Increased Sarcolipin Expression and Adrenergic Drive in Humans with Preserved Left Ventricular Ejection Fraction and Chronic Isolated Mitral Regurgitation

Zheng et al: Sarcolipin and Adrenergic Signaling Pathways in Human MR

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Abstract

Background—There is currently no therapy proven to attenuate left ventricular (LV) dilatation and dysfunction in the volume overload induced by isolated mitral regurgitation (MR). To better understand molecular signatures underlying isolated MR, we performed LV gene expression analyses and overlaid regulated genes into Ingenuity Pathway Analysis in patients with isolated MR.

Methods and Results—Gene arrays from LV tissue of 35 patients, taken at the time of surgical repair for isolated MR, were compared to 13 normal controls. Cine-magnetic resonance imaging (MRI) was performed in 31 patients before surgery to measure LV function and volume from serial short axis summation. LV end-diastolic volume was 2-fold (p=0.005) higher than normals and LV ejection fraction (EF) was 64±7% (50-79%) in MR patients. Ingenuity pathway analysis identified significant activation of pathways involved in β-adrenergic, cyclic AMP, and G-protein coupled signaling; while there was downregulation of pathways associated with complement activation and acute phase response. SERCA2a and phospholamban protein were unchanged in MR vs. control LVs. However, mRNA and protein levels of the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} ATPase (SERCA) regulatory protein sarcolipin, which is predominantly expressed in normal atria, were increased 12- and 6-fold respectively. Immunofluorescence analysis confirmed the absence of sarcolipin in normal LVs and its marked upregulation in MR LVs.

Conclusions—These results demonstrate alterations in multiple pathways associated with β-adrenergic signaling and sarcolipin in the LVs of patients with isolated MR and LVEF > 50%, suggesting a beneficial role for β-adrenergic blockade in isolated MR.

Key Words: mitral regurgitation, microarray, β-adrenergic signaling, human left ventricle
Isolated mitral regurgitation (MR) is characterized by left ventricular (LV) dilation and augmented stroke volume mediated by the Starling mechanism and facilitated by LV ejection into the low pressure left atrium. These favorable conditions for LV shortening dynamics can falsely elevate LV ejection fraction (EF) despite underlying cardiomyocyte contractile impairment. Therefore, in order to preserve LV systolic function and improve survival, corrective mitral valve surgery is recommended if LVEF falls below 60%. Nevertheless, a decrease in LV systolic function remains a risk after mitral valve repair and the mechanisms involved in the transition to irreversible cardiomyocyte damage in chronic isolated MR remain elusive. There is currently no effective medical therapy that attenuates progressive LV remodeling in isolated MR and mechanisms of LV myocardial remodeling specific to chronic MR are poorly understood.

Measurement of plasma or LV interstitial fluid catecholamines has identified increased sympathetic drive early and throughout the course of isolated MR in both animal models and humans, respectively. There is evidence of myocardial dysfunction from LV muscle strips and derangement of calcium handling proteins in patients with isolated MR despite LVEFs > 55%. Although a comprehensive analysis of LV tissue in human isolated MR is lacking, there is evidence that β1-adrenergic receptor blockade (β1-RB), initiated either immediately or three months after MR induction in the dog, improves LV contractility and cardiomyocyte calcium transients and cardiomyocyte fractional shortening. In a retrospective analysis of patients with severe MR and normal LVEF, β-RB imparts a significant independent survival benefit with or without coronary artery disease. Further, results of a recent Phase IIb clinical trial demonstrate a beneficial effect of β1-RB vs. placebo on LVEF in patients with isolated MR over a two year follow-up period.
To determine whether β-adrenergic signaling, or other signaling pathways, is important in patients with isolated MR, we determined the cardiac transcriptome from LV biopsies taken from patients with isolated MR and mean LVEF > 60% at the time of mitral valve corrective surgery. This analysis in patients with largely Class I-II NYHA symptoms demonstrates increased activity of adrenergic signaling pathways, leading further support for early β-RB treatment in isolated MR.

