Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396

# Deletion of osteopontin enhances $\beta_2$-adrenergic receptor-dependent anti-fibrotic signaling in cardiomyocytes

Celina M. Pollard[1], Victoria L. Desimine[1], Shelby L. Wertz[1], Arianna Perez[1], Barbara M. Parker[1], Jennifer Maning[1,3], Katie A. McCrink[1,4,5], Lina A. Shehadeh[2], and Anastasios Lymperopoulos[1]*

From the [1]Laboratory for the Study of Neurohormonal Control of the Circulation, Department of Pharmaceutical Sciences (Pharmacology), College of Pharmacy; Nova Southeastern University, Fort Lauderdale, FL 33328, USA; [2]University of Miami Miller School of Medicine, Miami, FL 33136, USA.

[3]Present address: Jackson Memorial Hospital, Miami, FL 33136, USA.

[4]Present address: Massachusetts General Hospital, Boston, MA 02114, USA.

[5]American Foundation for Pharmaceutical Education (AFPE) "Gateway to Research" Scholar.

***Correspondence to: Anastasios Lymperopoulos, PhD, FAHA, FESC, Associate Professor of Pharmacology,** Department of Pharmaceutical Sciences, Nova Southeastern University College of Pharmacy, 3200 S. University Dr., HPD (Terry) Bldg/Room 1338, Fort Lauderdale, FL 33328-2018, USA. al806@nova.edu

**Telephone:** +1-954-262-1338

**Fax:** +1-954-262-2278

**Abstract:** Cardiac $\beta_2$-adrenergic receptors (ARs) are known to inhibit collagen production and fibrosis in cardiac fibroblasts and myocytes. The $\beta_2$AR is a Gs protein-coupled receptor (GPCR) and, upon its activation, stimulates generation of cyclic 3`, 5`-adenosine monophosphate (cAMP). cAMP has two effectors: protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac). Epac1 has been shown to inhibit cardiac fibroblast activation and fibrosis. Osteopontin (OPN) is a ubiquitous pro-inflammatory cytokine, mediating also fibrosis in several

tissues, including the heart. OPN underlies several cardiovascular pathologies, including atherosclerosis and cardiac adverse remodeling. We have found that the cardiotoxic hormone aldosterone transcriptionally upregulates OPN in H9c2 rat cardiac myoblasts, an effect prevented by endogenous $\beta_2$AR activation. Additionally, CRISPR-mediated OPN deletion enhances cAMP generation in response to both $\beta_1$AR and $\beta_2$AR activation in H9c2 cardiomyocytes, leading to upregulation of Epac1 protein levels. These effects render $\beta_2$AR stimulation capable of completely abrogating transforming growth factor (TGF)-β-dependent fibrosis in OPN-lacking H9c2 cardiomyocytes. Finally, OPN interacts constitutively with $G_\alpha$s subunits in H9c2 cardiac cells. Thus, we have uncovered a direct inhibitory role of OPN in cardiac $\beta_2$AR anti-fibrotic signaling via cAMP/Epac1. OPN blockade could be of value in the treatment and/or prevention of cardiac fibrosis.

**Keywords:** $\beta_2$-adrenergic receptor; cAMP; cardiac myocytes; CRISPR; Epac1; fibrosis; osteopontin; signal transduction.

## 1. Introduction

Cardiac fibrosis is a fundamental process mediating adverse remodeling of the heart post-myocardial infarction (MI) or other ischemic injury and in heart failure (HF) [1]. The main cell type driving the fibrotic process is activated myofibroblasts, with significant contributions by monocytes/macrophages, lymphocytes, mast cells, vascular endothelial cells and cardiomyocytes, all of which produce and secrete key pro-fibrotic factors, such as reactive oxygen species, proteases and metalloproteinases, and fibrosis-promoting growth factors, mainly transforming growth factor (TGF)-β [2].

β-adrenergic receptors (ARs) are the main receptors mediating most of the actions of the catecholomine hormones norepinephrine and epinephrine in the heart [3-5]. All three βAR subtypes are expressed in the adult mammalian myocardium with the

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



58 $\beta_1$AR being the most abundant and mediating the positive inotropic and
59 chronotropic actions of the sympathetic nervous system, whereas the less abundant
60 $\beta_2$AR exerts several cardio-protective effects in the post-MI heart, such as inhibition
61 of apoptosis, inflammation, fibrosis, etc. [4, 6]. Indeed, $\beta_2$AR inhibits collagen
62 production and fibrosis in cardiac fibroblasts [7-9]. All ARs belong to the G protein-
63 coupled receptor (GPCR) superfamily and both $\beta_1$AR & $\beta_2$AR activate the Gs protein
64 signaling pathway in cardiac cells, leading to activation of adenylyl cyclase (AC) and
65 subsequent cyclic 3`, 5`-adenosine monophosphate (cAMP) synthesis [3]. cAMP is a
66 major second messenger inside cells and, in cardiac myocytes, it is responsible for
67 stimulation of cardiac contractility thanks, mainly, to activation of its effector kinase
68 PKA (protein kinase A or cAMP-dependent protein kinase), which phosphorylates
69 a variety of substrates to facilitate contraction in the cardiac myocyte [4, 10, 11]. In
70 addition to PKA, cAMP also directly activates the exchange protein directly
71 activated by cAMP (Epac) [12, 13]. Two Epac isoforms have been characterized,
72 Epac1 and Epac2. Epac1 is expressed ubiquitously and is quite abundant in the heart,
73 including cardiac fibroblasts [14]. This Epac isoform has been documented to
74 decrease collagen expression in response to βAR activation in rat cardiac fibroblasts
75 and its expression is downregulated in the heart post-MI and in response to pro-
76 fibrotic stimuli [12, 14, 16]. Epac1 blocks collagen and DNA synthesis in rat cardiac
77 fibroblasts and was very recently reported to also block atrial fibroblast activation,
78 migration, and secretion of fibrotic mediators in post-MI mice and in HF dogs [9,
79 14].
80 Osteopontin (OPN) is a member of the small integrin-binding ligand N-linked
81 glycoprotein (SIBLING) family and is expressed in normal mineralized tissues and
82 cell types including osteoblasts, macrophages, lymphocytes, vascular smooth
83 muscle cells (VSMCs), renal and cardiac fibroblasts, and several neoplastic tissues
84 [17-19]. As a pro-inflammatory protein, OPN induces VSMC inflammation in the
85 brain, pancreas, kidney, and heart [20]. It also participates in cell adhesion and
86 migration processes via interacting with various integrins and CD44, and is a potent
87 chemokine for mononuclear cells and VSMCs [20]. Thus, it comes as no surprise that

OPN has been implicated in various pathologic conditions, including atherosclerosis [18, 21-23], wound healing [19], and cardiac adverse remodeling [24]. In the present study, we sought to investigate OPN`s role in βAR-regulated cardiomyoblast fibrosis. Using the H9c2 rat cardiac myoblast cell line as our cell model, which endogenously expresses both $\beta_1$AR & $\beta_2$AR and OPN [25, 26], we found that OPN substantially impedes $\beta_2$AR-induced, cAMP/Epac1-dependent anti-fibrotic signaling in cardiac cells.

## 2. Results

### *2.1. Regulation of OPN expression by aldosterone and the $\beta_2$AR in H9c2 cardiomyocytes*

Since aldosterone is a well-documented cardio-toxic hormone mediating adverse remodeling, including cardiac fibrosis [27], we first examined the effect of this mineralocorticoid on OPN expression in the H9c2 cardiac cells. As shown in **Figure 1**, aldosterone treatment for 2 hours caused a rapid upregulation of OPN mRNA levels, indicating that OPN is an immediate/early response gene for aldosterone in H9c2 cardiac cells, consistent with previous studies demonstrating this in renal and in other cell/tissue types [20, 28].

