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## Phosphodiesterase-5 is Elevated in Failing Single Ventricle Myocardium and Affects Cardiomyocyte Remodeling *in vitro*

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### Abstract

**Background:** Single ventricle congenital heart disease (SV) is fatal without intervention and eventual heart failure (HF) is a major cause of morbidity and mortality. While there are no proven medical therapies for the treatment or prevention of HF in the SV population, phosphodiesterase-5 inhibitors (PDE5i), such as sildenafil, are increasingly utilized. While the pulmonary vasculature is the primary target of PDE5i therapy in patients with SV, the effects of PDE5i on the SV myocardium remain largely unknown. We sought to determine PDE5 expression and activity in the single right ventricle (RV) of SV patients relative to non-failing (NF) controls, and to determine if PDE5 impacts cardiomyocyte remodeling using a novel serum based *in vitro* model.

**Methods and Results:** PDE5 expression (n=9 NF, n=7 SV), activity (n=8 NF, n=9 SV) and localization (n=3 SV) were determined in explanted human RV myocardium. PDE5 is expressed in SV cardiomyocytes and PDE5 protein expression and activity are increased in SV RV compared to NF RV. Isolated neonatal rat ventricular myocytes (NRVMs) were treated for 72 hours with NF or SV patient serum ± sildenafil. RT-qPCR (n=5 NF, n=12 SV) and RNAseq (n=3 NF, n=3 SV) were performed on serum-treated NRVMs and demonstrated that treatment with SV sera results in pathological gene expression changes which are attenuated with PDE5i.

**Conclusions:** PDE5 is increased in failing SV myocardium and pathologic gene expression changes in SV serum-treated NRVMs are abrogated by PDE5i. These results suggest that PDE5 represents an intriguing myocardial therapeutic target in this population.

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Disclosures

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## Introduction

Congenital heart defects (CHD) are the single most common type of birth defect and a leading cause of infant death in the United States<sup>1</sup>. Single ventricle heart disease (SV) is the most common form of severe CHD<sup>2</sup> and comprises a spectrum of congenital cardiac malformations defined by severe underdevelopment of one ventricle. SV is universally fatal without intervention, and although outcomes are improving, more than 30% of SV patients die or require transplant within the first year of life and 10-year survival in this population is only 39–50%<sup>2,3</sup>. While the single ventricle can be a morphologic right ventricle (RV), left ventricle (LV) or of indeterminate morphology, single RV lesions such as hypoplastic left heart syndrome (HLHS) may be inherently at risk for heart failure presumably due to inherent limitations in the RV's ability to tolerate increased afterload<sup>3–6</sup>. As such, eventual heart failure (HF) is a leading cause of death and indication for heart transplant in the SV population<sup>3</sup>. While surgical techniques and perioperative care for SV continue to improve, long-term survival and quality of life ultimately depend on preservation of RV function. However, the molecular mechanisms underlying failure of the single RV are poorly understood, limiting the ability to identify effective therapies. Moreover, the ability to investigate mechanisms involved in SV HF or effects of pharmacologic interventions are limited by the lack of a postnatal animal model of SV and the difficulties associated with performing *in vivo* research in children.

Pharmacological treatment of HF in the pediatric population is extrapolated from the adult HF experience, and treatment guidelines are based primarily on expert consensus<sup>7</sup>. In recent clinical trials, the SV population demonstrated a detrimental trend in response to carvedilol<sup>8</sup>, and a lack of benefit in preventing HF with enalapril<sup>9</sup>, medications commonly used to treat adult HF. Given the divergent response to these adult HF medications in the pediatric SV population, age-related mechanisms governing HF, as well as fundamental differences in RV versus LV failure likely exist.

While there are currently no proven medical therapies for the treatment or prevention of HF in the SV population, selective and competitive phosphodiesterase type-5 inhibitors (PDE5i), such as sildenafil, are increasingly used in children and adults with SV. However, while sildenafil has been associated with improved hemodynamics, exercise tolerance and myocardial function by echocardiography in small series of SV patients, its direct effects outside of the pulmonary vasculature remain largely unknown<sup>10–13</sup>. PDE5 expression is prominent throughout the pulmonary vasculature and in the coronary vessels, but there is virtually no detectable PDE5 expression in the normal adult myocardium<sup>14</sup>. Thus, the rationale for the use of PDE5i in the SV population is PDE5 inhibition in the pulmonary vasculature, thereby promoting vascular smooth muscle relaxation, augmenting pulmonary blood flow and secondarily SV cardiac output<sup>15</sup>. Correspondingly, PDE5i's have been shown to lack any significant direct effects on the myocardium of normal, non-failing (NF) human and animal hearts *in vitro*<sup>16,17</sup>. However, recent data in both human HF patients and in animal models of HF, suggest that myocardial and cardiomyocyte-specific PDE5 expression is increased under conditions of cardiac stress, and is physiologically significant<sup>18–20</sup>. Increased myocardial PDE5 for example, has been shown to be associated

with maladaptive myocardial responses, and its inhibition both globally and in cardiomyocytes specifically, can have direct beneficial myocardial effects<sup>21,22</sup>.

In the present study, we sought to determine myocardial PDE5 expression and activity in SV patients relative to NF controls, and to evaluate PDE5 localization within the myocardium. Additionally, we utilized a novel model of cardiomyocyte remodeling, in which primary cardiomyocytes are treated with SV patient sera +/- sildenafil, to assess changes in pathologic cardiac gene expression and the impact of PDE5i *in vitro*.

## Methods

The data, analytic methods, and study materials (with the exception of patient samples, as these represent a limited resource) will be made available to other researchers for purposes of reproducing the results or replicating the procedure.

### Human Samples

All subjects gave informed consent and donated their hearts and/or blood to the Institutional Review Board-approved Investigations of Pediatric Heart Disease tissue bank at the University of Colorado, Denver. NF RV tissues were from pediatric (<18 years of age) organ donors with normal heart structure and function, whose hearts could not be placed for technical reasons (size or blood type mismatch). Samples of NF sera were obtained from control children with normal heart function. SV RV tissue and sera were from patients transplanted with SV of RV morphology who were transplanted secondary to systolic heart failure (single ventricle disease of LV or indeterminate morphology were excluded). Inclusion criteria for SV patients were: age <18 years and abnormal SV systolic function on echocardiogram. Patients that were transplanted primarily for SV lacking these defined clinical characteristics were excluded. At the time of cardiac explant, the heart tissue was rapidly dissected in the operating room, flash frozen, and stored at -80°C until further use.

### PDE activity assays

Approximately 150 mg of RV myocardium was homogenized and separated into nuclear, cytosolic, and sarcoplasmic reticulum-enriched microsomal fractions by differential centrifugation. cGMP-hydrolytic activity in the cytosolic fraction was quantified at 30°C by the 2-step snake-venom method with (<sup>3</sup>H) cGMP (0.1 μmol/L) as substrate<sup>23,24</sup>. Total cGMP-hydrolytic activity was quantified by measuring activity without addition of PDE inhibitor. PDE5 activity was quantified by measuring activity in the absence and presence of 0.01 μmol/L sildenafil, a concentration that inhibits recombinant PDE5 submaximally (IC46) in order to minimize possible inhibition of other PDE families<sup>24</sup>. PDE5-specific activity was calculated by dividing the difference in activity between the absence and presence of sildenafil by the fractional inhibition of PDE5 activity at this concentration. The amount of protein used per assay were adjusted to ensure that 20% (±15%) of the total cGMP was hydrolyzed during the assay.

## Western blots

Western blots were performed as described<sup>25</sup>. Protein was isolated from 10- to 25-mg frozen RV tissue in isoelectric focusing buffer at 4°C. PDE5A (Cell Signaling) and GAPDH (Santa Cruz Biotechnology) were quantified on the same blot. Blots were quantified using ImageJ (U.S. National Institutes of Health).

