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Phosphoproteomic analysis identifies phospho-Threonine-17 site of phospholamban important in low molecular weight isoform of fibroblast growth factor 2-induced protection against post-ischemic cardiac dysfunction

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Abstract

Rationale: Among its many biological roles, fibroblast growth factor 2 (FGF2) protects the heart from dysfunction and damage associated with an ischemic attack. Our laboratory demonstrated that its protection against myocardial dysfunction occurs by the low molecular weight (LMW) isoform of FGF2, while the high molecular weight (HMW) isoforms are associated with a worsening in post-ischemic recovery of cardiac function. LMW FGF2-mediated cardioprotection is facilitated by activation of multiple kinases, including PKCalpha, PKCepsilon, and ERK, and inhibition of p38 and JNK.

Objective: Yet, the substrates of those kinases associated with LMW FGF2-induced cardioprotection against myocardial dysfunction remain to be elucidated.

Methods and Results: To identify substrates in LMW FGF2 improvement of post-ischemic cardiac function, mouse hearts expressing only LMW FGF2 were subjected to ischemia-reperfusion (I/R) injury and analyzed by a mass spectrometry (MS)-based quantitative phosphoproteomic strategy. MS analysis identified 50 phosphorylation sites from 7 sarcoendoplasmic reticulum (SR) proteins that were significantly altered in I/R-treated hearts only expressing LMW FGF2 compared to those hearts lacking FGF2. One of those phosphorylated SR proteins identified was phospholamban (PLB), which exhibited rapid, increased phosphorylation at Threonine-17 (Thr17) after I/R in hearts expressing only LMW FGF2; this was further validated using Single Reaction Monitoring-based MS workflow. To demonstrate a mechanistic role of

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DISCLOSURES

None

phospho-Thr17 PLB in LMW FGF2-mediated cardioprotection, hearts only expressing LMW FGF2 and those expressing only LMW FGF2 with a mutant PLB lacking phosphorylatable Thr17 (Thr17Ala PLB) were subjected to I/R. Hearts only expressing LMW FGF2 showed significantly improved recovery of cardiac function following I/R ($p<0.05$), and this functional improvement was significantly abrogated in hearts expressing LMW FGF2 and Thr17Ala PLB ($p<0.05$).

Conclusion: The findings indicate that LMW FGF2 modulates intracellular calcium handling/cycling via regulatory changes in SR proteins essential for recovery from I/R injury, and thereby protects the heart from post-ischemic cardiac dysfunction.

Keywords

phospholamban phosphorylation; ischemia-reperfusion injury; fibroblast growth factor; mass spectrometry; proteomics and genomics

1. INTRODUCTION

Myocardial infarction (MI), a leading cause of death in the United States and other industrialized nations, is often followed by depressed cardiac function, which is associated with increased morbidity and mortality. Despite the widespread incidence of MI, there are few therapeutics that directly target the heart muscle to ameliorate the functional distress associated with ischemia and reperfusion (I/R) injury.

One promising molecule is fibroblast growth factor 2 (FGF2). Our laboratory and others have established that FGF2 can protect the heart from acute I/R injury, reducing infarct size and/or improving post-ischemic cardiac dysfunction independent of increasing blood flow, when administered exogenously or overexpressed[1–4]. FGF2 consists of high molecular weight (HMW) and low molecular weight (LMW) isoforms, expressed in both humans and rodents. HMW FGF2 is comprised of two isoforms in the mouse (21.5 and 22 kDa) and four in humans (21.5, 22, 24 and 34 kDa), and contains a nuclear localization sequence that leads it to be primarily detectable in the nuclei of cells. Both mice and humans have one 18 kDa LMW FGF2 isoform, which is found primarily in the cytosol and extracellular space. Recent work from our laboratory demonstrates that protection from post-ischemic dysfunction is mediated by LMW FGF2, but not HMW FGF2[5–7]. This improvement in cardiac function following I/R occurs, in part, via PKCα signaling to the contractile apparatus, altering the phosphorylation state of contractile proteins and their function[7]. Yet, it is unknown whether SR proteins are altered in expression or activity by LMW FGF2 to facilitate or “prime” the cardiac muscle for an ischemic insult, leading to improved post-ischemic cardiac function.

The current study uses a hypothesis-driven phosphoproteomics and genomics approach both to identify SR (phospho) proteins and genes regulated by cardiac FGF2 signaling and to link them to post-ischemic recovery of ventricular function. Here, we identify several SR phosphoproteins, including phospho-Threonine-17 phospholamban (PLB), that are modulated by LMW FGF2 during reperfusion. The present study demonstrates a direct role of PLB phosphorylation at Threonine-17 (Thr17) as a downstream mediator of LMW FGF2-mediated cardioprotection.

2. METHODS

2.1 Mouse Models and Exclusion Criteria

Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the NIH *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Mice with all isoforms of FGF2 ablated (*Fgf*2 KO) or with expression of only LMW FGF2 (HMWKO) were generated on a mixed 50% 129/Black Swiss background by the Doetschman laboratory, using a tag and exchange construct, as previously described[8,9]. Mice lacking expression of PKCα (*Pkcα*KO) were generated on a mixed 129/Black Swiss/FVBN mixed background as previously described by the Molkentin lab[10] (see Supplelmental Table 1 and Supplemental Figure 2). In addition, mice expressing only T17A modification of phospholamban (PLB), in which the amino acid Threonine is converted to Alanine at amino acid position 17 of PLB to inhibit phosphorylation at this site, were generated on a 129/CF-1 background as previously described by the Kranias laboratory[11], and bred to HMWKO mice to generate LMW FGF2 expressing PLB T17A mutant mice. Expression of the FGF2 isoforms and modification of PLB expression and phosphorylation represent the following genotypes: 1) HMWKO= only LMW FGF2 present along with native PLB, 2) *Plb* KO= both LMW and HMW FGF2 present but no endogenous murine phospholamban, 3) *Plb* KO/PLB T17A Tg= both LMW and HMW FGF2 present but endogenous murine PLB absent and overexpression of modified unphosphorylatable PLB at site threonine-17, and 4) HMWKOx*Plb* KO/PLB T17A Tg= only LMW FGF2 present and endogenous murine PLB absent and overexpression of modified unphosphorylatable PLB at site threonine-17 (see Supplemental Figure 1 and Supplemental Table 1 and Supplemental Figure 2). To ensure that Ser16 phosphorylation capability was intact, mice were injected intraperitoneally with a dose and time course of isoproterenol previously reported to selectively phosphorylate only the Ser-16 site[12] and immunoblotting was subsequently used to examine phosphorylation (see immunoblotting section and Online supplement).

A total of 8 mice were excluded from the I/R injury study, with 30 mice completing the I/R. Exclusion from the ischemia-reperfusion study was based on the signs of aortic or pulmonary vein leak in the working heart preparation. Aortic leak was defined as an aortic pressure <60 mmHg on Langendorff, retrograde perfusion mode. Pulmonary vein leak was noted as an aortic flow <2.0 mL/min, low (<4 mmHg) atrial pressure, and a blood gas pO_2 >380 mmHg or a visible leak (i.e., hole in ventricle or atrium) in the heart. Additionally, hearts that were unable to reach a stable baseline after 1 hour on the isolated heart apparatus were excluded as well.

2.2 Isolated Working Heart Ischemia/Reperfusion Injury Model (see Online supplement)

Excised murine hearts were subjected to I/R injury as previously described by our laboratory[2,5–7,13–15] (for experimental design, see Supplemental Figure 2). Our laboratory previously demonstrated that mouse hearts subjected to 30 minutes of ischemia and 30 minutes of reperfusion (reversible injury) or to 60 minutes of ischemia and 120 minutes of reperfusion (irreversible injury) had a similar degree of post-ischemic functional

recovery[2]; therefore, the current study employed the irreversible model of I/R injury (60 minutes of ischemia) which includes myocardial dysfunction and infarction. Percent (%) recovery of contractility or relaxation is +dP/dt or -dP/dt, respectively, at 120 minutes post-reperfusion normalized to baseline.

The current study only assessed the post-ischemic recovery of cardiac function based on previous findings from our lab[5,6] and others[16] which demonstrated, using gain-of-function and loss-of-function genetic mouse models, that all endogenous FGF2 isoforms were necessary for protection against myocardial infarction[5,6]; whereas, HMW and LMW FGF2 isoforms played opposing roles in the recovery of the heart from I/R injury[5,6]. Studies using genetically-modified mice with only LMW FGF2 expression (HMWKO), or wildtype mouse hearts with LMW FGF2 exogenously added, showed that this isoform was protective against post-ischemic dysfunction[5,6,16], while hearts with only the HMW isoforms (LMWKO), or with HMW FGF2 overexpressed, significantly lowered post-ischemic cardiac function[5].

2.3 Time Course I/R Studies

Mouse hearts were subjected to the I/R protocol as described above, and hearts were arrested at designated points: 1) 30 minutes of equilibration with no ischemia (sham), 2) 30 minutes of equilibration followed by 5 minutes of ischemia, 3) 30 minutes of equilibration followed by 60 minutes of ischemia + 5 minutes of reperfusion, and 4) 30 minutes of equilibration followed by 60 minutes of ischemia +120 minutes of reperfusion (Supplemental Figure 2), and snap-frozen in liquid nitrogen for further analysis of downstream targets of LMW FGF2-mediated cardioprotection.

2.4 Candidate Selection and Quantitative Mass Spectrometry Analysis by Selected Reaction Monitoring (SRM) (see Online Supplement Figure 4)

In many physiologic or pathophysiologic events, endogenously expressed LMW and HMW FGF2 have opposing effects on the biological activities of one another, as previously shown by our laboratory[5,6,17] and others[18–25]. WT hearts express both isoform classes of FGF2. Therefore, comparing the HMWKO (expressing only LMW) directly to the WT simply evaluates the effect HMW FGF2 loss, not the effect of LMW FGF2. Although these may be similar in effect, the mechanisms by which each isoform acts may be different and add additional complexity to initial studies to determine the basic signaling of each class of isoforms. Therefore, in order to examine the direct signaling effects of LMW FGF2 without interference from HMW FGF2 in initial mass spectrometry assays, the best comparison is HMWKO to FGF2KO. As reported previously by our laboratory from data generated using a new global phosphoproteomics workflow[26], 15 unique phosphorylation sites from 10 proteins were demonstrated to be significantly upregulated in LMW FGF2-expressing (HMWKO) mouse hearts as compared to *Fgf2*KO hearts that has been subjected to 60 minutes of ischemia and 5 minutes of reperfusion[26]. In that study, our laboratory reported the phosphorylation sites that showed a significant change in at least 4 of the 5 unique biological replicates and validated the data for Ser282 on cardiac myosin binding protein C using an SRM-based quantitative method. In this study, the original global phosphorylation data set (n=5 per genotype) were evaluated further via manual inspection of the comparative

profiles for all sites associated with SR proteins. Among these sites, proteins that showed differential response in at least 3 of the 5 cardiac samples were taken as potentially significant and subjected for further evaluation.

