Overexpression of junctophilin-2 does not enhance baseline function but attenuates heart failure development after cardiac stress

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Heart failure is accompanied by a loss of the orderly disposition of transverse (T)-tubules and a decrease of their associations with the junctional sarcoplasmic reticulum (jSR). Junctophilin-2 (JP2) is a structural protein responsible for jSR/T-tubule docking. Animal models of cardiac stresses demonstrate that down-regulation of JP2 contributes to T-tubule disorganization, loss of excitation-contraction coupling, and heart failure development. Our objective was to determine whether JP2 overexpression attenuates stress-induced T-tubule disorganization and protects against heart failure progression. We therefore generated transgenic mice with cardiac-specific JP2 overexpression (JP2-OE). Baseline cardiac function and Ca^{2+} handling properties were similar between JP2-OE and control mice. However, JP2-OE mice displayed a significant increase in the junctional coupling area between T-tubules and the SR and an elevated expression of the Na^{+}/Ca^{2+} exchanger, although other excitation-contraction coupling protein levels were not significantly changed. Despite similar cardiac function at baseline, overexpression of JP2 provided significantly protective benefits after pressure overload. This was accompanied by a decreased percentage of surviving mice that developed heart failure, as well as preservation of T-tubule network integrity in both the left and right ventricles. Taken together, these data suggest that strategies to maintain JP2 levels can prevent the progression from hypertrophy to heart failure.

cardiac dyads | in situ Ca^{2+} imaging | electron microscopy

In working ventricular myocytes, normal excitation-contraction (E-C) coupling requires precise communication between voltage-gated L-type Ca^{2+} channels (Cav1.2) located in clusters within transverse (T)-tubules and, less frequently, on the plasmalemma, and Ca^{2+} release channels/ryanodine receptor channels (RyRs) that are also clustered on the junctional sarcoplasmic reticulum (jSR) membrane (1–4). In normal hearts, flat jSR cisternae containing a continuous row of polymerized calsequestrin (CsQ2) either wrap around a T-tubule segment or abut against the plasmalemma (5, 6) and are coupled to the surface membranes via apposed clusters of RyR2 and Cav1.2 (7). These junctional sites are called dyads. However, although the jSR cisternae constitute a single continuous compartment, the clusters of RyR2 do not occupy the whole jSR surface but are in smaller groups (8, 9). Hence, each dyad is composed of several smaller RyR2/Cav1.2 complexes, also called couplons. Functional interaction between Cav1.2 and RyR2 at these sites ensure synchronous SR Ca^{2+} release and coordinated contraction (1, 10, 11). There is evidence that impaired cardiac E-C coupling/Ca^{2+} handling is a key mediator of heart failure (12, 13). One underlying mechanism for the defective Ca^{2+} release is the progressive loss of T-tubule network organization and of the relationship between RyR2 and Cav1.2 (14–16). Therefore, preventing loss of jSR/T-tubule junctions and of T-tubule organization may represent a new strategy for therapeutic intervention in heart failure.

In normal cardiomyocytes, the formation of dyads requires junctophilin 2 (JP2), a structural protein that provides a physical connection between the T-tubule and SR membranes (17). JP2's eight N-terminal "membrane occupation and recognition nexus" domains bind to the plasmalemma (T-tubules), and its C-terminal transmembrane domain tethers the opposite end to the SR membrane (17). Decreased JP2 levels have been observed in human heart failure patients and in failing hearts from animal models of cardiac disease (16, 18–22). Knockdown of JP2 results in acute heart failure that is associated with the loss of junctional membrane complex, disrupted T-tubule organization, and Ca^{2+} handling dysfunction (23). In addition, embryonic myocytes with JP2 deficiency have defective cardiac dyads, including more SR segments with no T-tubule couplings as well as reduced intracellular Ca^{2+} transients (17). These data collectively suggest that loss of JP2 contributes to the functional defects in heart failure. Therefore, interesting questions are: Is the JP2 deficiency effect linked to the resultant disruption of jSR/T-tubule junctions and of T-tubule network integrity, as suggested by previous findings (16–18, 23)? Conversely, could exogenous overexpression of JP2 in cardiomyocytes improve Ca^{2+} handling and protect against the development of heart failure?

Significance

Normal cardiac function requires coordinated contraction of working myocytes, which is initiated by a specific communication between Ca^{2+} channels on the transverse (T)-tubule membrane and ryanodine receptors on the sarcoplasmic reticulum (SR) membrane. Junctophilin-2 (JP2) is a structural protein that induces docking of SR to T-tubules to form dyads and that indirectly stabilizes the T-tubule network in ventricular cardiomyocytes. JP2 is frequently down-regulated in heart failure, in parallel with a disruption of the T-tubule network and loss of normal excitation-contraction coupling. Here we show that overexpression of JP2 stabilizes the T-tubule network and attenuates heart failure after cardiac stress. These data suggest that future treatment of heart disease may include strategies to stabilize the architecture of T-tubules and cardiac dyads.