## Immunohistochemistry and confocal microscopy

Briefly, freshly procured RV tissue was fixed in 4% paraformaldehyde at 4°C for 4–12 hours, rinsed in PBS and immersed in 30% sucrose solution for 6–12 hours at 4°C for cryoprotection. Fixed tissue was embedded and frozen in OCT media, and tissue was cryosectioned at –18 to –20°C into 6–10µm sections and mounted on microscope slides. Slides were stored at –80°C for no longer than 1 month before processing. Sections were fixed in ice cold acetone for 10 minutes, rinsed in PBS, and blocked for 1 hour in blocking buffer (2% normal goat sera, 1% BSA, and 0.1% NP-40 in PBS). Primary antibodies rabbit PDE5A (1:100, Abcam) and mouse sarcomeric  $\alpha$  actinin (1:400, Sigma Aldrich) were incubated overnight at 4°C, and Alexa Fluor- conjugated secondary antibodies (1:1000, Alexa 555 goat anti mouse and Alexa 488 goat anti rabbit, Sigma Aldrich) were incubated for 1 hour at room temperature. Nuclei were stained with DAPI (1µM, Invitrogen) for 10 minutes at room temperature. Secondary antibody-only staining confirmed lack of nonspecific binding for all antibodies used. Slides were mounted in Prolong Diamond anti-fade mounting media (Thermo Fisher Scientific) and images were acquired on the Olympus FLUOVIEW FV1000 confocal laser scanning microscope at 60X magnification (FITC: 488nm excitation, 520nm emission; TRITC: 543nm excitation, 618nm emission; DAPI: 405nm excitation, 461nm emission).

## Cell culture and serum treatment

Neonatal rat ventricular myocytes (NRVMs) were isolated from the ventricles of 1- to 2-day-old Sprague Dawley rats (Charles River) by enzymatic digestions as described<sup>26</sup>. NRVMs were treated with 2% human serum for 72 hours as described<sup>26</sup>. NRVMs were pre-incubated with 1.5µM sildenafil for 30 minutes prior to serum treatment. Efficiency of NRVM response was tested by treatment of cells with 10<sup>-5</sup>M phenylephrine (PE) for 72 hours as a positive control. All animal protocols are in accordance with PHS Animal Welfare Assurance, ID A3269–01, and approved by the University of Colorado, Denver - Animal Care and Use Committee.

## RNA isolation and RT-qPCR

NRVMs were homogenized in Qiazol (Qiagen), and RNA was extracted as per manufacturer's protocol. Human RV samples were homogenized in Qiazol, RNA was extracted using the RNeasy plus mini kit (Qiagen), and following extraction, RNA was treated with TURBO DNase (Thermo Fisher Scientific) as per manufacturer's protocol. cDNA was synthesized by using the Verso cDNA synthesis kit (Thermo Fisher Scientific) according to manufacturer's instructions. Gene expression was measured by RT-qPCR as described<sup>27</sup>, with Power Sybr Green PCR Master Mix (Life Technologies). Expression

levels of all transcripts were normalized to 18S rRNA, and all RT-qPCR data are represented on a Log<sub>2</sub> scale. RT-qPCR primers sequences are listed in supplemental Table S2.

### RNA-seq and Transcriptome Analysis

1X150 directional mRNA sequencing was performed on an Illumina hiSEQ 4000 (HT Mode), with an average of 38–44 million mapped reads per sample. Samples were de-multiplexed and aligned to the reference genome *R. norvegicus* (Rnor\_5.0) using gsnap<sup>28</sup>. HTSeq was used to produce gene counts for each of our 12 samples (N=3 NF serum treated NRVMs ±PDE5i and N=3 SV serum treated NRVMs ±PDE5i, from 3 different NRVM preparations)<sup>29</sup>. Counts of reads generated by HTSeq were normalized using edgeR<sup>30</sup>. While RNA-seq identified more than 20,000 genes, counts of 10 in any one sample were necessary for inclusion. After removal of low counts, 7,690 genes remained. Significant changes in gene expression were calculated using an ANOVA and a post-hoc Tukey with a non-stringent threshold of p<0.05. RNAseq on NRVMs was performed to generate hypotheses in regards to the human SV myocardium, therefore there was no correction for multiplicity of measured values. Differentially expressed genes of interest were validated in a cohort of human samples. Hierarchical clustering and heatmap generation were performed using R (The R Foundation). Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO) categorization using PANTHER were performed to investigate molecular pathways, biological processes and toxicity functions associated with differentially regulated genes.

### Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software), and statistical significance was set a priori at p<0.05. Normality of data was assessed, and non-normally distributed groups were Log<sub>2</sub> transformed, after which all data were approximately normally distributed. Comparisons between 2 normally distributed groups were conducted using an unpaired t-test. Comparisons of 3 or more normally distributed groups were conducted using a 1-way ANOVA; if the overall comparison reached significance, Holm-Sidak multiple comparisons post-hoc tests were performed. Quantitative results in figures are shown as mean±SEM, and in the text as mean±SD. For log-transformed data, the mean of the log-transformed data is reported.

## Results

### Patient Characteristics

Aggregate characteristics for patients included in this study are listed in Table 1. A more detailed description of individual subject characteristics is included in Table S1. The NF tissue and blood group included 17 subjects with a median age of 8 years, an interquartile range (IQR) of 3.1–13.6, and was 65% male. The SV tissue and blood group had 18 subjects with a median age of 2.6 years, an IQR of 0.9–4.8, and was 67% male. As expected based on limited young donor availability, the SV group is significantly younger than the NF group (p=0.004).

### PDE5 Expression and Activity is Increased in Human SV Myocardium

We quantified RV PDE enzymatic activity in the cytosolic fraction of 9 RV samples from patients with SV and in 8 RV samples from normal NF pediatric donor hearts (Figure 1A, B). While total RV cytosolic PDE activity was similar between SV and NF groups [NF=48.1±8.9, SV=48.8±15.2, p=0.73], PDE5 specific activity was significantly increased in the SV group, with the average PDE5-specific cGMP hydrolyzing activity more than 3 times higher in SV patients compared to NF controls [NF=4±3.1, SV=12.4±7.4, p=0.009]. RV PDE5 protein expression was also quantified in 7 hearts from patients with SV and in 9 NF pediatric donor hearts (Figure 1C). RV PDE5 expression was significantly increased in SV relative to NF, with the mean signal intensity of PDE5 relative to the loading control GAPDH more than 8 times higher in SV myocardium compared to NF myocardium [NF=-0.28±1.3, SV=2.8±1.2, p=0.0002]. A subset of pediatric RV samples, 3 SV and 1 NF RV, were subjected to histologic staining for PDE5 localization (Figure 2, Supplementary Figure S1). We determined that PDE5 is expressed in pediatric RV myocardium and is localized primarily within cardiomyocytes as determined by co-localization with  $\alpha$ -actinin in the z-disk region of the sarcomere.

### SV Serum Induces Pathological Gene Expression Changes in Primary Cardiomyocytes

As an *in vitro* model of SV-related pathological cardiomyocyte remodeling, primary cardiomyocytes (NRVMs) were treated with serum from SV patients and serum from NF controls (n=5 NF, n=12 SV in a total of 7 NRVM preparations). The  $\alpha$ -adrenergic receptor agonist PE was used as a positive control, as it results in robust activation of the fetal gene program, a hallmark of pathologic remodeling<sup>25</sup>. As compared to NF serum treatment, SV serum treatment results in significant induction of prototypical pathological cardiac fetal genes brain natriuretic peptide (BNP) and atrial natriuretic factor (ANF), as well as a significant reduction in the ratio of  $\alpha$ -Myosin Heavy Chain ( $\alpha$ MHC) to  $\beta$ -Myosin Heavy Chain ( $\beta$ MHC) in NRVMs (Figure 3).