Validation of the Thr17 phosphorylation changes on phospholamban (PLB) was achieved using an SRM-based mass spectrometry methodology on a new comparative set (n=3 per genotype) of cardiac tissue without (*Fgf2* KO) or with (HMWKO) expression of LMW FGF2 subjected to 60 minutes of ischemia + 5 minutes of reperfusion as described above. Unlike the global profile method that identified pThr17 of PLB as a potential site of interest, the SRM method is a targeted quantitative method that relies on the addition of a heavy isotope internal standard (IS) phosphopeptides for quantitation. After adding the IS to the trypsin-digested cardiac extracts, followed by titanium dioxide enrichment of phosphopeptide and reductive alkylation with acetone, quantitatation of pThr17 was achieved by nano-liquid chromatography (LC)-mass spectrometry (MS) analysis, through measurement of the pThr17 peptide versus the IS all as previously described by our laboratory [26]. One minor variation on this workflow compared to the previous description was the optimization and validation of the heavy isotope IS for quantification of the Thr17 phosphorylation of PLB. Specifically, the internal standard HeavyPeptide™ AQUA Basic phosphopeptide (ThermoFisher) with the sequence AS(pT)IEMPQQAR($^{13}C_6$;$^{15}N_4$), representing the identified tryptic peptide containing Thr17 phosphorylation site of PLB, was custom synthesized and was initially used to determine retention time, optimal collision energy, and optimal transition for SRM quantification of the phosphorylation site (see Supplemental Figure 4). Importantly, the y9 fragment ion could be used to conclusively identify the specific phosphorylation at Thr17, thus allowing for this heavy peptide to be used as an IS for quantification of the phosphorylation at Thr17 by comparing the SRM signal from the PLB isolated from the cardiac tissue (y9 at m/z=1055.54) versus a known amount (1 pmol) of the heavy isotope internal standard added to each sample (y9 at m/z=1065.54). These tryptic peptide solutions with IS were analyzed using an nano LC-MS/MS SRM methodology[26] where candidate ions m/z, 677.3 (2+) for unlabeled phosphopeptide and 682.3 (2+) for the heavy labeled IS peptide were subjected to fragmenting and quantification of the phosphorylation site specific fragment ion.

2.5 Immunoblotting (see Online supplement)

Levels of total and phosphorylated calcium handling proteins (i.e., total and/or phosphorylated levels of SERCA, calsequestrin, phospholamban, ryanodine receptor, or calcium-camodulin kinaseIIa [CaMKIIa]) were measured as briefly described in cardiac homogenates from snap-frozen hearts after 30 minutes of baseline equilibration (sham), 30 minutes of equilibration followed by 5 minutes ischemia (5'I), 30 minutes of equilibration followed by 60 minutes ischemia + 5 minutes reperfusion (60'I +5'R) or 120 (60'I + 120'R) minutes reperfusion. The homogenate was centrifuged at 15,000g for 15 minutes and the supernatant collected.

Protein concentration was determined via Biorad DC protein assay, and equal amounts of protein were loaded onto a sodium-dodecyl sulfate-polyacrylamide gel for electrophoresis, then transferred onto a nitrocellulose membrane and stained with a Ponceau reagent

to confirm transfer and check for equal protein loading. Primary antibodies used were SERCA (1:1000, ABR), calsequestrin (1:2500, ABR), phospholamban (1:1000, ABR), phospho-Thr17 phospholamban (1:1000, Badrilla), phospho-Ser16 phospholamban (1:1000, Badrilla), ryanodine receptor-2 (1:5000, Badrilla), phospho-calcium camodulin kinase II (CaMKIIa, 1:500, Santa Cruz), and CaMKIIa (1:500, Santa Cruz). Phosphorylation and total level of SR proteins were visualized by chemiluminescence. Densitometry of bands was evaluated by measuring the average Integrated Density Value (IDV) per unit area using an Alpha Innotech imager and software.

2.6 Statistical Analysis

All values in the text and figures were represented as mean±SEM of n independent experiments. All the data presented are individual mice or tissue samples and not pooled samples. Statistical significance, assigned to where probability is less than or equal to 5 percent ($p<0.05$), is indicated where present. Comparison between single data points in two groups was done with a Student's t-test; this includes differences in baseline or post-ischemic contraction and relaxation, and differences in protein levels for untreated or untreated hearts between genetically-modified and wildtype hearts. Time course measurements between untreated hearts of two different genotypes, including functional data over the course of the I/R experiments and protein phosphorylation evaluated by SDS-PAGE were analyzed with a one-way analysis of variance (ANOVA) followed by a post-hoc Student's t-test. Functional data over the course of the I/R experiments and protein phosphorylation evaluated by SDS-PAGE from double knockout studies were analyzed by a two-way ANOVA followed by a post-hoc Student's t-test. For quantitative SRM studies, PeakView™ software (version 1.1.0.0) from Sciex, was used to generate extracted ion chromatograms (XICs) for the pThr site specific transition pair—y9+ ion (MH+=1055.54) and AQUA heavy y9+ (13C6;15N4) ion (MH+=1065.54). The phosphorylation site specific transition pair peak areas were manually extracted and used to calculate the abundance of the phosphopeptide—AS(pT)IEMPQQAR relative to the IS standard AQUA heavy phosphopeptide, AS(pT)IEMPQQAR (13C6;15N4) for each biologically distinct replicate pair. All XICs were subjected to a 5 point Gaussian smoothing function that is available in PeakView™ software to better perform and illustrate the relative quantifications. Quantitative abundance differences of the Phosphopeptide—AS(pT)IEMPQQAR between the two cardiac types were identified using standard Student's t-test (2-tailed distribution and two-sample unequal variance).

3. RESULTS

3.1 Identification of sarco(endo)plasmic reticulum phosphoproteins involved in LMW FGF2-induced cardioprotection via phosphoproteomic discovery analysis

Our laboratory[5,7] and others[16,27] have previously demonstrated that the LMW isoform of FGF2 is important for the improved post-ischemic recovery of cardiac function. Loss of FGF2 expression significantly reduces ±dP/dt after I/R injury, while the expression of only LMW FGF2 results in markedly higher function after I/R. Previous work from our laboratory revealed that LMW FGF2 enhanced cardiac function following I-R injury via PKCα regulation of the contractile apparatus[7]. Yet, whether LMW FGF2 modulates SR

protein function to influence post-ischemic cardiac function remains to be elucidated. Data from the phosphoproteomic discovery phase using reductive alkylation by acetone (RABA) tagging from hearts not expressing FGF2 (*Fgf2*KO) and hearts only expressing LMW FGF2 (HMWKO) subjected to 60 minutes ischemia + 5 minutes reperfusion showed the successful identification of nearly 2300 phosphopeptides with high confidence (>95%)[26]. 50 phosphorylation sites were noted on seven sarcoplasmic reticulum (SR)-associated proteins. From these 50 phosphorylation sites, 7 sites (highlighted in red) showed differential phosphorylation response, including positions on junctophilin-2 (Jnpn), ryanodine receptor-2 (RyR2), calnexin (Clxn), and phospholamban (PLB), in at least 3 of the 5 cardiac samples (Table 1, Figure 1, Supplemental Figure 6). As PLB is an important regulator of the kinetics of cardiac Ca^{2+} transients and of contractility and relaxation[28–30] and the Threonine (Thr)-17 site was identified as a possible target, via our phosphoproteomic analysis, the following proteomic and biological analyses were pursued to demonstrate a role of phospho-Threonine-17 PLB in LMW FGF2-induced cardioprotection against post-ischemic dysfunction.

3.2 SRM-based mass spectrometry indicates elevated phospho-Threonine-17 phospholamban in reperfused LMW FGF2 (HMWKO) expressing hearts

Since the effects of LMW FGF2 are observed acutely after the onset of reperfusion, it was hypothesized that these actions may be related to the post-translational modifications of phospholamban during I/R injury. The phosphorylation of phospholamban at Threonine-17 was examined due to the importance of this site in the development of ischemic injury in the heart[31–33] and its role as a target for indirect regulation by PKC isoforms[34,35]. To further ascertain endogenous Thr17-phospholamban phosphorylation induction by LMW FGF2 for cardioprotection, SRM-based mass spectrometry was performed using the workflow that our laboratory has described previously[26] with a new set of mouse hearts subjected to I/R injury with and without LMW FGF2 expression (n=3). The level of phosphorylation in each sample was quantified for the relative abundance of the phosphopeptide, 14-AS(pT)IEMPQQAR-25 (corresponding to the phosphorylated Threonine-17 site of phospholamban) by comparison to the 1 pmole of internal standard of AQUA heavy phosphopeptide, AS(pT)IEMPQQAR (13C6;15N4) spiked into each sample just after the trypsin digestion (Figure 2A, Supplemental Figure 4). Note that, as indicated by the color key at the top of panel A, the pink traces all represent the signals for the internal quantitation standards spiked into each sample, while the blue traces represent the detected levels of the pThr17 peptide in each sample. Collectively, a 2.3X higher abundance of the phosphopeptide, 14-AS(pT)IEMPQQAR-25 was observed in FGF2 HMWKO hearts (0.950 + 0.152) vs. *Fgf2*KO (0.409 + 0.174) hearts ($p<0.02$, Figure 2B).

