Differential Expression of PDE5 in Failing and Non-Failing Human Myocardium

Shan et al: PDE5 Up-Regulation in Failing Human Ventricles

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Abstract

Background—Recognizing that inhibitors of phosphodiesterase type 5 (PDE5) are increasingly employed in patients with pulmonary hypertension and right ventricular failure, we examined PDE5 expression in the human right ventricle (RV) and its impact on myocardial contractility.

Methods and Results—Tissue extracts from the RV of 20 patients were assayed for PDE5 expression using immunoblot and immunohistochemical (IHC) staining. Tissues were selected from groups of non-failing (NF) organ donors and transplant recipients with end-stage ischemic cardiomyopathy (ICM) or idiopathic dilated cardiomyopathy (DCM). Among DCM patients, subgroups with mild or severe RV dysfunction (RVD) and prior LV assist devices (LVAD) were analyzed separately. Our results showed that PDE5 abundance increased more than four-fold in the RVs of the ICM compared to NF group. In DCM, PDE5 up-regulation was more moderate and varied with the severity of RV dysfunction. IHC confirmed that cardiac myocytes contributed to the up-regulation in the failing hearts. In functional studies, PDE5 inhibition produced little change in developed force (DF) in RV trabeculae from NF hearts, but produced a moderate increase in RV trabeculae from failing hearts.

Conclusions—Our results showed the etiology- and severity-dependent up-regulation of myocyte PDE5 expression in the RV and the impact of this up-regulation on myocardial contractility. These findings suggest that RV PDE5 expression could contribute to the pathogenesis of RV failure and direct myocardial responses to PDE5 inhibition may modulate the indirect responses mediated by RV afterload reduction.

Key Words: PDE5, cGMP, heart failure, myocardium, contractility
Phosphodiesterases (PDE) are a super-family of enzymes that hydrolyze cyclic AMP (cAMP) and cyclic GMP (cGMP) to AMP and GMP, respectively. Both cyclic nucleotides are essential second messengers in regulating the contraction and relaxation of vascular smooth muscle cells and cardiac myocytes. Cyclic GMP exerts its functions through downstream effectors, including protein kinase G (PKG), cGMP-binding proteins, and cyclic nucleotide-gated channels. PDE5 is a subtype of PDEs that preferentially hydrolyzes cGMP. The functional enzyme exists as a homodimer with each monomer containing both regulatory cGMP-binding GAF domains and a catalytic domain. Previous studies have shown that PDE5 activity can be regulated through phosphorylation and cellular cGMP levels. PDE5 has been shown to exist in many tissue types with high abundance in smooth muscle. By hydrolyzing cGMP, PDE5 modulates the cellular level of the secondary messenger that critically regulates the tone of the smooth muscle, and PDE5 inhibitors are increasingly employed as therapy for ameliorating pathological increases in pulmonary arterial resistance.

While the function of PDE5 in the pulmonary vasculature has been well-characterized, the physiological roles of myocardial PDE5 expression are more obscure, partially due to the much lower PDE5 mRNA expression in the myocardium. In animal studies using PDE5 inhibitors to explore cardiac functions of PDE5 and its potential as a drug target in treating cardiac hypertrophy, the effects of PDE5 inhibition have been inconsistent: beneficial under certain hypertrophic circumstances but not under the others. Also the beneficial effects of PDE5 inhibition tend to be sensitive to the magnitude of cardiac stresses used in creating the animal models.
In humans, two previous studies have reported increased PDE5 abundance in the left ventricle of patients with heart failure\textsuperscript{17,18}, and one study found increased right ventricular PDE5 in the hearts of neonates and young children with congenital heart anomalies\textsuperscript{14}. In each study the impact on cardiac function has been explored in animal models, with increased PDE5 expression tending to depress myocardial contractile functions. Inhibition of PDE5 has been reported to be beneficial for patients with pulmonary arterial hypertension who are prone to right ventricular (RV) failure and for patients with stable systolic heart failure\textsuperscript{9,19}. PDE5 inhibitors are pulmonary vasodilators, and may augment right ventricular function via reduced afterload. However, a direct inotropic action of PDE5 inhibitors on human myocardium has not been established at this point. Also, the effects of disease severity and etiologies of cardiomyopathy on PDE5 expression have not been explored to date. Accordingly, the present study examined PDE5 expression in the RV of hearts with severe ischemic cardiomyopathy (ICM) and nonischemic dilated cardiomyopathy (DCM) with mild or severe right ventricle dysfunction. Our data support a functionally significant increase in PDE5 abundance in failing RV myocardium. These findings indicate direct myocardial effects of PDE5 inhibitors during their in vivo administration to patients with RV failure and support a possible therapeutic role in the absence of increases in RV afterload.
Methods

Chemicals. All chemicals used were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated.

