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Remodeling of the Sarcomeric Cytoskeleton in Cardiac Ventricular Myocytes during Heart Failure and after Cardiac Resynchronization Therapy

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Abstract

Sarcomeres are the basic contractile units of cardiac myocytes. Recent studies demonstrated remodeling of sarcomeric proteins in several diseases, including genetic defects and heart failure. Here we investigated remodeling of sarcomeric α -actinin in two models of heart failure, synchronous (SHF) and dyssynchronous heart failure (DHF), as well as a model of cardiac resynchronization therapy (CRT). We applied three-dimensional confocal microscopy and quantitative methods of image analysis to study isolated cells from our animal models. 3D Fourier analysis revealed a decrease of the spatial regularity of the α -actinin distribution in both SHF and DHF versus control cells. The spatial regularity of α -actinin in DHF cells was reduced when compared with SHF cells. The spatial regularity of α -actinin was partially restored after CRT. We found longitudinal depositions of α -actinin in SHF, DHF and CRT cells. These depositions spanned adjacent Z-disks and exhibited a lower density of α -actinin than in the Z-disk. Differences in the occurrence of depositions between the SHF, CRT and DHF models versus control were significant. Also, CRT cells exhibited a higher occurrence of depositions versus SHF, but not DHF cells. Other sarcomeric proteins did not accumulate in the depositions to the same extent as α -actinin. We did not find differences in the expression of α -actinin protein and its encoding gene in our animal models. In summary, our studies indicate that HF is associated with two different types of remodeling of α -actinin and only one of those was reversed after CRT. We suggest that these results can guide us to an understanding of remodeling of structures and function associated with sarcomeres.

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Disclosures

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Keywords

Heart failure; cardiac resynchronization therapy; remodeling; cardiac recovery; alpha actinin

1 Introduction

The cytoskeleton is a complex intracellular network of proteins essential for determining the shape and mechanical properties of cells [1]. It also helps to coordinate the function of subcellular proteins in all cell types. These functions range from anchoring cellular organelles such as the Golgi apparatus, nuclei and mitochondria to transmission of extracellular signals and coordinating contraction [1, 2]. Cytoskeletal proteins form the sarcomeres, which are the basic contractile units of striated muscle cells. Within each sarcomere is a complex arrangement of proteins [2–4]. Each sarcomere is bounded by the Z-disk [5]. The Z-disk is crucial for maintaining sarcomeric structure and function. The main component of the Z-disk is the protein α -actinin, even though it accounts for less than 20% of Z-disk weight [4]. Alpha-actinin is an anti-parallel homodimer that anchors the actin filaments, which are essential for contraction in myocytes. It was initially believed that α -actinin solely provided an actin binding site [6]. However, subsequent studies demonstrated that α -actinin links to several transmembrane receptors, regulatory proteins, adherens junctions, focal adhesion sites and stress fibers [6]. Further studies revealed that α -actinin plays a pivotal role in the assembly of sarcomeres and the regular arrangement of myofilaments [7, 8]. The studies suggest that sarcomere assembly is initiated by small Z-bodies comprising complexes of α -actinin and associated proteins. Subsequently, the Z-bodies expand, fuse and align in Z-bands. Similar as other proteins in the Z-disk α -actinin exhibits a surprisingly dynamic exchange with the cytosolic pool [8]. Exchange rates were larger in Z-bodies than Z-disks indicating that molecular interactions increase the stability of Z-disks. Four different isoforms of α -actinin exist. The genes ACTN-1 and ACTN-4 encode α -actinin isoforms that are expressed in non-muscle cells, where these isoforms of α -actinin contribute to the actin cytoskeleton. ACTN-2 and ACTN-3 encode isoforms specific to the Z-disks of sarcomeres found in striated muscle fibers [4, 9–11], with α -actinin-2 being the only cardiac specific isoform [4].

Remodeling of sarcomeric proteins in cardiac disease has been implicated in reduced ventricular function [2]. In particular, it has been suggested that the transition from hypertrophy to heart failure (HF) occurs in two consecutive stages. The first stage is reversible and involves an accumulation of cytoskeletal proteins to counteract the increased strain imposed on the myocardium. The latter stage becomes irreversible and is characterized by a loss of contractile filaments and crucial sarcomeric proteins, including α -actinin, titin, and myomesin. Several cardiac diseases have been associated with remodeling of α -actinin and the Z-disk [9–12]. Melo et al. demonstrated that *Trypanosoma cruzi* in mouse myocytes caused α -actinin distributions to lose their periodic structure and to localize to focal adhesion sites [11]. Hein et al. found depositions of α -actinin-1 in failing myocardium [10], while Oxford et al. showed an accumulation of electron dense material around Z-lines in canines with arrhythmogenic cardiomyopathy. Cardiac disorders have also

been linked to mutations in the gene responsible for α -actinin-2, specifically dilated cardiomyopathy [13] and hypertrophic cardiomyopathy [9].

Here we investigated remodeling of α -actinin in two models of HF, synchronous (SHF) and dyssynchronous heart failure (DHF), as well as in a model of cardiac resynchronization therapy (CRT). Approximately 40% of patients suffering from HF develop delays of ventricular electrical activation that result in a dyssynchronous mechanical contraction of the ventricles. The electrical dyssynchrony results in a widened QRS interval, which has been shown to be an independent predictor of mortality and sudden cardiac death [14]. CRT is an established clinical therapy to treat DHF. CRT resynchronizes ventricular mechanical and electrical activity via biventricular pacing and has been proven effective at improving quality of life and reducing mortality in about 55% of patients [15]. A number of studies showed that CRT is associated with restoration of cardiac structure and function, for instance, the transverse tubular system and excitation-contraction coupling [16] as well as the electrophysiological and contractile properties of myocytes [17, 18]. However, little is known about the reorganization of sarcomeric structures and associated protein distributions in DHF and CRT.

