vs HF.



**Figure 7.** Beneficial effects of S1PR1 gene delivery on cardiac angiogenesis. **A.** Total myocardial blood flow measured at basal condition and after maximal coronary dilatation; **B.** Bar graph showing capillary density on mm<sup>2</sup> ratio evaluated by Lectin *Bandeiraea simplicifolia* I (BS-I) staining of capillaries in cardiac section obtained from HF control groups and HF plus rAAV6-S1PR1. Statistical significance between groups was determined by repeated measure ANOVA followed by Bonferroni post-hoc correction. N= 10 for each group. \*p<0.05 vs SHAM; †p<0.05 vs HF.

**Table 1**

Echocardiographic, hemodynamic and physical parameters at 12 weeks after rAAV6-Mediated Cardiac Gene Delivery

<b>Echocardiography</b>	<b>SHAM</b>	<b>HF</b>	<b>HF+AVV6-S1PR1</b>
FS%	37.4±0.7	13.6±0.8*	19.7±1.1*†
EF%	65.4±0.9	26.9±1.6*	38.3±1.8*†
Awd	1.68±0.05	1.38±0.06*	1.69±0.04†
Aws	2.66±0.09	1.79±0.09*	2.34±0.13*
LVIDd	8.5±0.2	10.92±0.18*	10±0.18*†
LVIDs	5.42±0.14	9.46±0.2*	8.08±0.19*†
PWd	1.73±0.04	2.03±0.06*	2.43±0.09*†
PWs	2.84±0.1	2.52±0.15	2.95±0.1
<b>LV catheterization, basal</b>			
	<b>SHAM</b>	<b>HF</b>	<b>HF+AVV6-S1PR1</b>
HR, bpm	330.5±11.6	322±9.6	290±8.1*†
LV dP/dt, mm Hg/s	6494.8±244	4461±179.8*	5260±245.7*†
LV -dP/dt, mm Hg/s	6968±327.8	3626±106.1*	4251.4±128.8*†
LVEDP, mm Hg	2.2±0.3	13.5±1.1*	7.7±0.9*†
LVESP, mm Hg	132.2±3.6	104.1±4.9*	107.8±3.3*
<b>Isoproterenol (333 ng/kg BW)</b>			
HR, bpm	400±20.7	381±12.7	341.1±4.3*†
LV dP/dt, mm Hg/s	15316±416.8	7476.5±140*	10036.1±272.4*†
LV -dP/dt, mm Hg/s	8629.8±365	5144.9±166.3*	7297.1±244.1*†
LVEDP, mm Hg	1.4±0.8	11.4±0.9*	6.6±0.7*†
LVESP, mm Hg	126.7±2	99.7±2.7*	110.8±3.2*†
BW, Kg	0.46±0.01	0.48±0.01	0.47±0.01
HW/BW, g/Kg	1.12±0.02	3.03±0.08*	3.55±0.13*†

Data are presented as mean ± SEM. Effect of *S1PR1* gene therapy on LV function evaluated at 12 weeks after gene delivery is shown. *In vivo* Ejection fraction (EF), Anterior wall diastolic thickness (Awd), Anterior wall systolic thickness (Aws), LVIDd, LV internal diameter at systole (LVIDs), PWd, Posterior wall systolic thickness (PWs), Heart rate (HR), LV dP/dt, LV -dP/dt, LV end-diastolic pressure (LVEDP), LV end-systolic pressure (LVESP), were assessed in: SHAM (n=10), HF+saline (n=10), HF+rAAV6-S1PR1 (n=10) and HF+rAAV6-GFP (n=10) rats. Ratio of heart weight to body weight and fractional shortening (FS%) was also measured in all groups ANOVA analysis and Bonferroni test were used between all groups.

\* p<0.05 vs SHAM;

† p<0.05 vs HF control groups.