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T-tubule remodeling during transition from hypertrophy to heart failure

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Abstract

Rationale—The transverse tubule (t-tubule) system is the ultrastructural substrate for excitation-contraction coupling in ventricular myocytes; t-tubule disorganization and loss are linked to decreased contractility in end stage heart failure (HF).

Objective—To examine 1) if pathological t-tubule remodeling occurs early in compensated hypertrophy and, if so, how it evolves during the transition from hypertrophy to HF; 2) the role of junctophilin-2 in t-tubule remodeling.

Methods and Results—We investigated t-tubule remodeling in relation to ventricular function during HF progression using state-of-the-art confocal imaging of t-tubules in intact hearts, using a thoracic aortic banding (TAB) rat HF model. We developed a quantitative t-tubule power (TT_{power}) index to represent the integrity of t-tubule structure. We found that discrete local loss and global reorganization of the t-tubule system (leftward shift of TT_{power} histogram) started early in compensated hypertrophy in left ventricular (LV) myocytes, prior to LV dysfunction, as detected by echocardiography. With progression from compensated hypertrophy to early and late HF, t-tubule remodeling spread from the LV to the right ventricle (RV), and TT_{power} histograms of both ventricles gradually shifted leftward. The mean LV TT_{power} showed a strong correlation with ejection fraction and heart weight to body weight ratio. Over the progression to HF we observed a gradual reduction in the expression of a junctophilin protein (JP-2) implicated in the formation of t-tubule / sarcoplasmic reticulum junctions. Furthermore, we found that JP-2 knockdown by gene silencing reduced t-tubule structure integrity in cultured adult ventricular myocytes.

Conclusions—T-tubule remodeling in response to TAB stress begins prior to echocardiographically detectable LV dysfunction and progresses over the development of overt structural heart disease. LV t-tubule remodeling is closely associated with the severity of cardiac

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hypertrophy and predicts LV function. Thus, t-tubule remodeling may constitute a key mechanism underlying the transition from compensated hypertrophy to HF.

Keywords

t-tubule; myocardial remodeling; hypertrophy; heart failure; confocal microscopy

Introduction

The transverse tubules (t-tubules) are orderly invaginations of surface membrane along the Z-line regions, with regular spacing (~2μm) along the longitudinal axis of mammalian ventricular myocytes. The widely distributed, highly organized t-tubule system is essential for rapid electrical excitation, initiation and synchronous triggering of sarcoplasmic reticulum (SR) Ca^{2+} release, and therefore coordinated contraction of each contractile unit throughout the entire cytoplasm. The t-tubule system is thus an important determinant of cardiac cell function. [1-3]

Heart failure (HF) is characterized by reduction of myocyte contractile function and defects in Ca^{2+} handling (i.e., blunted and dyssynchronous SR Ca^{2+} release) in myocytes from HF models (including human) [4-6]. The cause of Ca^{2+} handling defects in HF is likely multifaceted, with contribution from reduced SR Ca^{2+} content, ryanodine receptor (RyR) hyperphosphorylation, and change in action potential shape, among other factors [7-10]. Recent studies from several groups have provided compelling evidence supporting a concept that t-tubule structural remodeling is directly linked to SR Ca^{2+} release dysfunction in myocytes from animal models of HF [11-16] and in artificially detubulated myocytes [17-18]. Although there is no doubt that t-tubule remodeling correlates with Ca^{2+} handling defects, it is unknown whether t-tubule alterations are an early or late event in HF. The lack of studies of t-tubule changes prior to HF onset is thus a critical knowledge gap in understanding HF. It is also not clear whether there is a relationship between t-tubule structure and cardiac function during the progression from hypertrophy to HF. In addition, little is known about the potential molecular mechanisms underlying t-tubule remodeling in heart disease.

We investigated t-tubule structure and LV function during progression of cardiomyopathy in a thoracic-aortic banding (TAB) sprague-dawley rat model of pathological left ventricular (LV) afterload. We adapted our confocal imaging system to examine the in situ epicardial t-tubule structure from Langendorff perfused intact hearts. A quantitative and sensitive method (t-tubule power index, TT_{power}) was developed to characterize the overall integrity of the t-tubule system. Surprisingly, we found that t-tubule remodeling starts prior to echocardiographically detectable LV systolic dysfunction during compensated hypertrophy, and is seen as patchy t-tubule loss in local regions of the LV as well as global t-tubule reorganization. With disease progression, t-tubule structural remodeling penetrates from the left to the right ventricle. TT_{power} is closely associated with LV systolic function during disease progression, and corresponds to the loss of JP-2 expression. Moreover, in vitro gene silencing experiments show that JP-2 knockdown reduces t-tubule integrity in cultured ventricular myocytes. These data support the hypothesis that t-tubule remodeling is a critical early event marking the transition from compensated hypertrophy to decompensated HF during the progression of HF.

Methods

Animal experiments were performed according to the protocol approved by the University of Iowa Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (~60 gram) were subjected to pressure overload by TAB surgery [19]. Transthoracic

echocardiograms were performed in the University of Iowa Cardiology Animal Phenotyping Core Laboratory, using a Sonos 5500 Imager (Phillips Medical Systems, Andover, MA) 20. In situ confocal imaging of epicardial myocyte t-tubule structure on intact heart, power spectrum analysis of t-tubule structure, western blotting protein quantification, JP-2 knockdown experiments in cultured mouse adult myoctes were described in detail in Online Supplemental Materials.

