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Deletion of osteopontin enhances β_2-adrenergic receptor-dependent anti-fibrotic signaling in cardiomyocytes

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Abstract: Cardiac β_2-adrenergic receptors (ARs) are known to inhibit collagen production and fibrosis in cardiac fibroblasts and myocytes. The β_2AR 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

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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 β_2AR activation. Additionally, CRISPR-mediated OPN deletion enhances cAMP generation in response to both β_1AR and β_2AR activation in H9c2 cardiomyocytes, leading to upregulation of Epac1 protein levels. These effects render β_2AR stimulation capable of completely abrogating transforming growth factor (TGF)-β-dependent fibrosis in OPN-lacking H9c2 cardiomyocytes. Finally, OPN interacts constitutively with G_αs subunits in H9c2 cardiac cells. Thus, we have uncovered a direct inhibitory role of OPN in cardiac β_2AR anti-fibrotic signaling via cAMP/Epac1. OPN blockade could be of value in the treatment and/or prevention of cardiac fibrosis.

Keywords: β_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

β_1AR being the most abundant and mediating the positive inotropic and chronotropic actions of the sympathetic nervous system, whereas the less abundant β_2AR exerts several cardio-protective effects in the post-MI heart, such as inhibition of apoptosis, inflammation, fibrosis, etc. [4, 6]. Indeed, β_2AR inhibits collagen production and fibrosis in cardiac fibroblasts [7-9]. All ARs belong to the G protein-coupled receptor (GPCR) superfamily and both β_1AR & β_2AR activate the Gs protein signaling pathway in cardiac cells, leading to activation of adenylyl cyclase (AC) and subsequent cyclic 3`, 5`-adenosine monophosphate (cAMP) synthesis [3]. cAMP is a major second messenger inside cells and, in cardiac myocytes, it is responsible for stimulation of cardiac contractility thanks, mainly, to activation of its effector kinase PKA (protein kinase A or cAMP-dependent protein kinase), which phosphorylates a variety of substrates to facilitate contraction in the cardiac myocyte [4, 10, 11]. In addition to PKA, cAMP also directly activates the exchange protein directly activated by cAMP (Epac) [12, 13]. Two Epac isoforms have been characterized, Epac1 and Epac2. Epac1 is expressed ubiquitously and is quite abundant in the heart, including cardiac fibroblasts [14]. This Epac isoform has been documented to decrease collagen expression in response to βAR activation in rat cardiac fibroblasts and its expression is downregulated in the heart post-MI and in response to pro-fibrotic stimuli [12, 14, 16]. Epac1 blocks collagen and DNA synthesis in rat cardiac fibroblasts and was very recently reported to also block atrial fibroblast activation, migration, and secretion of fibrotic mediators in post-MI mice and in HF dogs [9, 14].

Osteopontin (OPN) is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family and is expressed in normal mineralized tissues and cell types including osteoblasts, macrophages, lymphocytes, vascular smooth muscle cells (VSMCs), renal and cardiac fibroblasts, and several neoplastic tissues [17-19]. As a pro-inflammatory protein, OPN induces VSMC inflammation in the brain, pancreas, kidney, and heart [20]. It also participates in cell adhesion and migration processes via interacting with various integrins and CD44, and is a potent chemokine for mononuclear cells and VSMCs [20]. Thus, it comes as no surprise that

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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 β_1AR & β_2AR and OPN [25, 26], we found that OPN substantially impedes β_2AR-induced, cAMP/Epac1-dependent anti-fibrotic signaling in cardiac cells.