### PDE5 Inhibition Attenuates SV Serum-Induced Pathological Gene Expression Changes in Primary Cardiomyocytes

Additionally, we utilized the SV-serum based *in vitro* model of cardiomyocyte remodeling to assess the intracellular consequences of PDE5 inhibition (n=4 NF+PDE5i, n=9 SV+PDE5i in a total of 7 NRVM preparations). While PDE5i had no significant effect on untreated NRVMs or those treated with NF patient serum, PDE5i significantly attenuated SV serum-induced pathological cardiac gene expression changes (Figure 4). Namely, BNP and ANF expression were significantly attenuated in the SV+PDE5i treated group, as expression levels were not significantly different from NF+PDE5i controls and were significantly less than SV serum treatment alone. There was an increase in the ratio of  $\alpha$ -MHC to  $\beta$ -MHC in the SV+PDE5i group, such that expression levels were not significantly different from NF +PDE5i controls.

## Transcriptome Profiling Indicates SV Serum Induces Significant Transcriptional Changes and Dysregulated Cardiotoxicity Pathways in Primary Cardiomyocytes, that are Normalized by PDE5i

Next generation sequencing (RNAseq) on a subset of serum-treated NRVMs  $\pm$  the PDE5i sildenafil (N=3 NF serum-treated NRVMs $\pm$ PDE5i and N=3 SV serum-treated NRVMs  $\pm$ PDE5i, from 3 different NRVM preparations) identified more than 20,000 genes. Genes with counts <10 were removed, and further analysis was performed using the resulting 7,690 genes. Of these, 1,135 were significantly differentially expressed between NF and SV serum-treated groups ( $p<0.05$ ) (Figure 5A-B). Of the 1,135 differentially expressed genes, 607 genes were significantly upregulated and 528 genes were significantly downregulated (Figure 5A). Unsupervised hierarchical clustering separated NF and SV serum-treated cells (Figure 5B). A list of all 1,135 differentially expressed genes is listed in supplemental Table S3.

Pathway analysis performed using Qiagen Ingenuity Pathway Analysis (IPA) software revealed multiple dysregulated canonical pathways associated with SV serum treatment (Figure 5C). Most prominently, signaling pathways involved in: axonal guidance, ceramide, peroxisome proliferator-activated receptor (PPAR), p38 mitogen-activated protein kinase (MAPK), phospholipase C (PLC), cardiac hypertrophy, aryl hydrocarbon receptor, B cell receptor, actin cytoskeleton, and Ephrin A signaling were significantly enriched ( $p<0.01$ ) in SV serum-treated NRVMs relative to NF serum-treated NRVMs. In addition to cardiac hypertrophy signaling, a more detailed IPA analysis of cardiac specific pathways revealed multiple dysregulated cardiotoxicity functions, including significant differential expression ( $p<0.05$ ) of pathways related to cardiac dysfunction, arteriopathy, cardiac damage, cardiac fibrosis, infarction, heart failure, congenital heart anomalies, cardiac cell death and arrhythmias (Figure 5D).

PANTHER was used to further categorize the 1,135 differentially expressed transcripts based on implicated biological processes. The top 3 categorizations indicate a majority of the differentially expressed genes are involved in cellular processes (28%, e.g., cell communication, cell cycle and movement of cellular components), metabolic processes (23%, e.g., primary metabolism and nitrogen compound metabolism) and response to stimuli (9%, e.g., stress response and immune response) (Figure 5E).

Additionally, more than half of the 1,135 differentially expressed transcripts were normalized ( $p>0.05$ ) by PDE5i, and unsupervised hierarchical clustering separated SV and SV+PDE5i serum-treated cells. (Figure 6A). Namely, 335 (55%) of the 607 significantly upregulated genes in the SV serum-treated group were no longer significantly different in the SV+PDE5i group relative to NF+PDE5i (Figure 6B), while 314 (60%) of the 528 significantly downregulated genes in the SV group were no longer significantly different in the SV+PDE5i group relative to NF+PDE5i (Figure 6C). IPA analysis of all 649 genes that were normalized with PDE5i in SV serum treated NRVMs predicted normalization of many differentially activated signaling pathways, including normalization of ceramide, p38 MAPK, PLC, cardiac hypertrophy, actin cytoskeleton, and oxidative stress response signaling (Figure 6D). IPA analysis also predicted normalization of SV serum-induced cardiotoxicity pathways, including: normalization of cardiac and ventricular hypertrophy

signaling, as well as normalization of genes involved in cardiac dysfunction (Figure 7A-C). A list of all 649 genes that were normalized by PDE5i are included in supplemental Table S4.

Representative genes from the top 6 most significantly differentially regulated signaling pathways activated by SV serum-treatment including ceramide, p38 MAPK, PLC, cardiac hypertrophy, actin cytoskeleton, and oxidative stress response signaling (Figure S2) were chosen for further analysis. Confirmatory analysis by RT-qPCR was performed on human RV tissue for representative genes in each pathway, including (1) ceramide kinase CERK<sup>31</sup>, (2) cyclic AMP-responsive transcription factor CREB<sup>32,33</sup>, (3) non-receptor tyrosine kinase FYN<sup>34,35</sup>, (4) cardiac ankyrin repeat domain ANKRD1<sup>36,37</sup>, (5)  $\alpha$ -actinin isoform ACTN2<sup>38,39</sup>, and (6) transcription factor MAFK<sup>40</sup>. RT-qPCR revealed that these representative genes were significantly upregulated in SV RV relative to NF control RV, consistent with the SV-sera NRVM treatment findings (Figure 8).

## Discussion

Long-term survival and quality of life in SV patients ultimately depends on preservation of RV function, particularly as more children with complex CHD are surviving into adulthood<sup>41</sup>. There are no proven therapies to treat or prevent RV failure in SV and persistence of RV dysfunction may reflect irreversible adverse remodeling that is presumably a precursor to eventual HF<sup>42</sup>. There remains a critical need to better understand the mechanisms of SV failure, and to identify clinically relevant pathways, biomarkers of disease progression, and therapeutic targets. In this study we demonstrate that myocardial PDE5 expression and activity are significantly elevated in SV patients relative to NF controls, and that PDE5 is expressed in SV cardiac myocytes. Moreover, *in vitro* treatment of primary cardiomyocytes with SV patient sera induces gene expression changes indicative of pathological myocardial remodeling, cardiac hypertrophy and cardiac dysfunction, which are abrogated by PDE5i. Finally, confirmatory analysis in human RV tissue revealed that gene expression changes induced by SV serum in primary cardiomyocytes are similar to those seen in SV myocardium. Together these data provide an important foundation for future mechanistic studies regarding PDE5i in SV and highlight a novel SV serum-based model that can be used to identify potential drug targets relevant to myocardial remodeling in the pediatric SV population.

### PDE5 Upregulation in SV

There is mounting preclinical and clinical evidence regarding the potentially beneficial cardiac effects of PDE5i, however, their use in children with heart disease remains primarily focused on modulating pulmonary blood flow. While PDE5i therapy in failing SV patients has been associated with improved hemodynamics, exercise tolerance and cardiac function<sup>10,12,43</sup>, the direct effects of PDE5i on the SV myocardium are unknown. Given the significant increase of PDE5 mRNA<sup>20</sup>, protein and activity in SV RV and the presence of PDE5 in SV cardiomyocytes, these findings suggest that in addition to effects on the pulmonary vasculature, PDE5i may directly target the myocardium. The specific mechanisms by which PDE5i may augment cardiac function remain to be determined.



## PDE5-Mediated Cardiomyocyte Remodeling

We and others have previously reported that circulating factors present in patient serum or plasma can be important paracrine modulators of cardiac gene expression<sup>26,44</sup>. Here we show that treatment of primary cardiomyocytes with SV patient serum induces changes in gene expression with similar directionality as in SV hearts<sup>45</sup> and a pediatric rodent model of RV hypertrophy<sup>46</sup>. Paracrine effects on cardiac remodeling can be mediated by a myriad of factors present in the circulation, and the specific factors modulating SV serum-induced cardiomyocyte remodeling remain to be determined.