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



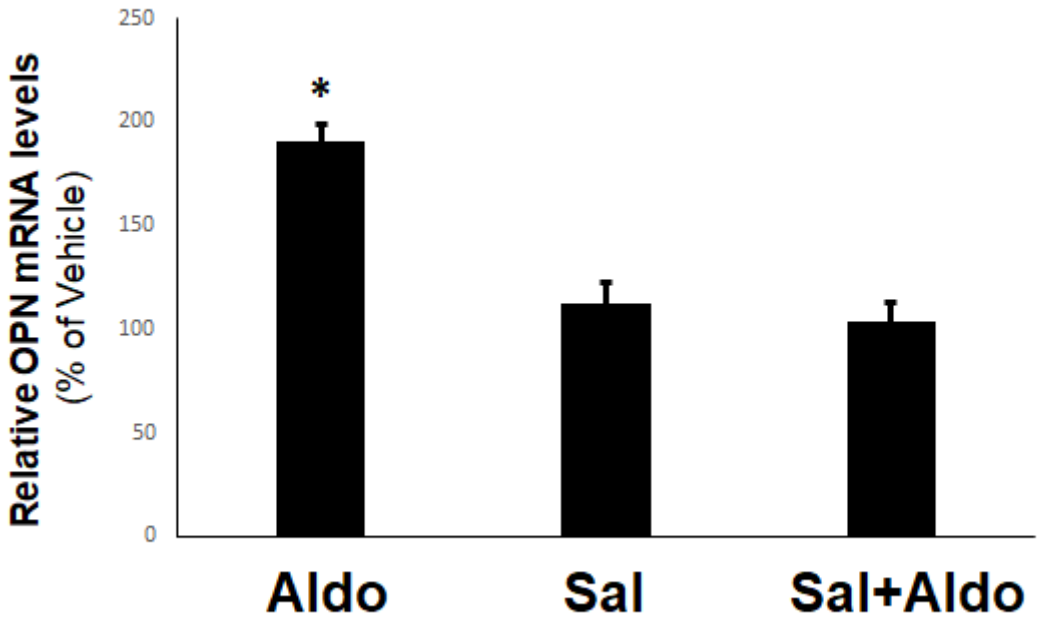


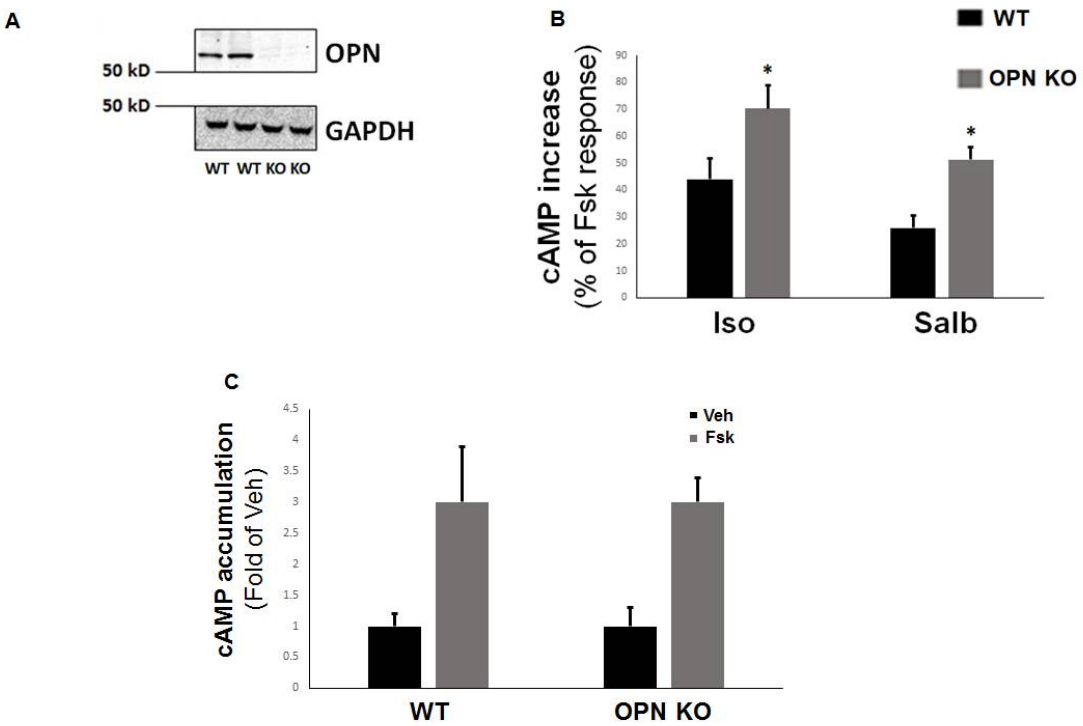

**Figure 1. $\beta_2$AR inhibits aldosterone-induced OPN upregulation in H9c2 cardiomyocytes.**

H9c2 cells were treated with 10 nM aldosterone (Aldo), 10 μM salbutamol (Sal) or 10 nM aldosterone in the presence of 10 μM salbutamol (Sal+Aldo) for 2 hours. At the end of this 2-hr period, cells were harvested, total RNA isolated and real-time PCR for OPN mRNA quantitation was performed. *, $p<.05$, vs. any other treatment; n=4 independent experiments/condition.

Additionally, this effect of aldosterone on OPN upregulation was mineralocorticoid receptor (MR)-dependent (data not shown), i.e. a classic genomic/transcriptional effect of aldosterone in H9c2 cardiomyocytes. Interestingly, $\beta_2$AR activation with salbutamol (albuterol), applied together with aldosterone, completely prevented the OPN mRNA induction by the latter (**Figure 1**). This finding strongly suggests that selective $\beta_2$AR stimulation may inhibit (at least some of) the genomic, MR-dependent effects of aldosterone in H9c2 cardiac cells.

122 *2.2. OPN opposes β2AR cAMP signaling in H9c2 cardiomyocytes*

123 Since the $\beta_2$AR inhibits OPN upregulation in H9c2 cardiomyocytes, an effect
124 consistent with this receptor`s anti-fibrotic actions in the heart, we posited that there
125 is perhaps a negative feedback loop operating in cardiac myocytes allowing OPN to
126 reciprocally regulate (inhibit) $\beta_2$AR function and signaling. To this end, we deleted
127 the OPN gene in H9c2 cells via CRISPR and compared the extent of cAMP
128 accumulation, the major second messenger generated by βARs regulating cellular
129 fibrosis [16], between cells having OPN deleted (OPN-knockout (KO) cells) and wild
130 type (WT) cells. After confirming the genetic deletion of OPN at the protein level
131 (**Figure 2A**), we stimulated the cells with either isoproterenol to activate both $\beta_1$ARs
132 & $\beta_2$ARs or salbutamol to selectively activate only the $\beta_2$ARs and measured acute
133 cAMP generation.




134

135 **Figure 2. Enhanced $\beta_2$AR-dependent cAMP accumulation in the absence of OPN**
136 **in H9c2 cardiomyocytes.**

137 (**A**) Western blotting to confirm OPN CRISPR-mediated deletion in H9c2 cells
138 infected with OPN-specific CRISPR lentivirus (KO) or mock CRISPR lentivirus (wild

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



type, WT). Blotting for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also shown as loading control. Representative blots from three independent experiments per condition with similar results are shown. (**B**) cAMP accumulation in response to 10 μM isoproterenol (Iso) or 10 μM salbutamol (Salb) in control WT and in OPN-depleted (OPN KO) H9c2 cells, expressed as % of the respective cAMP production induced by 10 μM forskolin (Fsk). *, $p<.05$, vs. WT; n=3 independent experiments/condition/cell clone. (**C**) cAMP accumulation in response to 10 μM forskolin (Fsk) or vehicle (Veh) in control WT and in OPN-depleted (OPN KO) H9c2 cells. No significant differences were observed between WT-Fsk and OPN KO-Fsk at $p=0.05$ (n=3 independent experiments/condition/cell clone).