2. Results

2.1. Regulation of OPN expression by aldosterone and the β_2AR 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].

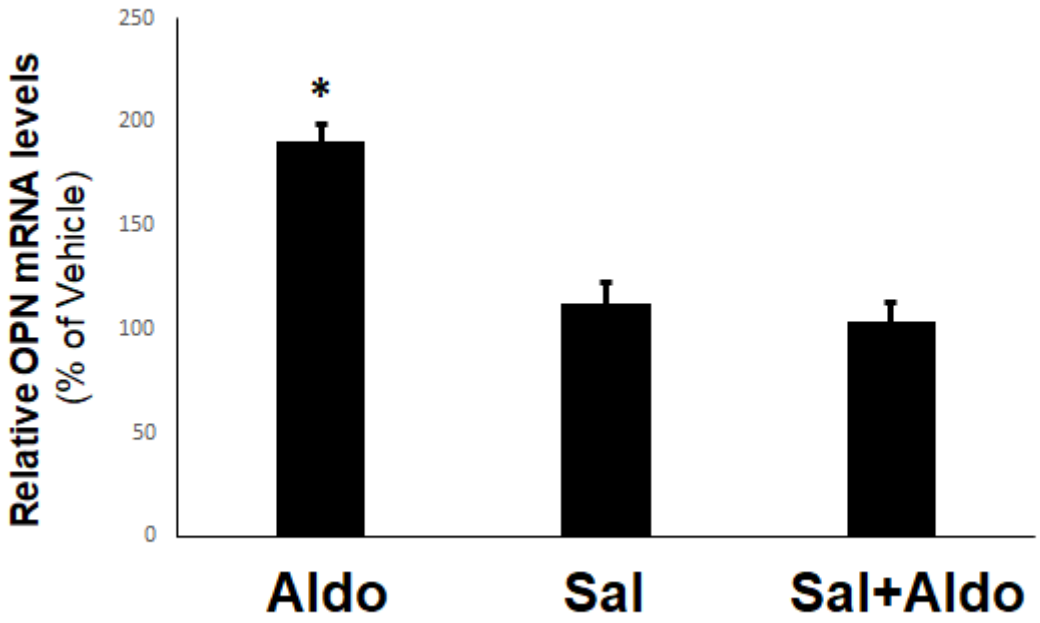

Figure 1. β_2AR inhibits aldosterone-induced OPN upregulation in H9c2 cardiomyocytes.

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, β_2AR 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 β_2AR stimulation may inhibit (at least some of) the genomic, MR-dependent effects of aldosterone in H9c2 cardiac cells.

2.2. OPN opposes β_2AR cAMP signaling in H9c2 cardiomyocytes

Since the β_2AR inhibits OPN upregulation in H9c2 cardiomyocytes, an effect consistent with this receptor`s anti-fibrotic actions in the heart, we posited that there is perhaps a negative feedback loop operating in cardiac myocytes allowing OPN to reciprocally regulate (inhibit) β_2AR function and signaling. To this end, we deleted the OPN gene in H9c2 cells via CRISPR and compared the extent of cAMP accumulation, the major second messenger generated by βARs regulating cellular fibrosis [16], between cells having OPN deleted (OPN-knockout (KO) cells) and wild type (WT) cells. After confirming the genetic deletion of OPN at the protein level (**Figure 2A**), we stimulated the cells with either isoproterenol to activate both β_1ARs & β_2ARs or salbutamol to selectively activate only the β_2ARs and measured acute cAMP generation.