Our hypothesis is that DHF associated remodeling of sarcomeric organization is reversed after CRT. We used α -actinin as a marker of sarcomeric organization. We applied high-resolution three-dimensional confocal microscopy to image α -actinin distributions in ventricular tissues and cells. Analyses of image stacks allowed us to provide quantitative data on the structural arrangement of α -actinin and its remodeling. Our studies revealed alterations of the spatial regularity of α -actinin. We applied Fourier analysis to characterize remodeling of the spatial arrangement of α -actinin. Also, our studies revealed longitudinal depositions of α -actinin in HF. Using methods of pattern detection we quantified their occurrences. For further insights into the composition of the longitudinal depositions we investigated colocalization of α -actinin with sarcomeric proteins and measured the density of α -actinin based on super-resolution microscopy. To shed light on α -actinin expression in our animal models we performed gene expression analyses and Western blotting.

2 Methods

2.1 Animal Model, Tissue and Isolated Cell Preparation

All procedures involving the handling of animals were approved by the Animal Care and Use Committees of the Johns Hopkins University and the University of Utah. Protocols complied with the published *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health.

The applied animal models have been described previously [16, 19–21]. In previous studies, successful implementation of the DHF and CRT models was confirmed by increased and normalized QRS duration, respectively (Table S1) [17]. In brief, adult male mongrel dogs were used as control and models of SHF, DHF and CRT. SHF and DHF animals underwent right atrial pacing for 6 weeks.

DHF was caused by left bundle-branch radiofrequency ablation. CRT animals underwent left bundle-branch radiofrequency ablation and 3 weeks of right atrial pacing followed by 3 weeks of resynchronization via biventricular pacing. The pacing rate for SHF, DHF and CRT animals was 180 to 200 bpm. Hemodynamic data were measured at the time of explantation (Table S2).

Lateral and anterior tissue samples from left ventricular subepicardial myocardium were taken shortly after explantation of the heart. Tissue was fixed with paraformaldehyde for confocal imaging or flash frozen in liquid nitrogen for western blot analysis. Cardiac myocytes were isolated enzymatically via retrograde Langendorff perfusion [17]. Myocytes were sampled from the anterior and lateral mid-epi myocardium of the left ventricle.

2.2 Immunohistochemistry

The sarcolemma of isolated myocytes was labeled using wheat germ agglutinin (WGA) conjugated to Alexa Fluor-555 (Invitrogen, Carlsbad, California, USA). After WGA labeling, cells were fixed and prepared for immunolabeling as previously described [16]. For studies of the subcellular distribution of α -actinin we incubated myocytes with a primary antibody of anti- α -actinin (ab9465, clone# EA-53, Abcam, San Francisco, California) diluted 1:200 in phosphate buffered saline (PBS) solution containing 2% bovine serum albumin, 2% normal goat serum and 0.05% Triton-X 100 overnight at 4°C. We then applied a secondary goat anti-mouse IgG (H+L) antibody attached to Alexa Fluor-488 (Invitrogen) for confocal microscopy or Alexa Fluor-647 (Invitrogen) for super-resolution imaging. Incubation with either secondary antibody was performed for 1 h at room temperature. For colocalization analyses of α -actinin with proteins of the sarcomeric cytoskeleton we labeled for titin (anti-titin-T12 provided by Dr. Elizabeth Ehler, King's College, London), filamentous actin (A22284, Invitrogen) and heavy chain myosin (ab15, Abcam). Each colocalization study involved staining for α -actinin and one other sarcomeric protein at a time. For dual labeling of α -actinin and titin or heavy chain myosin, we used a polyclonal rabbit anti-sarcomeric α -actinin antibody (ab137346, Abcam) instead of the EA-53 mouse monoclonal antibody. These antibodies were diluted 1:100 and incubated overnight at 4°C using the same PBS incubation solution as above. The secondary antibodies consisted of either a secondary goat anti-mouse IgG (H+L) antibody attached to Alexa Fluor-488 or 633 (Invitrogen) or goat anti-rabbit IgG (H+L) antibody attached to Alexa Fluor-488 or 633 (Invitrogen). Incubation with either secondary antibody was performed for 1 h at room temperature.

Tissue preparations were fixed in PBS containing 4% paraformaldehyde for 4 h at room temperature. The preparations were immersed in distilled water containing 30% sucrose for 2–3 d. The tissue was then flash-frozen using tissue-freezing medium (Triangle Biomedical Sciences, Durham, North Carolina, USA) at –20°C and sectioned into 80 μ m slices using a Leica CM1850 (Leica Biosystems, Wetzlar, Germany) cryostat. Afterwards tissue sections were stored in PBS, labeled with WGA and for α -actinin as described for isolated myocytes except with the incubation time for the secondary antibody prolonged to overnight at room temperature.