Results

Echocardiographic assay of cardiac function

TAB rats developed hypertrophy or HF at around 8-12 weeks, as determined by echocardiography (Online Table I). TAB rats with compensated hypertrophy exhibited significant increases in heart weight (HW), heart weight to body weight ratio (HW/BW), LV mass, LV mass to body weight ratio (LVmass/BW), lower end diastolic volume to LV mass ratio (Vol/Mass), but normal end diastolic volume (EDV), end systolic volume (ESV) and highly comparable ejection fraction (EF), in comparison to those of sham operated control rats. In contrast, HF rats displayed enlarged hearts with markedly higher HW/BW ratio and Vol/Mass ratio, massive increase in end systolic and diastolic volumes, and therefore significant reduction in EF (52.8±2.3%, N=14 HF rats vs 81.8±1.2%, N=7 sham rats, $p<0.001$). Figure 1A shows typical echocardiographic images at end systole and end diastole from different stages of TAB rats and sham control. We divided HF rats into early and advanced HF groups: those rats with EF higher or equal to the median EF (54.4%) were assigned to early HF group (with a mean EF=59.4±1.8%, N=7), and those rats with EF lower than the median EF were assigned to advanced HF group (with a mean of 45.0±4.4%, N=7) (Figure 1B).

In addition, pericardial effusion was often observed in HF rats (7 out of 29 TAB rats). Pulmonary edema was apparent in HF but not in hypertrophy rats, as the lung weight to body weight ratio was significantly increased in failing rats (11.1±1.0 mg/g, ranging from 5.5 to 16.2 mg/g, N=14 HF rats verse 4.4±0.1 mg/g, N=7 hypertrophy rats and 4.3±0.2 mg/g, N=5 sham rats, $p<0.001$ between HF and hypertrophy or between HF and sham group). Furthermore, there was a significant difference in lung weight to body weight ratio between early HF and advanced HF group (8.4±1.0 mg/g vs 13.8±0.7 mg/g, $p<0.01$).

In situ t-tubule imaging in Langendorff perfused hearts

We performed in situ t-tubule imaging on intact hearts using a Langendorff perfusion system adapted to laser scanning confocal microscopy. This approach provides high resolution imaging of myocytes within ~70 μm depth of the epicardium. Each frame covered a 202×202 μm^2 area and contained 10-20 well-demarcated myocytes (Figure 2A). Figure 2B shows a 3D image of the t-tubule system reconstructed from 25 stacks of t-tubule images acquired sequentially at 0.2 μm interval. This in situ imaging of t-tubule system on Langendorff-perfused intact hearts avoids possible damage of myocyte membranes and biased loss of cardiac myocytes during enzymatic isolation (see discussion), providing a novel, high resolution imaging assay for studying the t-tubule system in normal and diseased hearts.

The most important characteristics of myocyte t-tubule system are the highly organized, periodic striations with almost identical spacing (~2 μm distance). Figure 2C shows an example of power spectrum analysis of t-tubule signals from Figure 2A. The dominant frequency at ~0.5 μm^{-1} corresponds to the anticipated spatial distance of ~2 μm 12, representing t-tubule spacing in intact heart. We used the peak power value (in arbitrary unit, a.u.) at the dominant frequency as a quantitative index of t-tubule integrity (TT_{power}).

On average, TT_{power} of sham-operated healthy hearts was 2.08±0.03 (N=7 hearts). In contrast, atrial cells from control rat hearts, which had sparse irregular t-tubule system (both left and right atria), did not show a dominant peak in FFT power spectrum (Online Figure I).

Impairment of T-tubule integrity begins before LV systolic dysfunction

We next sought to determine the stage at which t-tubules begin to remodel after TAB, and the features of early t-tubule remodeling. Surprisingly, t-tubule remodeling began early at the compensated hypertrophy stage prior to LV dysfunction (Figure 3B, EF=77%). At the onset, this process was manifested as subcellular t-tubule loss limited to discrete local regions in some LV myocytes (arrows in Figure 3B). By using TT_{power} as a sensitive method to quantify the overall t-tubule integrity, we were able to detect subtle t-tubule structural reorganization, even in those with no visually discernible change in the regularity of t-tubule network (Figure 3B). Specifically, this reorganization was detected as a significant reduction in the average TT_{power} from in situ LV images (Figure 3E, 2.08±0.03, N=7 / sham LV versus 1.66±0.07, N=12 hearts / hypertrophy LV, $p<0.01$) (see also below and Figure 5). Our findings strongly suggest that t-tubule remodeling is an important early event that occurs in the LV at the stage of compensated hypertrophy, even though there is no ventricular dysfunction (as detected by echocardiography).

Progressive t-tubule deterioration during HF progression

Next we sought to investigate how t-tubule remodeling evolves during the transition from hypertrophy to HF in intact heart. Figure 3CD shows representative t-tubule images at different stages during HF progression. As the hypertrophied heart became de-compensated with compromised LV function (Figure 3C, EF=61%), the t-tubule system showed a marked increase in the irregularity of t-tubule structure. At the advanced HF stage, the t-tubule system was severely disrupted, with dramatic loss of t-tubule signals and loss of the regular striated pattern (Figure 3D, EF=49.5%). This phenomenon was consistently observed in all of the hearts examined in each group. We defined images with TT_{power} equal or less than 1.3 as having a massively disrupted t-tubule system, and compared images across different stages. We found that from sham to advanced HF the percentages of acquired images from the LV with massive t-tubule disruption were 0% (0 out of 70), 10% (13 out of 129), 76% (62 out of 81) and 86% (67 out of 78) in sham, hypertrophy, early and advanced HF, respectively. On average, TT_{power} showed a continuous, decreasing function in LV myocytes during HF development (Figure 3E). These data demonstrate that the LV t-tubule system undergoes progressive deterioration during the developmental course from hypertrophy to HF.