In support of the SRM-based phosphorylation state of phospholamban mediated by LMW FGF2, phosphorylation at Threonine-17 and Serine-16 were assessed over the course of ischemia-reperfusion injury by Western immunoblotting for phosphorylation-specific sites vs. total protein (Figure 3). In hearts only expressing the LMW isoform of FGF2, no differences in basal levels of phospholamban phosphorylation at sites Threonine-17 or Serine-16 were observed (Figures 3A and 3B, respectively). However, the phosphorylation of Threonine-17 phospholamban was significantly higher at early ischemia in LMW

FGF2 hearts than in hearts not expressing FGF2 ($p<0.05$, Figure 3A). This elevation continued through early reperfusion until late reperfusion, where levels return to that seen during baseline. The phosphorylation status of Serine-16 phospholamban was significant lower in LMW FGF2 (HMWKO) hearts compared to wildtype (WT) and *Fgf2* knockout at early reperfusion ($p<0.05$, Figure 3B). This was similar to the finding from the phospho-proteomic analysis showing a lack of phosphorylation status on Serin-16 site of phospholamban (Table 1). No difference in levels of phospho-Serine-16 phospholamban was observed at any other timepoint of ischemia or reperfusion between the different genotypes (Figure 3B).

No differences in the total protein levels of sarcoendoplasmic reticulum calcium ATPase (SERCA), calsequestrin, ryanodine receptor-2 (RyR2), and phospholamban (PLB) were seen in HMWKO hearts compared to wildtype or total *Fgf2* knockout (*Fgf2*KO) hearts (Figure 4), indicating that LMW FGF2 does not regulate expression of these proteins, but does influence the post-translational modification (i.e., phosphorylation state) of a number of these SR and calcium handling proteins.

3.3 Phospholamban phosphorylation during I/R in hearts only expressing LMW FGF2 is not altered in the absence of PKCα

PKCα indirectly regulates phospholamban phosphorylation via inhibitor-1 and protein phosphatase-1 activity[10]. Since both the phosphorylation of phospholamban and the activation of PKCα increases at early ischemia, it was examined whether this increase in the phosphorylation of phospholamban at Threonine-17 observed at early ischemia or early reperfusion was dependent on the presence of PKCα in hearts only expressing LMW FGF2. There was no significant difference in the ratio of phosphorylated phospholamban to total phospholamban when PKCα was ablated ($p<0.05$, Figure 5A), suggesting that PKCα is not involved in the differential phosphorylation of phospholamban at Threonine-17 during I/R.

3.4. CamKIIa phosphorylation during ischemia is higher in hearts only expressing LMW FGF2 compared to wildtype

As the Threonine-17 residue of PLB is known to be phosphorylated by calcium-calmodulin kinase IIa (CamKIIa), it was hypothesized that the difference in phosphorylation at this site during early ischemia was due to an increase in CamKIIa activation (i.e., phosphorylation) in hearts only expressing LMW FGF2. In wildtype hearts, levels of phosphorylated CamKIIa drop significantly at early ischemia ($p<0.05$, Figure 5B). In hearts only expressing LMW FGF2, there was a significantly higher ratio of phosphorylated/total CamKIIa, compared to wildtype at this same time point which corresponds to the time point at which higher levels of phosphorylated Thr17 PLB were seen as well ($p<0.05$, Figure 3A).

3.5 LMW FGF2-only expressing hearts with unphosphorylatable Threonine-17 site of phospholamban has poorer recovery of post-ischemic cardiac function than hearts expressing only LMW FGF2 with native phospholamban

The availability of *in vivo* mouse models developed by the Kranias laboratory[11] to assess the role of site-specific phosphorylation of phospholamban allowed our laboratory to determine the biological importance of Threonine-17 (Thr17) phosphorylation in LMW

FGF2-mediated cardioprotection against post-ischemic cardiac dysfunction. Therefore, to determine if the protective effects of endogenous LMW FGF2 against cardiac dysfunction are mediated by phospholamban phosphorylation, in particular, at Thr17 site, HMWKO mice (only expressing LMW FGF2) were bred with mice only expressing Thr17Ala (T17A) PLB, which eliminates the phosphorylatable Threonine-17 residue, but retains the PKA phosphorylatable serine-16 residue (see Supplemental Figure 1). Mice only expressing LMW FGF2 showed significantly improved recovery of cardiac function (determined as percent recovery of +dP/dt and -dP/dt) following I/R ($p<0.05$, Figure 6), and this functional improvement was significantly abrogated in hearts expressing LMW FGF2 and the PLB mutant lacking phosphorylatable Thr17 (Thr17Ala). Mice with normal levels of FGF2 (both LMW and high molecular weight [HMW]) with no PLB (*Plb* knockout) had poor post-ischemic recovery of cardiac function ($p<0.05$, Figure 6) compared to HMWKO (LMW FGF2-only) and wildtype; this is in support of previous findings[31,32,36,37]. Similarly, mice with normal levels of FGF2 (both LMW and high molecular [HMW]) and the normal PLB replaced by Thr17Ala PLB had poor post-ischemic recovery of cardiac function ($p<0.05$, Figures 6) compared to HMWKO (LMW FGF2-only) and wildtype. In this study, wildtype mice had a similar recovery of post-ischemic cardiac function as previously published[2,5,6,16]. Although a number of cardioprotection studies have demonstrated a sex-specific outcome[38–47], we have previously found no statistically significant differences between male and female mice in the protective effect of LMW FGF2[6,7], and the current study making use of smaller groups of animals did not further reveal any sex-specific differences in post-ischemic recovery of cardiac function or other measures (data not shown).

Other pre-ischemic and post-ischemic functional parameters for Wt, *Plb* KO, *Plb* KO/PLB Thr17Ala Tg, HMWKO and HMWKOx *Plb* KO/PLB Thr17Ala Tg hearts are shown in Supplemental Table 3. During ischemia-reperfusion injury, there was significant systolic and diastolic dysfunction as measured by left ventricular systolic (LVSP), diastolic (LVDP) and end-diastolic (LVEDP) pressures, time to peak left ventricular pressure (TPP), and half relaxation time (RT1/2).

4. DISCUSSION

The present study, using phosphoproteomics and genomics techniques, has uncovered a number of new potential effector targets of LMW FGF2 in the heart. Our findings demonstrate that a number of phosphoproteins of the SR may be involved in LMW FGF2-induced protection against post-ischemic cardiac dysfunction. Among these, the data presented in this manuscript demonstrates, for the first time, that phospholamban phosphorylation contributes to LMW FGF2-mediated cardioprotection. Therefore, because of the importance of phospholamban in cardiac contractility and relaxation and the validation via immunoblotting data of the proteomics/mass spectrometry identification of elevated phospho-Threonine-17 during late ischemia and early reperfusion, our laboratory pursued the role of phospho-Threonine-17 PLB in LMW FGF2-induced cardioprotection against post-ischemic cardiac dysfunction. Nonphosphorylatable Threonine-17 phospholamban mutation (T17A) bred into hearts only expressing LMW FGF2 (HMWKO) results in significant post-ischemic cardiac dysfunction compared to

LMW FGF2 hearts with native phospholamban activity. Overall, these findings are the first to elucidate downstream targets via phosphoproteomic analysis which demonstrate a role for calcium handling proteins/genes involved in LMW FGF2-induced protection against cardiac dysfunction.

FGF2 has been shown in a number of cell types to modulate calcium signaling[48–56]. Our laboratory[5–7] and others[16] have determined that LMW FGF2, both endogenously expressed or exogenously administered to the myocardium, protects the heart from post-ischemic dysfunction through extracellular paracrine/autocrine activation of FGFR. Given that FGFR activation can modulate Ca^{2+} homeostasis through the regulation of several intracellular kinases, including PKC, MAPK and Akt[57], whether FGF2 may promote cardioprotection through calcium regulation during I/R becomes an important question.

Contractility and relaxation are controlled by careful regulation of calcium handling proteins that release calcium from intracellular stores in the sarcoplasmic reticulum (SR) during systole, such as the ryanodine receptor (RyR2), as well as those that remove and sequester calcium away from the cytosol during diastole, such as the sarco-endoplasmic reticulum calcium ATPase (SERCA) and its regulator phospholamban (PLB), and calsequestrin (CSQ) which binds to calcium and keeps it localized near SR calcium channels[58]. As the heart undergoes ischemia, the calcium concentration inside the cell rises sharply, negatively affecting both contractile mechanics and metabolism, and resulting in dysfunction. This injury has been associated with post-ischemic regulation of calcium handling proteins, and targeting these proteins in mouse models of I/R has been shown to affect post-ischemic function[37,59–64]. However, the levels of these proteins were not found to be significantly changed between wildtype mice and those only expressing the LMW isoform of FGF2, or lacking expression of all isoforms of FGF2 (Figure 4).

Phosphoproteomics identified phospholamban as an important potential downstream target of LMW FGF2 during I/R injury. No changes in phospholamban were observed in hearts that had not been subjected to ischemia between wildtype, hearts only expressing LMW FGF2, and *Fgf2* knockout mice. However, when ischemia was induced, hearts expressing LMW FGF2 showed significantly higher phosphorylation at Threonine-17 compared to hearts lacking FGF2 expression, suggesting that LMW FGF2 expression promotes the phosphorylation of phospholamban at this residue. The phosphorylation of phospholamban at this residue is highly suggestive of a mechanism by which calcium modulation is rapidly altered early during I/R injury, with high significance for the degree of resultant injury. Phosphoplamban phosphorylation has been identified by Mattiazzi and investigators[31,32,36] as a dynamically regulated post-translational modification, with an extremely rapid phosphorylation at the onset of ischemia that is reversed within 10 minutes. Despite the transient nature of this phosphorylation, the introduction of a non-phosphorylatable residue in place of Threonine-17 is sufficient to drive significant injury. Further, the level of phosphorylation of phospholamban at Threonine-17 has been shown to be essential for its protective impact against ischemia-induced cardiac dysfunction. For example, Said and colleagues[36] report that an approximately 50% reduction in isoproterenol-induced PLB Threonine-17 phosphorylation by CamKII inhibition abolished the recovery of an isolated mouse heart after 20 minutes of ischemia and 60 minutes of

reperfusion. These finding suggest that phosphorylation of this residue during the first few minutes of ischemia is critical for recovery of the heart from I/R injury To test whether this transient change in phospholamban phosphorylation at Threonine-17 is similarly critical in our model of LMW FGF2-mediated protection from post-I/R dysfunction, we tested whether the loss of this phosphorylation site would prevent rescue from I/R injury observed in the LMW FGF2- expressing mice. In the present study, HMWKO (LMW FGF2 only) hearts expressing T17A phospholamban are no longer protected from post-ischemic cardiac dysfunction (Figure 6). This suggests that phospholamban phosphorylation at Threonine-17, originally identified by MS-based phosphoproteomic analyses, is one of the key SR phosphoproteins involved in LMW FGF2 cardioprotection. Another phosphorylation site, Serine-16 (Ser16) of PLB, was detected in the discovery phase of the comparative proteomics profiling study (Table 1) but was not shown to be significantly changed. However, an SRM-based assessment to evaluate changes in Ser16 phosphorylation in LMW expressing hearts following I/R injury showed about a 2-fold increase (data not shown). Yet, a timecourse to evaluate, by Western immunoblotting, changes in PLB Ser16 phosphorylation during I/R injury show decrease in Ser16 phosphorylation between LMW FGF2 expressing hearts and wildtype or those hearts that don't express FGF2 (Figure 3B), and even though the Serine-16 site can be phosphorylated in the non-phosphorylatable PLB Threonine-17 mutant (see Supplement Figure 1), cardioprotection against post-ischemic cardiac dysfunction was still absent (see Figure 6). This supports the finding by Mazziati's group[36] which demonstrated that the phosphorylation of PLB Threonine-17 was functionally important in the post-ischemic recovery of cardiac function, but the phosphorylation of PLB Serine-16 was not. Albeit, it is unclear from this study what direct role, if any, the phospho-Ser16 site on PLB has in the mechanism of LMW FGF2-induced cardioprotection against cardiac dysfunction.