Methods

Study Subjects

The protocol was approved by the University of Alabama at Birmingham Institutional Review Board and informed consent was obtained from all participants. The study group consisted of 35 patients (age mean 44 ± 14, median 38, range 21–71) with severe isolated MR secondary to degenerative mitral valve disease, who were referred for corrective MV surgery. Patients with obstructive coronary artery disease (> 50% stenosis), aortic valve disease, or concomitant mitral stenosis were excluded from the study. Severe isolated MR was documented by echocardiogram/Doppler studies and 31 patients underwent cine-Magnetic Resonance Imaging (MRI). Each patient with severe isolated MR had cardiac catheterization prior to surgery. Results of cine-MRI of the MR group was compared to a normal healthy cohort from a previous studies in our laboratory15,17,18 (age 40 ± 3, median 38, range 21–62 years), who have no history of cardiovascular disease.

At the time of surgery, LV tissue was taken from the lateral endocardial wall of the LV at the level of the tips of the papillary muscles in all patients. Portions of the biopsy sample were then placed in RNA later or frozen and stored at -80°for future analysis.
Cardiac Magnetic Resonance Imaging

A 1.5-T MRI scanner (Sigma GE Healthcare, Milwaukee, Wisconsin) optimized for cardiac application was used to perform all MRIs (see Figure 1).17-19 LV volumes were computed by summation of volumes defined by contours in each short axis slice, as previously described in our laboratory.17-19

RNA isolation and microarray

Total RNA was extracted from LV biopsies using Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen Sciences, MD) and cleaned by Ambion TURBO™ DNase. The integrity of RNA was evaluated by the BioRad Experion (Bio-Rad Laboratories, CA). Samples containing an OD ratio of 260/280 >1.8 and 28S/18S >1.7 were selected for microarray processing. Thirteen normal human RNA samples (males 12, female 1, ages: 41, 44, 21, 25, 23, 27, 29, 36, 27, 24, 21, 66, 50) were extracted from LV tissue from motor vehicle accident subjects (purchased from BioChain Institute, Hayward, CA). The Agilent One color human RNA microarrays were performed at Beckman Coulter Genomics (Danvers, MA).

Verification of Gene Expression Using Real-Time RT-PCR

Quantitative real-time PCR was performed using the Bio-RAD iCycler iQ™ system (Bio-Rad Laboratories, CA) on 500 ng total RNA from microarray samples to verify array data. Supplementary Table 1 demonstrates the selected genes and primer sequences (Sigma-Genosys, Woodlands, TX). GAPDH was chosen as an endogenous control.

Immunohistochemistry

Slides containing 5µm sections were deparaffinized in xylene and rehydrated in graded solutions of ethanol. After blocking with 5% normal serum, sections were incubated with sarcolipin (SLN) antibody (Santa Cruz Biotechnology, Inc CA; 1:100) and sarcoplasmic reticulum Ca^{2+} ATPase
(SERCA2) (Santa Cruz Biotechnology, Inc CA; 1:100) respectively for 1 hour at room
temperature. Sections were incubated in Alexa Fluor conjugated secondary antibody (Molecular
Probes, Eugene, OR; 1:200) for 1 hour at room temperature. The slides were mounted with
Vectashield Mounting Medium with DAPI for nuclear staining (Vector Laboratories,
Burlingame, CA). Image acquisition and intensity measurements were performed on a Leica
DM6000 epifluorescence microscope with SimplePCI software (Compix, Inc., Cranberry
Township, PA). Images were adjusted appropriately for background fluorescence.