Transcriptome analysis of SV serum-treated primary cardiomyocytes suggests dysregulation of genes involved in various cellular and metabolic processes including a high number of genes involved in cardiac remodeling, hypertrophy and dysfunction. Pathway analysis of differentially expressed genes in SV serum-treated cardiomyocytes also suggests enrichment of several other pathways, including significant activation of: ceramide, p38 MAPK, PLC, actin cytoskeleton, and oxidative stress response signaling. Many of these dysregulated canonical pathways have known roles in cardiac hypertrophy and dysfunction. For example, ceramide signaling has been implicated in a variety of physiological functions<sup>31</sup>, and a specific species of ceramide has been shown to promote cardiomyocyte hypertrophy through generation of reactive oxygen species (ROS)<sup>47</sup>, while inhibition of ceramide signaling using a sphingosine kinase-1 inhibitor in a mouse model of hypoxia-induced pulmonary arterial hypertension resulted in reduced RV hypertrophy and cardiomyocyte death, without affecting vascular remodelling<sup>48</sup>. Additionally, PLC enzymes are important for intracellular signaling, and can be activated by non-receptor tyrosine kinases such as FYN<sup>34,35</sup>. It has been shown that cardiomyocyte hypertrophy and cardiac dysfunction can occur via stimulation of PLC, resulting in generation of potent phospholipid-derived second messenger lipid molecules that can alter cardiomyocyte signaling<sup>49,50</sup>. Moreover, the p38 MAPK signaling pathway is known as one of the key sensors of cellular stress, and signal transduction occurs via a cascade culminating in activation of key transcription factors including those in the CREB family (e.g., CREB5)<sup>33</sup>. p38 MAPK signaling has a variety of roles in the cardiovascular system including regulation of cardiomyocyte proliferation, apoptosis and hypertrophy<sup>32,51,52</sup>. Additionally, structural cytoskeletal alterations (e.g., alterations in anchor proteins such as  $\alpha$ -actinins)<sup>53</sup>, both within cardiac myocytes and of the extracellular matrix contribute to ventricular remodeling and cardiac dysfunction<sup>38,53,54</sup>. Finally, under pathological conditions, oxidative stress in cardiomyocytes is considered a major stimulant of signal transduction, and can induce inflammatory cytokines and MAP kinases (e.g, MAFK)<sup>40,55</sup>.

Our transcriptome analysis of serum-treated primary cardiomyocytes revealed that more than half of the differentially expressed transcripts between SV-serum treated NRVMs and NF serum treated NRVMs were normalized by PDE5i, including normalization of ceramide, p38 MAPK, PLC, actin cytoskeleton, and oxidative stress signaling, as well as normalization of SV serum-induced cardiotoxicity pathways such as cardiac hypertrophy and dysfunction. Thus, these pathways may serve as potential therapeutic targets in this population and warrant future study. In summary, the *in vitro* model used in this study recapitulates some relevant SV myocardial gene expression patterns, poses no risk to children and provides a

useful mechanistic platform to investigate PDE5, as well as other signaling pathways altered by SV physiology.

### Limitations

There are important limitations of this study. The tissue bank-based aspect of this study is inherently cross-sectional, and as such, proof of mechanistic associations or knowing whether changes are pathologic or compensatory is not possible. Because of the relative rarity of SV, it is not possible for us to determine the influence of age, prior surgical procedures, or the temporal relationship of expression/activity changes in our findings. Secondary to the current widespread use of PDE inhibitors in the SV population, it is not possible to exclude all patients on a PDE5i, or to determine whether PDE5 upregulation is secondary to PDE3i (millrinone) treatment. It is important to note however, that there was no significant difference in PDE5 activity or expression between SV patients based on treatment with the PDE5i sildenafil. Because our experiments were performed using explanted RV myocardium, we acknowledge that the data are not necessarily representative of cardiomyocytes alone, and contributions of the extracellular matrix, fibroblasts and endothelial cells are unknown. Finally, we acknowledge that the unadjusted p-value ( $p < 0.05$ ) used in our primary cardiomyocyte RNA-seq data analysis may result in false positives. However, our goal was to identify a larger gene set to generate hypotheses regarding gene expression changes relevant in the human SV myocardium.

### Conclusions

Findings from this study suggest that elevated PDE5 in failing SV myocardium may contribute to adverse myocardial remodeling, and in addition to effects on the pulmonary vasculature, PDE5i may provide direct myocardial benefit in this population. These results underscore the importance of pediatric-specific investigations and justify attempts to improve the understanding of SV-specific molecular mechanisms leading to HF.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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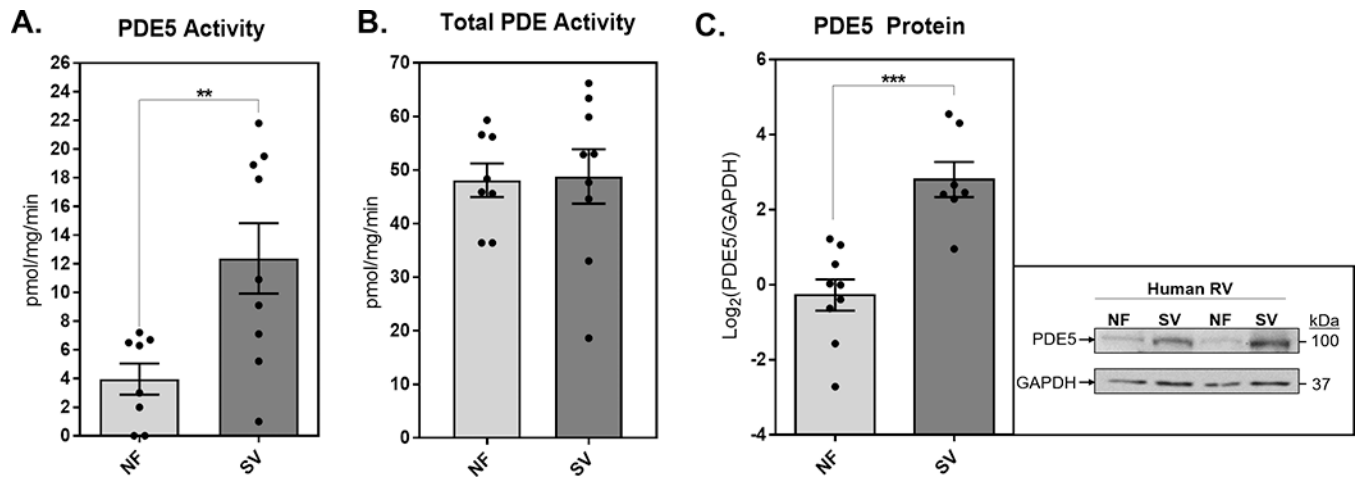
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**Clinical Impact Commentary****What is new?**

- Phosphodiesterase-5 expression and activity are increased in the right ventricular tissue of children with single ventricle heart disease, compared to that in right ventricles of normal children.
- Treatment of rat heart muscle cells (cardiomyocytes) with sera from children with single ventricle heart disease results in similar pathologic gene expression changes as are seen in single ventricle heart tissue.
- Phosphodiesterase-5 inhibitor treatment results in normalization of some of the transcriptional changes seen in the single ventricle serum-treated cardiomyocytes.