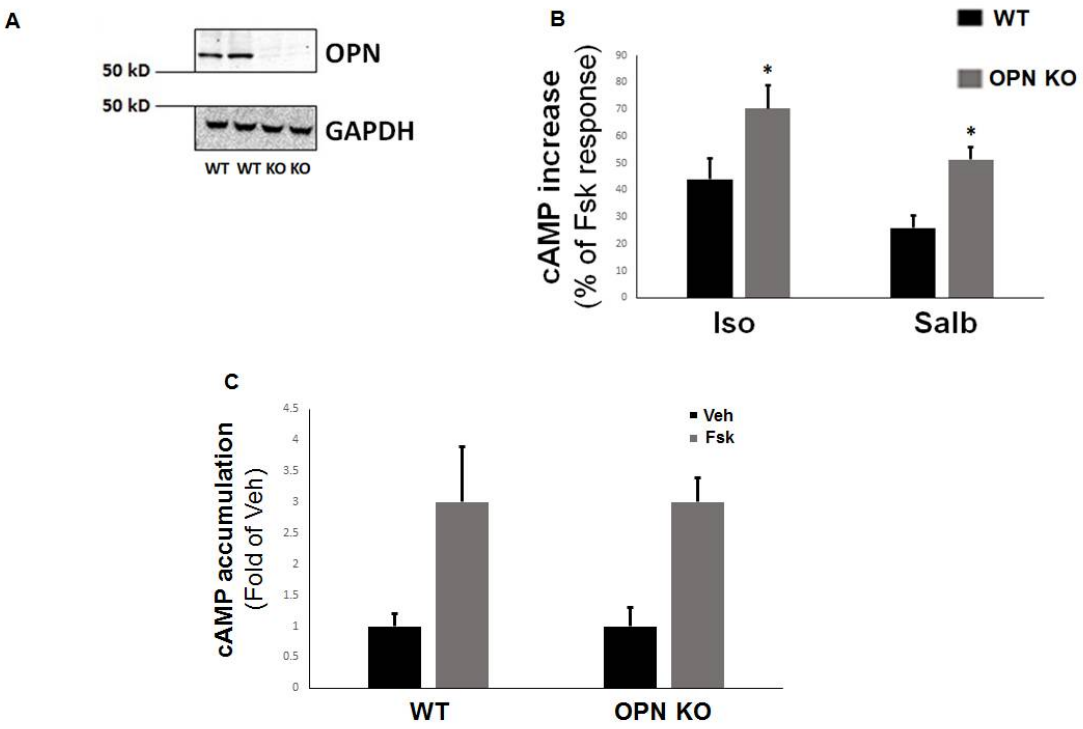

Figure 2. Enhanced β_2AR-dependent cAMP accumulation in the absence of OPN in H9c2 cardiomyocytes.

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

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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 β_2AR-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.