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To answer this question, we generated transgenic mice with cardiac-specific overexpression of JP2. Moderate overexpression of JP2 led to a significant increase in the junctional coupling area between T-tubule and SR membrane, but surprisingly, it did not enhance cardiac function or increase SR Ca^{2+} release at baseline. However, interestingly, JP2-overexpressing mice were resistant to left ventricular pressure overload-induced heart failure, demonstrating that JP2 overexpression is protective. These data suggest that preventing the loss of JP2 could be a potential therapeutic strategy for heart failure treatment.

Results

Cardiac-Specific Overexpression of JP2 Does Not Alter Baseline Contractile Function. Cardiac-specific JP2 overexpression (JP2-OE) mice were generated using a bitransgenic system with αMHC-driven cardiac specific JP2 overexpression, controlled by a tetracycline (Tet)-off system (Fig. 1*A*). First, we generated αMHC-JP2 mice, in which the JP2 cDNA is downstream of a modified αMHC promoter containing the tet-operon (αMHC-JP2 mice). These mice were then crossed with αMHC-tTA (tetracycline-controlled transactivator) mice, which express tTA under the control of a standard αMHC promoter, to generate double-transgenic JP2-tTA mice (JP2-OE mice). In this study, we used mice with constitutive JP2 overexpression beginning at the embryonic stage. Littermate αMHC-tTA mice served as controls. We first confirmed a threefold overexpression of JP2 protein in heart lysates from JP2-OE mice compared with littermate controls (Fig. 1*B*). Assessment of ejection fraction and left ventricular mass by echocardiography demonstrated no significant difference between JP2-OE and littermate control mice at 2, 4, and 8 mo of age (Fig. 1 *C* and *D*). Ex vivo studies of Langendorff-perfused hearts at various extracellular Ca^{2+} concentrations also revealed similar hemodynamic profiles (maximum left ventricular pressure and rate of pressure development) in JP2 overexpression and control mice (Fig. 1 *E* and *F*). Thus, JP2 overexpression has no significant effect on baseline cardiac function.

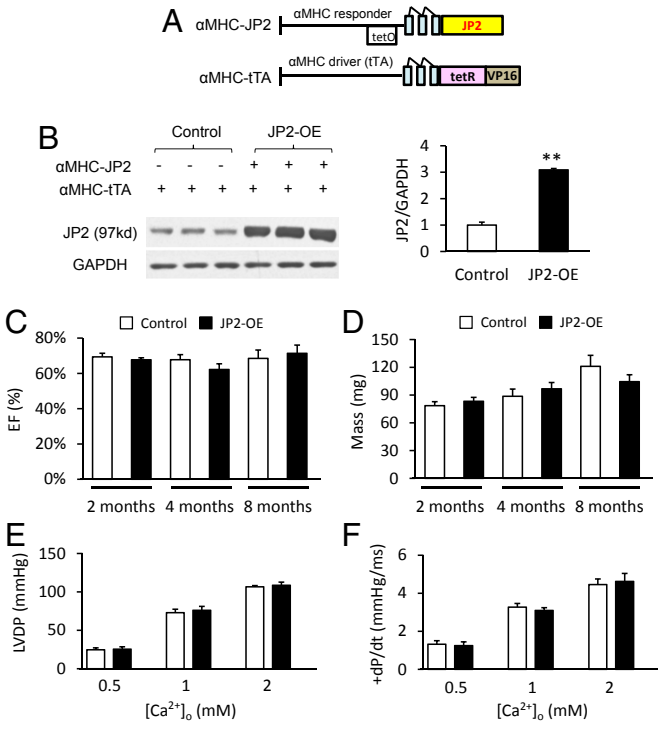

Fig. 1. Mice with cardiac-specific JP2-OE have normal baseline cardiac function. (*A*) Schematic of the bitransgenic system for cardiac-specific JP2-OE. The α-MHC-JP2 mice harbor a transgene composed of JP2 cDNA downstream of a modified αMHC promoter containing the tet-operon (tetO). The α-MHC-JP2 mice were crossed with α-MHC-tTA mice, which express tTA, under the control of a standard α-MHC promoter, to generate double-transgenic JP2-tTA (JP2-OE) mice. (*B*) Protein levels of JP2 in hearts from 2-mo-old JP2-OE and α-MHC-tTA (control) mice. $^{**}P < 0.01$, Student *t* test. (*C* and *D*) Electrocardiography of LV EF (*C*) and LV mass (*D*); $n = 8$–12 mice/group. (*E* and *F*) Ex vivo hemodynamic analysis of Langendorff-perfused hearts under indicated extracellular Ca^{2+} concentrations. LVDP, LV developed pressure; +dp/dt, maximum rate of pressure increase; $n = 5$–7 mice/group. Data shown are mean ± SEM.