OPN KO led to a significant enhancement of cAMP synthesis in response to both isoproterenol and salbutamol stimulations in H9c2 cardiomyocytes, compared to WT myocytes (**Figure 2B**). Importantly, this was not due to differences in AC activity between WT and OPN KO cells, since forskolin, a direct AC activator, produced comparable levels of cAMP accumulation in both H9c2 cell clones (**Figure 2C**). Taken together, these results suggest that OPN opposes βAR-stimulated cAMP signaling, including $\beta_2$AR-induced cAMP production, in H9c2 cardiomyocytes.

*2.3. Epac1 upregulation by OPN CRISPR-mediated deletion in H9c2 cardiomyocytes*

Since Epac1 plays an inhibitory role in cell/tissue fibrosis, including cardiac fibrosis [9, 16], next we examined the effects of OPN KO on Epac1 protein expression. Treatment of WT and OPN KO H9c2 cardiac cells with either isoproterenol (or salbutamol, data not shown) for 24 hours led to a significant upregulation of Epac1 protein levels in the absence of OPN, compared to WT cells (**Figures 3A & 3B**). This finding suggests that OPN not only blocks βAR-dependent cAMP production but also impedes βAR-induced Epac1 activation and upregulated expression in cardiac myocytes.

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



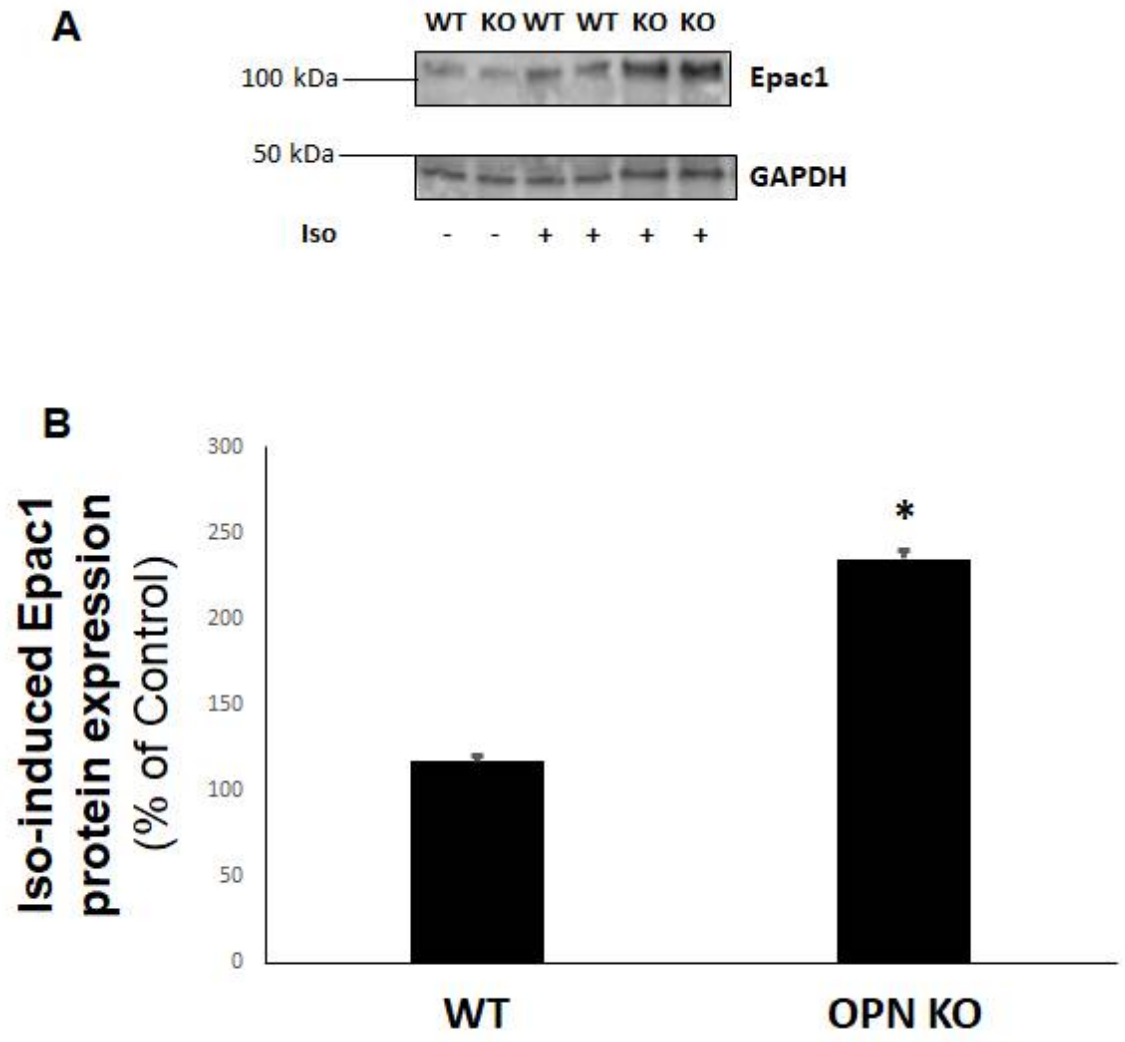



**Figure 3. Enhanced βAR-dependent Epac1 protein levels in the absence of OPN in H9c2 cardiomyocytes.**

H9c2 cells were treated with 10 μM isoproterenol (Iso) or vehicle for 24 hours in the presence (WT) or absence (KO) of OPN, then cells were harvested and total protein extracts prepared for Epac1 immunoblotting. Representative blots are shown in (**A**), including GAPDH as loading control, and the densitometric quantitation of three independent experiments per condition performed in duplicate is shown in (**B**). *, $p<.05$, n=3.

*2.4. Prevention of pro-fibrotic gene expression by OPN genetic ablation in H9c2 cardiomyocytes*

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



To examine the impact of OPN`s $\beta_2$AR signaling effects on fibrosis of H9c2 cardiomyocytes, we measured the $\beta_2$AR-dependent inhibition of collagen types I, III, and IV, as well as of fibronectin, mRNA induction in response to the classic pro-fibrotic stimulus TGF-β1 in the presence (WT) and absence (OPN KO) of OPN in H9c2 cells.