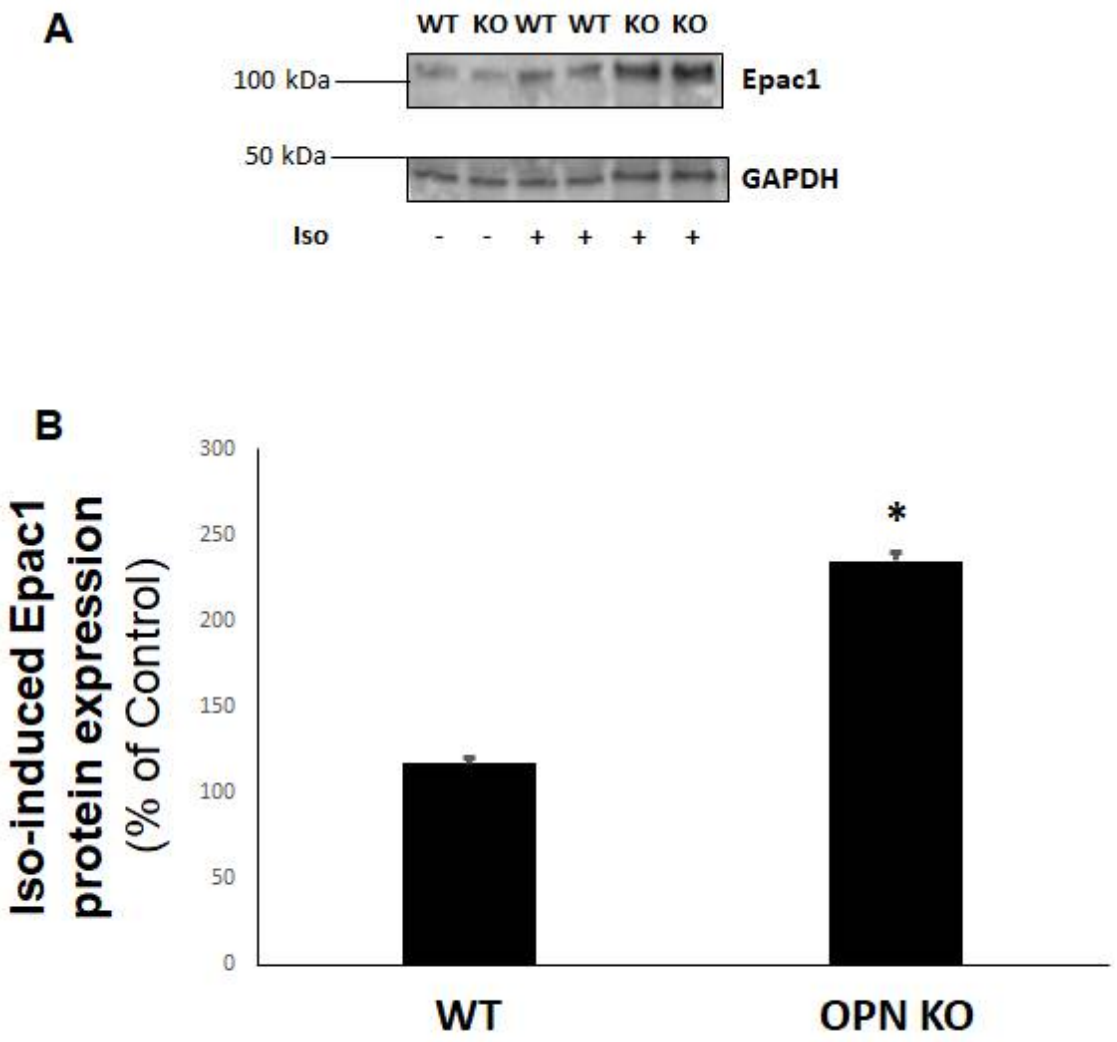

Figure 3. Enhanced βAR-dependent Epac1 protein levels in the absence of OPN in H9c2 cardiomyocytes.

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

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To examine the impact of OPN`s β_2AR signaling effects on fibrosis of H9c2 cardiomyocytes, we measured the β_2AR-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.