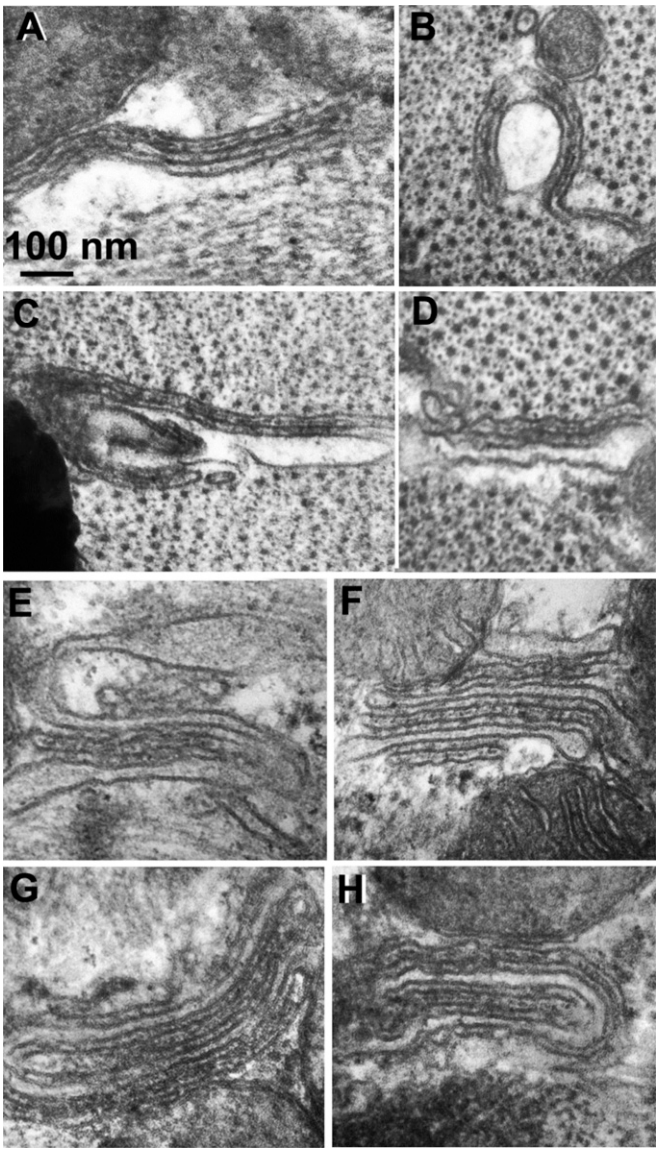

Fig. 2. The structure of dyads in control and JP2-OE myocardium from 2-mo-old mice. (*A*–*D*) Typical profiles of dyads in sectioned control myocardium from the LV. The T-tubule is either flattened (*A* and *D*) or expanded (*B* and *C*). A single flat jSR cisterna containing calsequestrin wraps around the T-tubules, often appearing as two separate profiles in the section. (*E*–*H*) About 10% of dyads in the JP2-OE hearts are constituted of convoluted T-tubules and multiple jSR contacts. The remaining dyads are similar to control but are more frequent and more extensive (Tables 1 and 2).

JP2 Overexpression Induces Increases in jSR/T-Tubule Contact Length and Dyad Frequency. Because JP2 is crucial for the docking of SR to surface membrane (plasmalemma and T-tubules) (17), we assessed the effects of JP2 overexpression on frequency and size of dyadic contacts between the two sets of membranes, using the

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Table 1. Length of jSR/T-tubule contacts in thin sections

Samples	Length of jSR/T-tubule contact, nm (mean ± SD)
Control*	294 ± 132
Junctophilin-2 overexpression†	553 ± 389‡

*From 3 hearts, 180 dyads.
†From 3 hearts, 175 dyads.
‡$P < 0.00001$ versus control, Student *t* test.