Delayed t-tubule remodeling in RV myocytes in pressure overload-induced cardiomyopathy

Normally, the t-tubule system of LV and RV myocytes in healthy heart appears to be indistinguishable and displays near identical TT_{power} (Figure 3A and 4A). Pressure overload induced cardiac hypertrophy due to TAB starts from the LV. During the compensated hypertrophy stage, LV myocytes showed loss of t-tubules with significantly reduced TT_{power}, but the t-tubule structure in RV myocytes remained unaffected (Figure 4AB). This indicates differential properties between LV and RV t-tubule remodeling in response to pressure overload at the hypertrophy stage. Interestingly, with the progression of disease t-tubule remodeling spreads from the LV to the RV. Figure 4 displays RV t-tubule images from hearts at different stages. As shown in Figure 4C, RV myocyte loss of t-tubules and their regular organization is first detectable at the early HF stage. However, at the advanced stage of HF (Figure 4D), the RV myocyte t-tubule system also showed severe disruption, similar to that seen in the LV. The percentages of RV t-tubule images with massive disruption (TT_{power}≤1.3) were 0% (1 out of 75), 1% (2 out of 136), 33% (24 out of 72) and

82% (62 out of 76) in sham ($p>0.05$ vs LV sham, Chi-test), hypertrophy ($p<0.001$ vs LV hypertrophy), early HF ($p<0.001$ vs LV early HF) and advanced HF ($p>0.05$ vs LV advanced HF), respectively. The summary data of TT_{power} (Figure 4E) further support the notion of differential temporal remodeling of the RV t-tubule system during the progression of TAB-induced HF.

Global reorganization of t-tubule system in TAB induced hypertrophy and HF

The fact that a fraction of myocytes display massive t-tubule disruption during the progression of TAB-induced hypertrophy and HF raises an intriguing question: is there a subset of myocytes that are more vulnerable to the pressure overload stress? Or all myocytes are globally affected by the stress, in terms of t-tubule remodeling? To discriminate between these two possibilities, we analyzed the histogram distribution of TT_{power} from individual images of both LV and RV at different disease stages.

Under control conditions, TT_{power} in either LV or RV myocyte populations exhibited a single-mode (centered at 2.0-2.3), bell-shaped distribution (Figure 5A&E), suggesting that TT_{power} from all myocytes belong to one single population in the unstressed hearts. After the TAB stress, the entire histogram of LV TTpower shifted leftward in hypertrophied hearts. (centered between 1.7-2.0, Figure 5B). Nevertheless, the histogram retained its single-mode configuration, suggesting that the entire population of myocytes undergo t-tubule remodeling in a similar fashion. In particular, it is noteworthy that the highest LV TT_{power} (≥ 2.6) was decreased from 16% in sham to 1% in the hypertrophic hearts (Figure 5B). These data strongly suggest that, in addition to overt subcellular loss of t-tubules in a fraction of myocytes, the entire population of epicardial myocytes undergoes global t-tubule remodeling (in a manner that may not be detectable visually) early at the hypertrophy stage in response to TAB stress. As the disease progressed to early and advanced HF, we found that the TT_{power} histogram further shifted leftward while retaining a single mode of distribution in the LV (Figure 5C&D), further supporting the notion that the entire myocyte population examined behaves similarly during the progression of cardiomyopathy. Similar results were obtained with RV myocytes, except that the leftward shift of TT_{power} histogram in RV showed a clear delay than that of LV myocytes (Figure 5E&F). Taken together, these findings demonstrate global t-tubule structure reorganization at hypertrophy (LV) and HF stages (LV and RV) in myocyte response to pressure overload stress.

T-tubule remodeling correlates with LV function

The role of the t-tubule system in EC coupling, particularly in triggering synchronous SR Ca^{2+} release and the consequence of t-tubule remodeling in isolated failing myocytes (e.g., dys-synchrony of SR Ca^{2+} release) are well recognized 11¯15. However, the functional relationship between t-tubule structure and global LV systolic function is not well understood. We examined the correlation between the LV mean TT_{power} (which represents the overall t-tubule status of myocardium, assuming the mid- and endocardium myocytes share similar patterns in t-tubule remodeling during HF progression) with the corresponding cardiac morphometrics and performance. We found that LV TT_{power} displayed a strong negative correlation ($R^2=0.80$, $p<0.001$) with HW/BW ratio (Figure 6A), an important index of cardiac hypertrophy, suggesting t-tubule remodeling is closely associated with the development of hypertrophy. Moreover, we found that LV TT_{power} correlated with LV EF in a positive linear function ($R^2=0.68$, $p<0.001$, Figure 6B), suggesting that such t-tubule remodeling exerts an adverse effect on contractile function. Furthermore, we showed that the relationship between LV TT_{power} and end systolic volume (ESV, $R^2=0.57$, $p<0.001$, Figure 6C), and end diastolic volume (EDV, $R^2=0.38$, $p<0.001$, Figure 6D) was nicely fitted with a power function. These results reveal a heretofore unappreciated structure-function

correlation between subcellular t-tubule remodeling and whole-animal cardiac performance during disease development.

JP-2 down-regulation in TAB hearts

To investigate the molecular mechanism of t-tubule remodeling in pressure overload cardiomyopathy, we examined the protein level of JP-2, a member of the junctophilin protein family important for the formation of t-tubule – SR junctions 21, in LV tissues from frozen hearts. There was a progressive decrease in JP-2 protein level in LV tissues, starting from hypertrophy stage (Figure 7AB).

To examine if JP-2 loss contributes to t-tubule remodeling observed in pressure overload disease, we used recombinant lentivirus carrying JP-2 shRNA to silence JP-2 expression in cultured adult mouse ventricular myocytes. After 64 hours of transfection, JP2-shRNA knocked down the expression level of JP-2 to 51% of that of myocytes transfected with control vectors containing a scrambled shRNA sequence (control) (Figure 8AB). JP-2 shRNA infected myocytes displayed a significant reduction in t-tubule power compared to the control group (Figure 8CD, n=43 and 57 cells for scramble control and JP-2 shRNA groups, respectively, $p<0.01$), with a leftward shift in the t-tubule power histogram (Figure 8EF). These data indicate that JP-2 downregulation may be involved in t-tubule remodeling in pressure overload cardiomyopathy.