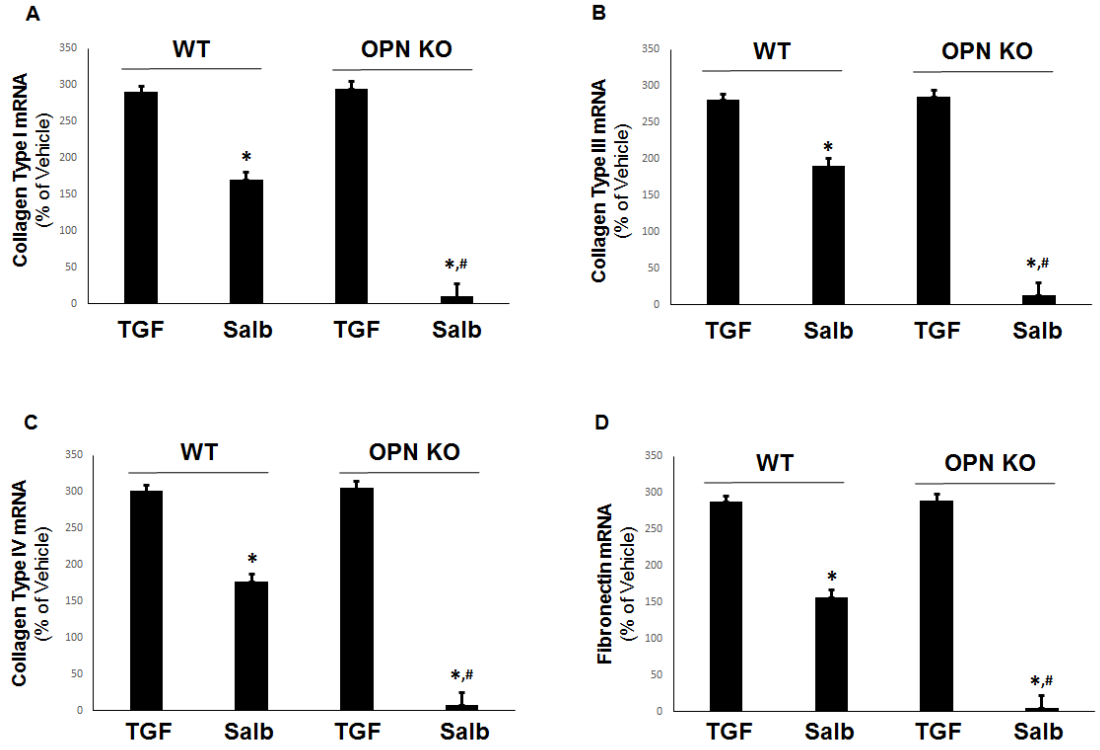

Figure 4. Absence of OPN potentiates the β_2AR-mediated inhibition of TGFβ-dependent profibrotic factor mRNA induction in H9c2 cells.

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-β_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, β_2AR 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

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enabled salbutamol to completely abrogate the TGFβ-dependent expression of all of these pro-fibrotic factors: collagen I (**Figure 4A**), collagen III (**Figure 4B**), collagen IV (**Figure 4C**), and fibronectin (**Figure 4D**). Thus, OPN significantly hinders the ability of the β_2AR to block fibrosis via cAMP and Epac1 in cardiac myocytes.

2.5. OPN inhibits β_2AR cAMP signaling by directly interacting with the G_αs/olf protein subunit in H9c2 cardiomyocytes

In an attempt to begin to dissect the molecular mechanism by which OPN perturbs β_2AR`s cAMP-mediated anti-fibrotic signaling in H9c2 cardiomyocytes, we examined OPN`s interaction with the G_αs/olf protein subunit, previously reported to occur in osteoblastic cells resulting in β_2AR dysfunction [29]. Indeed, co-immunoprecipitation experiments indicated that OPN and the G_αs subunit protein interact with each other constitutively (in the absence of any stimulus), albeit weakly, in H9c2 cardiac cells (**Figure 5A**). Furthermore, this interaction is unaffected by β_2AR activation, since salbutamol neither enhances nor reduces it (**Figures 5A & 5B**). Thus, similarly to bone cells, OPN physically interacts with G_αs subunits inside cardiac cells impeding downstream β_2AR signaling to AC, cAMP and Epac1.

3. Discussion

In the present study, we report, for the first time to our knowledge, the adverse role OPN plays at hindering β_2AR-dependent, cAMP/Epac1-mediated anti-fibrotic signaling and function in rat cardiac myocyte-like cells. By physically interacting constitutively with the G_α subunit of the Gs heterotrimeric protein, intracellular OPN reduces the cAMP generating capacity of the β_2AR in cardiac cells, thereby reducing both the activity (acutely) and protein expression (long-term) of the cAMP effector Epac1, which is normally anti-fibrotic in several tissues, including in the heart (**Figure 6**) [16]. Additionally, we found that aldosterone, a powerful pro-fibrotic and adverse remodeling-associated hormone in the heart that is also elevated during post-MI HF progression [30], directly induces OPN transcriptional

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upregulation to mediate fibrosis (**Figure 6**). Notably, this aldosterone-induced OPN upregulation is blocked by the β_2AR, 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 β_2AR-dependent, cAMP/Epac1-mediated signaling, and on the other hand, the β_2AR opposes the aldosterone/MR-mediated OPN induction (**Figure 6**).