Human Tissue Procurement. The use of human tissues in this study was approved by the Institutional Review Board at University of Pennsylvania and all patients provided prospective informed consent. RV tissues were obtained from explanted non-failing and failing human hearts at the time of organ donation or heart transplantation. High potassium cardioplegia was administered immediately before or after cardiectomy, and hearts were promptly transported to the laboratory on wet ice where they were immediately processed for molecular, histological or physiological characterization.

Echocardiography. Quantification of 2-D and Doppler echocardiographic data including left heart chamber dimensions, left ventricular ejection fraction (LVEF) and the degree of mitral and tricuspid regurgitation were performed in the standard manner. Right ventricular (RV) function was quantified by measuring the tricuspid annular plane systolic excursion (TAPSE) as described previously. TAPSE values < 1.5 cm were considered severe RV dysfunction, values 1.5-1.8 cm were considered moderate RV dysfunction, values 1.81-2.1 cm were considered mild RV dysfunction, and values > 2.1 cm were considered normal. The Doppler estimation of pulmonary artery systolic pressure was determined using the peak TR velocity in the standard manner.

Protein Extraction and Immunoblot Analysis. About 100 mg frozen human RV and LV tissues were minced briefly into small chunks and homogenized using the FastPrep System (BIO 101, Inc., Vista, CA) with 3 mm Zirconium beads (OPS Diagnostics, LLC, Lebanon, NJ) in 20 mM Tris pH7.5/100 mM NaCl/1 mM EDTA/1 mM PMSF/ protease inhibitor cocktail (Sigma).
Triton X-100 was added to the homogenates to the final concentration of 1% and incubated on ice for 30 min. At the end of the incubation, the homogenates were centrifuged at 13 kG force at 4°C for 15 min. The supernatants were collected and the protein content in each sample was determined using the BCA assay (Pierce, Rockford, IL). An equal amount of protein from each sample was used for SDS-PAGE. PDE5 and GAPDH expression levels in the samples were assayed according to a standard immunoblotting procedure, using a polyclonal rabbit anti-PDE5 antibody (Cell Signaling Technology, Danvers, MA) and a polyclonal rabbit anti-GAPDH antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). ECL substrates (GE Healthcare Piscataway, NJ) were used to develop the blots and protein bands recognized by the antibodies were visualized after exposure to X-ray films (Denville Scientific, Metuchen, NJ). The intensities of the bands were quantified using Alpha-imager (Alpha Innotech, San Leandro, CA).

**Immunohistochemistry.** Isolated human right ventricular tissues were immediately fixed in 4% formaldehyde followed by paraffin-embedding. Immunohistochemical staining was carried out using the ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Briefly, sections were de-paraffinized and re-hydrated. A citrate buffer, 10mM citric acid, 0.05% Tween 20, pH 6.0, was used to unmask antigens at 100°C for 20’ and the endogenous peroxides was quenched by incubating the sections in PBS/2% H2O2 for 15’ at RT. After blocking with 10% normal goat serum/PBS/0.05% T-20, PDE5 and myosin heavy chain expression in the sections were detected with antibodies against the proteins. Rabbit polyclonal anti-PDE5 was from Abcam (Cambridge, MA) and mouse monoclonal anti-myosin heavy chain antibody was from Developmental Studies Hybridoma Bank (Iowa City, IA). HRP-conjugated secondary antibodies to rabbit and mouse were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). DAB was used to visualize myosin heavy chain staining and
hematoxylin was used for nuclei counterstaining (Vector Laboratories, Burlingame, CA) while DAB-Ni was used to visualize PDE5 staining. Background controls employed only the secondary antibodies.