Western blot analyses

Total protein homogenates were prepared from the LV tissues of control (n = 3 males, ages: 42,
49, 42 years) and MR patients. Control LV tissue for this protein analysis was purchased from
Imginex Laboratories (San Diego, CA) from subjects succumbing from motor vehicle accidents.
This tissue had a normal histological examination and all subjects had no history of
cardiovascular disease and no reported evidence of medications at the time of death. LV tissue
from 3 normal and 4 MR hearts was electrophoretically separated on 8% [for SERCA2a and
calsequestrin (CSQ)] or 14% [for phospholamban (PLN) and triadin] sodium-dodecyl sulfate-
polyacrylamide gel electrophoresis or 16.5% glycerol-Tricine gel [for sarcolipin (SLN)] and
electrophoretically transferred to nitrocellulose membranes. The membranes were
immunoprobed with primary antibodies [anti-rabbit SLN, 1:3000; anti-rabbit SERCA2a, 1:5000;
anti-rabbit PLN, 1:3000; anti-rabbit phospho-PLN, 1:10,000; anti-rabbit CSQ, 1:5000], followed
by HRP-conjugated secondary antibodies.²⁰ Signals were detected by Super Signal WestDura
substrate (Pierce) and quantitated by densitometry and normalized to CSQ levels.

Protein in sample buffer was first normalized to Coomassie staining. The levels of CSQ
did not differ in control and MR samples using GAPDH as loading control. The Western blot for
SERCA, PLN, triadin, and SLN were done separately but on the same samples and using CSQ as well as GAPDH as loading controls.

**Genespring, Ingenuity Pathway Analysis (IPA) and statistical analysis**

Microarray data was analyzed by Genespring GX 11.5. Raw data were log2 transformed and then normalized to the 75th percentile of all values on a chip. The MR and normal samples were compared using t-test to examine for differentially expressed genes. A list of genes with ≥ 2.0-fold change was generated first and tested by Benjamini-Hochberg Multiple Testing Correction. Significant genes were selected with a cut-off of $p < 0.05$ and fold change $≥ 2.0$. The list of altered genes was then imported into IPA for pathway analysis as previously described in our laboratory. The Fisher’s Exact Test was applied by IPA to predict the likelihood that the association between the set of altered genes and a related pathway is not due to random association. RT-PCR and western blot were analyzed by t-test. MRI volumes and function between control subjects and MR patients were compared using Student’s two sample t test. Significance was set at $p < 0.05$.

**Results**

**Clinical Characteristics**

Clinical characteristics of the 51 control subjects and 35 MR patients are outlined in Table 1. The MR group is older than controls (55 ± 12 vs 44 ±14 vs. yr, $p < 0.0001$). There are no significant differences in body surface area (BSA) and gender between the two groups. Heart rate and diastolic blood pressure are similar in the two groups. Table 1 also summarizes individual patient medications, history of hypertension, NYHA functional class.
Magnetic Resonance Imaging

Thirty-one MR patients had MRI performed within one month prior to surgery (Table 2). MR patients have greater LV end-diastolic volume (EDV), LV end-systolic volume (ESV), and LV stroke volume (SV) normalized to BSA compared to controls. MR patients have higher LVED and LVES dimensions, similar LVEF, but a higher LV mass vs. controls. Figure 1 demonstrates the spherical remodeling and thinning of the LV wall in a representative MR patient.

Microarray Analysis

The microarray analysis identified 724 differentially expressed genes (at least 2-fold change) in MR vs. controls ($p < 0.05$), including 353 upregulated and 371 downregulated genes. The heatmap in Figure 2a demonstrates a consistent pattern of change of these genes in the 35 MR LVs and 13 normal LVs. A Principal Components Analysis (PCA) plot (Figure 2b) verifies the quality of the array. In this plot, samples representing the same experimental conditions are more similar to each other than to samples representing different experimental conditions. Supplementary Table 2 lists genes well established in the pathophysiology of cardiovascular disease identified by IPA. Among the 724 genes, the gene with the highest fold-increase (22-fold) is natriuretic peptide A (NPPA); NPPB is also increased by 5.13-fold. The upregulation of these marker genes for hypertrophy underscores the quality of the gene expression profiles from the patients with severe MR and higher LV mass compared to control LVs.