The intermediary steps by which LMW FGF2 expression results in increased phosphorylation of phospholamban at Threonine-17, a target of CaMKIIa[65], remain unknown. Despite the fact that CaMKIIa may be modulated by PKCα[34], this PKC isoform that is activated early enough to impact phospholamban phosphorylation at this time point[7] does not appear to regulate phosphorylation of phospholamban at early ischemia or early reperfusion (Figure 5A). In fact, the activation of PKCα at this timepoint may be more of an effect than a cause of phospholamban regulation; phosphorylation at Threonine-17 is expected to relieve phospholamban's inhibition on SERCA, promoting increased calcium uptake and higher amplitude of calcium release during systole, which may in turn result in higher activation of PKCα, a conventional calcium-activated kinase. Alternately, the reduction in CaMKIIa activation observed in wildtype hearts is mitigated in hearts only expressing LMW FGF2 (Figure 5B), suggesting that maintained CaMKIIa activity could be responsible for the increase in PLB phosphorylation. The data presented here suggest that the levels of phosphorylated CaMKIIa decrease in wildtype hearts during I/R injury, which is supported by Yu and colleagues[35], who demonstrate decreased CaMKIIa activity under conditions of hypoxia in rat hearts. However, in the presence of only LMW FGF2 expression, the phosphorylation state of CaMKIIa remains constant through ischemia, and is significantly higher than wildtype cohorts during early ischemia, suggesting that LMW FGF2 may be positively regulating CaMKIIa's activity. While both WT and CamKIIa

phosphorylation levels decrease over time during ischemia (Figure 5B), acidosis, sodium overload, and oxidation is expected to result in an increase of activity[33,66]; it may be that CaMKIIa activity is finely regulated during ischemia, and LMW FGF2 expression alters the balance in favor of higher activation, leading to the observed result of higher phosphorylation at Thr17 phosphorylation in both groups at early ischemia (Figure 3A). Future studies are necessary to determine if this is the case. The impact of CaMKIIa activation in I/R has been shown to be injury-dependent[31,32,67,68]. Studies by Mattiazzi and colleagues[31,32,67] have shown a protective role for this kinase in an I/R model of reversible stunning through phospholamban phosphorylation at threonine-17. Conversely, CaMKIIa has been observed to act detrimentally in a model of irreversible ischemic injury, in a manner dependent on the activity of the sodium calcium exchanger[68]. It is, therefore, important to delineate which pathways integrating CamKIIa are stimulated in FGF2-mediated cardioprotection. The expression of only LMW FGF2 has been previously determined to provide protection from post-I/R dysfunction[5–7], with no protection from irreversible infarct development. Data from our current study suggest that CaMKIIa activation plays a protective role similar to the stunning model described by Mattiazzi and colleagues[31,32,67]. Indeed, an increase in PLB phosphorylation is observed here in the presence of LMW FGF2, suggesting that a similar mode of cardioprotection is produced in this model. The loss of LMW FGF2-mediated protection from stunning in the absence of Thr17-phosphorylatable PLB provides strong support for this hypothesis (Figure 6).

Cardioprotection stimulated by LMW FGF2 has previously been found by our laboratory also to involve phosphorylation of myofibril proteins by isoforms of protein kinase C[7]. The results presented here broaden our previous findings to suggest a multi-faceted mechanism of protection, at both the level of the myofibril, via PKCs, and the SR, via phospholamban phosphorylation. These mechanisms may act in parallel, with each of these pathways independently necessary for protection by FGF2. Either blocking PKCα and ε activity at the myofibril, or preventing phosphorylation of PLB are both sufficient to abrogate the improved post-IR function observed in the HMWKO compared to WT or KO. This suggests that the cardioprotection stimulated by LMW FGF2 works at several levels simultaneously.

In addition to the integral role that phospholamban phosphorylation plays in FGF2-mediated cardioprotection, microrarray and mass spectrometry experiments have uncovered several other calcium-handling proteins altered by expression of LMW FGF2, that future studies may also reveal to play a role in altered calcium handling during I/R (Table 1, Supplemental Table 2). Sarcolipin, a regulator of SERCA with homology to phospholamban[69–71], is upregulated 13-fold in non-ischemic LMW FGF2-only (HMWKO) hearts vs. hearts with no FGF2 (*Fgf2*KO), and this upregulation is confirmed by RT-qPCR (see Supplemental Figure 8). An increase in sarcolipin production would be expected to result in decreased SERCA activity[69,72]. In addition, this increase in sarcolipin may also contribute towards the priming of the heart, by reducing changes in calcium amplitudes in the heart during I/R. Similarly, fibroblast growth factor homologous factor-1 (*Fhf1* or *Fgf12*), a putative member of the FGF family that is thought to mediate its effects intracellularly[73–75], and regulates ion homeostasis, was upregulated in hearts only expressing LMW FGF2 compared to *Fgf2* KO hearts (see Supplemental Figure 9). There is recent evidence

that FGF12 modulates voltage-gated sodium channel and/or cardiac L-type voltage-gated calcium channel trafficking and kinetics, respectively, leading to cardiac conduction and rhythm complications[76–79]. Whether FGF12 has a similar effect in heart muscle function as it does in regulating heart conduction remains to be seen. Junctophilin-2 coordinates the L-type voltage-gated calcium channel and the ryanodine receptor 2 (RyR2) in the T tubule to efficiently promote calcium-induced calcium release from the SR[80–82]. The discovery phase of the phosphoproteomic analysis demonstrates, for the first time, a significant increase in JP2 phosphorylation in LMW FGF2-only hearts following I/R injury (Table 1 and Supplemental Figure 6), suggesting that LMW FGF2 may regulate JP2 function in calcium-induced calcium release to improve post-ischemic contractile function. In this current study, nine novel phosphorylation sites on JP2 were identified, of which four are to be regulated by LMW FGF2, although the mechanism of phosphorylation is unclear. A recent phosphoproteomic analysis of HL-1 cardiomyocytes demonstrated that Akt2 isoform enhanced the Ser179 site of JP2[83]; LMW FGF2 activates Akt signaling, suggesting that this kinase may modulate the LMW FGF2-induction of junctophilin-2 phosphorylation in our model of I/R injury. Similarly, in skeletal muscle, junctophilin-2 could be phosphorylated by PKC[84]. Decreased phosphorylation of junctophilin-2 has been associated with uncoupling of calcium entry into the cell and RyR-associated calcium release[84], which suggests that hyperphosphorylation in the environment of I/R injury would assist in the preservation of EC coupling, and the rapid sequestration of excess extracellular calcium into the SR. Additionally, LMW FGF2 may regulate intracellular calcium handling via phosphorylation changes of other SR proteins, ryanodine receptor-2 (RyR2) and calnexin (Clnxn) also identified by our phosphoproteomic analysis, that may be essential for recovery from I/R injury (Supplemental Table 1, Supplemental Figure 6). With its phosphorylation status of RyR2 at serine 2807 enhanced in mouse hearts expression LMW FGF2 (HMWKO hearts, also see Supplemental Figures 6 and 7), this finding suggests that RyR2 activity may be important in the improved post-ischemic cardiac contractility induced by LMW FGF2. Calnexin is a membrane protein of the sarco(endo)plasmic reticulum[85,86] involved in endoplasmic reticulum (ER) quality control[87] and ER stress[87], and it can be phosphorylated by a number of kinases including ERK, casein kinase CK2 and PKC[87]. A study by Bousette and colleagues demonstrated that calnexin (Clxn) is not only regulates ER stress and apoptosis, but also is important in cardiac contractility by modulating calcium cycling via L-type calcium channel and SERCA activity[88]. In addition to calnexin, calreticulin is a related chaperone protein that is highly expressed in the heart to also ensures proper folding of (glyco)proteins destined to the cell surface[88–90]. Calreticulin mRNA was elevated in hearts expressing LMW FGF2 based on the microarray analysis, but no differences were seen at the protein level using immunoblot (Supplemental Figure 10); therefore, the role and relationship of these chaperone proteins in LMW FGF2-induced cardioprotection against cardiac dysfunction is still unclear.

Although the data presented here gives evidence for a role for LMW FGF2 regulation of calcium cycling in mice, a limitation of this work may be its relevance to humans. Calcium cycling in mouse and human ventricular myocytes have been shown to be different, with a much larger role for the SR in mice compared to humans[91]. Nevertheless, the importance

of phospholamban regulation in cardiac disease has been well-established in human patients, in spite of the SR's diminished role in normal cardiac function[92,93].