myofibrils as reference points. At sites of close proximity to the T-tubule, where the functional junctions are established, the jSR cisternae are typically flat and filled with beads of CsQ2. The appearances of "normal" dyads and the jSR/T-tubule contacts that constitute them are quite variable in thin sections of control hearts, in part because of the actual variability in the contact geometry and in part because of the orientation of the section relative to the membrane profiles. Mostly, a single T-tubule profile, either collapsed (Fig. 2 *A* and *B*) or wide open (Fig. 2 *B* and *C*), is seen in each image. The jSR may appear to contact the T-tubule on one side (Fig. 2 *A* and *D*), but in fact, it partially wraps around it, so that two, sometimes apparently separated, jSR profiles are often seen on the two sides of a single T-tubule profile (Fig. 2 *B* and *C*). Double profiles of T-tubules, resulting from a convolution of the tubule, are rare in control hearts. Three easily detectable effects of the overexpression are seen. One is the presence of ∼10% dyads that are "convoluted" because of multiple T-tubule profiles that join jSR on several sides, and sometimes in multiple layers (Fig. 2 *E*–*H*). The second change is the significant, almost twofold, increase in the total length of jSR/T-tubule contacts at each dyad (Table 1), involving an increase in the profile length of CsQ2- filled jSR cisternae closely apposed to T-tubules. A third change is in the frequency of dyad profiles in the location between the myofibrils at the level of the Z-line (Table 2). It is not clear whether the latter increase is a result of a combination of new contact sites or simply a result of the increase in the extent of each contact site noted earlier. These data confirm the function of JP2 as a physical bridge, facilitating the formation of jSR/T-tubule and jSR/PM junctions (17, 23). In addition, ryanodine receptors (feet) are clearly present in the gap between jSR and T-tubules in all cases, but we could not determine whether their frequency is changed from normal.

Effect of JP2 Overexpression on E-C Coupling Proteins in Cardiomyocytes. E-C coupling proteins congregate in the vicinity of cardiac dyads. It is then necessary to examine whether an increased jSR/T-tubule contact area resulting from JP2 overexpression results in changes in the expression levels of E-C proteins in cardiomyocytes. Using whole lysates from left ventricles of control and JP2-OE mice, interestingly, we found that Na^+/Ca^{2+} exchanger 1 (NCX1) protein level was threefold higher in JP2-overexpressing hearts (Fig. 3). However, we did not detect significant differences in the expression levels of RyR2, Cav1.2, sarcoplasmic reticulum calcium ATPase 2a (SERCA2a), CsQ2, and junctin (Fig. 3). These data suggest that JP2 overexpression minimally affects the E-C coupling proteins (except NCX1 protein), despite the significant increase in the coupling area between T-tubules and jSR membranes.

Effect of JP2 Overexpression on Ca^{2+} Handling Function in Cardiomyocytes. We postulated that the JP2-OE-mediated increase in coupling sites and contact length would lead to an enhancement of cardiomyocyte E-C coupling. To determine whether up-regulation of JP2 affects normal E-C coupling function in cardiac myocytes, we analyzed the Ca^{2+} transients of ventricular myocytes by in situ imaging in perfused intact hearts. Surprisingly, neither the amplitudes nor the kinetics of in situ Ca^{2+} transients were altered dramatically by JP2 overexpression (Fig. 4 *A* and *B*). Moreover, we observed no difference in any parameters of elementary Ca^{2+} release events, Ca^{2+} sparks, between control and JP2-OE myocytes (Fig. 4 *C* and *D*). These data suggest that up-regulation of JP2 has no obvious effect on cardiomyocyte E-C coupling function and gating properties of RyRs, consistent with the normal contractile function of JP2-overexpressing hearts.

JP2 Overexpression Protects Hearts from Developing Heart Failure Under Left Ventricular Pressure Overload. Although the baseline cardiac function is not altered by JP2 up-regulation, we hypothesized that JP2 overexpression may exert protective effects under pathological conditions. JP2-OE or control male mice were subjected to transaortic banding (TAB) at 9 wk of age. After 5 wk of TAB, 14 of 22 control mice survived, whereas the survival rate was increased to 18 of 22 in JP2-OE mice (Fig. 5*A*). The heart weight/body weight ratio and lung weight/body weight ratio were both markedly attenuated (Fig. 5 *B* and *C*), whereas ejection fraction (EF) was significantly improved in JP2-OE mice (Fig. 5*D*) compared with in control mice after TAB stress. The proportion of surviving JP2-OE and control mice with either hypertrophy or heart failure were further analyzed. Surviving mice with lung weight/body weight ratio >7 (mg/g) and EF value <55% were considered to have developed heart failure (see *SI Materials and Methods* for details on definition of heart failure), whereas the other mice were considered to have cardiac hypertrophy. For the control group, 10 of 14 surviving mice were classified as having heart failure. JP2 overexpression decreased the fraction of mice with heart failure to 5 of 18 (Fig. 5*E*).