Validation of Microarray with Quantitative PCR

Supplementary Table 3 demonstrates microarray results validated by PCR for PLN, SLN, NPPA, 5'-AMP-activated protein kinase subunit beta-2 (PRKAB2), (natriuretic peptide receptor C) NPR3, peroxidoredoxin 3 (PRDX3), desmocollin 1 (DSC1), Kv channel interacting protein 2
(KCNIP2), and FOS. There is excellent agreement between microarray and quantitative PCR (Supplementary Table 3).

IPA Canonical Pathway Analysis

Activation of the cardiac β-adrenergic signaling in the MR hearts

The 724 altered genes are analyzed by Ingenuity Pathway Analysis (IPA). The top network with the score of 38 is associated with cardiovascular disease. Canonical pathway analysis identifies the significant activation of cardiac β-adrenergic signaling pathway in the MR hearts (Figure 3a). Figure 3b demonstrates that the altered genes and their relation with calcium channel regulation.

PLN is a 52-amino acid sarcoplasmic reticulum membrane protein expressed abundantly in cardiac muscle. In its dephosphorylated form, PLN interacts with SERCA2a to inhibit Ca\(^{2+}\) transport by lowering SERCA2a’s affinity to Ca\(^{2+}\). When PLN is phosphorylated, its inhibitory effect on SERCA2a is relieved. The 31-amino acid sarcoplasmic reticulum (SR) membrane protein, SLN, has a similar ability to inhibit either SERCA1a or SERCA2a. In the human MR heart, the mRNA of PLN and SLN are increased by 2.5- and 12.4-fold respectively. Protein kinase A, cAMP dependent, regulatory type 1α (PRKAR1A) and PRKA anchor protein 7 (AKAP7), which direct or indirectly bind to PLN and regulate its phosphorylation, are increased 2-fold. There is a significant decrease in phosphodiesterase 4D (PDE4D) and PDE3B that degrade cAMP and cGMP, which inactivate PKA.

Activation of intercellular and second messenger signaling in MR hearts

Adrenergic receptors belong to G-protein coupled receptor family and a major activity of β-adrenergic receptors modulates the level of the second messenger cAMP. Thus, we analyzed the activation of the canonical intercellular and second messenger signaling in MR hearts. Figure 4 demonstrates the significant activation of G-protein coupled receptor and cAMP signaling. The
corresponding heat maps represent the normalized signal intensity values for genes in these two signaling pathways in NL and MR LVs. The significant activation of cAMP-mediated and G-protein coupled receptor signaling further supports the highly activated β-adrenergic signaling in the MR hearts.

Down-regulation of the humoral immune response and cytokine signaling in MR hearts

We further analyze the regulation of inflammatory signaling pathway in the MR heart. Figure 5 demonstrates an extensive down-regulation of humoral immune response and cytokine signaling in these patients with isolated MR and predominantly Class I-II symptoms and well preserved LVEF.

Quantification of SLN and PLN proteins in the normal and MR heart by western blot

The increase in SLN transcription is further verified by a 6-fold increase in SLN protein in the MR LV (Figure 6 a & b), while PLN protein levels are not altered in the MR heart. The protein levels of SERCA2a, CSQ and triadin are also unaltered in the MR LV compared to the control group.

To determine the influence of age on the microarray results, we performed a linear regression analysis between SLN or PLN mRNA expression and the ages of the 35 patients. Supplemental Figure 1 A & B demonstrates no significant correlation between age and expression of SLN or PLN mRNA expression in the MR hearts. In order to rule out the influence of gender on the gene array results, SLN and PLN mRNA expression was compared between the male control (n=12) and male MR (n=24) hearts. Supplemental Figures 2A and 3A demonstrate that SLN and PLN mRNA expression is significantly higher in the male MR vs. male normal hearts. SLN and PLN mRNA expression was then compared in male and female patients. Supplemental Figures 2B and 3B demonstrate that SLN and PLN mRNA expression do not
differ between the male and female MR patients. Taken together, these results demonstrate that gender does not influence the expression of SLN or PLN.