This study reveals a new perspective by which LMW FGF2 may have an impact on post-ischemic cardiac function (Figure 7). The understanding that LMW FGF2 activity may result in regulatory changes in protein(s), leading to modulation of intracellular calcium signaling in the heart essential for recovery from I/R injury, opens up the possibility that LMW FGF2 may directly affect calcium cycling in the myocyte to protect the heart from stunning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

5'I	5 minutes ischemia
60'I +5'R	60 minutes ischemia + 5 minutes reperfusion
ANOVA	120 (60'I + 120'R) minutes reperfusion analysis of variance
CaMKIIa	calcium camodulin kinase II
CLXN	calnexin
CSQ	calsequestrin
XIC	extracted ion chromatogram
FGF2	fibroblast growth factor 2
FHF	fibroblast growth factor homologous factor
HMW	high molecular weight
I/R	ischemia-reperfusion
JP2	junctophilin-2

KO	knockout
LC	liquid chromatography
LMW	low molecular weight
MS	mass spectrometry
MI	myocardial infarction
PLB	phospholamban
RABA	reductive alkylation by acetone
RyR2	ryanodine receptor
SR	sarcoendoplasmic reticulum
SERCA	sarcoendoplasmic reticulum calcium ATPase
SRM	selected reaction monitoring
Ser16	serine-16
Thr17	threonine-17

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HIGHLIGHTS

- LMW FGF2 improves post-ischemic recovery of cardiac function
- Phosphoproteomic/SRM mode identified SR proteins in LMW FGF2 cardioprotection
- Threonine-17 site of PLB important for LMW FGF2 effect against ischemic dysfunction
- LMW FGF2 improves post-ischemic recovery via multiple calcium handling proteins/genes

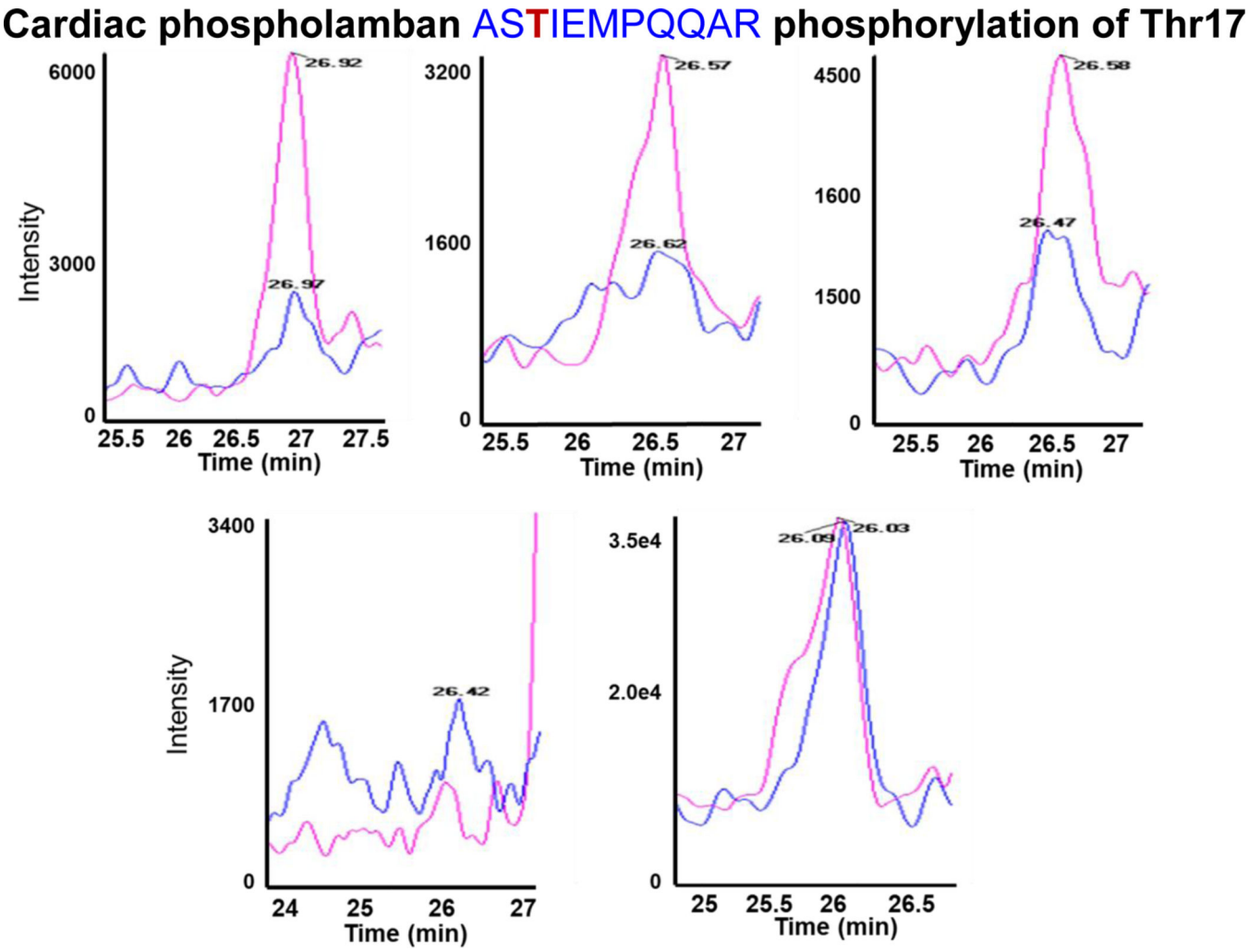


Figure 1.

Phosphorylation changes identified in phospholamban from *Fgf2*KO and FGF2 HMWKO hearts that were subjected to 60 minutes of ischemia and 5 minutes of reperfusion. The cardiac tissues were solubilized, trypsin digested, RABA-tagged, enriched on TiO2 and subjected to nano-liquid chromatography-coupled mass spectrometry as described in the Methods section. Each profile represents an overlay of the extracted ion profiles (XIC) for the *Fgf2*KO (blue trace) and the FGF2 HMWKO (pink trace) for the indicated sites for phosphorylation. All XICs are presented after a 2-point Gaussian smooth. n=5 for each genotype.

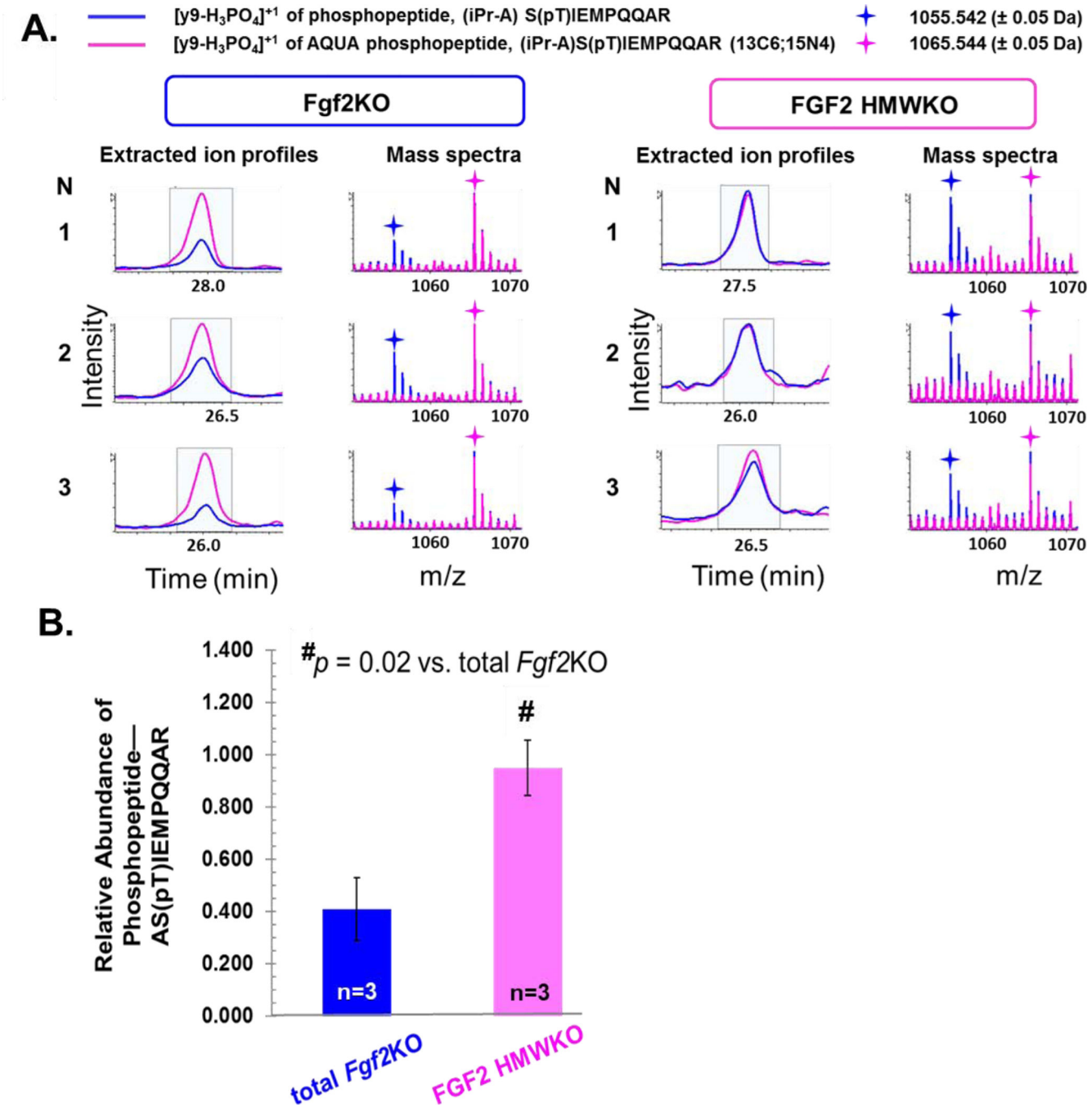


Figure 2.