2.3 Confocal Imaging and Image Preprocessing

Imaging was performed using a Zeiss LSM 5 Live Duo (Carl Zeiss, Jena, Germany) confocal microscope equipped with a 63×/1.4 Numerical Aperture oil immersion objective. Tissue sections were imaged with a size of 1024×1024×~220 voxels. Voxel sizes were 0.1 μm×0.1 μm×0.1 μm. Segments of isolated myocytes from control (n=11), SHF (n=7), DHF (n=8), and CRT (n=11) animals were imaged with a size of 512×256×~256 voxels with the same resolution. We oriented image stacks so that the longitudinal axis of the cells was parallel to the y-axis of the stack (Fig. S1A). Image stacks were cropped to remove empty regions. Afterwards, image stacks were pre-processed for correction of noise, background, attenuation and convolution [16, 22, 23]. As reported previously, we found that the signal-to-noise ratio of image data decreases significantly with increasing distance from the glass slide [16]. Thus only the first 150 slices (15 μm) of each image stack were used for analysis. We used the α-actinin distribution to identify the cell interior. We dilated the α-actinin signal along the y-axis for 6 iterations followed by 10 dilation and erosion steps using a 6-neighbor mask. Subsequent analyses were restricted to the cell interior. The mask of the cell interior was used to calculate a Euclidian distance map. Here, each voxel value represented the distance to the edge of the cell.

2.4 Fourier Analysis

Images were transformed into the Fourier domain using the equation:

$$G(u, v, w) = \frac{1}{NMP} \sum_{x=0}^{N-1} \sum_{y=0}^{M-1} \sum_{z=0}^{P-1} g(x, y, z) e^{-2\pi i (\frac{xu}{N} + \frac{yv}{M} + \frac{zw}{P})}$$

Here G describes the image in the Fourier domain, while g is the image in the spatial domain. N , M and P are the number of voxels in the x-, y- and z-directions, respectively. We cropped a three-dimensional range in the Fourier domain containing only spatial frequencies 0.4 to 0.66 μm⁻¹. This section was then binned into 10° increments from 0–180° with 90° defined along the y-axis. A schematic representation of the binning process in 2D is presented in Fig. S1B. The summary intensity in each bin was calculated and normalized across all bins. The resulting histogram provides a measure for the spatial organization and regularity of image components along the specified angles. In Fig. S2 we provide examples for this analysis on four synthetically created images with specified spatial regularity. Fig. S2A represents an image with regular transverse sheets. The patterns in Figs. S2B-D become increasingly more irregular. In particular, Fig. S2D has randomly sized offsets creating a significantly irregular pattern. Fig. S2E shows the results of the Fourier analysis explained above applied to the images.

2.5 Detection of Longitudinal Depositions

Longitudinal depositions in α-actinin were detected using a series of cross-correlations and thresholding steps (Fig. S3). Cross-correlation was performed in three dimensions using the equation:

$$H(x, y, z) = \sum_{\alpha} \sum_{\beta} \sum_{\gamma} f^*(\alpha, \beta, \gamma) g(x + \alpha, y + \beta, z + \gamma)$$

where H is the resulting image, f^* is the complex conjugate of a Gaussian function f and g is the original image. The Gaussian function was defined as:

$$f(x, y, z) = e^{-\left(\frac{x^2}{2\sigma_x^2} + \frac{y^2}{2\sigma_y^2}\right)}$$

with the parameters σ_x and σ_y . We used two separate functions to extract longitudinal (along y-axis) and transversal components (along x-axis) in the image. The function for extracting longitudinal components had a σ_x of 0.1 μm and a σ_y of 1 μm . The function for extracting transversal components used a σ_x of 1 μm and a σ_y of 0.1 μm . The resulting functions were discretized into matrices with dimensions of 17, 17, and 1 in x-, y- and z-direction, respectively. Each matrix was correlated separately with the pre-processed image data yielding two image stacks: one with enhanced transversally oriented features (I_{TRANS}) and one with enhanced longitudinally oriented features (I_{LONG}). Three separate conditions were tested to identify longitudinal depositions. First, local extrema in the I_{LONG} image were detected that satisfied the following equation:

$$I_{LONG}(x, y, z) \geq MODE_{TRANS} + STD_{TRANS}$$

where $I_{LONG}(x, y, z)$ is the voxel value at the point (x, y, z) in the I_{LONG} image stack, $MODE_{TRANS}$ and STD_{TRANS} are the mode and standard deviation of the I_{TRANS} image stack, respectively. Points were then checked to satisfy the following two equations:

$$I_{LONG}(x, y, z) > I_{TRANS}(x, y, z) + STD_{TRANS} \quad I_{PRE}(x, y, z) > MODE_{PRE} + STD_{PRE}$$

where $I_{TRANS}(x, y, z)$ is the voxel value at the point (x, y, z) in the I_{TRANS} image stack while $I_{PRE}(x, y, z)$ is the voxel value at (x, y, z) in the pre-processed α -actinin image stack. $MODE_{PRE}$ and STD_{PRE} are the mode and standard deviation of the preprocessed image stack, respectively.

2.6 Super-Resolution Imaging

Super-resolution imaging of isolated cardiomyocytes was performed with a Vutara SR 200 (Vutara, Inc., Salt Lake City, UT). The imaging is based on fluorescence photoactivation localization microscopy (FPALM) [24, 25]. Cells labeled for α -actinin with Alexa Fluor-647 (Invitrogen) were immersed in photoswitching buffer. The buffer comprised of 50 mM cysteamine and oxygen scavengers (glucose oxidase and catalase) in 50 mM Tris+10 mM buffer at pH 8.0. Fluorescence was achieved using a 647 nm excitation laser and a 405 nm activation laser. Images were recorded using a 60 $\times$ /1.2 NA water immersion objective. Image resolution was 20 nm in the xy plane and 50 nm in z-direction. A confidence value

for each point was calculated as the geometric mean of the photon count and z-offset for that particular point.