**Immunofluorescence staining of SLN**

Figure 6c demonstrates immunofluorescence staining of SLN in the normal and MR heart. Protein expression of SLN (red) is very low in the normal LV, but dramatically increases in the MR LV. Furthermore, immunohistochemical co-staining (Figure 6d) with SERCA2 (green) demonstrates that SERCA2 and SLN (red) are co-localized in the MR heart.

**Discussion**

The results of the current investigation support the significant role played by the adrenergic nervous system and the expression and a potential functional role of SR Ca$^{2+}$ regulatory protein SLN, in the nonfailing LV of patients with isolated MR. The new finding of SLN upregulation, which is normally an atrial specific SERCA regulator, has important implications for its role in mediating human cardiac muscle SR Ca$^{2+}$ transport and LV function in patients with isolated MR.

To gain a comprehensive insight into cellular processes in these patients with isolated MR and well preserved LVEF, we imported the list of altered genes into IPA for pathway analysis to predict the association between the set of altered genes and a related function. The statistical analysis for this process is presented in Figure 3, which demonstrates a significant activation of cardiac β-adrenergic signaling in the MR heart that is corroborated by a significant activation of cAMP and G-protein couple receptor signaling (Figure 4). Adrenergic receptors belong to G-protein coupled receptor family and a major activity of β-adrenergic receptors modulates the level of the second messenger cAMP. Thus, we analyzed the activation of the
canonical intercellular and second messenger signaling in MR hearts. Figure 4 demonstrates the significant activation of G-protein coupled receptor and cAMP signaling. The corresponding heat maps represent the normalized signal intensity values for genes in these two signaling pathways in NL and MR LVs. The significant activation of cAMP-mediated and G-protein coupled receptor signaling further supports the highly activated β-adrenergic signaling in the MR hearts.

As opposed to the end-stage dilated cardiomyopathy, where there is global decrease in β-adrenergic signaling,22,23 the current study includes MR patients with a well preserved LVEF and the majority of the patients presenting in Class I or II NYHA Heart Failure (Class I n=18, Class II n=13, Class III n=4). It is of interest that previous studies report that in patients with isolated MR, who largely present in Class III to IV heart failure, there is a down regulation of SERCA2a LV protein levels and a negative correlation between SERCA2a protein levels and LV end-diastolic diameter.10,11 Thus, as with the failing heart, the decompensated MR heart has decreased SERCA2a expression, whereas in our patients who largely presented in Class I and II symptoms, β-adrenergic signaling is increased and SERCA2a mRNA and protein expression is unchanged at this earlier stage in isolated MR.

Among the 724 altered genes, SLN is the second most upregulated gene, increasing from a very low intensity of 144 in normal to 3085 in MR LVs. Sarcolipin is normally expressed at high levels in atria and its expression is at very low/undetectable levels in the ventricles.24-29 However, the role of SLN in ventricular function in normal and diseased myocardium in human or in animal models is not understood. In patients with Tako-Tsubo cardiomyopathy, sarcolipin is upregulated acutely in the dysfunctional LV segment and returns to normal at the 12 day recovery phase when LVEF returned to normal.30 Our study is the first to demonstrate the SLN protein upregulation in the LV of patients with isolated MR. Studies using transgenic and
knockout mouse models suggest that SLN functions as a regulator of SERCA2a by lowering its Ca\(^{2+}\) affinity.\(^{25-30}\) These studies also show that the inhibitory function of SLN is independent of PLN. Thus, increased expression of SLN in MR hearts may be an earlier compensatory alteration in the setting of increased adrenergic drive. Nevertheless, the question remains whether ventricular expression of SLN is a detrimental effect since its expression, although to a lower level, is present in the basal state.