Because disorganization of the T-tubule network is a hallmark of heart failure, we assessed the T-tubule architecture in the control and JP2-OE mice subjected to TAB to define whether protection against heart failure is coupled to protection of T-tubule network integrity. In images from both the left ventricle (LV) and right ventricle (RV) of control and JP2-OE mice at baseline, the T-tubules marked by a lipophilic dye (MM 4–64; AAT Bioquest, Inc.) form well-aligned transverse bands with sarcomeric spacings. Quantitative analysis of T-tubule architecture using AutoTT (24) shows that overexpression of JP2 does not change the T-tubule architecture in hearts at baseline condition (Fig. S1). After TAB (Fig. 6*A*, *Upper*), T-tubule profiles are less frequent and quite disordered, with loss of periodic spacings. JP2 overexpression, in contrast, attenuated T-tubule disarray, in response to TAB, and both LV and RV show prominent transverse bandings of labeled T-tubules (Fig. 6*A*, *Bottom*). Further

Table 2. Frequency of cardiac dyads

Samples	Frequency of "normal" dyads (number of dyads/number of intermyofibrillar spaces; mean ± SD)	Frequency of "convoluted" dyads (number of dyads/number of intermyofibrillar spaces; mean ± SD)	Total frequency of dyads (number of dyads/number of intermyofibrillar spaces; mean ± SD)
Control*	0.33 ± 0.07	0	0.33 ± 0.07
JP2-OE†	0.53 ± 0.10‡	0.05 ± 0.06‡	0.58 ± 0.13‡

*From 3 hearts, 136 sites.
†From 4 hearts, 187 sites.
‡$P < 0.00001$ versus control, Student *t* test.

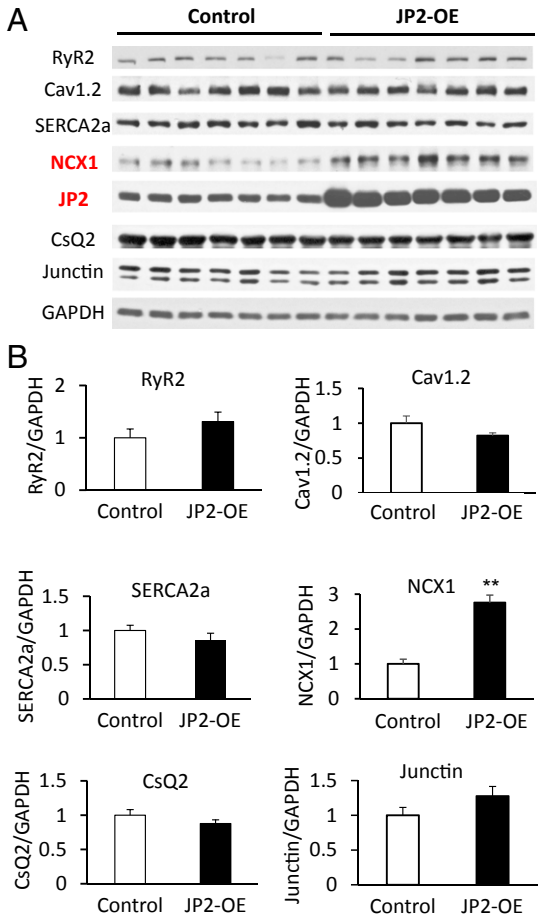

Fig. 3. Effect of JP2-OE on expression of other E-C coupling proteins. (*A*) Representative Western blotting bands of E-C coupling proteins RyR2, Cav1.2, SERCA2a, NCX1, CsQ2, and junctin and loading control GAPDH from tissue lysates of control and JP2-OE hearts from 2-mo-old mice. (*B*) Average data on protein levels of RyR2, Cav1.2, SERCA2a, NCX1, CsQ2, and junctin normalized to GAPDH; $n = 7$ samples per group. $^{**}P < 0.01$ versus control, Student t test. RyR2 in the JP2-OE group was 20% higher than in control mice but did not reach significant difference between the groups.

analysis with AutoTT provides a quantitative evaluation of the protective effects of JP2-OE on stress-induced T-tubule remodeling. The density of transverse elements (TE) of T-tubules (TE density; Fig. 6*B*), T-tubule regularity (Fig. 6*C*), and index of T-tubule integrity [a combined measure of T-tubule density and regularity (24); Fig. 6*D*] are significantly higher in both LV and RV myocytes of JP2-overexpressing hearts compared with control hearts, indicating JP2 overexpression preserves T-tubule architecture in response to pressure overload stress.