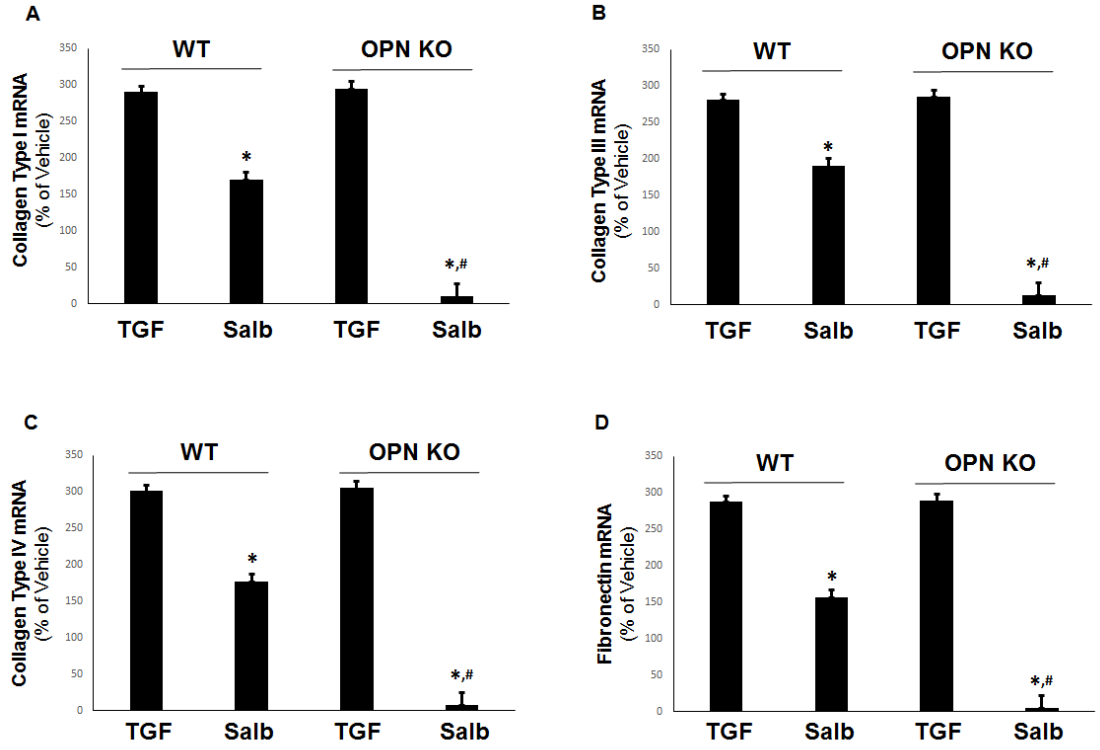



**Figure 4. Absence of OPN potentiates the $\beta_2$AR-mediated inhibition of TGFβ-dependent profibrotic factor mRNA induction in H9c2 cells.**

mRNA levels of type I collagen (**A**), type III collagen (**B**), type IV collagen (**C**), and fibronectin (**D**), in WT or OPN-depleted (OPN KO) H9c2 cells treated with 10 ng/ml TGF-$\beta_1$ (TGF) with or without 10 μM salbutamol (Salb). *, $p<.05$, vs. TGF; #, $p<.05$, vs. WT-Salb; n=3 independent experiments per condition (two-way ANOVA with Bonferroni test).

Although, as expected, $\beta_2$AR activation inhibited partially the transcriptional upregulation of TGFβ-induced collagen type I (**Figure 4A**), type III (**Figure 4B**), type IV (**Figure 4C**), and fibronectin (**Figure 4D**) in normal WT cells, the absence of OPN

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



196 enabled salbutamol to completely abrogate the TGFβ-dependent expression of all of
197 these pro-fibrotic factors: collagen I (**Figure 4A**), collagen III (**Figure 4B**), collagen IV
198 (**Figure 4C**), and fibronectin (**Figure 4D**). Thus, OPN significantly hinders the ability
199 of the $\beta_2$AR to block fibrosis via cAMP and Epac1 in cardiac myocytes.
200
201 *2.5. OPN inhibits $\beta_2$AR cAMP signaling by directly interacting with the $G_\alpha$s/olf protein*
202 *subunit in H9c2 cardiomyocytes*
203 In an attempt to begin to dissect the molecular mechanism by which OPN perturbs
204 $\beta_2$AR`s cAMP-mediated anti-fibrotic signaling in H9c2 cardiomyocytes, we
205 examined OPN`s interaction with the $G_\alpha$s/olf protein subunit, previously reported
206 to occur in osteoblastic cells resulting in $\beta_2$AR dysfunction [29]. Indeed, co-
207 immunoprecipitation experiments indicated that OPN and the $G_\alpha$s subunit protein
208 interact with each other constitutively (in the absence of any stimulus), albeit
209 weakly, in H9c2 cardiac cells (**Figure 5A**). Furthermore, this interaction is unaffected
210 by $\beta_2$AR activation, since salbutamol neither enhances nor reduces it (**Figures 5A &**
211 **5B**). Thus, similarly to bone cells, OPN physically interacts with $G_\alpha$s subunits inside
212 cardiac cells impeding downstream $\beta_2$AR signaling to AC, cAMP and Epac1.
213
214 **3. Discussion**
215 In the present study, we report, for the first time to our knowledge, the adverse role
216 OPN plays at hindering $\beta_2$AR-dependent, cAMP/Epac1-mediated anti-fibrotic
217 signaling and function in rat cardiac myocyte-like cells. By physically interacting
218 constitutively with the $G_\alpha$ subunit of the Gs heterotrimeric protein, intracellular
219 OPN reduces the cAMP generating capacity of the $\beta_2$AR in cardiac cells, thereby
220 reducing both the activity (acutely) and protein expression (long-term) of the cAMP
221 effector Epac1, which is normally anti-fibrotic in several tissues, including in the
222 heart (**Figure 6**) [16]. Additionally, we found that aldosterone, a powerful pro-
223 fibrotic and adverse remodeling-associated hormone in the heart that is also
224 elevated during post-MI HF progression [30], directly induces OPN transcriptional

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



upregulation to mediate fibrosis (**Figure 6**). Notably, this aldosterone-induced OPN upregulation is blocked by the $\beta_2$AR, so it appears that there is a closed negative feedback loop operating in cardiac myocytes, in which OPN is on one hand induced by aldosterone to promote fibrosis, in part via blockade of $\beta_2$AR-dependent, cAMP/Epac1-mediated signaling, and on the other hand, the $\beta_2$AR opposes the aldosterone/MR-mediated OPN induction (**Figure 6**).