We have previously reported xanthine oxidase, lipofuscin accumulation, and myofibrillar loss in these patients with isolated MR;\(^{16}\) while in the current study, IPA pathway analysis demonstrates a significant downregulation of genes associated with acute phase response, in particular cytokines, and the complement system (Figure 5). Oral and coworkers report a relationship between TNF-\(\alpha\) expression and severity of LV remodeling, suggesting that TNF-\(\alpha\), and an increase in cytokines in general, may occur in the decompensated LV in patients with isolated MR.\(^{31}\) In support of this contention, we have previously reported gene array results from the volume overload of aortocaval fistula in the rat where 24 hours,\(^{32}\) 2 and 15 week ACF time points are marked by global inflammatory gene expression, while the 5 week interval was relatively quiescent for inflammatory gene expression.\(^{33}\) In the MR heart, there is an extensive down-regulation of humoral immune response and cytokine signaling in these patients with isolated MR and predominantly Class I-II symptoms and well preserved LVEF (Figure 6). Thus, the expression of inflammatory cytokines in the MR heart may be dependent upon the clinical stage of LV remodeling and/or heart failure symptoms; while cardiomyocyte oxidative stress may be a persistent finding throughout the course of volume overload.

There are several potential limitations of the current study. In addition to a small sample size, the RNA and protein control samples are not obtained from the same hearts. To avoid
potential differences of LV region on gene array results in the MR hearts, biopsies were taken from the same region of the LV in all patients in the lateral LV endocardial wall at the tip of the papillary. However, the mean age of the control (33 yr) was younger than the MR patient tissue samples (55 yr), which may have affected the results. In addition, heart samples from deceased donors may have unpredictable warm ischemic time before collection. To ensure the reliability of the microarray data and the comparability of the expressional profiles between the normal and MR hearts: 1) RNA samples are screened by checking their integrity and genomic contamination. Only the samples that meet the requirement of an OD ratio of 260/280 >1.8 and 28S/18S >1.7 were selected for microarray processing. 2) The excellent homogeneity within control and MR cohort demonstrated by cluster heatmap (Figure 2a) and the PCA plot (Figure 2b) in the MR hearts indicates the high quality of RNA samples used in this study. 3) The marked increase of cardiac hypertrophic markers NPPA and NPPB in the MR heart compared to the control samples also ascertains the trustworthiness of the microarray data. Finally, mRNA and protein analysis was not performed on the same samples. However, for the purposes of defining the status of SLN, we feel that the similar RNA, protein and immunohistochemistry findings truly reflect the fact that the LV has little Sarcolipin expression in the control hearts and is markedly upregulated in the MR heart.

The patient population of the current investigation is homogeneous in the marked degree of LV remodeling defined by MRI-derived volumes, a preserved LVEF, all but four patients with Class I-II symptoms, and no significant epicardial coronary artery disease. However, the majority of patients were taking either beta-receptor blockers or renin-angiotensin system blockers. Unfortunately, the small number of patients does not allow for an evaluation of gene response in the face of drug therapy. Despite this variability of drug therapy, the results of the
gene array clearly identify enhanced β-adrenergic receptor signaling and related cyclic AMP and G protein coupled signaling. The new finding is the marked upregulation of SLN, a key regulator of atrial SERCA pump. Further work will fill a large gap in knowledge about the processes leading to cardiac dysfunction in response to volume overload, in particular the question of how SLN expression is related to Ca^{2+} homeostasis in the setting of increased adrenergic drive in isolated MR.

Sources of Funding

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Disclosures

None.