Discussion

In the present study, using TAB rat model in combination with state-of-the-art confocal imaging technique and a novel method to analyze t-tubule integrity, we examined the developmental changes of myocyte t–tubule system during the progression of hypertrophy and HF. The major findings of our study are as the following: 1) Myocytes at the compensated hypertrophy stage already display disruptive t-tubule remodeling, characterized by discrete local t-tubule loss and global t-tubule structural reorganization; 2) This deleterious structure remodeling progressively worsens as the disease develops, with massive t-tubule disruption at advanced HF; 3) A differential t-tubule remodeling process was observed between LV and RV myocytes; 4) T-tubule structural remodeling is highly correlated with the degree of hypertrophy; 5) The severity of t-tubule disorganization is strongly correlated with global LV function and loss of JP-2 expression.

In situ confocal imaging of t-tubule structure

The t-tubule system is a specialized membrane network, playing important roles in membrane excitation, SR Ca^{2+} release activation, muscle contraction, and signal transduction 22. Early studies on t-tubule structure were based on electron microscopy (EM). EM studies with high spatial resolution have provided important information about t-tubule structure in normal myocardium 23¯24, and in hypertrophied heart 25. However, the sophisticated EM technique cannot provide a complete picture of the t-tubule system. Recently, several groups have used confocal microscopy to investigate t-tubule structure in health and disease 11¯15, 26¯33. All these studies were performed in single isolated myocytes. Enzymatic dissociation of myocytes may impair the t-tubule membrane of healthy cells 34. It is also very likely that those myocytes with severely damaged t-tubule membrane may be more fragile due to enzyme digestion, mechanical stirring, Ca^{2+} unloading and reloading during myocyte isolation process 35. Thus, it is possible that enzymatic isolation may yield predominantly (relatively) healthy myocytes and myocytes isolated from healthy hearts may display varying degrees of t-tubule change (see Figure 2A of reference 15, by Heinzel et al, 2008). Taken together, these factors intrinsic to the isolation of cardiomyocytes are an obstacle to identifying subtle changes in t-tubule

membrane structure, as we observed here in hypertrophied hearts. Membrane damage due to cell isolation techniques could explain the inconsistent results reported previously in human failing hearts 29, 33. In this study, we examined myocyte t-tubule structure using a novel in situ confocal imaging technique combined with Langendorff-perfused hearts. In doing so, we were able to avoid damage to the t-tubule system linked to myocyte dissociation. Additionally, this technique allows us to visualize a large number of myocytes collectively and to measure myocytes from different epicardial regions in situ. It also allowed measurement of early, subtle alterations and severe disruption of t-tubule structure (Figures 3 and 4).

(Ultra)-structural T-tubule remodeling in heart diseases

In animals, He et al. first identified a significant loss of t-tubule density (without t-tubule disorganization) in ventricular myocytes from a dog model with pacing-induced HF 28. Subsequently, other studies (including ours 12) using other animal HF models have reported profound t-tubule remodeling (loss and/or disorganization) in single isolated failing myocytes. These other animal models include spontaneous hypertensive rats with overt HF 12, mouse myocardial infarction 13, pig myocardial infarction 15, sheep rapid pacing HF model 16. In human, early reports with histological examination in failing heart tissue sections showed t-tubule dilation with an increase 36 or decrease 37 in the density of t-tubules. Other preliminary reports also presented inconsistent findings 29, 33. A very recent study using both scanning ion conductance microscope and confocal microscope helped resolve the discrepancy in early reports. T-tubule loss was pronounced in failing ventricular myocytes from all HF patients (ischemic heart disease, idiopathic dilated cardiomyopathy, and hypertrophic obstructive cardiomyopathy) 14. From a spectrum of etiological settings in both human and animal models, these studies had drawn similar conclusions, indicating that t-tubule remodeling is a principal problem in many heart diseases, especially at end stage HF. However, all these studies (including those human studies and our previous 12–15, 27–29, 33) were done in myocytes at advanced HF stage. Our findings showed that t-tubule remodeling occurs prior to the onset of HF, suggesting t-tubule remodeling is not a secondary modification after HF, rather an important early event during HF progression.

T-tubule remodeling in the transition from hypertrophy to HF

The deleterious consequence of t-tubule remodeling on EC coupling in HF has been carefully explored in several elegant studies 12–13, 15. T-tubule loss and / or disorganization has been directly linked to dyssynchronous Ca^{2+} sparks, reduced and slowed Ca^{2+} transients during EC coupling in failing myocytes 12–13, 15. Using spontaneously hypertensive rats in HF, we showed that t-tubule disorganization led to an increase in orphaned RyRs and loss of local control of CICR, resulting in decreased EC coupling efficacy and increased dyssynchrony of SR Ca^{2+} release in failing myocytes. A recent study from Sipido's group reported similar findings in an ischemic cardiomyopathy pig model where t-tubule loss was associated with reduced synchrony of Ca^{2+} release and reduced efficiency of coupling Ca^{2+} influx to Ca^{2+} release 15. Very recently, t-tubule remodeling and Ca^{2+} release dysfunction were also found in atrial myocytes from a rapid-pacing induced sheep HF model 16. These experimental findings were also supported by computer modeling, in which t-tubule reorganization can reduce the synchrony of Ca^{2+} spark production, lead to the appearance of late Ca^{2+} sparks and greater nonuniformity of intracellular Ca^{2+} 38.