Quantitative analysis of Threonine-17 phosphorylation of phospholamban by selected reaction monitoring (SRM)-based mass spectrometry. Hearts that were subjected to 60 minutes of ischemia and 5 minutes of reperfusion from both *Fgf2*KO and FGF2 HMWKO mice were digested and prepared for quantitative analysis using a heavy isotope internal standard peptide as described in the Methods section. A) Extracted ion chromatograms (XIC) and respective mass spectra for $[y9\text{-}H_3PO_4]^{+1}$ fragment ions indicating relative abundance of phosphopeptide, (iPr-A)S(pT)IEMPQQAR from each of the 3 *Fgf2*KO (left

panels) and FGF2 HMWKO (right panels) hearts where iPr indicates the N-terminal addition of an isopropyl group from the reductive alkylation by acetone step in the workflow. The XIC are all presented after a 5 point Gaussian smooth. Pink trace is the signal for the internal quantitation standards spiked into each sample. Blue trace is detected levels of the pThr-17 peptide in each sample. (B) Quantitation of the relative abundance of Thr17 phosphorylation as the average relative abundance of $[y9\text{-}H_3PO_4]^{+1}$ ion, relative to AQUA heavy $[y9\text{-}H_3PO_4]^{+1}$ (13C6;15N4) from the XIC peak areas in panel A for each of the cardiac genotypes. #p<0.02 vs. *Fgf2* KO. n=3 for each genotype.

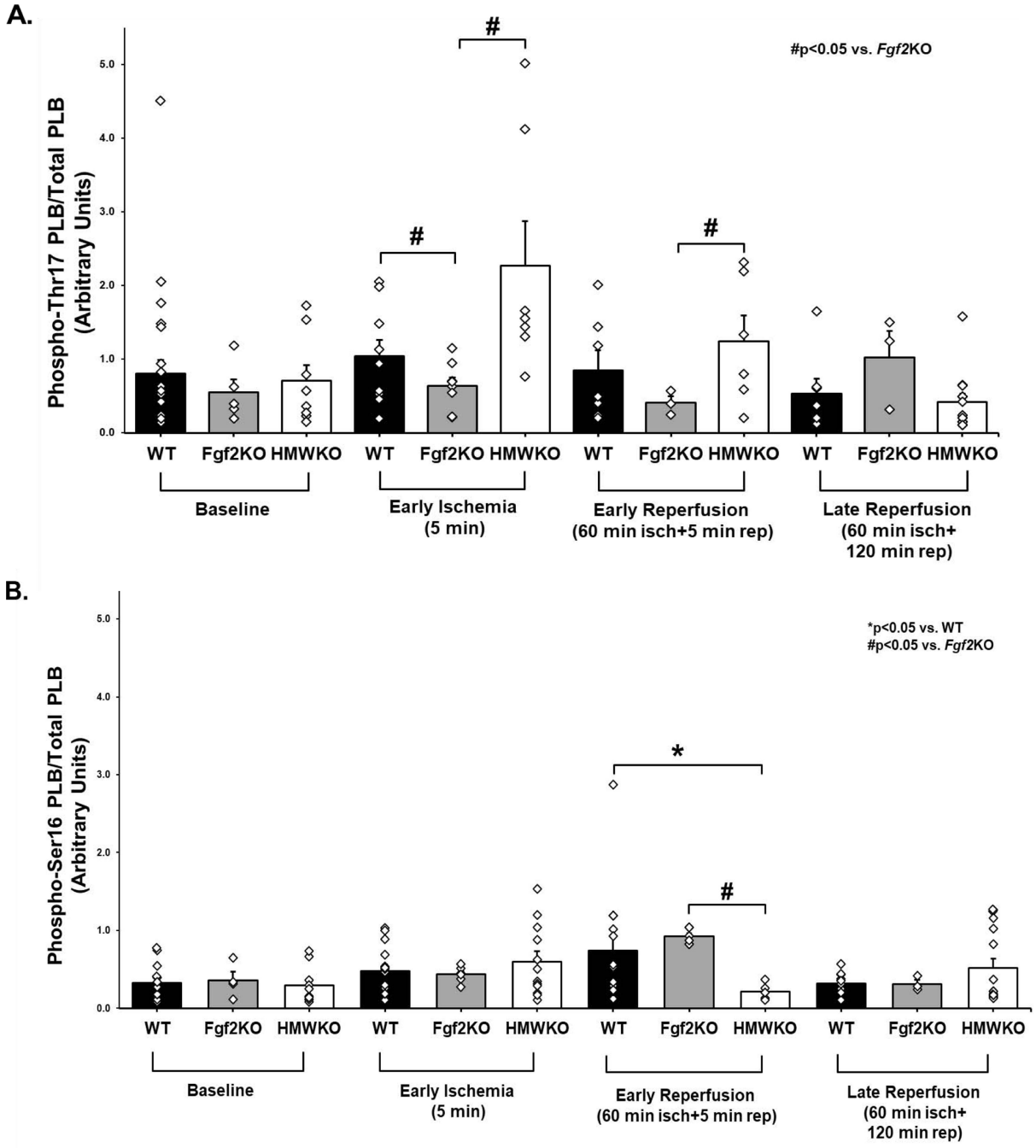


Figure 3.

Phospholamban (PLB, panel A and B) at baseline (sham), early ischemia (5 minutes ischemia), early reperfusion (60 minutes ischemia + 5 minutes reperfusion) and late reperfusion (60 minutes ischemia + 120 minutes reperfusion). Phosphorylation was measured after immunoblotting as the ratio of phosphorylated phospholamban to total phospholamban in wildtype hearts (black bar), hearts with expression of all isoforms of FGF2 ablated (*Fgf2*KO, gray bar), or hearts only expressing LMW FGF2 (HMWKO, white bar). PLB phosphorylation at site threonine-17 (panel A) significantly increased in WT

and HMWKO hearts at early ischemia and early reperfusion compared to *Fgf2*KO hearts; however, there was less PLB phosphorylation at site serine-16 at early reperfusion in LMW FGF2 (HMWKO) compared to wildtype or *Fgf2*KO hearts (panel B). *p<0.05 vs. WT. #p<0.05 vs. *Fgf2*KO.

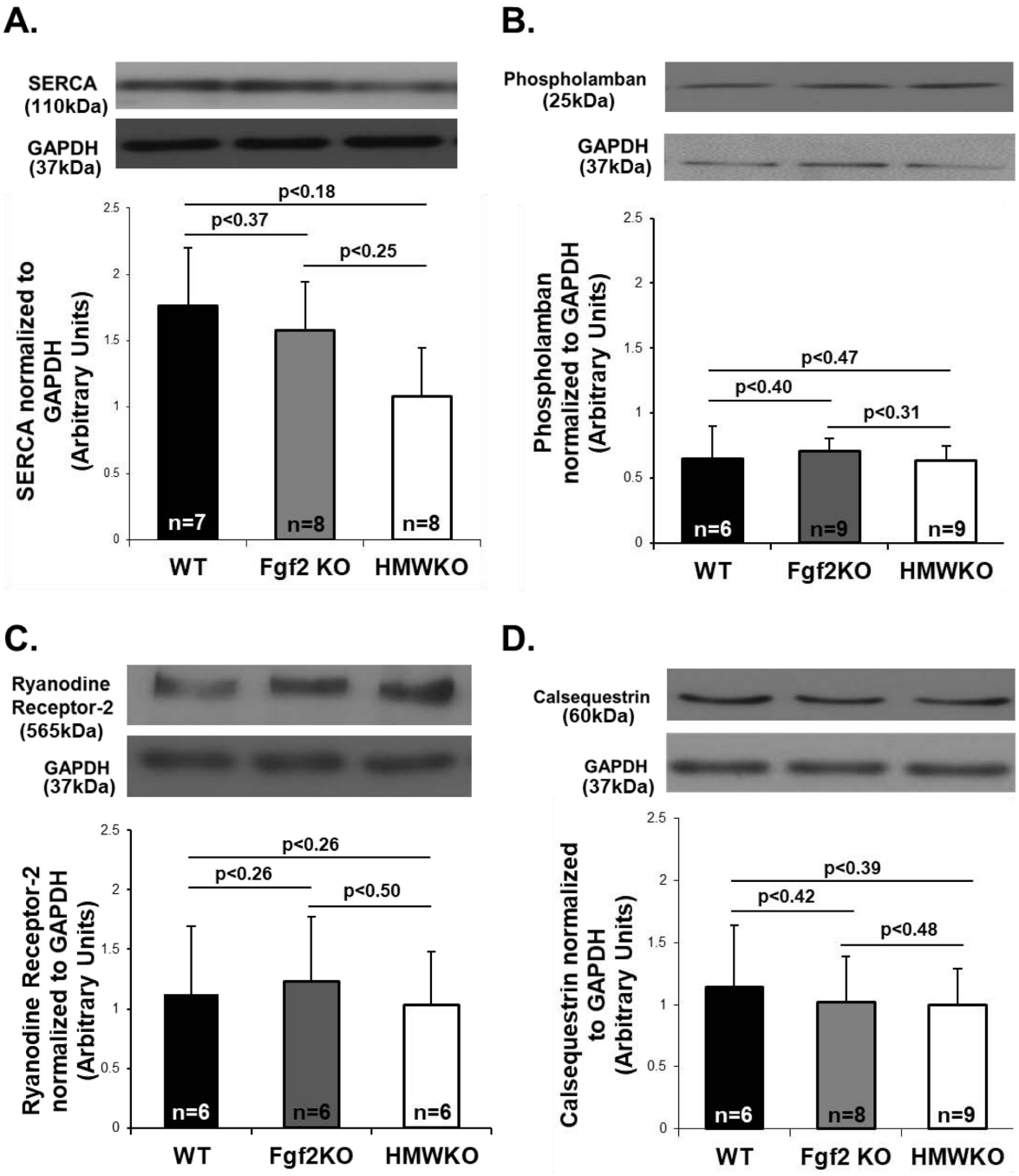


Figure 4.

Levels of calcium handling proteins in non-ischemic wildtype hearts (WT, black bar), hearts with FGF2 expression ablated (*Fgf2*KO, dark gray bar) and hearts only expressing LMW FGF2 (HMWKO, white bar). No significant changes were observed in the expression of sarcoendoplasmic reticulum calcium ATPase (SERCA, A), phospholamban (B), ryanodine receptor-2 (RyR2, C), or calsequestrin (D). Hearts were probed for SERCA, phospholamban, RyR2, or calsequestrin via immunoblotting and normalized to GAPDH. Representative immunoblot images were cropped for space limitations. No difference in

SR protein expression between the different genotypes. n=6–9 per group. Of note, work by Chu and colleagues (Ann NY Acad Sci 853: 49–62, 1998) demonstrated that the key SR proteins such as SERCA and CSQ were not altered; yet, RyR2 was significantly lowered via compensation in PLBKO hearts employed in the functional studies.