References


Table 1. Baseline Characteristics

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<th>Variable</th>
<th>Control (n=51)</th>
<th>MR (n=35)</th>
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<td>55±12</td>
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<td>53%</td>
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<td>ACEi, AT₁RB or BB</td>
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<td>Hear Rate, bpm</td>
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<td>Diastolic BP, mm Hg</td>
<td>75±10</td>
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Value is mean ± SD; BSA: body surface area; HBP: high blood pressure; ACEi: Angiotensin Converting Enzyme inhibitor; AT₁R Angiotensin II type I receptor blocker; BB: beta-blocker.
Table 2. MRI LV Volume and Function

<table>
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<th>Control (n=51)</th>
<th>MR (n=31)</th>
<th>p-value</th>
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<td>LVSV index, ml/m²</td>
<td>44±7</td>
<td>71±18</td>
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<td>LVEF, %</td>
<td>65±6</td>
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<td>LV Mass, mg</td>
<td>95±25</td>
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<td>LVED dimension, cm</td>
<td>5.04±0.46</td>
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<td>1.74±0.28</td>
<td>&lt;0.0001</td>
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Value is mean ± SD; LV: left ventricle; ED: end-diastolic; ES: end-systolic; BSA: body surface area; LVEDV index: LV ED volume/BSA; LVESV index: LVES volume/BSA; LVSV index: LV stroke volume/BSA; LVEF: LV ejection fraction.
Figure Legends

Figure 1. LV two chamber view of two representative examples from Control (left) and MR patient (right). The MR heart demonstrated the marked LV dilatation and wall thinning compared to the control heart. (ED: end-diastole; ES: end-systole)

Figure 2. Heat map and Principal Components Analysis. a) Heat map generated by Genespring GX.11 demonstrates 724 genes altered \( \geq 2 \) fold from 13 normal LVs and 35 LVs with severe mitral regurgitation. Blue branches on the top columns represent the normal group, red branches represent the MR patient group. (b) A Principal Components Analysis (PCA) plot demonstrates the quality of the array. Each dot represents an expression profile of an individual sample plotted by PCA score. In the plot, samples representing the same experimental condition are more similar to each other than to samples representing a different experimental condition. Blue dots represent normal LVs. Red dots represent MR LVs.

Figure 3. IPA analysis demonstrates the activation of the canonical cardiovascular signaling in MR patients. a. Stacked bar charts demonstrate IPA-generated cardiovascular signaling. Among the 724 gene altered, 11 genes are in cardiac \( \beta \)-adrenergic signaling pathway. The changes in these 11 genes results a significant activation of cardiac \( \beta \)-adrenergic signaling pathway (\( p = 0.017 \)). The height of the bars indicates the percentage of genes that changed in the particular pathway. Red bar: upregulated. Green bar: down regulated. Pathways (orange square and dotted line) to the right of the threshold (blue dashed line) are significantly activated. Heat map represents the normalized signal intensity values for genes in the selected signaling pathway in normal controls (left column) and MR patients (right column). Red indicates higher expression.
Figure 4. **IPA analysis demonstrates the activation of the canonical intercellular and second messenger signaling in MR patients.** As in Figure 3, stacked bar charts demonstrate IPA-generated intercellular and second messenger signaling.

Figure 5. **IPA analysis demonstrats the down-regulation of the canonical humoral immune response (a) and cytokine signaling (b) in MR patients.** As in Figures 3 and 4, stacked bar charts demonstrate IPA-generated humoral immune response and cytokine signaling.

Figure 6. **Western blots showing the protein levels of SLN, SERCA2, PLN, and triadin in control (C) and MR LVs (Panels a, b).** Protein was first normalized to Coomassie staining. The levels of CSQ did not differ between control and MR using GAPDH as loading. The Western blot for SERCA, PLN, triadin, and SLN were done separately but on the same samples using CSQ as well as GAPDH as loading controls. * indicates p<0.005; n=4 controls and n=6 for MR. c. Immunohistochemistry demonstrates ectopic expression of SLN (red) in human MR heart vs normal heart. d: SLN double-labeled with SERCA2 in the normal and MR heart demonstrates increased SLN in the MR heart.
Cardiac β-adrenergic signaling
Cardiomyocyte differentiation via BMP receptors
Nitric Oxide signaling in the cardiovascular system
Atherosclerosis signaling
eNOS signaling
Hypoxia signaling in the cardiovascular system
Extrinsic prothrombin activation pathway
Inhibition of angiogenesis by TSP1
Cellular effects of sildenafil
Intrinsic prothrombin activation pathway
P2Y purigenic receptor signaling pathway