It is surprising that hypertrophied heart with normal global function already showed some degree of t-tubule remodeling. This might be related to large cardiac reserve function: no apparent cardiac functional phenotype even when structural remodeling costs ever diminishing cardiac reserve function. Specifically, cardiac hypertrophy in response to pressure overload involves many compensatory, molecular and biochemical mechanisms

39-40 that may account for compensating and maintaining normal heart function during hypertrophy stage. For example, increase in cell size and in the number of sarcomere units 41-42 (originated from those molecular and biochemical signaling alterations), may also contribute to compensating the negative effect of t-tubule loss on SR Ca^{2+} release and myocyte contractility, resulting into a maintained myocardial function in hypertrophy. However, these compensating mechanisms ultimately are apparently ineffective for balancing the defects caused by more severely damaged t-tubule system as structural heart disease progresses. The progressive deterioration of t-tubule structure throughout the disease development, and the strong correlation between t-tubule remodeling and LV function support the notion that t-tubule remodeling is a critical factor during the transition from compensated hypertrophy to HF.

Molecular mechanisms underlying t-tubule remodeling in TAB induced cardiomyopathy

JP-2 is a member of the junctophilin protein family important for the formation of junctional membrane complex (i.e., the cardiac dyad) between t-tubule membrane and SR 21, 43-45. JP-2 mutations were associated with human hypertrophic cardiomyopathy 46-47 and JP-2 knockout mice have defective cardiac dyads, altered intracellular calcium transients, and embryonic lethality 21. JP-2 mRNA expression was reduced in hypertrophied rats 48 and mouse cardiomyopathy model 49, but it was unknown if JP-2 expression is affected in accord with t-tubular remodeling during progression of structural heart disease. Our results show that t-tubule remodeling correlates with the loss of JP-2 expression in left ventricular myocytes of pressure overload model and that JP-2 knockdown (by 50%) reduces t-tubule integrity in cultured myocytes, suggesting that reduced JP-2 expression may be important in mediating t-tubule remodeling in pressure overload cardiomyopathy. However, it's recognized that there is no report about the heterozygous phenotype of JP-2 mutants 21. Careful examination of JP-2 heterozygous mice (at rest and under stress) and, more ideally, future studies with inducible JP-2 knockout / knockdown mice should provide further critical information on JP-2 functions in cardiac health and disease.

In summary, for the first time we identified t-tubule adverse remodeling in intact hearts during compensated hypertrophy stage, and mapped the t-tubule change to the progression of myocardial disease in a temporally and functionally defined disease model. T-tubule disruptive remodeling is a critical event during the development of hypertrophy that are tightly associated with the declining of myocardial function and transition from compensated hypertrophy to HF.

Novelty and Significance

What is known?

- T-tubule system is an organized membrane network important for normal cardiac excitation-contraction coupling.
- End-stage heart failure of animal models or human patients associated with T-tubule remodeling / disorganization.
- T-tubule remodeling causes Ca^{2+} release and cell signaling dysfunction in isolated cardiomyocytes.

What new information does this article contribute?

- The first detailed methods for *in situ* t-tubule imaging on intact heart and quantitative analysis of t-tubule structure.
- T-tubule alteration starts early in compensated hypertrophy stage, featuring discrete local loss and global reorganization of the affected myocardium.

- T-tubule remodeling gradually deteriorates over the progression of heart disease, correlates with the severity of cardiac hypertrophy and predicts heart function.
- T-tubule remodeling represents a key mechanism underlying the transition from compensated hypertrophy to heart failure.
- Junctophilin-2 (JP-2) downregulation is likely responsible for t-tubule remodeling in cardiomyopathy.

Summary

T-tubule system is a highly organized membrane network in working mammalian ventricular myocytes. The maintenance of this specialized membrane structure is critical for normal contractile function and the pumping capability of the beating heart. Reduction in t-tubule density and organization has been linked to abnormal Ca^{2+} function and decreased contractility in end-stage heart failure. However, it is unknown whether t-tubule alterations are an early or late event in heart failure. The lack of studies of t-tubule changes prior to hear failure onset is a critical knowledge gap in understanding heart failure. It is also not clear whether changes in t-tubule structure are related to cardiac function during progression from hypertrophy to heart failure. In this study, we provided evidence showing that t-tubule remodeling is an important early event during heart failure progression, starting at compensated hypertrophy stage. Our data support the hypothesis that t-tubule remodeling may constitute an important mechanism underlying the transition from compensated hypertrophy to decompensated heart failure, indicating that preventing t-tubule remodeling during the hypertrophy stage may be clinically important for delaying progression to heart failure. Moreover, we present evidence linking for the first time a single molecule (JP-2) to reduced t-tubule integrity during heart failure. These findings suggest -new therapeutic strategies for the treatment of heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

t-tubule	Transverse-tubule
HF	Heart Failure
TAB	Thoracic aortic banding
TT_{power}	t-tubule power

LV	left ventricular / ventricle
RV	right ventricular / ventricle
JP-2	junctophilin-2
SR	sarcoplasmic reticulum
RyR	ryanodine receptor
EF	ejection fraction
ESV	End systolic volume
EDV	End diastolic volume
HW/BW	heart weight / body weight ratio