**What are the clinical implications?**

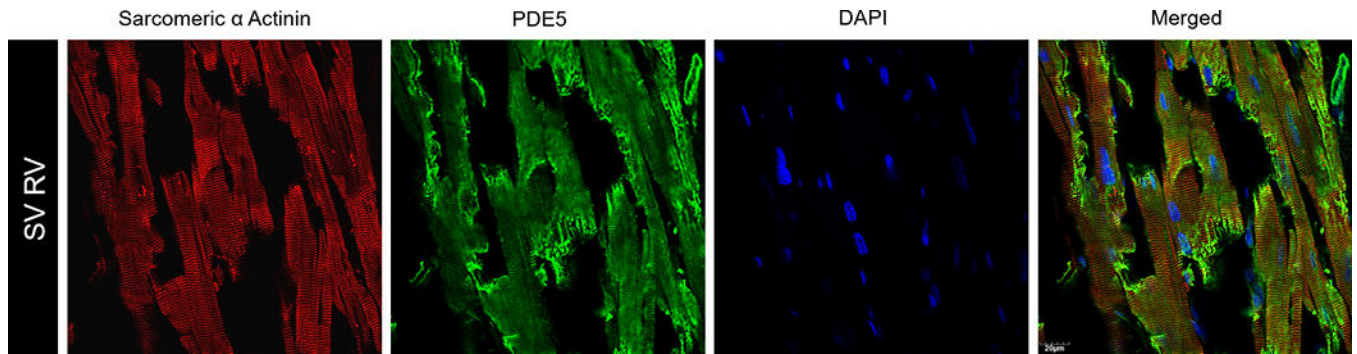
- Remodeling (pathologic hypertrophy and cardiac dysfunction) of the single ventricle heart can lead to heart failure and death or the need for heart transplant.
- There are no proven therapies and a limited understanding of the mechanisms underlying single ventricle heart failure.
- Elevation of phosphodiesterase-5 in heart tissue may contribute to single ventricle heart failure.
- Phosphodiesterase-5 inhibitor therapy may have beneficial effects specific to the heart muscle of children with single ventricle congenital heart disease.
- Treatment of primary cardiomyocytes with patient sera may represent a novel model for future studies of the mechanisms involved in single ventricle heart failure.



**Figure 1. PDE5 Expression and Activity is Increased in SV RV Myocardium.**

(A) PDE5 specific enzymatic activity in NF and SV. Bar equals mean $\pm$ SEM, each point represents individual patient values, n=7 NF and n=13 SV RV samples; asterisk (\*) denotes significant difference between groups, unpaired t-test, p<0.05. (B) Total PDE enzymatic activity in NF and SV. Bar equals mean $\pm$ SEM, each point represents individual patient values, n=7 NF and n=13 SV RV samples. (C) Quantification of PDE5 protein relative to GAPDH protein in NF and SV. Quantitative data were log<sub>2</sub> transformed, bar equals log<sub>2</sub>(mean $\pm$ SEM), each point represents individual patient values, n=9 NF and n=8 SV RV samples; asterisk (\*) denotes significant difference between groups, unpaired t-test, p<0.001. A representative blot of PDE5 and GAPDH protein in NF and SV RV is shown on the right.

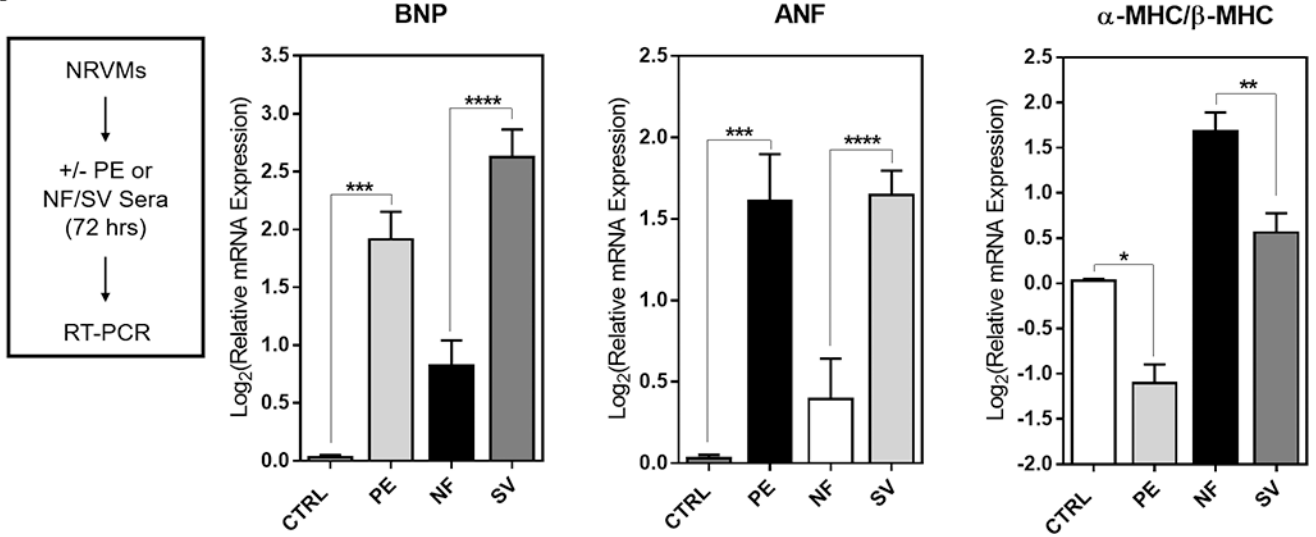




**Figure 2. PDE5 is Expressed in Pediatric SV RV Cardiomyocytes.**

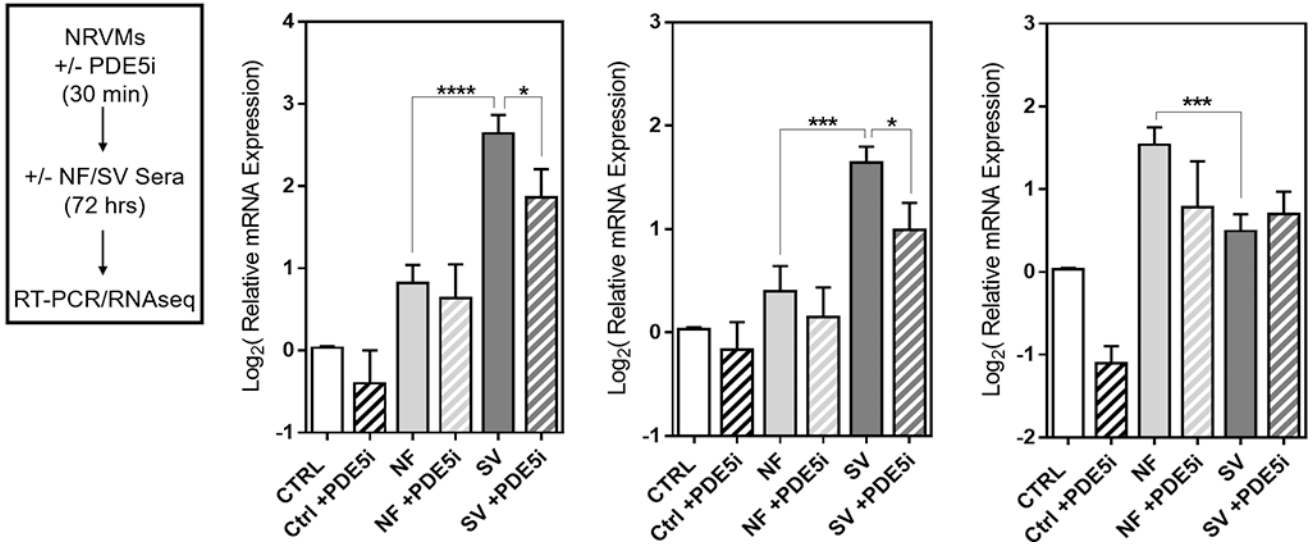
Sectioned SV tissue was stained with fluorescently labeled secondary antibodies to primary sarcomeric  $\alpha$  actinin (red), primary PDE5 (green) and nuclei were stained with DAPI (blue). The merged confocal laser microscopy images demonstrate that PDE5 is expressed in SV cardiomyocytes. All images are at 60X magnification, scale bar = 20 $\mu$ M.