To analyze these data, 16 spherical regions were visually selected in both transverse sheets and longitudinal depositions from a total of 4 SHF cells. These regions had a radius of 75 nm. The number of detected molecules (confidence>0.8) within that sphere was calculated for each region.

2.7 Colocalization of α -Actinin with Sarcomeric Proteins

Isolated myocytes labeled for α -actinin and an additional sarcomeric protein were studied using confocal microscopy as described above. Two-dimensional images with an isotropic resolution of 0.1 μm were acquired. Colocalization of α -actinin with titin, actin or myosin was measured using Pearson's correlation coefficient:

$$R_P = \frac{\sum_i (S_{1,i} - \bar{S}_1)(S_{2,i} - \bar{S}_2)}{\sqrt{\sum_i (S_{1,i} - \bar{S}_1)^2 \sum_i (S_{2,i} - \bar{S}_2)^2}}$$

with the image of α -actinin, S_1 , the image of a sarcomeric protein, S_2 , and the mean image intensity, $S_{0305;1}$ and $S_{0305;2}$, respectively. Pearson's correlation coefficient ranges between -1 and 1. A value of 1 implies an increasing linear relationship of pixel intensities in the measured images. A value of 0 indicates that pixel intensities are not linearly related. A value of -1 indicates a decreasing linear relationship. Colocalization was determined within entire imaged cell segments ($n=4$ for all models), in sarcomeres with and without longitudinal depositions of α -actinin (titin: $n=14$, actin: $n=13$, myosin: $n=6$) using Fiji with the Coloc 2 plugin [26].

2.8 Gene Expression Analysis

Gene expression data were retrieved from the Gene Expression Omnibus (GEO) database [27] (GSE14327 [1-color design data] and GSE14338 [2-color design data]). In previous work we generated these data from left ventricular subendocardial myocardium of 11 control, 10 DHF and 9 CRT canine models [28]. In brief, total RNA was isolated with TRIzol reagent (Invitrogen) from the sub-endocardium of the anterior and lateral left ventricular walls. Following a 1-color design, RNA from the anterior and lateral left ventricular myocardium was labeled with Cy3 and hybridized onto 2 separate Agilent 44K canine-specific microarrays. Studies were repeated in a subset of animals (6 control, 5 DHF, and 5 CRT canine) using a 2-color design. Corresponding anterior and lateral samples from the same heart were labeled with Cy3 and Cy5 and hybridized onto the same microarray to achieve a direct comparison of the relative gene expression in different regions of the same heart. ACTN2 and pro-atrial natriuretic factor (NPPA) were each represented on the microarray by a single specific oligonucleotide. In subsequent studies, we compared the DHF and CRT data to publicly available datasets of fetal and adult human myocardium (GEO accession numbers GSE2051, 2061, 2062). Unpaired 2-class significance analysis of microarrays (SAM) was used to determine differences in gene expression and correct for multiple testing [29]. Differences in gene expression were regarded as statistically

significant if a false discovery rate of $q < 0.05$ was achieved. Functional annotation of differentially expressed genes was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database [30] as previously described [28, 31].

2.9 Western Blotting

Tissue samples were collected from the isolated heart, flash frozen in liquid nitrogen and stored at -80°C . Samples were homogenized in 0.5 ml of NP40 Cell Lysis Buffer (Invitrogen) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri) for 10 min or until it flowed freely. A protein assay was performed using a Protein Assay Kit (Pierce, Rockford, Illinois). Samples were heated at 70°C for 10 min and then 10 μg of protein was loaded in each well of a 4–15% polyacrylamide 26 well Criterion TGX Precast Gel (Bio-Rad, Hercules, California, USA) with a standard ladder (Bio-Rad). The gel was electrophoresed for 2 h at 85 V. Samples were transferred to 0.2 μm nitrocellulose at 50 V for 45 min. The blot was incubated in a blocking solution comprised of 0.01 M Tris HCl, 0.15 M NaCl, 0.05% Tween-20 (TBS-T) with 5% skim milk powder overnight on a shaker platform at 4°C . Incubation with primary antibodies was performed in blocking solution containing the two primary antibodies: mouse monoclonal to sarcomeric α -actinin at 1:10,000 (Abcam) and mouse monoclonal to GAPDH at 1:10,000 (Abcam) for 2 h at room temperature. The blot was next incubated in goat polyclonal secondary antibody to mouse IgG1 heavy chain conjugated with horseradish peroxidase at 1:20,000 (Abcam) and Streptactin-HRP Conjugate at 1:20,000 (Bio-Rad) in TBS-T for 1 h on a shaking platform at room temperature. The blot was washed several times before addition of Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare, Waukesha, Wisconsin, USA) and imaged using a ChemiDoc XRS+ System (Bio-Rad).

Quantitative analysis was performed using Image Lab Software 4.0.1 (Bio-Rad) to obtain intensities for α -actinin and GAPDH for each lane. Normalization was performed for each lane by dividing the α -actinin signal intensity by the respective GAPDH intensity.