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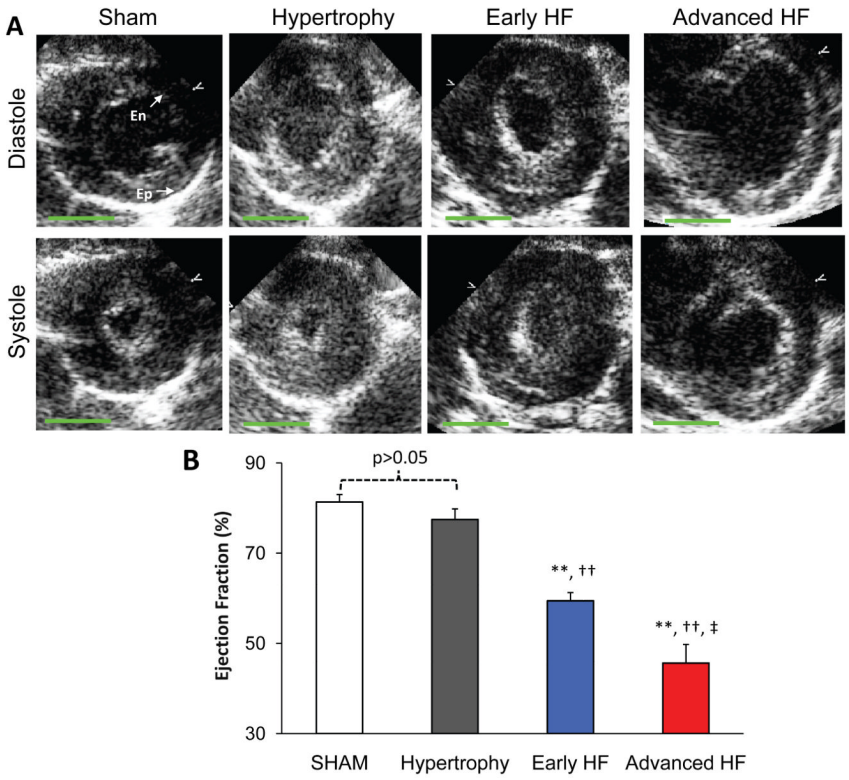

Figure 1. Echocardiographic results of TAB rat heart function

A. Representative echocardiographic images at end diastole and end systole from sham, hypertrophy, early HF and advanced HF rats, respectively. At advanced HF stage, the heart was dilated with severe systolic dysfunction. **B**. Summary data of EF from different groups. Note: En stands for endocardial border; Ep for epicardial border. **, $p<0.01$ vs sham; †, $p<0.05$; ††, $p<0.01$ vs hypertrophy; ‡, $p<0.05$; ‡‡, $p<0.01$ vs early HF. Green bar=5 mm.

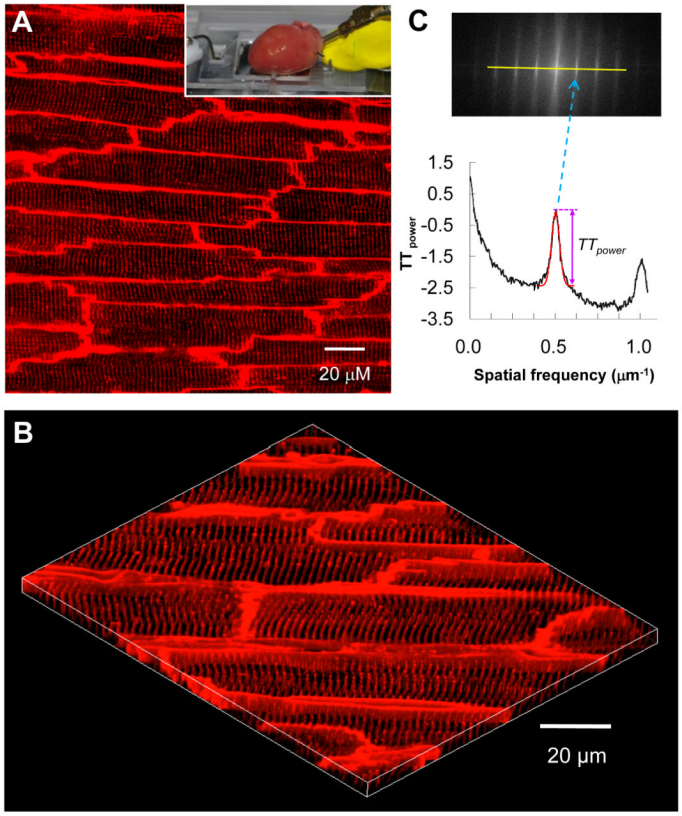

Figure 2. In situ confocal imaging of t-tubules on intact rat heart and t-tubule image analysis

A. A typical t-tubule image from the epicardium of Langendorff-perfused intact rat heart (control, sham-operated) loaded with lipophilic membrane indicator FM4-64. The inset illustrates a Langendorff-perfused heart attached to the inverted Zeiss 510 confocal microscope system. **B.** The periodically organized t-tubules were viewed from a 3D reconstruction of 25 confocal stacks at 0.2 μm interval. Scale bar: 20 μm. **C.** Power spectrum retrieved from a 2D Fourier transformation of confocal t-tubule image characterized the power magnitude of the regular organization of t-tubule system, in which the first peak located at spatial frequency of ~0.5 μm^{-1} corresponded to the ~2 μm interval of t-tubules. TT_{power} was measured as an absolute value using Gaussian fitting around the first peak (the red dashed curve).

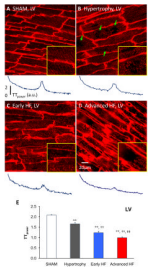

Figure 3. Progressive t-tubule remodeling of LV myocytes in TAB rat cardiomyopathy

Representative t-tubule images from LV of age-matched sham operated heart (**A**), hypertrophy (**B**), early HF (**C**) and advanced HF (**D**). The bottom panels showed the power spectrums obtained from 2D Fourier transform of the raw images above. At hypertrophy stage (**B**), discrete T-tubule loss (green arrows) was often observed with slight t-tubule disorganization, which caused a mild decrease in TT_{power}. In moderately de-compensated heart (**C**), LV myocytes exhibited widely impaired t-tubule system and further decrease in TT_{power}. At advanced HF stage (**D**), myocytes lost majority of t-tubules with striated pattern almost vanished, resulting in severe reduction in TT_{power}. Each yellow-framed inset is a zoom-in view of an area 40×40 μm from associated images. **E**. Summarized data of LV myocyte t-tubule power (N=7, 12, 7, 7 hearts for sham, hypertrophy, early HF and advanced HF, respectively). Note: **, $p<0.01$ vs sham; †, $p<0.05$; ††, $p<0.01$ vs hypertrophy; ‡, $p<0.05$; ‡‡, $p<0.01$ vs early HF.