**Contractile Response to PDE5 Inhibition.** Without antecedent fixation or freezing, thin subendocardial myocardial trabeculae were isolated from the right ventricular free wall and mounted in a slackened position in a custom designed isometric muscle chamber (Scientific Instruments, Heidelberg, Germany), as previously described. The Ca\(^{2+}\) concentration of physiologic buffer within the muscle chamber was incremented gradually to 1.75 mmol/L CaCl\(_2\). After a 60 minute equilibration period, acute studies were conducted at 37°C and at a stimulation frequency of 0.5 Hz. Experimental trabeculae length (L) was set to 80% of the difference between L\(_0\) and L\(_{max}\). Isometric twitches were recorded before and after administration of the PDE5 inhibitor, MY-5445, at 10 uM.

**Statistical Methods:** For immunoblots, PDE5 levels normalized to GAPDH were compared between patient groups using Kruskal-Wallis and Mann-Whitney tests, and correlation between levels in left and right ventricles was assessed using Spearman rank correlations. For muscle strip experiments, linear mixed-effects models with a group-by-time interaction term were used to compare mean contractile parameters before and after PDE5 inhibition in non-failing and failing hearts. Strip-specific and subject-specific random intercepts were used to account for the temporal correlation of responses within strips over time, and the correlation due to clustering of multiple strips within a subject. All analyses were completed using Stata 11.0 (StataCorp LP, College Station, Texas).
Results

**Patient Characteristics.** The Table summarizes the clinical characteristic of individuals providing myocardial tissue for these studies. Subjects were grouped into five categories: non-failing (NF), ischemic cardiomyopathy (ICM), end-stage idiopathic dilated cardiomyopathy (DCM) with mild RV dysfunction (RVD), DCM with severe RVD, and DCM with severe RVD and pre-explant support with a left ventricular assist device (LVAD). Non-failing control hearts from organ donors had normal RV and LV function based on echocardiography with minimal medication exposure at the time of tissue procurement. Each of the transplant recipients providing heart tissue was receiving multiple cardiovascular medications at the time of their transplantation. Of note, three of four LVAD patients were still requiring milrinone for RV support at the time of their transplants. Among the patients with ischemic cardiomyopathy, the severity of RV dysfunction varied from mild to severe.

**Expression levels of PDE5 in the RV.** To understand the regulation of PDE5 expression in response to cardiomyopathies, we examined PDE5 protein levels in the RV of hearts grouped according to the five categories listed above. Extracts from these tissues were subjected to immunoblot analysis (Figures 1A, 1C, and 1E) using antibody against PDE5 (see Methods for details). GAPDH level in each sample was also measured by immunoblot and used as a reference for normalization. A set of NF samples were loaded on each gel to allow the correction of gel to gel variations. PDE5 level in each sample was obtained by normalizing the band intensity of PDE5 with the band intensity of GAPDH (Figures 1B, 1D, and 1F). A comparison of PDE5 levels in the NF and failing group including all etiologies is shown in Figure 2A). PDE5 levels in each disease subgroup and NF group are shown in Figure 2B).
As shown in Figure 1 and 2, our results revealed that there is a significant change in PDE5 protein level among the failing hearts compared to NF. Specifically, PDE5 expression increased in the RV of ICM group compared to the NF group (4.6 fold and \( p < 0.001, n = 9 \) for ICM group and \( n = 4 \) for NF group). For DCM group, about 2 fold increase in PDE5 level was observed when RV dysfunction was mild (\( p < 0.008, n=4 \) for each group), when RV dysfunction was severe, a bigger increase in PDE5 expression was detected (3-fold and \( p < 0.003, n = 4 \) for each group). However, for DCM hearts with LVAD support, PDE5 expression in the RV was not statistically different from that of the NF group. Comparison of different RV failure subgroups suggests heterogeneity in the degree of PDE5 elevation in the failing RV depending on the degree of RV failure (Kruskal-Wallis \( P = 0.004 \)). Interestingly, across all samples and subgroups, PDE5 abundance in the RV was not correlated with PDE5 abundance in the LV (Figure 2C). These results suggest that response of PDE5 expression to cardiomyopathy were etiology dependent, with ICM having more impact on the PDE5 expression level than DCM. LVAD support did not appear to reduce the RV PDE5 expression associated with severe RVD.