2.10 Statistical Analysis

All results are listed and displayed as mean $\pm$ standard error. All statistical analyses were performed in Matlab R2012b (The Mathworks Inc., Natick, Ma, USA). Significance was established using a one-way analysis of variables (ANOVA) followed by a post-hoc Tukey-Kramer t-test. Only differences with a p-value less than 0.05 were considered significant. Significant differences in bar graphs were marked by brackets.

3 Results

3.1 Distribution of α -Actinin in Normal and Heart Failure Tissue

We imaged sections from control canine cardiac ventricular tissue. The preprocessed 3D confocal microscopic image stack is shown in Fig. 1A. Preprocessing consisted of deconvolution, background removal and attenuation correction. The corresponding unprocessed image stack is presented in Fig. S4A. In these images, α -actinin was primarily arranged in regular, parallel, transverse sheets. These sheets ran laterally and exhibited frequent gaps and extended throughout the myocytes. This pattern is consistent with

previous descriptions of α -actinin arrangement in striated muscle [3, 4, 6]. Fig. 1B shows a 3D reconstruction of the α -actinin signal. Here the sheet-like structure and repetitive nature of α -actinin is more apparent. We also found that where sheets were interrupted, the gaps were commonly filled with WGA signal (Fig. 1C). This illustrates that the transverse tubular system is closely associated with the α -actinin sheets.

The pre-processed XY images of tissue from SHF, DHF and CRT animals are presented in Figs. 1D, E and F, respectively. The corresponding unprocessed image stacks are presented in Fig. S4B, C and D, respectively. It is clear that the α -actinin distribution in these animals was remodeled versus control. The overall spatial regularity of the α -actinin signal became reduced. Furthermore, visual inspection of these images revealed longitudinal depositions of α -actinin spanning and connecting adjacent parallel sheets of α -actinin. While the decrease in spatial regularity is most noticeable in DHF, the longitudinal depositions are visible in all three models of HF. Examples for three-dimensional structure of the α -actinin distribution from SHF, DHF and CRT are presented in the expanded 3D views of G, H and I respectively. The 3D reconstructions depict that while transversal α -actinin sheets extended throughout the cell depth (z-direction), the longitudinal depositions only extended 1–2 micrometer in the z-direction.

3.2 Distribution of α -Actinin in Isolated Myocytes

Subsequently, we imaged isolated cells using the same methods as described above for imaging of tissue. Fig. 2 represents an isolated myocyte from an SHF animal labeled for α -actinin and with WGA. The patterns of α -actinin were consistent with those previously observed in tissue indicating that the α -actinin structure was not affected by the cell isolation procedure. We found transverse sheets running regularly throughout the entirety of the cell as well as the longitudinal depositions spanning and connecting adjacent transverse sheets. Furthermore, we noticed thick areas of α -actinin around the longitudinal and lateral edges of the cell.

We acquired 45, 53, 34 and 49 image stacks from segments of control, SHF, DHF and CRT cells, respectively. Figs. 3A–D show representative 3D preprocessed image stacks for these experimental groups. The corresponding unprocessed image stacks are presented in Fig. S5. The arrangement of α -actinin in these image stacks was similar to that in fixed tissue. In control cells (Fig. 3A) the α -actinin distribution maintained a regular parallel, transversely running, sheet-like pattern. We visually inspected this pattern in cells from our models of SHF, DHF and CRT (Figs. 3B–D) and found a decreased spatial regularity in DHF. Here, α -actinin sheets were broken down into smaller segments with vertical offsets being more common. In the models of SHF, DHF and CRT we found longitudinal depositions of α -actinin similar to those observed in tissue. These longitudinal depositions were most noticeable in the exemplary CRT cell (Fig. 3D). To illustrate differences between cells with a low occurrence of depositions and cells with a high occurrence of depositions, we created 3D reconstructions of the α -actinin distribution in control and CRT animals (Figs. 3E and 3F, respectively).

3.3 Spatial Regularity of α -Actinin Distributions

We applied Fourier analysis to assess spatial regularity of the α -actinin distribution in isolated myocytes (Figs. 4A and B). We focused on the intensity occurring along the 0° and 90° angles. In the transverse direction ($0\pm 5^\circ$) the averaged intensity in DHF cells was significantly lower than that in all other animal models (Fig. 4A). Here, decreases in intensity signify that the α -actinin sheets running transversely through the cells were irregular. Examples for irregular distributions are presented in Fig. S2. The examples show an increase in intensity along the transverse direction ($0\pm 5^\circ$) when the hyphenations are regular (Fig. S2B). However when the offsets are random and without regular pattern, we find a low intensity (Fig. S2D). We did not find significant differences between the control, SHF and CRT groups indicating that CRT restored the α -actinin distribution to a more regular structure after DHF.

Our study revealed significant differences between control and DHF regarding the regularity of α -actinin sheets in the longitudinal direction ($90\pm 5^\circ$). Here, control cells were different to DHF and SHF cells (Fig. 4B). CRT cells were not different versus control, again indicating that the regularity was partially restored after CRT. The full 180° intensity histogram is presented in Fig. S6. We found further differences between combinations of the control, CRT and DHF animal models along other angles ($20\pm 5^\circ$, $30\pm 5^\circ$, $140\pm 5^\circ$, $150\pm 5^\circ$ and $160\pm 5^\circ$). However, the intensities along those angles were mostly small and the differences negligible. Exceptions include the differences between control and DHF at $110\pm 5^\circ$ and $120\pm 5^\circ$.