**A.**



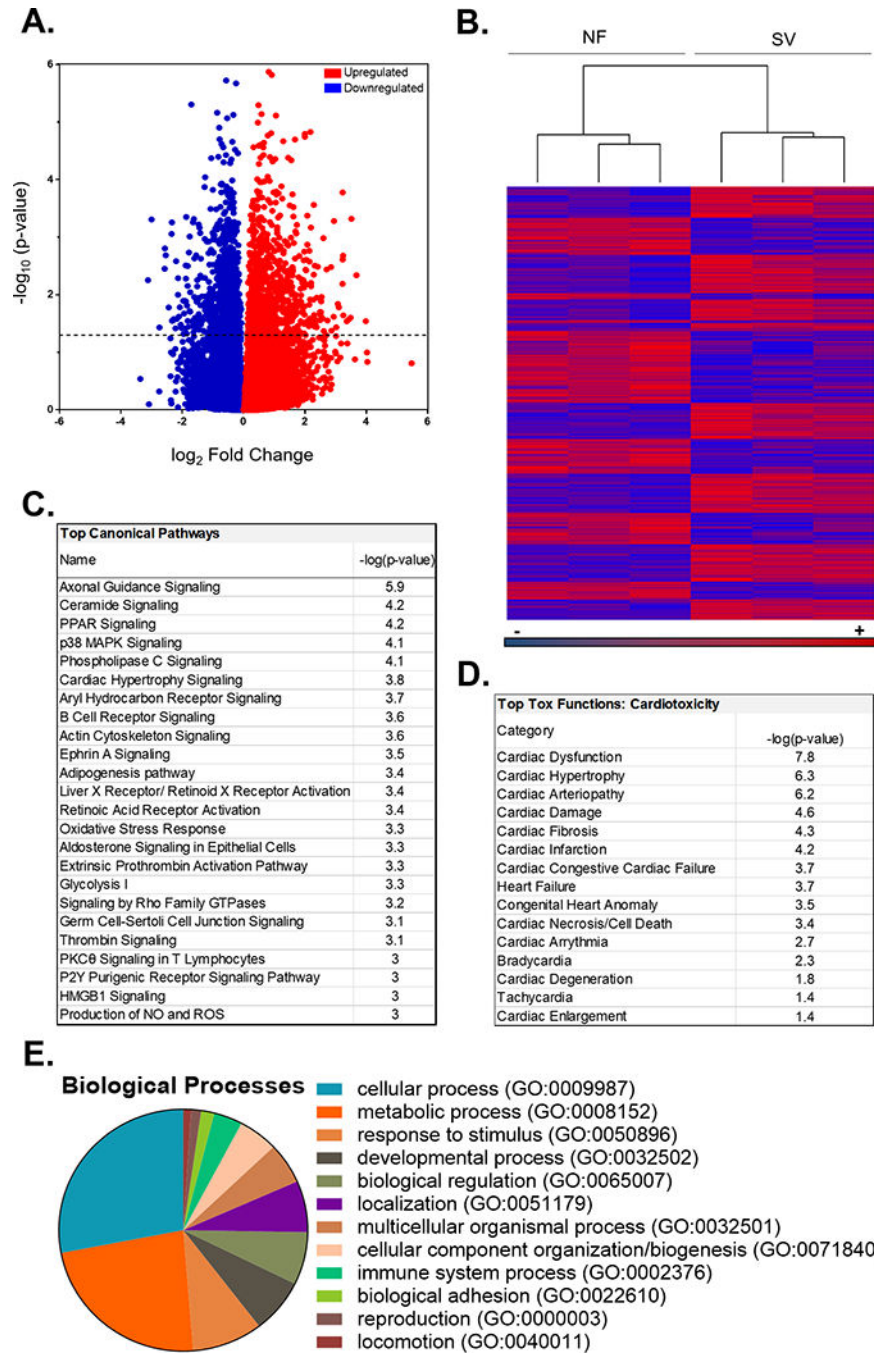
**Figure 3. SV Serum Induces Pathological Gene Expression Changes in Cardiomyocytes *in vitro*.** (A) NRVMs were treated with either phenylephrine (PE) or with sera from NF or SV patients for 72 hours. RNA was harvested for RT-qPCR. (B) Log<sub>2</sub> relative mRNA expression of prototypical pathological cardiac genes (BNP, ANF, βMHC and αMHC) in NRVMs. For all groups, bar equals log<sub>2</sub>(mean±SEM), asterisk (\*) denotes a significant difference between groups, one-way ANOVA, Holm-Sidak multiple comparisons (\*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001), n=5 NF, n=16 SV, n=10 PE treatments in a total of 7 NRVM preparations.

**A.**



**Figure 4. PDE5i Attenuates SV Serum-Induced Pathological Gene Expression Changes in Cardiomyocytes *in vitro*.**

(A) NRVMs were treated with sildenafil (PDE5i), and after 30 minutes cells were treated with sera from NF or SV patients for 72 hours. RNA was harvested for RT-qPCR and RNAseq. (B) Log<sub>2</sub> relative mRNA expression of prototypical pathological cardiac genes (BNP, ANF, βMHC and αMHC) in NRVMs. For all groups, bar equals log<sub>2</sub>(mean±SEM), asterisk (\*) denotes a significant difference between groups, one-way ANOVA, Holm-Sidak multiple comparisons (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001), n=5 NF, n=16 SV, n=4 NF+PDE5i, n=14 SV+PDE5i in a total of 7 NRVM preparations.



**Figure 5. SV Serum Induces Significant Transcriptional Changes and Dysregulated Cardiotoxicity Pathways *in vitro*.**

(A) Volcano plot representation of all the 20,398 transcripts detected by RNA-Seq depicted as the  $\log_2$  fold-changes in expression (x-axis) and the log odds of a gene being differentially expressed (y-axis), highlighting the 1,135 transcripts that are significantly differentially expressed between NF versus SV-serum treated NRVMs (above dotted line). ANOVA,  $-\log_{10}(\text{p-value}) > 1.3$  or  $\text{p} < 0.05$ ). (B) Heat map of the 1,135 significantly differentially expressed transcripts (raw Fragments Per Kilobase of transcript per Million mapped reads, FPKM values). Unsupervised hierarchical clustering separated NF (n=3) and