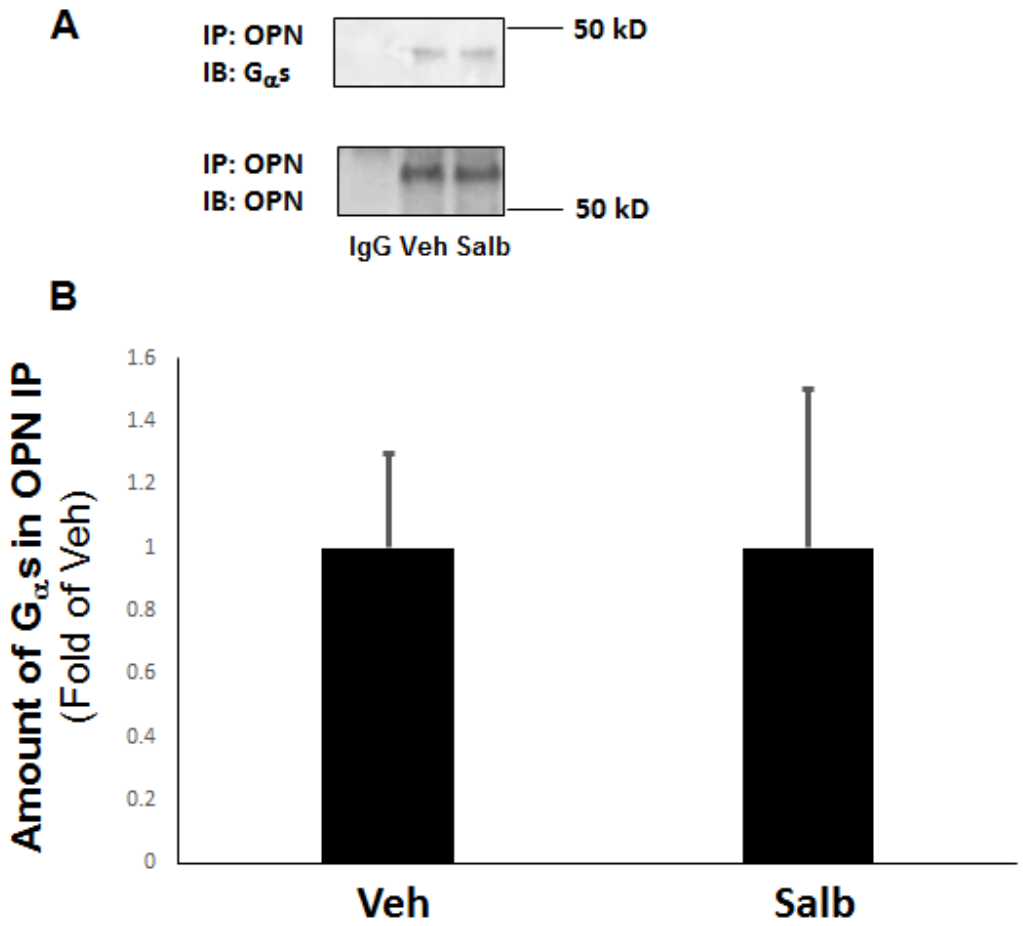

Figure 5. OPN opposes β_2AR signaling via physical interaction with G_αs in H9c2 cardiomyoblasts.

Co-immunoprecipitation (co-IP) of OPN with G_αs 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_αs co-IP`d with OPN was observed between Veh and Salb (n=3).

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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.