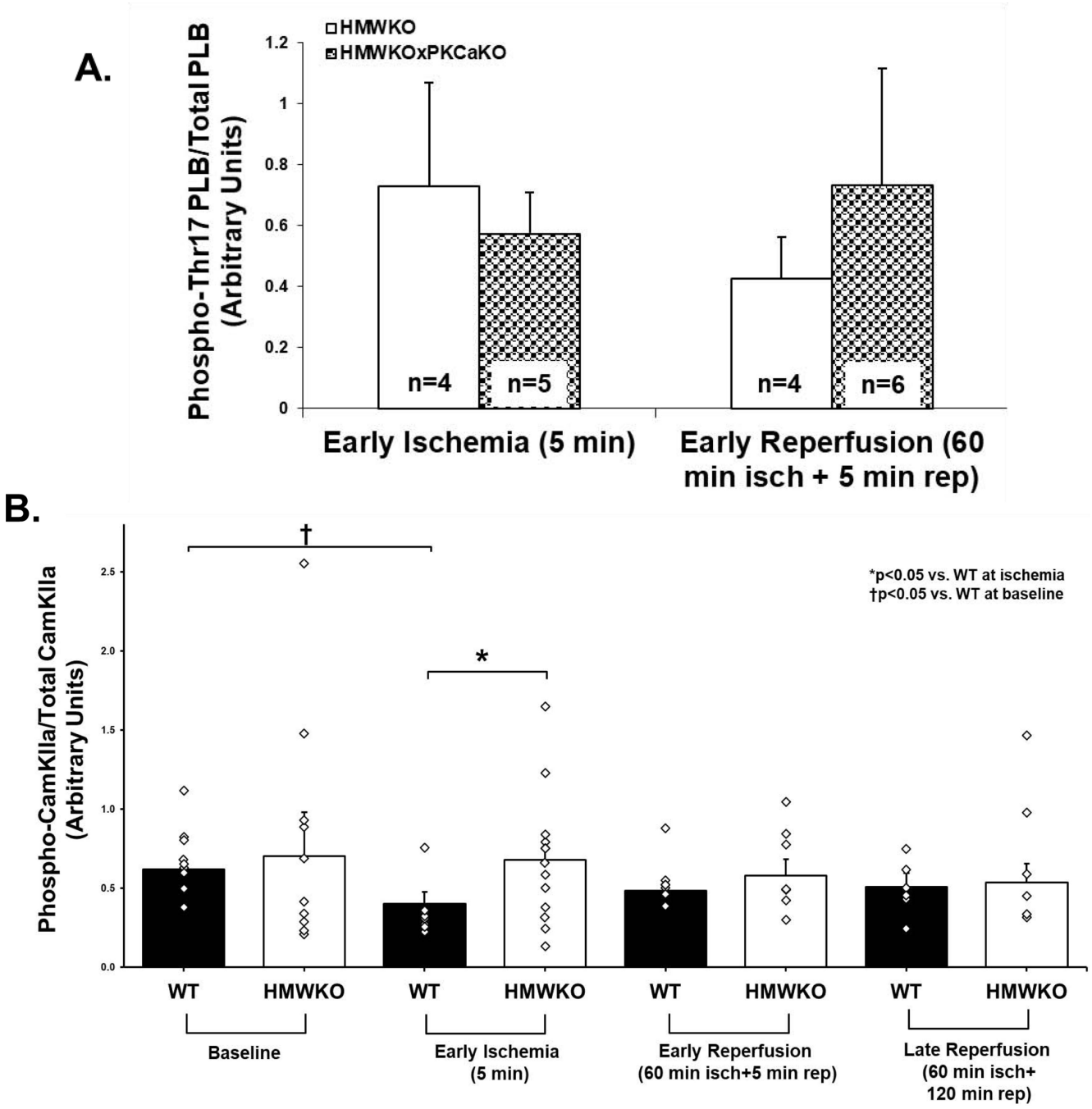
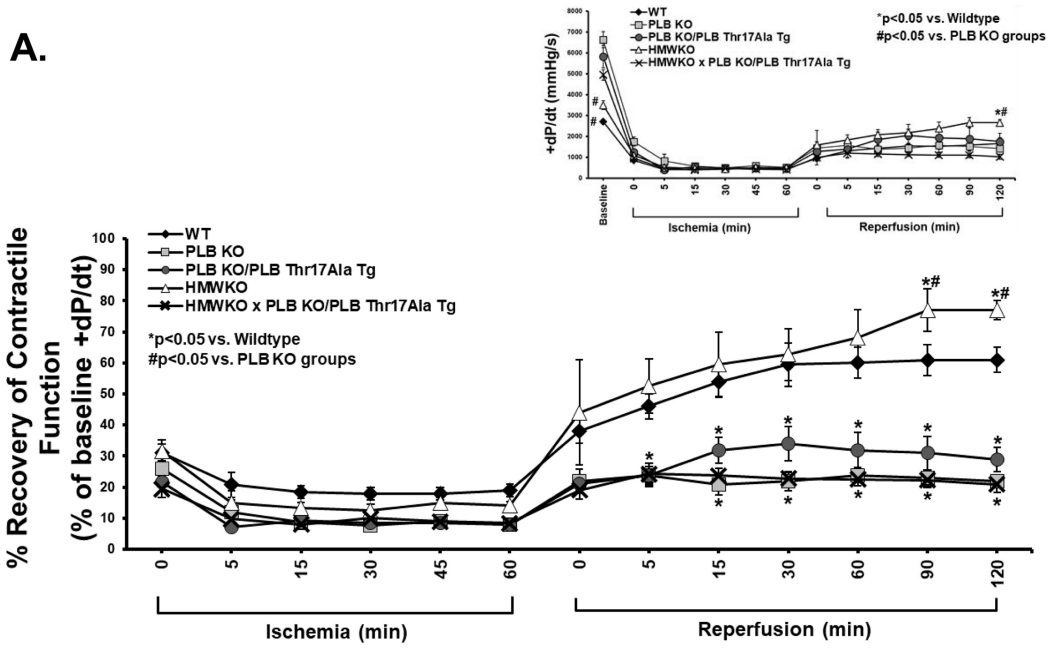
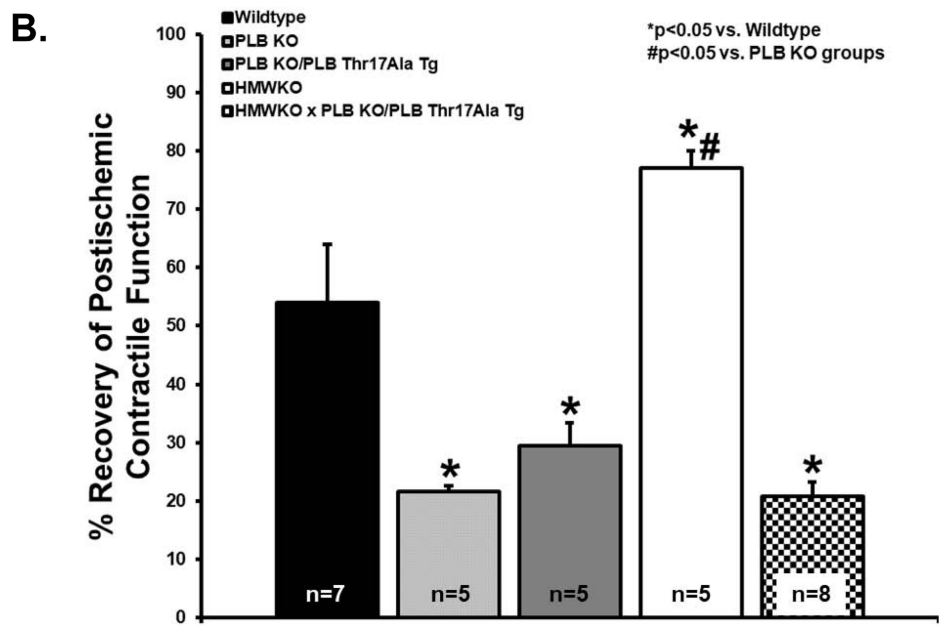


Figure 5.

(A) Phospholamban Threonine-17 phosphorylation during ischemia and reperfusion in hearts expressing only LMW FGF2 at early ischemia (5 minutes ischemia), early reperfusion (60 minutes ischemia + 5 minutes reperfusion), in the presence (white bar) and absence of PKCα (PKCaKO, patterned bar). Phosphorylation was measured as the ratio of phosphorylated phospholamban to total phospholamban. PKCα ablation did not reduce the phosphorylative state of phospholamban. n=4–6 hearts per group. (B) CamKII phosphorylation in hearts expressing only LMW FGF2 (HMWKO, white bar) and wildtype

(WT, black bar) hearts at baseline (sham), early ischemia (5 minutes ischemia), early reperfusion (60 minutes ischemia + 5 minutes reperfusion) and late reperfusion (60 minutes ischemia + 120 minutes reperfusion). Phosphorylation was measured as the ratio of phosphorylated CamKII to total CamKII. CamKII showed a higher degree of phosphorylation at early ischemia in HMWKO (LMW FGF2 only) hearts compared to wildtype. *$p<0.05$ vs. WT at ischemia. †$p<0.05$ vs WT at baseline.