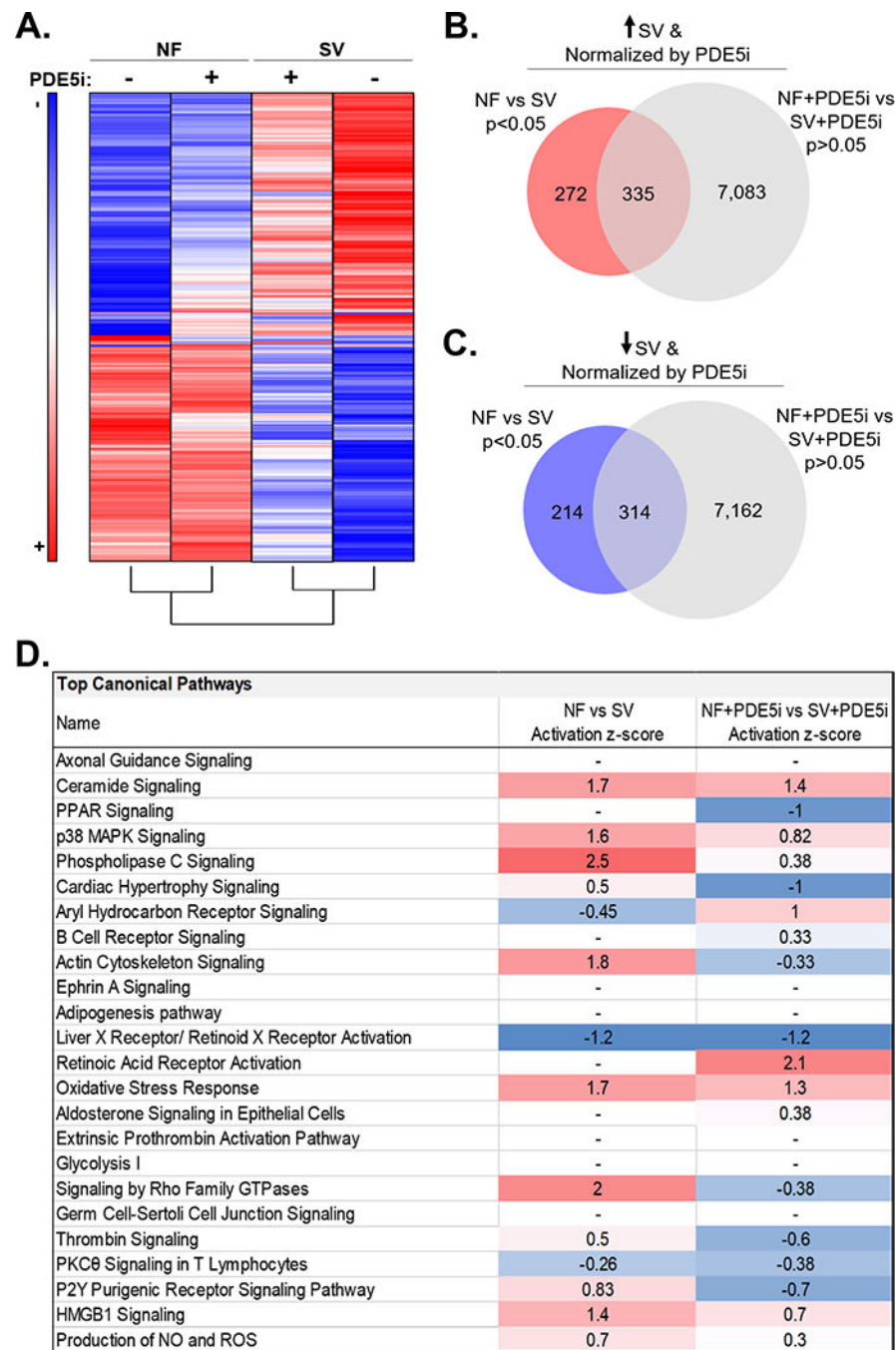
SV (n=3) serum-treated NRVM samples. (C) Significantly dysregulated canonical pathways in NF versus SV serum-treated NRVMs identified with IPA using the 1,135 transcripts that changed significantly in all 3 SV-treated cell groups. Fisher's exact test,  $-\log_{10}(\text{p-value}) > 2.9$  or  $p < 0.001$ . (D) Significantly dysregulated cardiotoxicity functions in NF versus SV serum-treated NRVMs identified with IPA using the 1,135 transcripts that changed significantly in all 3 SV-treated cell groups. Fisher's exact test,  $-\log_{10}(\text{p-value}) > 1.3$  or  $p < 0.05$ . (E) Categorization of Gene Ontology (GO) annotations for biological processes in NF versus SV serum-treated NRVMs identified with PANTHER using the 1,135 transcripts that changed significantly in all 3 SV-treated cell groups.

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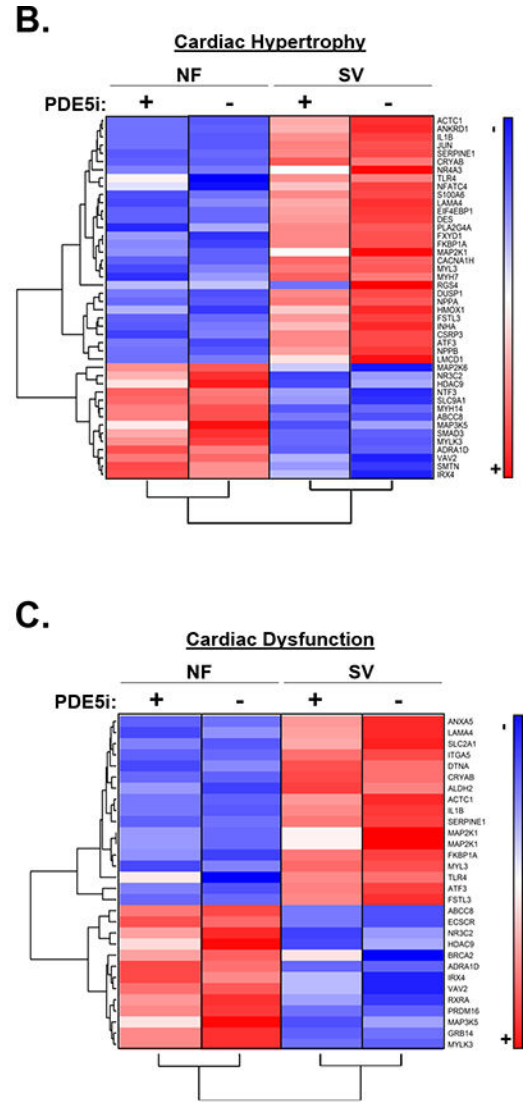
**Figure 6. PDE5i Normalizes SV Serum-Induced Transcriptional Changes *in vitro*.**

(A) Heat map summary of the 649 significantly differentially expressed transcripts that were normalized by PDE5i (average fold change relative to NF,  $n=3$  NF,  $n=3$  NF+PDE5i,  $n=3$  SV,  $n=3$  SV+PDE5i in a total of 3 NRVM preparations). Unsupervised hierarchical clustering separated NF, NF+PDE5i, SV, and SV+PDE5i serum-treated samples. (B) SV serum-induced upregulated gene expression changes were compared with gene expression changes that were not significantly different between NF+PDE5i and SV+PDE5i serum-treated groups. The Venn diagram indicates significantly upregulated genes that were normalized

with PDE5i. (C) SV serum-induced downregulated gene expression changes were compared with gene expression changes that were not significantly different between NF+PDE5i and SV+PDE5i serum-treated groups. The Venn diagram indicates significantly downregulated genes that were normalized with PDE5i. (D) Activation z-scores of significantly dysregulated canonical pathways in NF versus SV serum-treated NRVMs compared to NF +PDE5i vs SV+PDE5i serum-treated NRVMs.