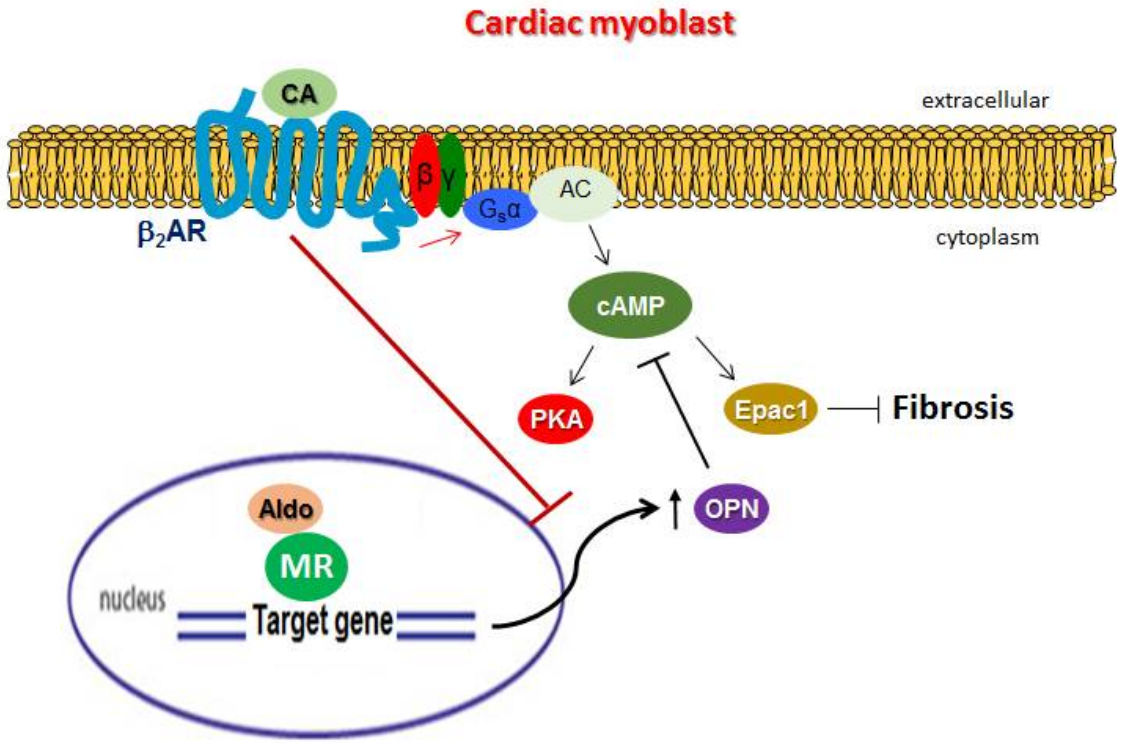

Figure 6. Schematic illustration of the proposed role of OPN in β_2AR ant-fibrotic signaling in H9c2 cardiomyocytes.

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,

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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 β_2AR. The β_2AR, in contrast with the β_1AR, 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 β_2AR 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 β_2AR-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 β_2AR 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 (β_2AR-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 β_2AR 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 β_2AR [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 β_2AR`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

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through which OPN opposes β_2AR anti-fibrotic signaling to cAMP/Epac1 is definitely worth pursuing and is the goal of our currently ongoing studies.

Another major question arising from our present findings pertains to the mechanism by which the β_2AR, in a negative feedback regulatory manner, opposes the aldosterone/MR-dependent OPN upregulation in cardiac myocytes (**Figure 6**). How does the β_2AR (a GPCR) block this aldosterone/MR transcriptional effect? The MR is a ~1,000-amino acid cytoplasmic (at rest) protein with three functional domains: the N-terminal domain (NTD) that regulates transcriptional activity of the receptor; the DNA-binding domain (DBD) involved in the binding of the promoter of the target gene; and the ligand-binding domain (LBD) responsible for hormone binding [37, 38]. In the nucleus, the MR depends on numerous molecular co-regulators to activate and regulate its target genes that carry the (shared with the glucocorticoid receptor) glucocorticoid response element (GRE) sequence in their promoters [39]. Importantly, the MR undergoes various post-translational modifications, such as phosphorylation, ubiquitination, etc., which play important roles in regulation of its nuclear translocation and of its transcriptional activity [40]. Indeed, the MR contains several serine and threonine residues that are substrates for kinases like PKA, whose phosphorylation of the MR has been reported to inhibit MR cytoplasm-to-nucleus trafficking [40, 41]. Given that PKA is activated by the β_2AR (**Figure 6**), β_2AR may inhibit MR-dependent OPN upregulation through PKA. Another possibility is that GRK5, the other major GRK isoform in cardiac cells [4, 35], which is also activated by the agonist-occupied β_2AR [42], inhibits the MR by directly phosphorylating it (A. Lymperopoulos, unpublished data & Ref. 27). We plan to investigate these possibilities and to delineate the exact mechanism of β_2AR`s regulation of aldosterone signaling in the heart in future studies.