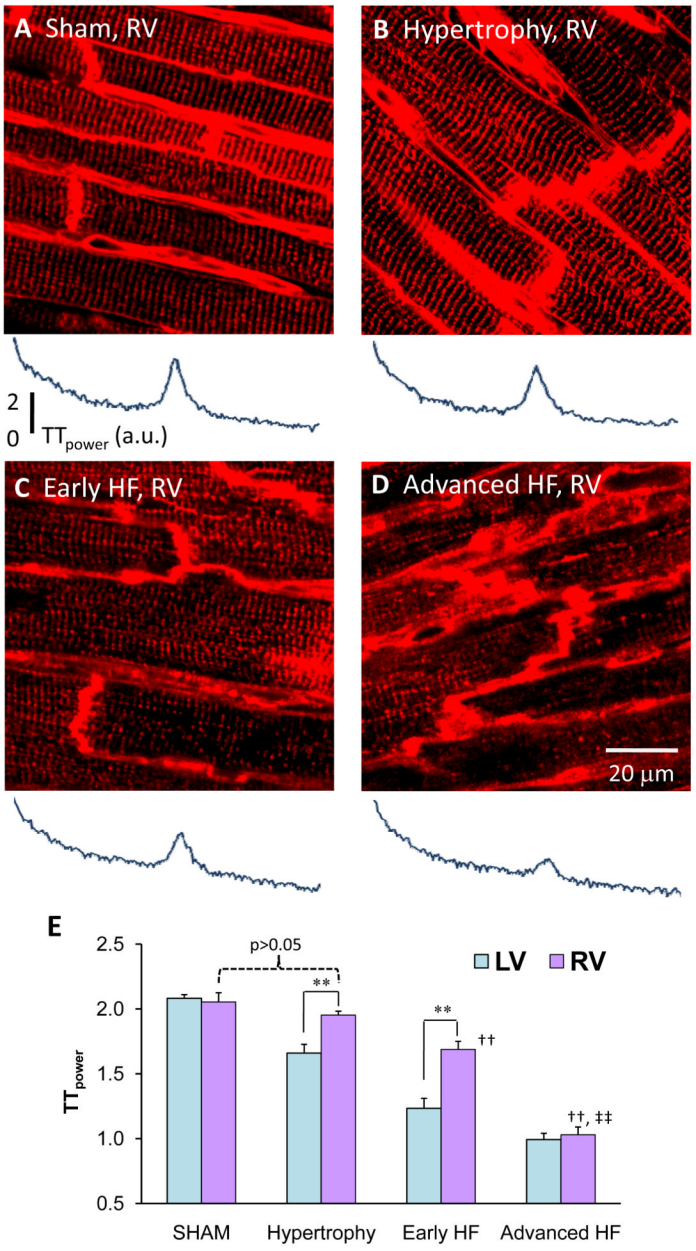

Figure 4. Delayed t-tubule remodeling in RV myocytes

Representative images acquired from RV epicardium at sham (**A**), hypertrophy (**B**), early HF (**C**) and advanced HF (**D**) stages. RV myocytes are highly comparable to those of LV in t-tubule structure organization (Figures 4A and 3A). Different from LV t-tubule remodeling (Figure 3B), RV myocytes remained unaffected in t-tubule structure at hypertrophy stage (4B). At early HF stage, RV myocytes started to lose t-tubules and their regular organization (4C), similar to that of LV myocytes at hypertrophy (3B). At advanced HF stages, both LV and RV myocytes were severely damaged (3D & 4D). Bottom panels beneath each image are corresponding power spectrum results. **E**. Comparison of the TT_{power} from LV and RV myocytes. RV myocytes from hypertrophy hearts had similar TT_{power} as RV of sham control (2.05±0.07, N=7 sham vs 1.95±0.03, N=12 hypertrophy, p>0.05), but had greater power than hypertrophied LV (1.66±0.07, p<0.01). At early HF stage, RV myocytes also had larger TT_{power} than LV (RV 1.69±0.06 vs LV 1.23±0.08, N=7, p<0.01). Note: **, p<0.01 for

comparison between LV vs RV; ††, $p<0.01$ vs hypertrophy (RV); ‡‡, $p<0.01$ vs early HF (RV).

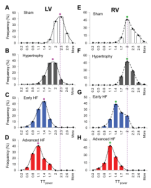

Figure 5. Histogram distribution of LV and RV myocyte TT_{power}

A-D (LV), The count numbers at each bin size were normalized by the total images of each group and were shown as frequency. Each panel represents over a thousand of myocytes. An unambiguous, global population shift as well as mode shift was present from sham (**A**) to advanced HF (**D**), indicating global t-tubule structure reorganization occurred in both hypertrophy and HF stages. **E-H (RV)**, Different from those of LV myocytes, the mode of RV TT_{power} distribution and global population were not shifted at hypertrophy stage (**E**&**F**). At early (**G**) and advanced HF (**H**) stages, a gradual leftward shift in RV TT_{power} distribution were observed, similar to that of LV myocytes.