**A.**

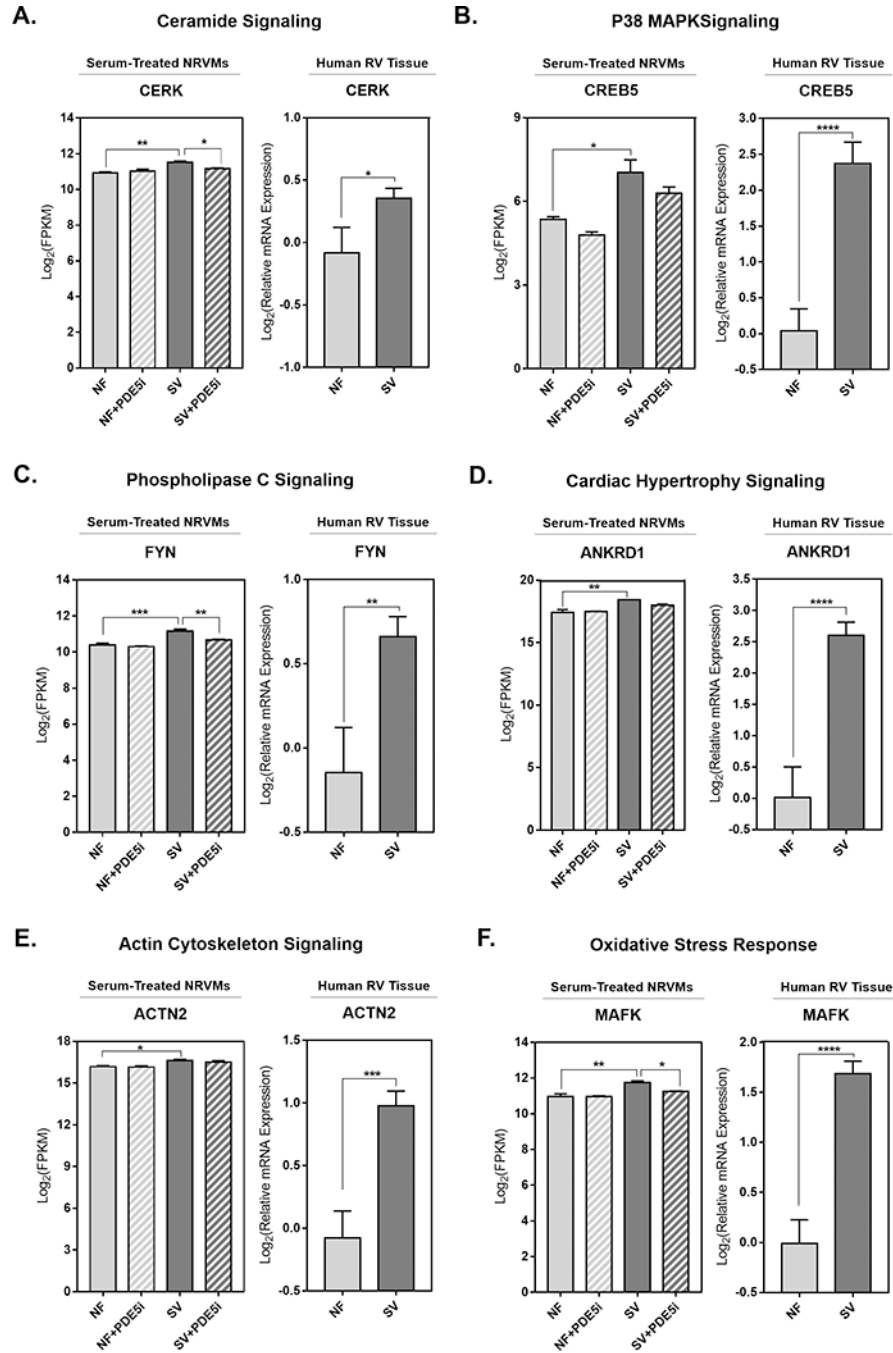
Top Tox Functions: Cardiotoxicity		
Name	NF vs SV Activation z-score	NF+PDE5i vs SV+PDE5i Activation z-score
<b>Cardiac Dysfunction</b>		
Dysfunction of Heart	0.57	0.08
Ventricular Dysfunction	1.1	-0.2
Hypertrophy of Ventricle	0.9	-
<b>Cardiac Hypertrophy</b>		
Hypertrophy of Heart	1	-
Hypertrophy of Cardiomyocytes	0.64	-
Hypertrophy of Cardiac Muscle	1	-
Ventricular Hypertrophy	2.1	-
<b>Cardiac Arteriopathy</b>		
Coronary Artery Disease	-	-
<b>Cardiac Damage</b>		
Damage of Heart	-0.78	-1.5
Injury of heart	0.1	-0.78
Damage of Cardiac Muscle	-1.2	-1.2
<b>Cardiac Fibrosis</b>		
Fibrosis of Heart	-0.93	-0.93
<b>Cardiac Infarction</b>		
Infarction of Heart	-0.31	-0.31
Myocardial Infarction	-0.7	-0.7
<b>Heart Failure</b>		
Congestive Heart Failure	0.7	0.7
<b>Congenital Heart Anomaly</b>		
Ventricular Septal Defect	1.2	1.2
Conotruncal Heart Malformations	1.7	1.7
Familial Congenital Heart Disease	1.1	1.1
Congenital Heart Disease	1.6	1.6
Persistent Truncus Arteriosus	1.5	1.5
<b>Cardiac Necrosis/Cell Death</b>		
Necrosis of Cardiac Muscle	-0.27	-0.43
Apoptosis of Cardiomyocytes	-0.6	-0.8
<b>Cardiac Arrhythmia</b>		
Arrhythmia	-	0.6
<b>Bradycardia</b>		
Bradycardia	-	1
<b>Cardiac Degeneration</b>		
Degeneration of Heart Tissue	-	-



**Figure 7. PDE5i Normalizes SV Serum-Induced Transcriptional Changes Associated with Cardiac Hypertrophy and Dysfunction *in vitro*.**

(A) Activation z-scores of significantly dysregulated cardiotoxicity pathways in NF versus SV serum-treated NRVMs compared to NF+PDE5i vs SV+PDE5i serum-treated NRVMs. (B) Heat map summary of the genes predicted to be involved in cardiac hypertrophy signaling based on IPA, that were normalized by PDE5i (average fold change relative to NF, n=3 NF, n=3 NF+PDE5i, n=3 SV, n=3 SV+PDE5i in a total of 3 NRVM preparations). (C) Heat map summary of the genes predicted to be involved in cardiac dysfunction signaling based on IPA, that were normalized by PDE5i (average fold change relative to NF, n=3 NF, n=3 NF+PDE5i, n=3 SV, n=3 SV+PDE5i in a total of 3 NRVM preparations).





**Figure 8. Representative Gene Expression Changes in SV Serum-Treated Primary Cardiomyocytes are Similar to Those in SV RV Myocardium.** Representative genes (A) Ceramide kinase (CERK), (B) Cyclic AMP-responsive element binding protein (CREB5), (C) Proto-oncogene tyrosine-protein kinase (FYN), (D) Cardiac ankyrin repeat domain 1 (ANKRD1), (E)  $\alpha$ -Actinin isoform ACTN2, and (F) MAF transcription factor K (MAFK) from each of the top most differentially regulated pathways (ceramide, p38 MAPK, PLC, hypertrophy, actin cytoskeleton and oxidative stress) activated by SV serum treatment in primary cardiomyocytes. For all serum-treated NRVM groups: data are depicted as log<sub>2</sub> FPKM values, bar equals log<sub>2</sub>(mean±SEM), asterisk (\*) denotes a

significant difference between groups, one-way ANOVA, Holm-Sidak multiple comparisons (\*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ),  $n = 3$  NF,  $n = 3$  NF+PDE5i,  $n = 3$  SV,  $n = 3$  SV+PDE5i in a total of 3 NRVM preparations. For all human RV tissue groups: data are depicted as  $\log_2$  relative mRNA expression, bar equals  $\log_2(\text{mean} \pm \text{SEM})$ , asterisk (\*) denotes a significant difference between groups, unpaired T-test (\*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ),  $n = 7$  NF,  $n = 14$  SV.

## Patient Characteristics

Table 1.

Experiment	Group	No. of Subjects	% Male	Median Age (years) [IQR]	% PDE3i	% PDE5i	% Non-PDEi Inotrope*	% Digoxin	% ACEi	% $\beta$ -Blocker	% Diuretic
PDE5 Activity	NF	8	62.5	7.7 [2.8–8.9]	0	0	50	0	0	12.5	0
	SV	9	55.6	2.8 [1–3.8]	88.9	22.2	11.1	66.7	77.8	0	77.8
Tissue PDE5 Protein	NF	9	66.7	7.4 [3.2–8.7]	0	0	33.3	0	0	0	0
	SV	7	71.4	2.3 [1.1–2.9]	71.4	14.3	0	57.1	85.7	0	85.7
Tissue PDE5 Histology	NF	1	100	2.9 [n/a]	0	0	100	0	0	0	0
	SV	3	100	2.9 [1.6–7.6]	66.7	33.3	0	100	66.7	0	66.7
NRVM + Serum RT-PCR	NF	5	40	11.5 [7–13.6]	0	0	0	20	0	0	0
	SV	12	58.3	2 [0.9–4.8]	50	33.3	0	66.7	50	0	75
NRVM + Serum RNAseq	NF	3	33.3	13.6 [12.5–14.5]	0	0	0	0	0	0	0
	SV	3	33.3	1.9 [0.9–3.2]	100	66.7	0	100	66.7	0	66.7
Tissue RT-PCR	NF	7	57.1	7 [2.3–7.7]	0	0	57.1	0	0	14.3	0
	SV	14	57.1	1.78 [0.8–3.6]	71.4	21.4	0	64.3	64.3	0	78.6
All Patients	NF	17	64.7	8.0 [3.1–13.6]	0	0	29.4	5.9	0	5.9	0
	SV	18	66.7	2.6 [0.9–4.8]	61.1	27.8	5.6	66.7	55.6	0	77.8

Aggregate characteristics for all patients included in this study. ACEi = angiotensin-converting enzyme inhibitor, PDEi = phosphodiesterase inhibitor.

\* Non-PDEi Inotrope includes: dopamine, dobutamine, epinephrine, norepinephrine.