**Localization of PDE5 in the Myocardium.** The adult myocardium is composed of various cell types including cardiac myocytes, fibroblasts, endothelial cells and vascular smooth muscle cells. The endothelial and smooth muscle cells are known to express PDE5 \(^8\). To assess if cardiac myocytes contributed to the observed change of PDE5 protein level in the RV tissue from hearts of ICM and DCM with mild RVD, we carried out immunohistochemical staining of PDE5 in paraffin-embedded tissue sections of RV fixed immediately after procurement (Figure 3A). Polyclonal rabbit anti-PDE5 antibody was used to detect the presence of the protein in the tissue. Monoclonal mouse anti-sarcomeric myosin heavy chain antibody was used to identify cardiac myocytes in consecutive sections (Figure 3C).
As shown in Figure 3A, vascular PDE5, a positive control, can be readily detected (see solid arrows) in the RVs of NF, DCM and ICM. In the area primarily occupied by myocytes, staining of comparable intensity as that of vasculature can be observed in the ICM sample. The connective tissue regions remain unstained (see open arrows), demonstrating the specificity of the staining assay. In the case of DCM from a heart with mild RVD, the PDE5 staining in the myocyte region is less intense compared to that of vasculature and lower in frequency compared to the ICM. This corresponds well with the observed PDE5 protein levels in ICM and DCM tissue samples by immunoblot. In the NF tissue, PDE5 staining is most intense in the vasculature, myocyte PDE5 staining intensity is considerably less, suggesting relative less contribution from myocyte PDE5 expression. The expression of PDE5 in cardiomyocytes was also shown by previous studies of Nagendran et al and Pokreisz et al.14,17. Taken together, the data indicate that more myocytes from the RV of ICM and DCM express PDE5 protein than from RV of NF, and that PDE5 from cardiac myocyte thus contributes substantially to the total tissue PDE5 content. Furthermore, the intensity of immunohistochemical staining among different samples correlated well with that observed with immunoblotting, suggesting that the difference in PDE5 protein levels between NF and ICM or DCM hearts can be attributed, at least in part, to the differences in the respective myocytes.

**PDE5 inhibition Increases Contractility in Isolated RV Trabeculae.** We next examined whether PDE5 directly modulates contractility in RV trabeculae obtained from three ICM, one DCM and two NF human hearts, with multiple trabeculae isolated from each RV, using a PDE5 inhibitor, MY-5445. As shown in Figure 4, PDE5 inhibition had a negligible effect on the isometric force generation in trabeculae from the non-failing RV (A, C). However, a positive inotropic effect was evident in trabeculae from the failing RV compared to the baseline (B, C).
Diastolic force, already elevated in the failing trabeculae, tended to increase further with PDE5 inhibition, but decreased in the NF trabeculae (D). The greater effect of PDE5 inhibition in the failing heart was also reflected in the rate of force development (E) and force decline (F). The association of increased contractile effects due to PDE5 inhibition with the differential PDE5 expression in the ICM myocardium suggests that the up-regulated PDE5 in the failing tissues is a functionally important regulator of basal contractility in these hearts.
Discussion

PDE5 is a cGMP specific phosphodiesterase that plays an important role in regulating cellular cGMP levels. The present study demonstrates that right ventricular PDE5 abundance is increased in failing human hearts compared with non-failing myocardium. Increased PDE5 expression was most apparent in failing hearts from patients with ischemic cardiomyopathy where right ventricular myocardium exhibited more than four-fold increases in PDE5 protein abundance. Localization of PDE5 via immunohistochemical staining demonstrated that increased expression was likely a result of increases in cardiac myocyte PDE5, with vascular smooth muscle PDE5 expression providing background levels in both NF and failing hearts. Among individuals with non-ischemic cardiomyopathy, degrees of PDE5 up-regulation in the RV seem to be dependent on the severity of RV dysfunction. Our studies showing differential responses to acute PDE5 inhibition in isolated RV trabeculae provides the first direct evidence that increased PDE5 expression in failing human myocardium may modulate RV contractility independent of preload or afterload.