3.4 Longitudinal Depositions of α -Actinin

Using the algorithm introduced in Section 2.5, we detected the longitudinal depositions in isolated cells. We calculated the occurrence of these depositions using the volume of the cell (Fig. 4C). The occurrence of longitudinal components was larger in both models of HF versus control. Furthermore, CRT cells had a higher occurrence versus control and SHF. We further analyzed this remodeling to examine if it was localized to a specific area of the left ventricle. Fig. S7 compares the occurrence of these longitudinal depositions in cells from the lateral and anterior left ventricle. We did not find a significant difference between these areas in any of the animal models.

Further analysis allowed us to provide insights into the intracellular distribution of longitudinal depositions. Using the distance map calculated from the intracellular mask, we measured the distance of each detected longitudinal deposition from the edge of the cell (Fig. 4D). In all animal models the majority of depositions were within $1\ \mu\text{m}$ of the cell edge. Here, control cells exhibited a smaller number of depositions versus SHF, DHF and CRT. At larger distances a persistent pattern appeared in the densities of these longitudinal depositions in the different animal models. At all distances control cells displayed the lowest occurrence of depositions, followed by SHF, DHF and then CRT.

3.5 Molecular Density of Longitudinal Depositions

We used super-resolution microscopy to further characterize and quantify the longitudinal depositions that we found. A representative 3D reconstruction (Fig. 5) reveals similar

features to those obtained with confocal microscopy, i.e. transverse sheets and longitudinal depositions of α -actinin. Since each point in these images represents a single detection, super-resolution microscopy allowed us to quantify the number of fluorophores present in different regions of the cell. We compared the number of detections in volumes centered at the transverse sheets versus longitudinal depositions. Longitudinal depositions were significantly less dense in detections than regions of the transverse sheets (1.0 ± 0.11 vs 0.49 ± 0.07 , normalized to the number of detections in the transverse sheets).

3.6 Remodeling of Sarcomeric Proteins

To shed light on remodeling of the sarcomeres we examined three additional cytoskeletal proteins. We investigated if these proteins undergo spatial remodeling as described above for α -actinin. Fig S8 shows exemplary images of titin, actin and myosin with α -actinin as well as intensity profiles through longitudinal depositions in CRT cells. The images indicate that the organization of titin, actin and myosin in a sarcomere in relationship to α -actinin is not altered. We applied Pearson's correlation coefficient to investigate if the cytoskeletal proteins accumulate in longitudinal depositions similar as described above for α -actinin (Fig. 6). Colocalization of titin with α -actinin was reduced in sarcomeres with longitudinal depositions versus sarcomeres without longitudinal depositions. This indicates that titin is not a major component in these depositions. Colocalization of actin and myosin with α -actinin was not different in sarcomeres with and without depositions. Inspection of intensity profiles (Fig. S8G, H, K, and L) suggests that these proteins are present in the depositions, but are not accumulated as α -actinin is.

3.7 Analyses of Gene Expression

Analysis of ACTN2 expression did not show significant transcriptional differences between control, DHF, and CRT animals in either anterior or lateral left ventricular myocardium (Fig S9). However, regional changes in ACTN2 expression were suggested by a significantly larger ratio of ACTN2 expression between the anterior and lateral left ventricle in DHF animals compared to controls. Importantly, the anterior-to-lateral ACTN2 ratio was restored to control levels by CRT (Fig. 7A).

Besides upregulation of natriuretic peptides, dysregulation of sarcomeric genes, including alpha- and beta-myosin heavy chain isoforms, is regarded as a hallmark of the fetal gene expression program, which is characteristic of HF. In our study, we found a negative correlation for the anterior-to-lateral ratios of ACTN2 and NPPA (natriuretic peptide A; coefficient of determination R^2 : 0.559; Fig. 7B). Of note, the similarities between the fetal gene expression program and our animal model of dyssynchronous and resynchronized HF extended beyond selected marker genes. When we examined expression of 160 KEGG pathways, we found a highly coordinated regulation of metabolic and signaling pathways. Regulation of pathways in CRT versus DHF was similar to the regulation in human adult versus fetal myocardium (Fig. 7C). In general, metabolic and cell signaling pathways in CRT versus DHF were downregulated and upregulated, respectively.

3.8 Western Blot Results

Based on our analyses of confocal and super-resolution microscopic images, we hypothesized that cells with longitudinal depositions have an up-regulation of α -actinin. To test this hypothesis we measured the expression of α -actinin in tissues from control and CRT animals since they represent the extreme cases of the occurrence of depositions (Figs. 4C and D). Fig. S10 shows a representative western blot stained for α -actinin and GAPDH. Differences between the averaged and normalized α -actinin to GAPDH signal ratios in control and CRT animals were not significant (1.0 ± 0.08 vs 1.08 ± 0.11 , normalized to the control ratio).

4 Discussion

Our study used three-dimensional microscopy and image analyses to reveal previously unknown remodeling of sarcomeres in cardiac ventricular myocytes from failing hearts. Visual inspection of images exposed remodeling associated with HF, in particular the irregular arrangement and occurrence of longitudinal depositions of α -actinin. We applied Fourier analysis to measure the decrease of spatial regularity of the α -actinin distribution. In DHF cells, we found decreased regularity of α -actinin sheets. Along the transverse direction, regularity was increased after CRT. In the longitudinal direction, the regularity of the α -actinin distribution was similar in CRT and control cells, while regularity in DHF was lower compared to control. These results support our hypothesis that DHF associated remodeling of sarcomeric organization is partly reversed after CRT. However, our studies also revealed longitudinal depositions of α -actinin in the applied HF models and after CRT. The depositions spanned between adjacent transverse sheets and their density was approximately half of that found in the transverse sheets. We found an increased occurrence of these depositions after CRT versus SHF. However, the difference between DHF and CRT was not significant. We also examined colocalization of proteins of the sarcomeric cytoskeleton with the longitudinal depositions. We found that these proteins do not accumulate in the depositions indicating that their major contributor is α -actinin.