Cardiomyocyte differentiation via BMP receptors
NO signaling in the cardiovascular system
Inhibition of angiogenesis by TSP1
Cellular effects of sildenafil
Intrinsic prothrombin activation pathway

Figure 3a

Figure 3b
Figure 4
**A**

- C  C  C  MR  MR  MR
- SERCA2a
- PLN
- CSQ
- triadin
- SLN
- GAPDH

**B**

- Control
- MR
- Fold Changes (normalized to CSQ)

**C**

Red: SLN; Green: myosin; Blue: DAPI

**D**

Red: SLN; Green: SERCA2; Blue: DAPI

**Note:** The image contains graphs and figures related to protein expression and localization in heart muscle, with specific focus on SERCA2a, PLN, CSQ, triadin, SLN, and GAPDH. The data suggests differences in expression levels between control and MR conditions.
Increased Sarcolipin Expression and Adrenergic Drive in Humans with Preserved Left Ventricular Ejection Fraction and Chronic Isolated Mitral Regurgitation


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**Supplementary Table 1.** Primer sequences for validating microarray by real-time PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer forward sequence 5’-3’</th>
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<tr>
<td>SLN</td>
<td>TAGCCAGGGTGTTGCTTT</td>
<td>AGAATGGCATCCTGTCGTAC</td>
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<td>AGTGGATTCGCTCTTGAC</td>
<td>GAGGGACACCTCATTCTCT</td>
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<td>PRKAB2</td>
<td>GCGTTTCGATCTGAGGAA</td>
<td>TCAAGCACAAATCAGCCTT</td>
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<td>NPR3</td>
<td>CCTGGAAAACATCGGGGAAT</td>
<td>TGTGAAATGCTCCCAAGTC</td>
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<td>PRDX3</td>
<td>CCTACGATCAAGCCAAGT</td>
<td>TTGCAGGAGTTACACGCT</td>
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<tr>
<td>DSC1</td>
<td>GTGGAAGAGCATTGAGGA</td>
<td>CCAGGGACTGAGCTCCTGA</td>
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<tr>
<td>KCNIP2</td>
<td>TTGAATTTGTCCACCGTGT</td>
<td>AAGTGCCATAGGTGCTGTG</td>
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<td>FOS</td>
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## Supplementary Table 2. Selected genes related to cardiovascular disease by IPA.

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<th>Name</th>
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<th>Signal Intensity</th>
<th>Description</th>
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<td>NPPB</td>
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<td>ABAT</td>
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</tbody>
</table>

The p-values are Benjamini-Hochberg FDR p-values.
Supplementary Figures

Supplementary Figure 1.

A

Supplementary Figure 2.

A

Supplementary Figure 3.
Supplementary Figure Legends

Supplementary Figure 1. Regression analysis demonstrates SLN and PLN expression in the MR hearts are not influenced by age. A, Correlation analysis of SLN signal intensity with patients’ ages (n=35). B, Correlation analysis of PLN signal intensity with patients’ ages (n=35).

Supplementary Figure 2. Gender does not influence the expression of SLN in MR heart. A, Comparison between male normal hearts (n=12) with male MR hearts (n=24) demonstrates SLN is significantly increased in MR patients. B, Comparison between male (n=24) and female (n=11) MR patients demonstrates no significant difference on SLN expression between the male and female patients.

Supplementary Figure 3. Gender does not influence the expression of PLN. A, Comparison between male normal hearts (n=12) and male MR hearts (n=24) demonstrates PLN is significantly increased in MR patients. B, Comparison between male (n=24) and female (n=11) MR patients demonstrates no significant difference in PLN expression between the male and female patients.