Discussion

Our work is the logical extension of earlier studies aimed at decreasing the level of JP2 expression in the heart. The pioneering JP2 knockout mouse (17) showed a 90% reduction in the frequency of jSR/T-tubule junctions and a 55% reduction in the average length of the junctional couplings. This general effect was further confirmed by silencing JP2 with shRNA in isolated myocytes (21), transgenic inducible JP2 knockdown in adult murine hearts (23), and exogenous expression of the JP2-suppressing microRNA, miR-24 (21). Our present results provide clear evidence that cardiac-specific overexpression of JP2 produces the converse effects in terms of an increase in the frequency and size of dyads.

Despite the increased frequency of junctional couplings, JP2 overexpression did not induce an enhancement of either global cardiac or Ca^{2+} handling function, perhaps related to the lack of significant effect on the expression levels of RyR2, Cav1.2, SERCA2a, and CsQ2. Surprisingly, JP2 overexpression resulted in a significant, threefold increase in expression of NCX1 protein. However, this moderate NCX1 overexpression did not affect baseline cardiac E-C coupling, which might have been predicted by the minimal effects of engineered 2.5-fold (25) or ninefold overexpression of NCX1 (26-28) and cardiac-specific NCX1 deletion (29) on cardiac function. Some compensatory effects, such as noted in the NCX1-deficient hearts, that allow continuity of heart function over time (30) are likely at work. NCX1 expression and/or activity are increased in many models of cardiac hypertrophy and failure (31, 32), yet it is unclear whether up-regulation of NCX1 is beneficial or detrimental in cardiac disease. In a recent study led by Cheung and colleagues, inducible overexpression of NCX1 corrected myocyte contractile and $[Ca^{2+}]_i$ transient abnormalities but had no effect on myocardial dysfunction induced by pressure overload (33). Based on these findings, one may conclude that the increased NCX1 expression/activity does not contribute to the development of heart failure. Our data show that the increase in NCX1 is coincident with cardiac protection in JP2-OE mice, where jSR/T-tubule

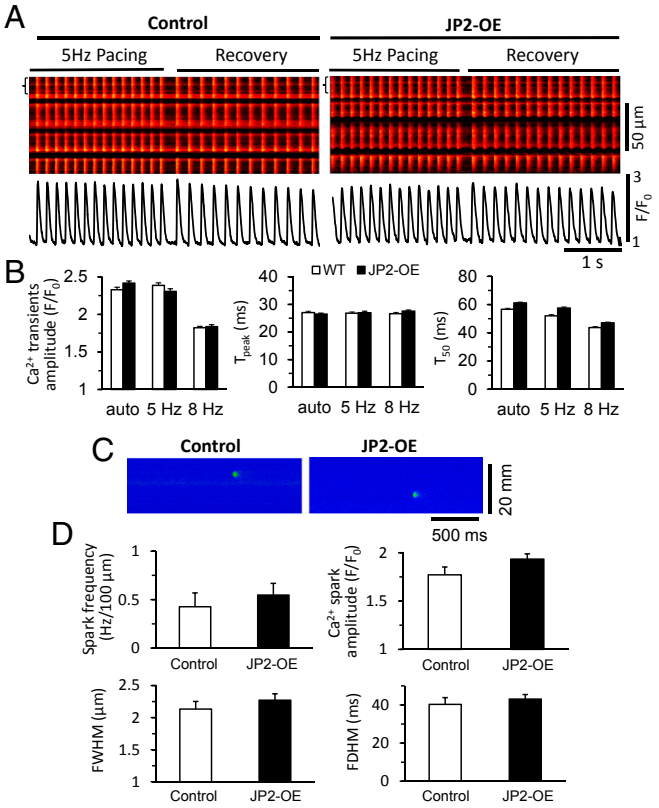

Fig. 4. JP2-OE does not alter cardiac Ca^{2+} handling function. (*A*) Representative images of Ca^{2+} transients recorded from epicardial myocytes of intact hearts from 2–3-mo-old control or JP2-OE mice. (*Bottom*) Time course profiles of Ca^{2+} transients from cells, as indicated by the bracket on the left side of the image. The signal was averaged over the region indicated by the bracket. (*B*) Average data of the amplitude and kinetics of Ca^{2+} transients from beating hearts under autonomous beating (auto), 5- or 8-Hz external stimulation; $n = 170–250$ cells from three to four hearts for each group. (*C*) Representative Ca^{2+} spark images from 2–3-mo-old control or JP2-OE mice. (*D*) Mean values of the frequency and other Ca^{2+} spark parameters. FWHM, full width at half maximum; FDHM, full duration at half maximum; $n = 27–52$ cells from 3 hearts for each group. Data shown are mean ± SEM.