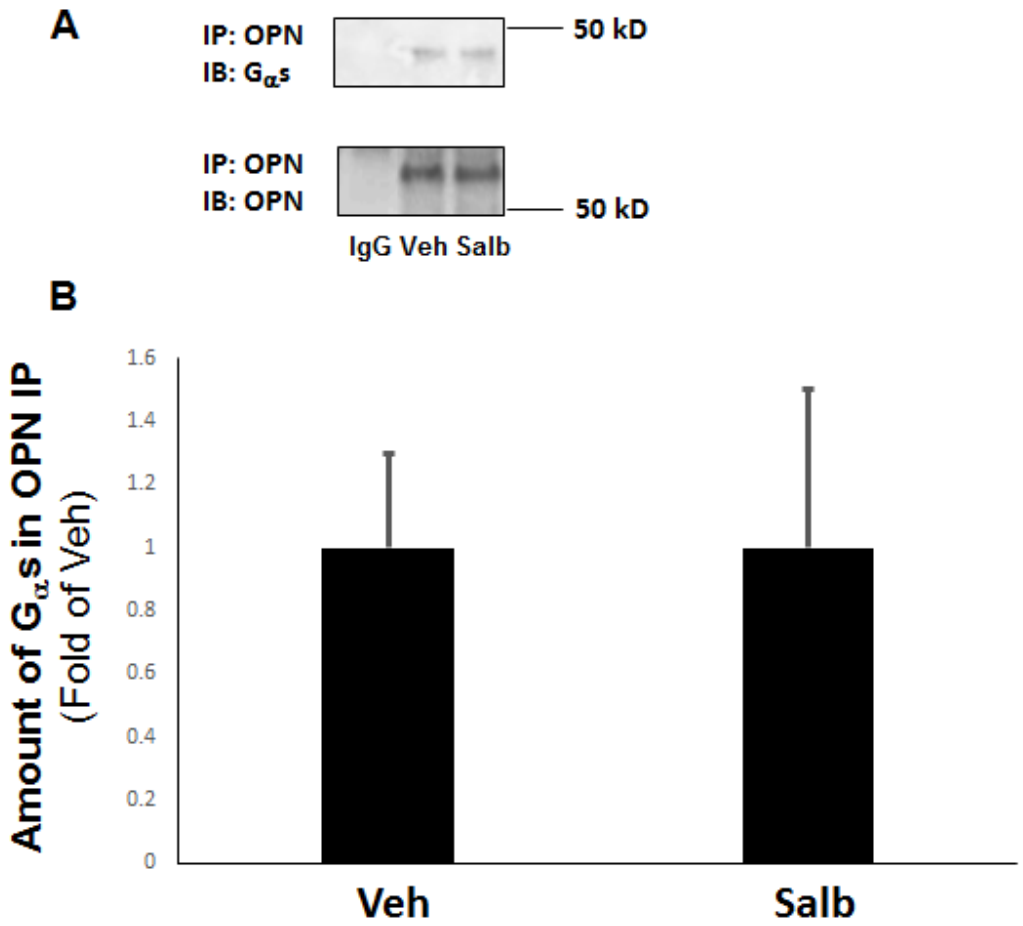



**Figure 5. OPN opposes $\beta_2$AR signaling via physical interaction with $G_\alpha$s in H9c2 cardiomyoblasts.**

Co-immunoprecipitation (co-IP) of OPN with $G_\alpha$s in native WT H9c2 cells, treated with vehicle (Veh) or 10 μM salbutamol (Salb). Representative blots are shown in (**A**) and the densitometric quantitation of three independent experiments is shown in (**B**). IB: Immunoblotting; IP: Immunoprecipitation; IgG: IP with a general IgG antibody (negative control for the OPN IP). No significant difference (at p=0.05) in the amount of $G_\alpha$s co-IP`d with OPN was observed between Veh and Salb (n=3).

OPN is a known downstream transcriptional target of aldosterone and its receptor, the steroid-responsive transcription factor MR [20, 28]. Immediate/early OPN mRNA induction promoting fibrosis in response to aldosterone has been reported in the kidneys [20], during VSMC proliferation and inflammation [31], in vascular endothelial cells [32], and in peripheral blood mononuclear cells [33]. Here we report that this is the case also in cardiac myoblasts.

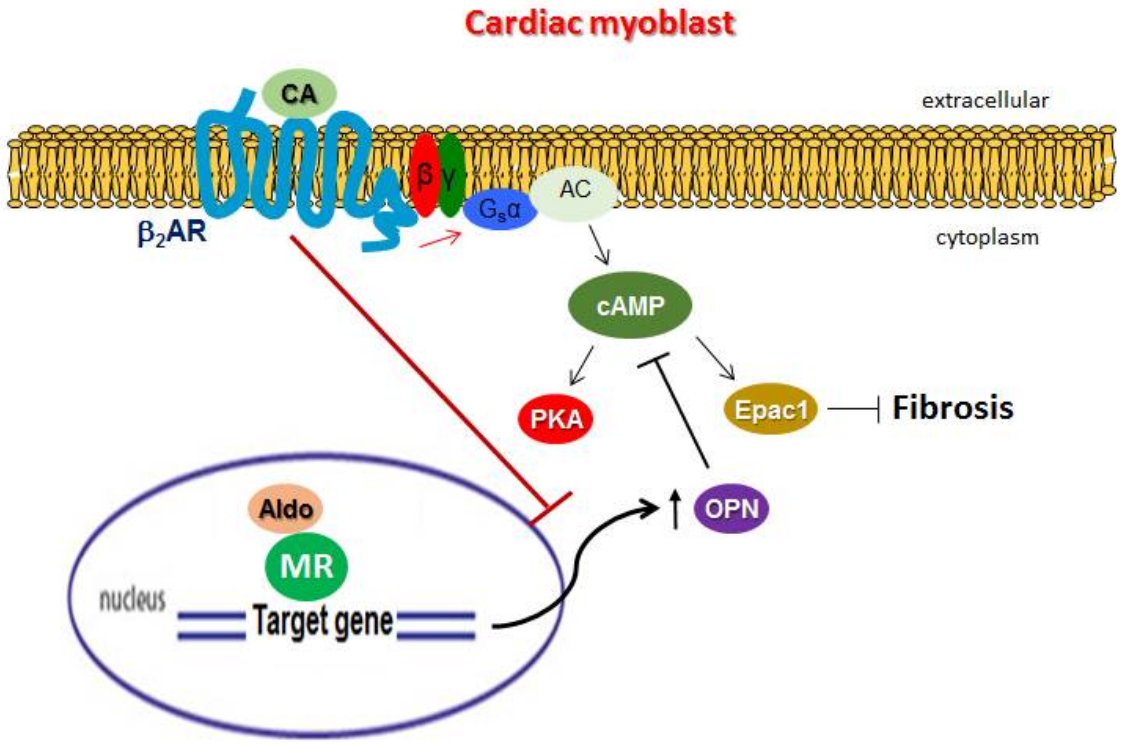



**Figure 6. Schematic illustration of the proposed role of OPN in $\beta_2$AR ant-fibrotic signaling in H9c2 cardiomyocytes.**

CA: Catecholamine. See text for details and for all other molecular acronym descriptions.

OPN is a ubiquitous, pro-inflammatory cytokine that is activated in response to a variety of hemodynamic, pro-inflammatory, oxidative stress-related, and pro-fibrotic (e.g. TGFβ) stimuli. It exists as both intracellular and extracellular and thus,

it can promote fibrosis via both intracellular TGFβ signaling facilitation and modulation of extracellular matrix remodeling [29]. Our present findings add a novel mechanism for OPN`s pro-fibrotic effects, at least in the heart: interference with the anti-fibrotic actions of the catecholamines through the $\beta_2$AR. The $\beta_2$AR, in contrast with the $\beta_1$AR, is considered cardio-protective in the post-MI failing heart, since it can facilitate infarct (wound) healing, promote cardiomyocyte survival and limit inflammation, apoptosis, and other adverse remodeling processes that ensue immediately after an MI [4, 34]. Indeed, cAMP and its effector Epac1, which are induced by the activated $\beta_2$AR in the heart, are known to exert anti-fibrotic effects in several cell types and tissues, including in the heart [16]. Our present findings indicate that OPN reduces $\beta_2$AR-dependent cAMP generation, and thus Epac1 levels and activity, in heart cells by physically interacting with the $G_\alpha s$ protein, the cognate signal transducer to which the $\beta_2$AR couples, in order to activate AC and induce cAMP synthesis (**Figure 6**). This is in line with an old study in bone cells reporting the exact same mechanism ($\beta_2$AR-dependent cAMP generation hindrance) underlying OPN`s role in the modulation of the sympathetic tone of bone mass regulation [29].

Of course, how exactly OPN inhibits $\beta_2$AR signaling to cAMP in cardiac myocytes remains to be elucidated in future studies. One plausible mechanism could be recruitment of GPCR-kinase (GRK)-2 (or some other GRK) to the OPN-$G_\alpha s$ complex. GRKs bind agonist-activated GPCRs and phosphorylate them to induce their functional desensitization (i.e. decoupling from G proteins) [35]. In fact, GRK2, the major GRK isoform regulating the cardiac $\beta_2$AR [4], has been reported to directly phosphorylate and inhibit Epac1 in the central nervous system of mice in vivo, thereby mitigating persistent, chronic inflammatory pain promoted by Epac1-to-Rap1 signaling [36]. Thus, GRK2 could have a dual role in inhibition of cardiac $\beta_2$AR`s anti-fibrotic signaling: a) direct desensitization (Gs protein decoupling) of the $\beta_2$AR itself, and b) direct blockade of Epac1`s anti-fibrotic activity. In any case, identification of the complete mechanism and of the additional molecular partners