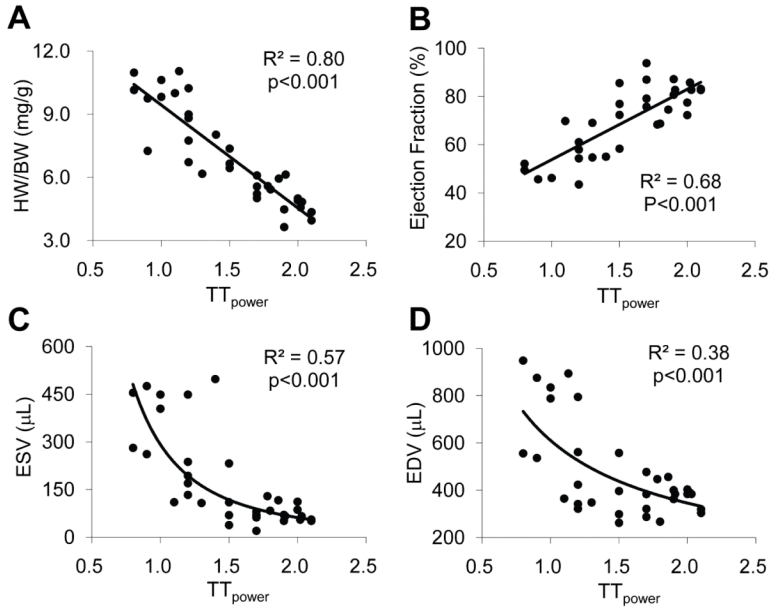

Figure 6. Correlation between TT_{power} and LV function

The LV TT_{power} was in good linear correlation with HW/BW ratio (R^2=0.80, p<0.001) (**A**) and EF (R^2=0.68, p<0.001) (**B**). Moreover, the LV TT_{power} was well fitted in power function with end systolic volume (ESV) (R^2=0.57, p<0.001) (**C**) and end diastolic volume (EDV) (R^2=0.38, p<0.001) (**D**).

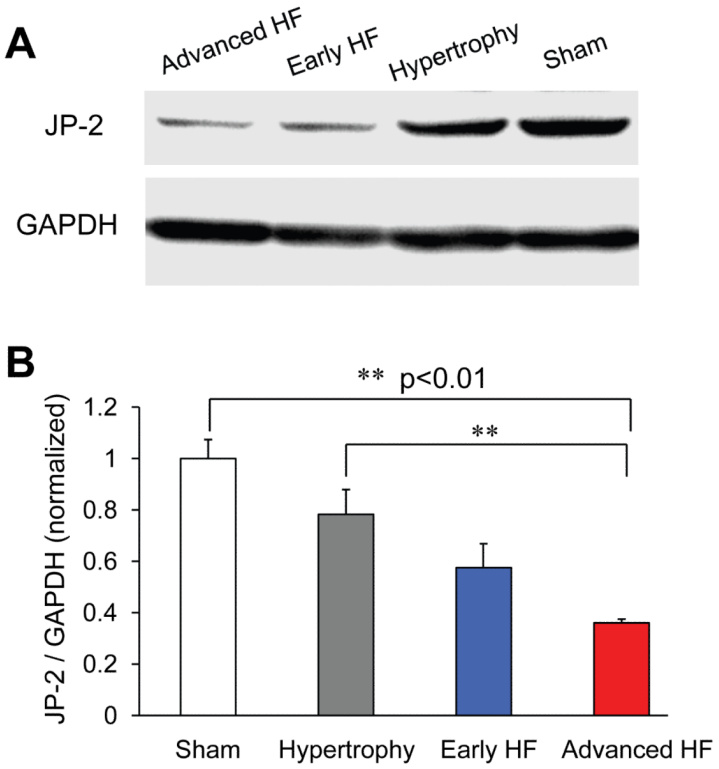

Figure 7. Junctophilin-2 (JP-2) down-regulation in TAB rats

A, representative examples of JP-2 protein Western Blotting from LV frozen tissues at different disease stages and sham control. **B**, Summary results of normalized JP-2 protein expression level, showing a progressively reduction in JP-2 protein level in rats in response to pressure overload stress (N=5, 4, 2, 3 hearts for sham, hypertrophy, early HF and advanced HF, respectively).

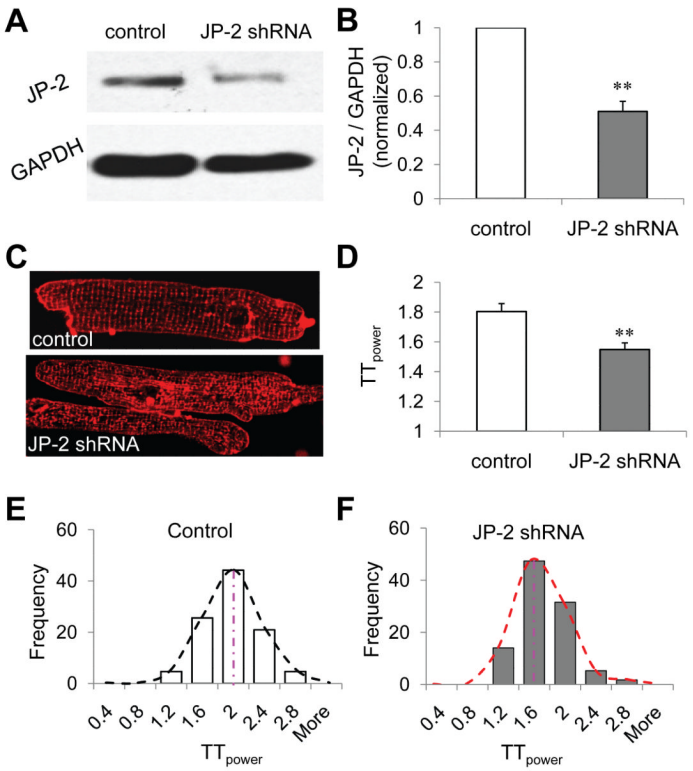

Figure 8. JP-2 knockdown caused a significant reduction in TT_{power} in cultured mouse ventricular myocytes

A&B, JP-2 shRNA (64 hrs) knocked down the protein expression level of JP-2 by 49% (n=4, 4 for both control and JP-2 shRNA groups, $p<0.01$). **C&D**, JP-2 shRNA transfected myocytes had worse t-tubule organization and a significant reduction in t-tubule integrity than control ($p<0.01$). **E&F**, JP-2 shRNA knockdown shifted the TT_{power} histogram leftward, in comparison with that of scrambled control shRNA infected myocytes (n=57 and 43 cells, respectively).