By selecting samples from different disease groups and examining the expression of PDE5 in the RV, we obtained insights into factors regulating PDE5 expression in the human myocardium. Unlike previous human studies focusing only on non-ischemic cardiomyopathy, our comparison of ischemic versus non-ischemic cardiomyopathy revealed significantly greater RV PDE5 expression in ICM. Moreover, the presence of variable degrees of RV dysfunction provided an opportunity to demonstrate that the severity of RV dysfunction affects the degree of RV PDE5 up-regulation among patients with DCM. As with previous studies, immunohistochemical staining demonstrated that increased overall myocardial PDE5 abundance in failing hearts was likely due to increased cardiac myocyte PDE5 expression with relatively
unchanged expression in vascular smooth muscle. Thus, our data is generally in agreement with
the result of earlier studies, but reveals further potential factors that may contribute to the
regulation of PDE5 expression during disease progression.

Taken together, intergroup comparisons indicate that the etiology and severity of RV
cardiomyopathy affect PDE5 expression while LVAD support does not significantly alter RV
PDE5 expression. Accordingly, it is reasonable to speculate that one or more of the many
hypertrophic signaling pathways activated in the failing myocardium directly or indirectly
contribute to increased PDE5 expression in the failing RV. For example, recent studies by
Mokni et al demonstrated that angiotensin II infusion induces increased PDE expression
(including PDE5) during the early phases of myocardial hypertrophy, while other studies have
linked oxidative stress to increased myocardial PDE5 expression.

Previously, no direct association between the up-regulation of PDE5 and contractility
alteration in human tissues have been reported. In studies that examined PDE5 up-regulation in
failing human myocardium, animal models were used to understand the impact on tissue
functions. Assessing impacts of the up-regulation on human tissue could, to a certain
degree, verifies the relevance of the results from the animal studies, given the reported difference
between human and animal in metabolizing c-GMP. Our results reported here showed that
PDE5 inhibitor, MY5445, acutely increases the contractility of muscle strips isolated from the
failing hearts independent of effects on preload or afterload. Our finding that acute PDE5
inhibition increases contractility and prior studies indicating that PDE5 inhibition protects
against cardiac hypertrophy in animal models, support the possibility that increased
PDE5 expression may exacerbate RV dysfunction and hypertrophy in the failing heart.

Important caveats concerning this possibility relate to difference in time frame and compensatory
responses in our studies versus clinical trials. For example, our data demonstrates functional actions of acute PDE5 inhibition during an interval of less than one hour while clinical trials examine responses over much longer intervals. With respect to compensatory responses, a strength of using isolated myocardial trabeculae is that intrinsic contractile responses to PDE5 can be assessed without potentially confounding changes in preload, afterload, perfusion or neurohormonal influences. In vivo, these influences may change and shape the overall myocardial response to PDE5 inhibition.

Together, our findings suggest that PDE5 inhibition in patients with pulmonary arterial hypertension may have both direct (via inotropy) and indirect (via reduced afterload) positive effects on right ventricular contractility. Such findings also suggest that up-regulated myocardial PDE5 expression may contribute to the progression of heart failure and support an emerging therapeutic role for PDE5 inhibitors for both right and left ventricular failure.
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Disclosures

Dr. Quaile is currently an employee of GlaxoSmithKline.

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RVD Grade indicates right ventricle dysfunction grade; Est PA Syst, estimated pulmonary artery systolic pressure; TR Grade, tricuspid regurgitation grade; LVEDD, left ventricle end diastolic diameter; LVEF, left ventricle ejection fraction; MR Grade, mitral regurgitation grade; LVAD Dur, duration of left ventricle assistant device implementation.

Medication abbreviations: A, Amiodarone; ACE, Angiotensin converting enzyme inhibitors; ARB, Angiotensin II receptor blockers; BB, Beta-adrenergic blocking agents; D, Diuretic; Dig, Digoxin; H, Hrydralazine; Mil, Milrinone; N, Nitrates; S, Statins; W, Warfarin.
**Figure Legends**

**Figure 1. PDE5 Expression in RV tissues of ICM, DCM and NF Human Hearts.**
Immunoblot analysis was carried out to examine PDE5 protein expression levels in the RV tissue extracts using an anti-PDE5 antibody. In each blot, a group of NF samples was included as a control for the disease groups and abundance of GAPDH was also determined to control for total protein content in each sample. A) DCM with mRVD vs. NF hearts; C) DCM with sRVD without or with antecedent VAD support vs. NF hearts; and E) ICM vs. NF hearts. In B), D) and F) GAPDH normalized PDE5 protein levels, measured by densitometry of protein bands in the blots, were used to compare the relative abundance of the protein in the samples. Sample orders in each group are listed in the Table. Abbreviations: NF-Non-failing, ICM-ischemic cardiomyopathy, DCM-dilated cardiomyopathy, mRVD-mild RV dysfunction, sRVD-severe-RV dysfunction, and VAD-left ventricular assist device.