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



285 through which OPN opposes $\beta_2$AR anti-fibrotic signaling to cAMP/Epac1 is
286 definitely worth pursuing and is the goal of our currently ongoing studies.
287
288 Another major question arising from our present findings pertains to the mechanism
289 by which the $\beta_2$AR, in a negative feedback regulatory manner, opposes the
290 aldosterone/MR-dependent OPN upregulation in cardiac myocytes (**Figure 6**). How
291 does the $\beta_2$AR (a GPCR) block this aldosterone/MR transcriptional effect? The MR
292 is a ~1,000-amino acid cytoplasmic (at rest) protein with three functional domains:
293 the N-terminal domain (NTD) that regulates transcriptional activity of the receptor;
294 the DNA-binding domain (DBD) involved in the binding of the promoter of the
295 target gene; and the ligand-binding domain (LBD) responsible for hormone binding
296 [37, 38]. In the nucleus, the MR depends on numerous molecular co-regulators to
297 activate and regulate its target genes that carry the (shared with the glucocorticoid
298 receptor) glucocorticoid response element (GRE) sequence in their promoters [39].
299 Importantly, the MR undergoes various post-translational modifications, such as
300 phosphorylation, ubiquitination, etc., which play important roles in regulation of its
301 nuclear translocation and of its transcriptional activity [40]. Indeed, the MR contains
302 several serine and threonine residues that are substrates for kinases like PKA, whose
303 phosphorylation of the MR has been reported to inhibit MR cytoplasm-to-nucleus
304 trafficking [40, 41]. Given that PKA is activated by the $\beta_2$AR (**Figure 6**), $\beta_2$AR may
305 inhibit MR-dependent OPN upregulation through PKA. Another possibility is that
306 GRK5, the other major GRK isoform in cardiac cells [4, 35], which is also activated
307 by the agonist-occupied $\beta_2$AR [42], inhibits the MR by directly phosphorylating it
308 (A. Lymperopoulos, unpublished data & Ref. 27). We plan to investigate these
309 possibilities and to delineate the exact mechanism of $\beta_2$AR`s regulation of
310 aldosterone signaling in the heart in future studies.
311 In summary, we report here that the ubiquitous pro-inflammatory cytokine OPN,
312 transcriptionally induced by aldosterone and the MR, mediates cardiac fibrosis in
313 part via perturbation of the $\beta_2$AR`s cAMP/Epac1-dependent anti-fibrotic signaling
314 in rat cardiac myoblasts. The $\beta_2$AR, in turn, inhibits the aldosterone-induced OPN
315 upregulation in the heart, thereby closing a feedback regulation loop. These findings

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



provide a previously unappreciated mechanism for OPN`s cardiac effects and suggest a direct involvement of OPN in cardiac βAR function and signaling. Finally, from a translational/therapeutic standpoint, our data suggest that OPN blockade, perhaps with a cell-permeable anti-OPN antibody or with OPN siRNA-mediated knockdown, can significantly boost the therapeutic efficacy of $\beta_2$AR agonists, already available in clinical practice, or of cAMP analogs and/or Epac1 small molecule activators, currently in therapeutic development, against cardiac fibrosis and adverse remodeling in general.

## 4. Materials and Methods

### *4.1. Materials*

All chemicals (aldosterone, isoproterenol, salbutamol, forskolin) were from Sigma-Aldrich (St. Louis, MO), except for human recombinant TGF$\beta_1$, which was purchased from Cell Signaling Technology (Danvers, MA).

### *4.2. Cell culture and CRISPR-mediated OPN KO*

The H9c2 rat cardiomyoblast cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described [43]. For CRISPR/Cas9-mediated OPN genetic deletion, a custom-made oligo targeting an mRNA exon of the rat OPN gene (Target mRNA RefSeqId: NM_012881/6, *Spp1* gene of the Rattus norvegicus species) was designed, synthesized, and inserted into a CRISPR lentiviral construct (Sigma-Aldrich). 48 hours after infection of H9c2 cells with this rat OPN-specific CRISPR lentivirus, protein extracts were prepared and the knockdown/knockout of OPN was verified via western blotting for OPN with an anti-rat OPN monoclonal antibody (MPIIIB10(1); Developmental Studies Hybridoma Bank-DSHB, Iowa City, IA).

### *4.3. Real-time qPCR*

Real-time quantitative PCR for OPN, collagen type I, III, and IV, and fibronectin was performed essentially as described [44, 45]. Briefly, total RNA was isolated from H9c2 cells with the Trizol reagent, according to the manufacturer`s instructions (Invitrogen, Carlsbad, CA), followed by quantitative real-time PCR in a MyIQ

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



Single-Color Real-Time PCR detection system (Bio-Rad Life Sciences Research, Hercules, CA) using SYBR Green Supermix (Bio-Rad) and 100 nM of gene-specific oligonucleotides. Quantification of mRNA included normalization to 18s rRNA levels. No bands were seen in control reactions in the absence of reverse transcriptase. Primer pairs used were (all from Sigma-Aldrich) [20]: 5`-TGGCAGTGGTTTGCTTTTGC-3` & 5`-CCAAGTGGCTACAGCATCTGA- 3′ for OPN; 5`-ATCTCCTGGTGCTGATGGAC-3` & 5`-ACCTTGTTTGCCAGGTTCAC-3` for collagen type I; 5`-AGGCAACAGTGGTTCTCCTG-3` & 5`-GACCTCGTGCTCCAGTTAGC-3` for collagen type III; 5`-GGCGGTACACAGTCAGACCAT-3` & 5`-TGGTGTGCATCACGAAGGA-3` for collagen type IV; 5`-CGAGGTGACAGAGACCACAA-3` & 5`-CTGGAGTCAAGCCAGACACA-3` for fibronectin; and 5′-TCGATGCTCTTAGCTGAGTG-3′ & 5′-TGATCGTCTTCGAACCTCC-3′ for 18S rRNA.

*4.4. cAMP accumulation determination*

cAMP accumulation was measured with the "Direct cAMP ELISA kit" (Product #ADI-900-066; Enzo Life Sciences, Farmingdale, NY), essentially as described previously [44].

*4.5. Co-immunoprecipitation and western blotting*

H9c2 cell extracts were prepared, as described previously [43], in a 20 mM Tris pH 7.4 buffer containing 137 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM PMSF, 1 mM $Na_3VO_4$, 10 mM NaF, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. Protein concentration was determined and equal amounts of protein per sample were used for immunoprecipitation (IP) or western blotting. Epac1 was detected via western blotting in total cellular extracts with an anti-Epac1 antibody (sc-28366; Santa Cruz Biotechnology, Santa Cruz, CA, USA), coupled with immunoblotting for GAPDH with an anti-GAPDH antibody (sc-25778; Santa Cruz Biotechnology) as protein loading control. For the OPN-$G_\alpha s$ co-IPs, OPN was immunoprecipitated by overnight incubation of H9c2 cell protein extracts with the mouse anti-OPN

Peer-reviewed version available at Int. J. Mol. Sci. 2019, 20, 1396; doi:10.3390/ijms20061396



antibody (DSHB) attached to Protein A/G-Sepharose beads (Sigma-Aldrich). The IPs were then subjected to immunoblotting for $G_{\alpha}s$/olf protein (sc-55545; Santa Cruz Biotechnology), and for OPN again, to confirm IP of equal amounts of endogenous OPN. All immunoblots were revealed by enhanced chemiluminescence (ECL, Life Technologies, Grand Island, NY, USA) and visualized in the FluorChem E Digital Darkroom (Protein Simple, San Jose, CA, USA), as described previously [6, 43-46]. Densitometry was performed with the AlphaView software (Protein Simple) in the linear range of signal detection (on non-saturated bands).

*4.6. Statistical analysis*

Data are generally expressed as means ± S.E.M. Unpaired two-tailed Student's t-test and one-way ANOVA with Bonferroni test were performed for statistical comparisons. For all tests, a p value $< 0.05$ was generally considered to be significant.

**Author Contributions:** C.M.P., V.L.D., S.L.W., and A.P. performed research. J.M. and K.A.M. contributed to the writing of the manuscript. L.A.S. provided reagents/materials for the study and assisted with the writing of the manuscript. A.L. designed and supervised the study and wrote the paper.