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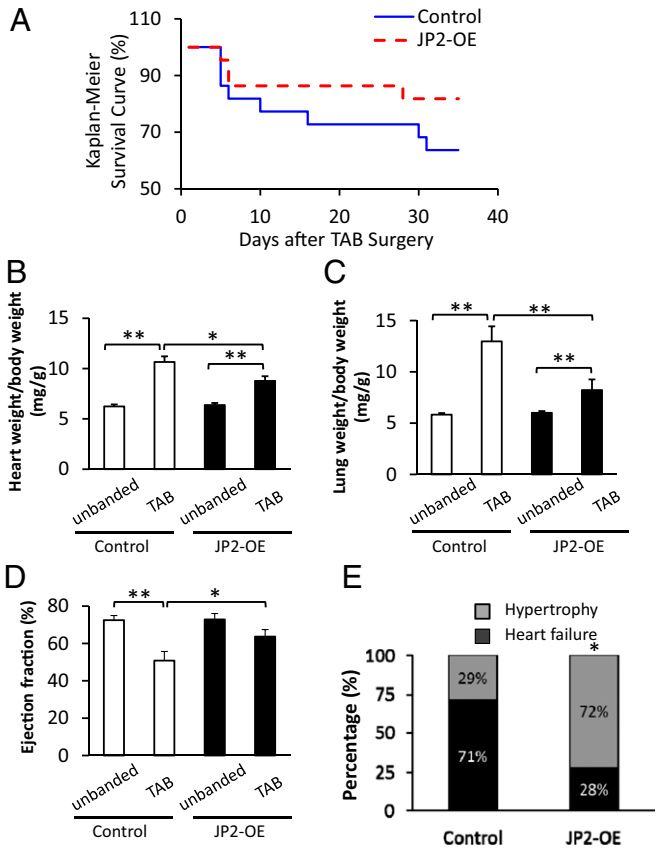

Fig. 5. JP2-OE protects against pressure overload-induced heart failure. (*A*) Kaplan–Meier survival curves for control and JP2-OE mice after TAB surgery. Fourteen control and 18 JP2-OE, out of 22 mice for each group, survived 5 wk after TAB. (*B* and *C*) Summarized heart weight/body weight (*B*), lung weight/body weight (*C*), and EF (*D*) of unbanded mice and TAB mice 5 wk after surgery; n = 11–18 mice for each group. $^{*}P < 0.05$; $^{**}P < 0.01$, as indicated, Kolmogorov–Smirnov rank test. (*E*) JP2-OE mice are less likely to develop heart failure in response to TAB. n = 14–18 mice. $^{*}P < 0.05$ versus control, χ^2 test.

contacts and dyad frequency are increased. We speculate that in the presence of tightly organized jSR/T-tubule couplings, a moderate increase of NCX1 could help prevent the development of heart failure by maintaining low Ca^{2+} concentration in the microdomain of cardiac dyads.

Of major relevance to cardiac structure and function is the connection between down-regulation of JP2 in the failing heart and integrity of the cardiac dyad and T-tubule system. Although induced by a variety of JP2 dysregulation mechanisms, from repression at the translational level by miR-24 (34) to Ca^{2+}-dependent calpain-mediated degradation (35, 36), to mistrafficking of JP2 from T-tubules to the cell periphery (37), the final effect indicates an implicit connection between the role of JP2 as a structural protein in the maintenance of T-tubules and dyads and the maintenance of normal calcium homeostasis. Our data directly link JP2 overexpression to the protection against deleterious T-tubule remodeling and E-C coupling dysfunction. The relationship between the two factors is best understood by considering how the normal jSR/T-tubule organization is achieved during differentiation (38). The "transverse" tubules initially form a disordered predominantly longitudinal net between the myofibrils, and their transition to an ordered transverse network follows their association with jSR elements (39). The dyads (in myocardium) and triads (in skeletal muscle) can be seen as the elements that tether the T-tubules (via JP2) to specific positions relative to the myofibrils, therefore guiding the T-tubule network into a predominantly transverse orientation. Seen in this context, the loss of T-tubule network organization in the failing heart is simply a reflection of the loss of dyadic jSR/T-tubule associations, and the latter is a direct consequence of a decline in JP2 expression levels (14, 16, 23) and/or a change in JP2 localization (37). Conversely, the protective effect of JP2 overexpression can be assigned to the increased jSR/T-tubule associations that we observe.