In summary, we report here that the ubiquitous pro-inflammatory cytokine OPN, transcriptionally induced by aldosterone and the MR, mediates cardiac fibrosis in part via perturbation of the β_2AR`s cAMP/Epac1-dependent anti-fibrotic signaling in rat cardiac myoblasts. The β_2AR, in turn, inhibits the aldosterone-induced OPN upregulation in the heart, thereby closing a feedback regulation loop. These findings

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 β_2AR 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 TGFβ_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

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

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.

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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.

3. Capote, L.A.; Mendez Perez, R.; Lymperopoulos, A., GPCR signaling and cardiac function. *Eur J Pharmacol* 2015, 763, (Pt B), 143-148.

4. Lymperopoulos, A.; Rengo, G.; Koch, W.J., Adrenergic nervous system in heart failure: pathophysiology and therapy. *Circ Res* **2013**, 113, (6), 739-753.

5. Desimine, V.L.; McCrink, K.A.; Parker, B.M.; Wertz, S.L.; Maning, J.; Lymperopoulos, A., Biased Agonism/Antagonism of Cardiovascular GPCRs for Heart Failure Therapy. *Int Rev Cell Mol Biol* **2018**, 339, 41-61.

6. Salazar, N.C.; Vallejos, X.; Siryk, A.; Rengo, G.; Cannavo, A.; Liccardo, D.; De Lucia, C.; Gao, E.; Leosco, D.; Koch, W.J.; Lymperopoulos, A., GRK2 blockade with βARKct is essential for cardiac β2-adrenergic receptor signaling towards increased contractility. *Cell Commun Signal* **2013**, 11, 64.

7. Meszaros, J.G.; Gonzalez, A.M.; Endo-Mochizuki, Y.; Villegas, S.; Villarreal, F.; Brunton, L.L., Identification of G protein-coupled signaling pathways in cardiac fibroblasts: cross talk between G(q) and G(s). *Am J Physiol Cell Physiol* **2000**, 278, (1), C154–C162.

8. Ostrom, R.S.; Naugle, J.E.; Hase, M.; Gregorian, C.; Swaney, J.S.; Insel, P.A.; Brunton L.L.; Meszaros, J.G., Angiotensin II enhances adenylyl cyclase signaling via Ca2þ/calmodulin. Gq-Gs cross-talk regulates collagen production in cardiac fibroblasts. *J Biol Chem* 2003, 278, (27), 24461–24468.

9. Surinkaew, S.; Aflaki, M.; Takawale, A.; Chen, Y.; Qi, X.Y.; Gillis, M.A.; Shi, Y.F.; Tardif, J.C.; Chattipakorn, N.; Nattel, S., Exchange protein activated by cyclic-adenosine monophosphate (Epac) regulates atrial fibroblast function and controls cardiac remodelling. *Cardiovasc Res* **2019**, 115, (1), 94-106.

10. Lymperopoulos, A.; Garcia, D.; Walklett, K., Pharmacogenetics of cardiac inotropy. *Pharmacogenomics* **2014**, 15, (14), 1807-1821.

11. Bers, D.M., Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* **2008**, 70, 23-49.

12. Bos, J.L., Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci* **2006**, 31, (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 β_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

 doi:10.20944/preprints201902.0266.v1

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