**Funding:** This research was funded by an NSU`s President`s Faculty Research & Development Grant (PFRDG) (to A.L.).

**Conflicts of Interest:** The authors declare no conflict of interest related to this publication.

## References

1. Berk, B.C.; Fujiwara, K.; Lehoux, S., ECM remodeling in hypertensive heart disease. *J Clin Invest.* **2007**, 117, (3), 568-575.

2. Kong, P.; Christia, P.; Frangogiannis, N.G., The pathogenesis of cardiac fibrosis. *Cell Mol Life Sci* 2014, 71, (4), 549–574.

403 3. Capote, L.A.; Mendez Perez, R.; Lymperopoulos, A., GPCR signaling and cardiac
404 function. *Eur J Pharmacol* 2015, 763, (Pt B), 143-148.
405 4. Lymperopoulos, A.; Rengo, G.; Koch, W.J., Adrenergic nervous system in heart
406 failure: pathophysiology and therapy. *Circ Res* **2013**, 113, (6), 739-753.
407 5. Desimine, V.L.; McCrink, K.A.; Parker, B.M.; Wertz, S.L.; Maning, J.;
408 Lymperopoulos, A., Biased Agonism/Antagonism of Cardiovascular GPCRs for
409 Heart Failure Therapy. *Int Rev Cell Mol Biol* **2018**, 339, 41-61.
410 6. Salazar, N.C.; Vallejos, X.; Siryk, A.; Rengo, G.; Cannavo, A.; Liccardo, D.; De
411 Lucia, C.; Gao, E.; Leosco, D.; Koch, W.J.; Lymperopoulos, A., GRK2 blockade with
412 βARKct is essential for cardiac β2-adrenergic receptor signaling towards increased
413 contractility. *Cell Commun Signal* **2013**, 11, 64.
414 7. Meszaros, J.G.; Gonzalez, A.M.; Endo-Mochizuki, Y.; Villegas, S.; Villarreal, F.;
415 Brunton, L.L., Identification of G protein-coupled signaling pathways in cardiac
416 fibroblasts: cross talk between G(q) and G(s). *Am J Physiol Cell Physiol* **2000**, 278, (1),
417 C154–C162.
418 8. Ostrom, R.S.; Naugle, J.E.; Hase, M.; Gregorian, C.; Swaney, J.S.; Insel, P.A.;
419 Brunton L.L.; Meszaros, J.G., Angiotensin II enhances adenylyl cyclase signaling via
420 Ca2þ/calmodulin. Gq-Gs cross-talk regulates collagen production in cardiac
421 fibroblasts. *J Biol Chem* 2003, 278, (27), 24461–24468.
422 9. Surinkaew, S.; Aflaki, M.; Takawale, A.; Chen, Y.; Qi, X.Y.; Gillis, M.A.; Shi, Y.F.;
423 Tardif, J.C.; Chattipakorn, N.; Nattel, S., Exchange protein activated by cyclic-
424 adenosine monophosphate (Epac) regulates atrial fibroblast function and controls
425 cardiac remodelling. *Cardiovasc Res* **2019**, 115, (1), 94-106.
426 10. Lymperopoulos, A.; Garcia, D.; Walklett, K., Pharmacogenetics of cardiac
427 inotropy. *Pharmacogenomics* **2014**, 15, (14), 1807-1821.
428 11. Bers, D.M., Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol*
429 **2008**, 70, 23-49.
430 12. Bos, J.L., Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci* **2006**, 31,
431 (12), 680–686.

13. de Rooij, J.; Zwartkruis, F.J.; Verheijen, M.H.; Cool, R.H.; Nijman, S.M.; Wittinghofer, A.; Bos, J.L., Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **1998**, 396, (6710), 474–477.

14. Yokoyama, U.; Patel, H.H.; Lai, N.C.; Aroonsakool, N.; Roth, D.M.; Insel, P.A., The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci USA* 2008, 105, (17), 6386–6391.

15. Metrich, M.; Lucas, A.; Gastineau, M.; Samuel, J.L.; Heymes, C.; Morel, E.; Lezoualc'h, F., Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res* **2008**, 102, (8), 959–965.

16. Insel, P.A.; Murray, F.; Yokoyama, U.; Romano, S.; Yun, H.; Brown, L.; Snead, A.; Lu, D.; Aroonsakool, N., cAMP and Epac in the regulation of tissue fibrosis. *Br J Pharmacol* **2012**, 166, (2), 447-456.

17. Lai, C.F.; Seshadri, V.; Huang, K.; Shao, J.S.; Cai, J.; Vattikuti, R.; Schumacher, A.; Loewy, A.P.; Denhardt, D.T.; Rittling, S.R.; Towler, D.A., An osteopontin-NADPH oxidase signaling cascade promotes pro-matrix metalloproteinase 9 activation in aortic mesenchymal cells. *Circ Res* **2006**, 98, (12), 1479–1489.

18. Liaw, L.; Almeida, M.; Hart, C.E.; Schwartz, S.M.; Giachelli, C.M., Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. *Circ Res* **1994,** 74, (2), 214–224.,

19. Liaw, L.; Birk, D.E.; Ballas, C.B.; Whitsitt, J.S.; Davidson, J.M.; Hogan, B.L., Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest* **1998**, 101, 1468–1478.

20. Irita, J.; Okura, T.; Jotoku, M.; Nagao, T.; Enomoto, D.; Kurata, M.; Desilva, V.R.; Miyoshi, K.; Matsui, Y.; Uede, T.; Denhardt, D.T.; Rittiling, S.R.; Higaki, J., Osteopontin deficiency protects against aldosterone-induced inflammation, oxidative stress, and interstitial fibrosis in the kidney. *Am J Physiol Renal Physiol* **2011**, 301, (4), F833-F834.

21. Giachelli, C.M.; Bae, N.; Almeida, M.; Denhardt, D.T.; Alpers, C.E.; Schwartz, S.M., Osteopontin is elevated during neointima formation in rat arteries and is a

461 novel component of human atherosclerotic plaques. *J Clin Invest* **1993**, 92, (4), 1686–
462 1696.
463 22. Golledge, J.; McCann, M.; Mangan, S.; Lam, A.; Karan, M., Osteoprotegerin and
464 osteopontin are expressed at high concentrations within symptomatic carotid
465 atherosclerosis. *Stroke* **2004**, 35, (7), 1636–1641.
466 23. Matsui, Y.; Jia, N.; Okamoto, H.; Kon, S.; Onozuka, H.; Akino, M.; Liu, L.;
467 Morimoto, J.; Rittling, S.R.; Denhardt, D.; Kitabatake, A.; Uede, T., Role of
468 osteopontin in cardiac fibrosis and remodeling in angiotensin II-induced cardiac
469 hypertrophy. *Hypertension* **2004,** 43, (6), 1195–1201.
470 24. Trueblood, N.A.; Xie, Z.; Communal, C.; Sam, F.; Ngoy, S.; Liaw, L.; Jenkins,
471 A.W.; Wang, J.; Sawyer, D.B.; Bing, O.H.; Apstein, C.S.; Colucci, W.S.; Singh, K.,
472 Exaggerated left ventricular dilation and reduced collagen deposition after
473 myocardial infarction in mice lacking osteopontin. *Circ Res* **2001**, 88, (10), 1080–1087.
474 25. Yano, N.; Suzuki, D.; Endoh, M.; Tseng, A.; Stabila, J.P.; McGonnigal, B.G.; Zhao,
475 T.C.; Padbury, J.F.; Tseng, Y.T., Beta-adrenergic receptor mediated protection
476 against doxorubicin-induced apoptosis in cardiomyocytes: the impact of high
477 ambient glucose. *Endocrinology* **2008**, 149, (12), 6449-6461.
478 26. Mlih, M.; Abdulrahman, N.; Gadeau, A.P.; Mohamed, I.A.; Jaballah, M.; Mraiche,
479 F., Na(+)/H (+) exchanger isoform 1 induced osteopontin expression in
480 cardiomyocytes involves NFAT3/Gata4. *Mol Cell Biochem* **2015**, 404, (1-2), 211-220.
481 27. Parker, B.M.; Wertz, S.L.; Pollard, C.M.; Desimine, V.L.; Maning, J.; McCrink,
482 K.A.; Lymperopoulos, A., Novel Insights into the Crosstalk between
483 Mineralocorticoid Receptor and G Protein-Coupled Receptors in Heart Adverse
484 Remodeling and Disease. *Int J Mol Sci* **2018**, 19, (12).
485 28. Kiyosue, A.; Nagata, D.; Myojo, M.; Sato, T.; Takahashi, M.; Satonaka, H.; Nagai,
486 R.; Hirata, Y., Aldosterone-induced osteopontin gene transcription in vascular
487 smooth muscle cells involves glucocorticoid response element. *Hypertens Res* **2011**,
488 34, (12), 1283-1287.