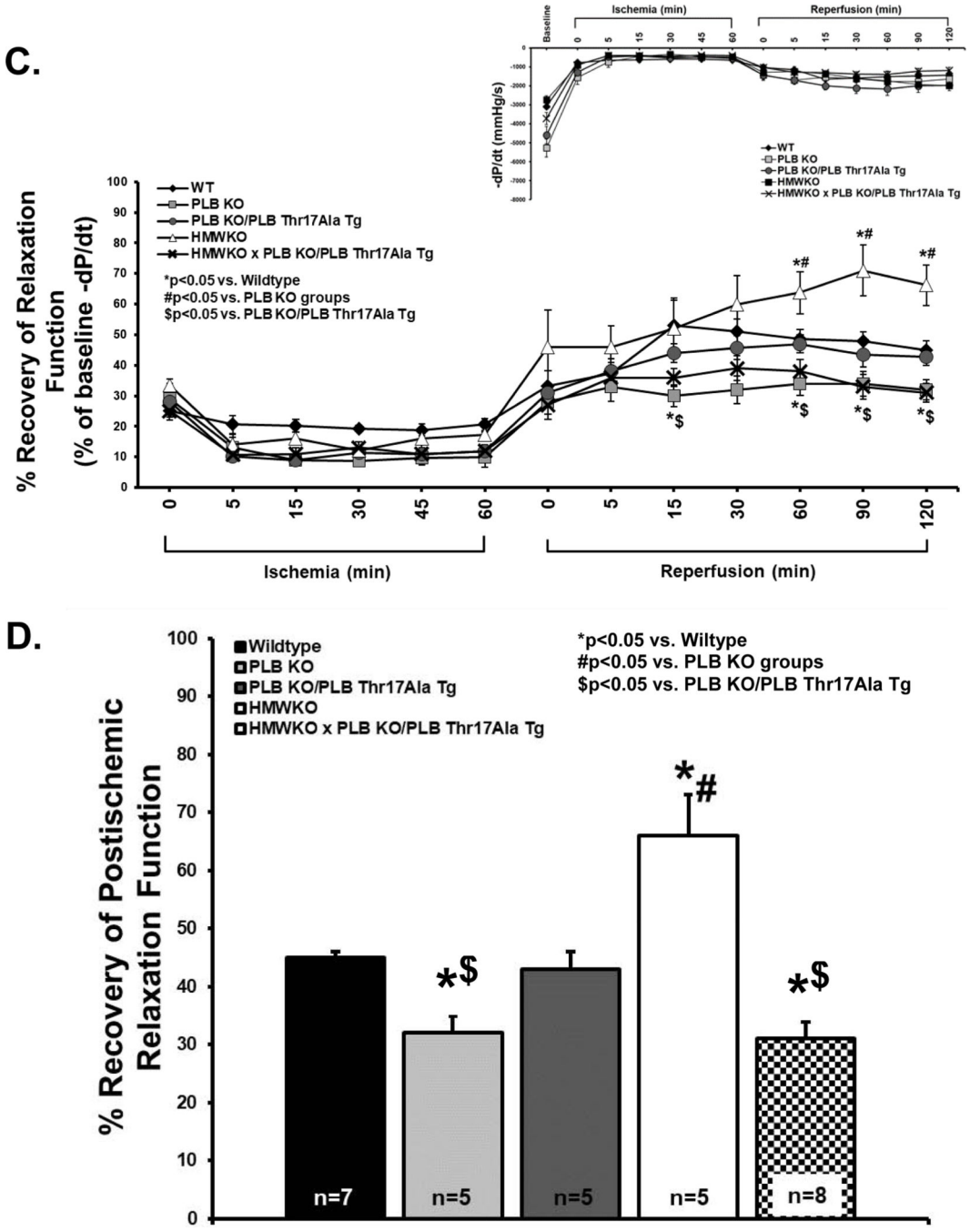


Figure 6.

LMW FGF2-mediated protection against cardiac dysfunction involves phospholamban phosphorylation at site Threonine-17. Mice only expressing LMW FGF2 showed significantly improved recovery of cardiac function (panels A and B, % recovery of contraction, +dP/dt; panels C and D, % recovery of relaxation, -dP/dt) during I/R ($p<0.05$), and this functional improvement was significantly abrogated in hearts expressing LMW FGF2 and the PLB mutant lacking phosphorylatable Thr17 (Thr17Ala). Percent (%) recovery of contractility or relaxation is +dP/dt or -dP/dt, respectively, over I/R (Panel A

and C) or at 120 minutes reperfusion normalized to baseline (Panels B and D). Insets for panel A and panel C depict +dP/dt and –dP/dt over time of I/R, respectively. n=5–8 per genotype.*p<0.05 vs. Wildtype. #p<0.05 vs. PLB KO groups. $p<0.05 vs. PLB KO/PLB Thr17Ala Tg.

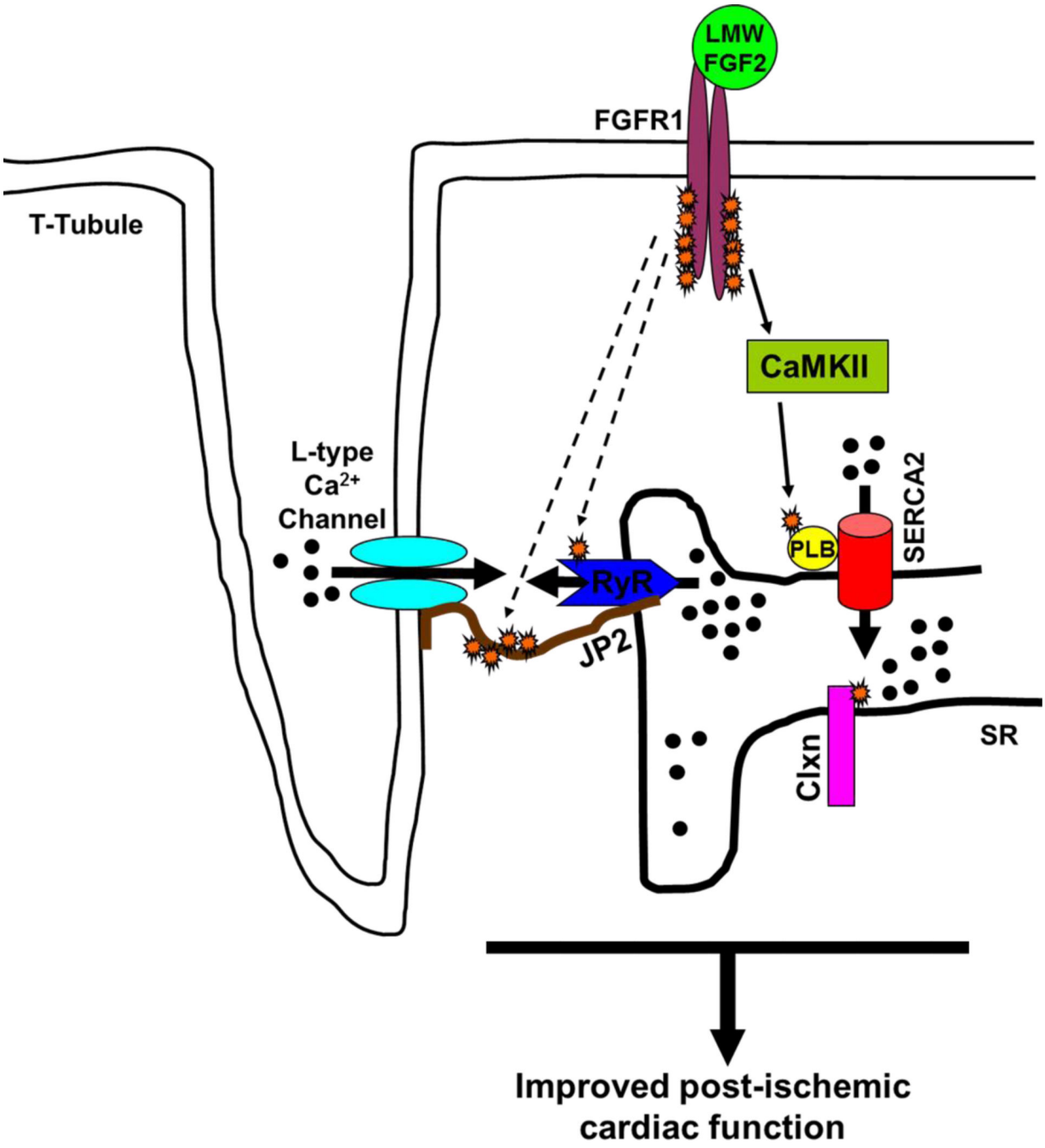


Figure 7.

Working model of LMW FGF2-induced protection against post-ischemic cardiac dysfunction. Evidence from our lab and others demonstrate that LMW FGF2 interacts with FGF receptor-1 (FGFR1). We have previously shown that LMW FGF2 activates PKCalpha which translocates to the contractile apparatus and phosphorylates troponins, ultimately resulting in improvement in post-ischemic contractile function. Our recent data indicate that LMW FGF2 increases calcium calmodulin kinase II (CaMKII) activation. Our phosphoproteomic evidence and post-ischemic cardiac function suggest that phosphorylation

(orange star) occurs at threonine-17 site of phospholamban (PLB). Additionally, LMW FGF2 may regulate intracellular calcium handling via phosphorylation (orange star) changes of other SR proteins, ryanodine receptor-2 (RyR2), junctophilin-2 (JP2), and calnexin (Clnxn) essential for recovery from I/R injury. These observations indicate that LMW FGF2 may protect the heart from post-ischemic cardiac dysfunction by directly affecting calcium cycling in the injured myocyte.

Table 1:

Phosphorylation sites identified on sarco(endo)plasmic reticulum (SR) phosphoproteins via phosphoproteomic discovery analysis. Phosphorylated SR proteins identified with high confidence (>95%) and relevant phosphopeptides identified during the exploratory evaluation of Fgf2KO and FGF2 HMWKO mouse cardiac tissue collected at 60 minutes of ischemia and 5 minutes of reperfusion. Red highlighted phosphorylation sites showed apparent relative changes in phosphorylation as judged by isotope tagging and mass spectrometry (see Figure 1 and Supplemental Figure 6).

Accessions	Protein	Number of Sites	Sites detected with high confidence (>95%)			
gi|133778931	Histidine rich calcium binding protein	20	Thr27	Ser129	Ser249	Ser466
			Ser49	Ser141	Ser324	Ser474
			Ser50	Ser150	Ser402	Ser517
			Ser54	Ser151	Thr403	Ser542
			Ser104	Ser228	Ser421	Ser591
gi|6806903 gi|158635979	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform b	3	Thr8 Ser661 Ser663			
gi|10947010	Junctophilin-2	9	Thr453	Thr483	Ser597	
			Ser462	Ser591	Ser600	
			Ser479	Ser593	Ser613	
gi|124430578	Ryanodine receptor 2, cardiac	7	Ser2367	Thr2809		
			Ser2692	Ser2810		
			Ser2693	Ser2813		
			Ser2807			
gi|6671664 gi|160333216 gi|160333212	Calnexin precursor	5	Ser553 Thr561 Ser563 Ser569 Ser582			
gi|34610235	Reticulon-4 isoform A	4	Ser105	Ser167		
			Ser165	Ser839		
gi|21351281 gi|12963503	Cardiac phospholamban	2	Ser16 Thr17			