**Figure 2. PDE5 Expression in Different Categories of Failing Human Hearts.** A) Right ventricular PDE5 abundance was elevated in heart failure (HF) compared to non-failing controls (NF); Mann-Whitney P = 0.003. B) Comparison of different RV failure subgroups suggests heterogeneity in the degree of PDE5 elevation in the failing RV depending on the degree of RV failure; Kruskal-Wallis P = 0.004. Data are in arbitrary units normalized to GADPH abundance (see Fig. 1). PDE5 expression level in the LV was determined in the same manor as that of RV’s. The GAPDH normalized PDE5 levels in paired RV and LV samples from each heart was plotted in Figure 2C demonstrating a lack of correlation of RV and LV PDE5 expression levels; Spearman rho = 0.04; P = 0.85. See Figure 1 for definitions of abbreviations.

**Figure 3. PDE5 Expression in Myocytes of NF, DCM and ICM Human Hearts.**
Consecutive paraffin embedded RV tissues sections from NF, DCM, and ICM hearts were used to examine PDE5 expression. The sections in A) were incubated first with a rabbit polyclonal
anti-PDE5 antibody followed by HRP conjugated goat anti-rabbit secondary antibody and then developed by using DAB-Ni as a substrate to visualize the bonded antibodies. Section B) was processed in parallel with A) but without using the primary anti-PDE5 antibody. An antibody against striated muscle myosin heavy chain was used to replace the anti-PDE5 antibody in A) to reveal the myocyte location in the tissue with DAB as substrate for HRP-conjugated goat anti-mouse IgG secondary antibody C). Hematoxylin nuclear counterstaining was only applied to section in C) to avoid false positives that could be introduced to A) due to the staining color similarity between hematoxylin and DAB-Ni.

**Figure 4. Contractile Response to Acute PDE5 Inhibition.** Representative twitch tracings are shown in A) non-failing and B) failing human RV trabeculae before and after administration of the PDE5 inhibitor MY-5445. Mean steady-state developed force C), diastolic force D), rate of force development E) and decline F) before and after application of the PDE5 inhibitor MY-5445 in non-failing and failing human right ventricular trabeculae were also determined. Three RV trabeculae from two nonfailing hearts, and nine trabeculae from four failing hearts (3 ICM and 1 DCM) were used. Non-bolded P-values compare parameters within each patient group before and after PDE5 inhibition, and bolded P-values compare the temporal changes in heart failure with the temporal change in non-failing hearts. Error-bars indicate 95% confidence intervals.
A) Force (mN/mm²) vs. Time (msec) with baseline and with MY-5445.

B) Force (mN/mm²) vs. Time (msec) with baseline and with MY-5445.

C) Developed Force (mN/mm²) for Heart failure vs. Non-failing at Baseline and MY-5445 10 mmol/L. P-values: Baseline: P < 0.001, MY-5445: P = 0.009.

D) Diastolic Force (mN/mm²) for Heart failure vs. Non-failing at Baseline and MY-5445 10 mmol/L. P-values: Baseline: P = 0.13, MY-5445: P = 0.36.

E) +dF/dt (mN/s/mm²) for Heart failure vs. Non-failing at Baseline and MY-5445 10 mmol/L. P-values: Baseline: P < 0.001, MY-5445: P = 0.024.

F) −dF/dt (mN/s/mm²) for Heart failure vs. Non-failing at Baseline and MY-5445 10 mmol/L. P-values: Baseline: P = 0.045, MY-5445: P = 0.009.
Differential Expression of PDE5 in Failing and Non-Failing Human Myocardium
Xiaoyin Shan, Michael P. Quaile, Jeffery K. Monk, Benjamin French, Thomas P. Cappola and Kenneth B. Margulies

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