29. Nagao, M.; Feinstein, T.N.; Ezura, Y.; Hayata, T.; Notomi, T.; Saita, Y.; Hanyu, R.; Hemmi, H.; Izu, Y.; Takeda, S.; Wang, K.; Rittling, S.; Nakamoto, T.; Kaneko, K.; Kurosawa, H.; Karsenty, G.; Denhardt, D.T.; Vilardaga, J.P.; Noda, M., Sympathetic control of bone mass regulated by osteopontin. *Proc Natl Acad Sci USA* **2011**, 108, (43), 17767-17772.

30. Lymperopoulos, A.; Rengo, G.; Zincarelli, C.; Kim, J.; Koch, W.J., Adrenal beta-arrestin 1 inhibition in vivo attenuates post-myocardial infarction progression to heart failure and adverse remodeling via reduction of circulating aldosterone levels. *J Am Coll Cardiol* **2011**, 57, (3), 356-365.

31. Cao, C.; Luo, X.; Ji, X.; Wang, Y.; Zhang, Y.; Zhang, P.; Zhong, L., Osteopontin regulates the proliferation of rat aortic smooth muscle cells in response to gingipains treatment. *Mol Cell Probes* **2017**, 33, 51-56.

32. Sugiyama, T.; Yoshimoto, T.; Hirono, Y.; Suzuki, N.; Sakurada, M.; Tsuchiya, K.; Minami, I.; Iwashima, F.; Sakai, H.; Tateno, T.; Sato, R.; Hirata, Y., Aldosterone increases osteopontin gene expression in rat endothelial cells. *Biochem Biophys Res Commun* **2005**, 336, (1), 163–167.

33. Miura, R.; Nakamura, K.; Miura, D.; Miura, A.; Hisamatsu, K.; Kajiya, M.; Hashimoto, K.; Nagase, S.; Morita, H.; Fukushima Kusano, K.; Emori, T.; Ishihara, K.; Ohe, T., Aldosterone synthesis and cytokine production in human peripheral blood mononuclear cells. *J Pharmacol Sci* **2006**, 102, (3), 288–295.

34. Xiang, Y.; Kobilka, B.K., Myocyte adrenoceptor signaling pathways. *Science* **2003**, 300, (5625), 1530-1532.

35. Lymperopoulos, A.; Rengo, G.; Koch, W.J., GRK2 inhibition in heart failure: something old, something new. *Curr Pharm Des* **2012**, 18, 186–191.

36. Singhmar, P.; Huo, X.; Eijkelkamp, N.; Berciano, S.R.; Baameur, F.; Mei, F.C.; Zhu, Y.; Cheng, X.; Hawke, D.; Mayor, F. Jr.; Murga, C.; Heijnen, C.J.; Kavelaars, A., Critical role for Epac1 in inflammatory pain controlled by GRK2-mediated phosphorylation of Epac1. *Proc Natl Acad Sci USA* **2016**, 113, (11), 3036-3041.

517 37. Belden, Z.; Deiuliis, J.A.; Dobre, M.; Rajagopalan, S., The Role of the
518 Mineralocorticoid Receptor in Inflammation: Focus on Kidney and Vasculature. *Am*
519 *J Nephrol* **2017**, 46, (4), 298-314.
520 38 Brown, N.J., Contribution of aldosterone to cardiovascular and renal
521 inflammation and fibrosis. *Nat Rev Nephrol* **2013**, 9, (8), 459-469.
522 39. Fuller, P.J.; Yang, J.; Young, M.J., 30 years of the mineralocorticoid receptor:
523 coregulators as mediators of mineralocorticoid receptor signaling diversity. *J*
524 *Endocrinol* **2017**, 234, (1), T23–T34.
525 40. Faresse, N., Post-translational modifications of the mineralocorticoid receptor:
526 How to dress the receptor according to the circumstances? *J Steroid Biochem Mol Biol*
527 **2014**, 143, 334–342.
528 41. Walther, R.F.; Atlas, E.; Carrigan, A.; Rouleau, Y.; Edgecombe, A.; Visentin, L.;
529 Lamprecht, C.; Addicks, G.C.; Haché, R.J.; Lefebvre, Y.A., A serine/threonine-rich
530 motif is one of three nuclear localization signals that determine unidirectional
531 transport of the mineralocorticoid receptor to the nucleus. *J Biol Chem* **2005**, 280, (17),
532 17549-17561.
533 42. Komolov, K.E.; Du, Y.; Duc, N.M.; Betz, R.M.; Rodrigues, J.P.G.L.M.; Leib, R.D.;
534 Patra, D.; Skiniotis, G.; Adams, C.M.; Dror, R.O.; Chung, K.Y.; Kobilka, B.K.;
535 Benovic, J.L., Structural and Functional Analysis of a $\beta_2$-Adrenergic Receptor
536 Complex with GRK5. *Cell* **2017**, 169, (3), 407-421.
537 43. McCrink, K.A.; Maning, J.; Vu, A.; Jafferjee, M.; Marrero, C.; Brill, A.; Bathgate-
538 Siryk, A.; Dabul, S.; Koch, W.J.; Lymperopoulos, A., β-Arrestin2 Improves Post-
539 Myocardial Infarction Heart Failure via Sarco(endo)plasmic Reticulum $Ca_{2+}$-
540 ATPase-Dependent Positive Inotropy in Cardiomyocytes. *Hypertension* **2017**, 70, (5),
541 972-981.
542 44. Bathgate-Siryk, A.; Dabul, S.; Pandya, K.; Walklett, K.; Rengo, G.; Cannavo, A.;
543 De Lucia, C.; Liccardo, D.; Gao, E.; Leosco, D.; Koch, W.J.; Lymperopoulos, A.,
544 Negative impact of β-arrestin-1 on post-myocardial infarction heart failure via

545 cardiac and adrenal-dependent neurohormonal mechanisms. *Hypertension* **2014**, 63,
546 (2), 404-412.
547 45. Jafferjee, M.; Reyes Valero, T.; Marrero, C.; McCrink, K.A.; Brill, A.;
548 Lymperopoulos, A., GRK2 Up-Regulation Creates a Positive Feedback Loop for
549 Catecholamine Production in Chromaffin Cells. *Mol Endocrinol* **2016**, 30, (3), 372-381.
550 46. Lymperopoulos, A.; Rengo, G.; Funakoshi, H.; Eckhart, A.D.; Koch, W.J., Adrenal
551 GRK2 upregulation mediates sympathetic overdrive in heart failure. *Nat Med* **2007**,
552 13, (3), 315-323.
553
554