In summary, our study revealed two types of structural remodeling: One identified by reduced regularity of the α -actinin distribution in DHF and normalized regularity after CRT and the other by the increased occurrence of longitudinal depositions in DHF and CRT. In previous work it has been established that DHF is associated with an increased QRS duration and CRT restores QRS duration to similar values as in control (Table S1). Comparative gene expression analyses indicated reversion of the fetal gene program in DHF by CRT. Our study suggests that decreased spatial regularity is correlated to prolonged QRS duration and activation of the fetal gene program.

Sarcomeric remodeling has been found in other forms of heart failure. Studies in a canine model elucidated ultrastructural changes in sarcomeric arrangement during arrhythmogenic right ventricular cardiomyopathy [12]. The structural changes commonly led to left bundle branch block, which is the basis for our DHF model. However, the fundamental mechanisms underlying block in this disease model are very different to the cause of block in our DHF model, which is based on ablation. Using electron microscopy, the study found an accumulation of electron dense material around the Z-disk that extended in various

directions and filled the entire area of the sarcomere. In this study [12], changes in α -actinin expression were not detected by western blot, which is consistent with our study. The study compared the finding of “Z band streaming” to a human skeletal muscle disease known as nemaline myopathy. Differences between our results, the accumulation of electron dense material in arrhythmogenic right ventricular cardiomyopathy and the ultrastructural phenotypes of nemaline myopathy include the extent and local features of remodeling [12, 32, 33]. In our study, the degree of disorganization of the Z-disk and local remodeling appears smaller. Nevertheless, the longitudinal depositions could be a special case of Z-band streaming with remodeling of α -actinin restricted to single sarcomeres.

It has also been shown that the Z-disk becomes altered in culture and during diseases other than heart failure. Cultured adult rat cardiomyocytes completely lose striation of α -actinin between 4–7 days [34]. In mouse cardiomyocytes, after 72 h of infection with the parasite *Trypanosoma cruzi*, the overall expression of α -actinin decreased by 32% [11]. Furthermore α -actinin completely lost its periodic arrangement and became localized to focal adhesion sites [11]. A genetic study on hypertrophic cardiomyopathy (HCM) in humans showed that all family members affected with HCM had a G to A missense mutation in exon 3 of *ACTN2*. Additionally, 3 further mutations in highly conserved regions of *ACTN2* were found in affected individuals. In this study, 4 of these mutations were identified as causes of HCM, supporting the notion that disruption of Z-disk proteins can lead to heart disease [9, 35].

Hein et al. suggested that sarcomeric remodeling during HF occurs in two main stages, an early reversible stage characterized by the accumulation of cytoskeletal proteins to offset the increased strain imposed on the myocardium, and a latter irreversible stage defined by the loss of contractile filaments [2]. We suggest that the remodeling seen here is part of the primary stage when the myocytes are still compensating for the increased strain that arises in HF.

A recent study reported significant reductions in the amount of sarcomere shortening in isolated cells from SHF and DHF animals [18]. The study also showed a decrease of about 40% in the strain magnitude of the anterior-septal and lateral walls in these models versus control [18]. Furthermore, it has been suggested that disorganization of sarcomeres leads to changes of contractility [36]. Thus, the sarcomeric remodeling observed in our study can help explain reduced sarcomere shortening in HF. Also, we suggest that the increase of sarcomere shortening in isolated CRT cells reported in [18] can be explained in part by restoration of sarcomere organization revealed in our studies. It is difficult to separate effects of sarcomere organization on contractility from other remodeling processes in HF and after CRT. Nevertheless, we suggest that remodeling of sarcomere organization plays a complementary role. Also, our studies indicate that longitudinal depositions have only a minor effect on passive mechanical properties and contractility of myocytes. We observed the highest occurrence of longitudinal depositions in CRT cells, which nevertheless have similar contractile properties as control cells.

We performed gene and protein expression analyses to test the hypothesis that the depositions are caused by upregulation of α -actinin. Our analyses rejected this hypothesis. Differences of *ACTN2* and α -actinin-2 expression between CRT and control myocardium

were not significant. However, the occurrence of depositions was increased in CRT versus control myocytes. We propose two mechanisms to explain why CRT cells exhibited the highest occurrence of longitudinal depositions of α -actinin. In our model of CRT the heart undergoes 3 weeks of DHF followed by 3 weeks of biventricular pacing. It is probable that the heart is experiencing new stressors after initiation of CRT. Therefore, depending on the timescale of the remodeling, our studies could be at the time of peak occurrence of these longitudinal depositions. The second mechanism is that the decreased sarcomere organization in DHF makes the cells more prone to creating longitudinal depositions when synchrony is restored by CRT. Both explanations point at a potential role of longitudinal depositions as a marker of previous and ongoing remodeling at the subcellular scale. We speculate that increased numbers of longitudinal depositions indicate that remodeling processes are initiated.