Stabilization of the dyad positions has a very striking direct effect in inhibiting the functional decay that leads to heart failure, indicating that orderly location of calcium release sites is of importance in the tightly controlled cardiac calcium homeostasis. Here, we showed that a moderate threefold specific overexpression of JP2 in transgenic mice alleviates pathological loss of orderly T-tubule structure and the transition from hypertrophy to heart failure in response to pressure overload stress. These findings are supported by a recent study demonstrating that silencing miR-24 with a miR-24 antagomir preserves myocardial function under cardiac stress through maintenance of JP2 expression, and thereby the cardiac dyad (40). In conclusion, strategies to either augment JP2 expression

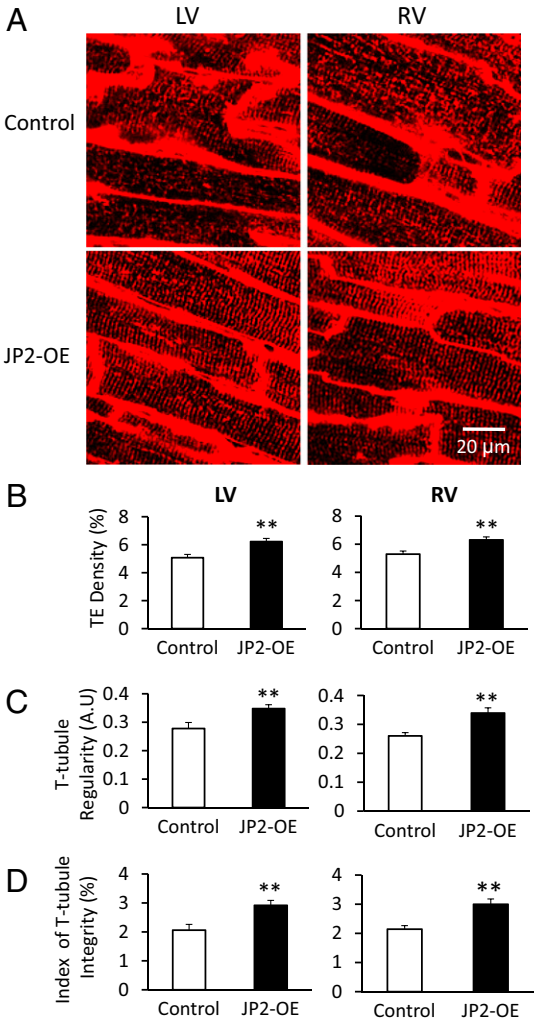

Fig. 6. JP2-OE attenuated myocyte T-tubule remodeling in response to pressure overload. (*A*) Representative LV and RV T-tubule images stained with lipophilic marker MM 4–64 from control and JP2-OE mice 5 wk after TAB. (*B*–*D*) Summarized data on the density of transverse elements (TE) of T-tubules (*B*), T-tubule regularity (*C*), and index of T-tubule integrity (*D*); n = 11 and 14 hearts for control and JP2-OE, respectively. $^{**}P < 0.01$ versus control.

or prevent its down-regulation, in combination with strategies to maintain the proper localization of JP2, clearly have a place in the future treatment of cardiac disease.

Materials and Methods

Animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Detailed materials and methods are presented as *SI Materials and Methods*.

Generation of Transgenic Animals, TAB Surgery, and Echocardiography. The cardiac-specific JP2 overexpression vector was constructed by subcloning mouse JP2 cDNA (a gift from Hiroshi Takeshima, Kyoto University, Kyoto) into a tet-operated αMHC transgenic vector, as described previously (41). These transgenic mice were crossed with αMHC-tTA (Jackson Lab) to generate double-transgenic mice (JP2-OE) carrying both αMHC-JP2 and αMHC-tTA. Nine-wk-old male JP2-OE mice and their control littermates were subjected to pressure overload by TAB surgery. Transthoracic echocardiograms were performed in the University of Iowa Cardiology Animal Phenotyping Core Laboratory, as described (18).

Electron Microscopy. Fixation, embedding, and sectioning for electron microscopy were done essentially as in Knollmann and colleagues (42), and images were obtained from sections of the LV ventricle papillary muscles. Dyads were counted in images from longitudinal sections at a magnification of 10,800×. Measurements of jSR/T-tubule contact length and RyR density in dyads were obtained from images of cross sections at magnifications of 57,800× and 133,000×, respectively. All morphometric analysis was based on Fiji (NIH) image processor software. All images were collected after "random sampling" procedures from 3 control and JP2-OE hearts.

Western Blotting. Western blots were performed as described previously (43).

In situ T-tubule and Ca^{2+} Imaging. Confocal Imaging of T-tubules and Ca^{2+} handling in Langendorff-perfused intact hearts was performed as described previously (16, 44). The density, regularity, and integrity of T-tubules were analyzed using AutoTT (24).

Statistics. Data were expressed as mean ± SEM; Student *t* tests, χ^2 test, rank test or one-way ANOVA were applied when appropriate; and $P < 0.05$ was considered statistically significant.

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