Limitations

Rapid pacing is widely used for animal models of non-ischemic HF. The models recapitulate major electrophysiological (action potential prolongation, high incidence of sudden cardiac death, atrial arrhythmias), morphological (ventricular hypertrophy), functional (depressed contractility) and genomic (re-expression of a fetal gene expression pattern) hallmarks of human HF. However, the models may not mimic all aspects of the complex clinical spectrum of congestive HF in patients. The changes in myocardial structure occurring with tachypacing may be dissimilar to clinical forms of HF due to chronic ischemia or hypertensive disease, which usually develop over a longer period of time. Thus, extrapolation of the findings from our HF models to clinical forms of HF require caution. Also, our CRT model is based on only 3 weeks of biventricular pacing. While studies indicated that 3 weeks of CRT restored some structural and functional properties of the heart [16, 17, 19], other properties, for instance, hemodynamic properties were not restored (Table S2). Membrane capacitance of isolated myocytes, which is a marker of their size, is increased after CRT versus control myocytes indicating persistent cellular hypertrophy after 3 weeks of CRT [17]. Our study suggests that CRT leads to partial restoration of sarcomeric organization. It is possible that the time scale of restoration of sarcomeric organization is longer than the given 3 weeks. The focus of our study was on characterization of sarcomeric α -actinin. We applied a monoclonal antibody for sarcomeric α -actinin. Similar antibodies have been characterized in previous studies [10, 37]. The antibody labels α -actinin 2 and 3, but not α -actinin 1 or 4. We did not find labeling of blood vessels in our images from tissue slices conforming that the antibody does not label smooth muscle α -actinin 1.

In summary, our study provides unique quantitative insights into remodeling of sarcomeric protein organization based on three-dimensional high-resolution confocal microscopy. We introduced new methods to characterize subcellular remodeling in HF and after CRT. The findings of this study will guide us towards developing studies to elucidate the mechanisms and effects of subcellular remodeling in cardiac disease and restoration after therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Our studies revealed that heart failure is associated with sarcomeric remodeling
- Remodeling included spatial irregularity and longitudinal depositions of α -actinin
- Sarcomeric remodeling occurred in anterior and lateral left ventricular myocardium
- Sarcomeric irregularity was reduced after cardiac resynchronization therapy
- Longitudinal depositions were not reduced after cardiac resynchronization therapy

Fig. 1.

Pre-processed 3D stacks of tissue labeled with WGA (blue) and for α -actinin (red). (A) Sections through stack acquired from control tissue. The yellow lines indicate the presented cross sections. (B) 3D reconstruction of α -actinin distribution marked with box in (A). (C) 3D reconstruction of α -actinin and tsystem labeled with WGA in the transverse sheet marked in (B). Pre-processed 2D XY slices of (D) SHF, (E) DHF and (F) CRT tissue sections. Images reveal frequent longitudinal depositions of α -actinin. (G-I) 3D reconstructions from regions marked in (D-F), respectively. The reconstructions indicate fragmentation and decreased spatial regularity of α -actinin sheets. Bar in (A) applies to (D-F).

Fig. 2.

XY cross section from a 3D image stack of an isolated ventricular myocyte from SHF model. The cell was labeled with WGA (blue) and for α -actinin (red). Arrows point to wide areas of strong α -actinin signal along the cell ends. Triangles point to longitudinal depositions.

Fig. 3.
Pre-processed 3D image stacks of sections of isolated cardiac myocytes from (A) control, (B) SHF, (C) DHF, and (D) CRT animals. Sections were labeled for α -actinin. (E, F) 3D reconstructions of the segments marked in (A, D).

Fig. 4.

Quantitative analyses of α -actinin distributions in control, SHF, DHF, and CRT cells. (A) Fourier analysis of image intensities in the transverse direction ($0\pm5^\circ$). (B) Fourier analysis of image intensities in the longitudinal direction ($90\pm5^\circ$). (C) Average occurrence of longitudinal depositions. (D) Occurrences of longitudinal depositions for each experimental group as a function of the distance from the cell surface.

Fig. 5. Three-dimensional image of α -actinin detections from super-resolution microscopy of an isolated cardiac myocyte from SHF model. The field of view is $\sim 22 \times 23 \times 8 \mu\text{m}$. Arrows point to longitudinal depositions spanning between transverse sheets in the cell interior.

Fig. 6.

Colocalization analysis of α -actinin with titin (A), actin (B) and myosin (C). Pearson correlation coefficients R_p were calculated for entire cell segments, regions of interest centered on sarcomeres without longitudinal depositions, and regions centered on sarcomeres with longitudinal depositions. Co-localization of α -actinin in sarcomeres and regions with longitudinal depositions was significantly reduced for titin only. For actin and myosin, differences of colocalization were not significant.

Fig. 7.

Gene expression analysis using a two-color microarray and labeling with Cy3 and Cy5. (A) The ratio of ACTN2 gene expression in Ant versus Lat samples is significantly higher in DHF than in control or CRT samples. Differences of NPPA ratios were not significant. (B) The ratio of ACTN2 expression in Ant and Lat samples is negatively correlated to the NPPA ratio. (C) Analysis of KEGG pathways in DHF versus CRT myocardium indicates a similar regulation of pathways as in fetal versus adult myocardium re-expression. The net expression of a KEGG pathway (number of upregulated minus downregulated genes within a study in relation to total number of genes per given KEGG pathway) is color-coded with yellow and blue, representing decreased and increased expression of the pathway genes, respectively. The net expression of the different pathways is depicted vertically and sorted

according to their similarity of gene expression of the pathway “oxidative phosphorylation” (OXPHOS).