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Remodeling of the Transverse Tubular System after Myocardial Infarction in Rabbit Correlates with Local Fibrosis: A Potential Role of Biomechanics

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Abstract

The transverse tubular system (t-system) of ventricular cardiomyocytes is essential for efficient excitation-contraction coupling. In cardiac diseases, such as heart failure, remodeling of the t-system contributes to reduced cardiac contractility. However, mechanisms of t-system remodeling are incompletely understood. Prior studies suggested an association with altered cardiac biomechanics and gene expression in disease. Since fibrosis may alter tissue biomechanics, we investigated the local microscopic association of t-system remodeling with fibrosis in a rabbit model of myocardial infarction (MI). Biopsies were taken from the MI border zone of 6 infarcted hearts and from 6 control hearts. Using confocal microscopy and automated image analysis, we quantified t-system integrity (I_{TT}) and the local fraction of extracellular matrix (f_{ECM}). In control, f_{ECM} was $18 \pm 0.3\%$. I_{TT} was high and homogeneous (0.07 ± 0.006), and did not correlate with f_{ECM} ($R^2 = 0.05 \pm 0.02$). The MI border zone exhibited increased f_{ECM} within 3mm from the infarct scar ($30 \pm 3.5\%$, $p < 0.01$ vs control), indicating fibrosis. Myocytes in the MI border zone exhibited significant t-system remodeling, with dilated, sheet-like components, resulting in low I_{TT} (0.03 ± 0.008 , $p < 0.001$ vs control). While both f_{ECM} and t-system remodeling decreased with infarct distance, I_{TT} correlated better with decreasing f_{ECM} ($R^2 = 0.44$) than with infarct distance ($R^2 = 0.24$, $p < 0.05$). Our results show that t-system remodeling in the rabbit MI border zone resembles a phenotype previously described in human heart failure. T-system remodeling correlated with the amount of local fibrosis, which is known to stiffen cardiac tissue, but was not found in regions without fibrosis. Thus, locally altered tissue mechanics may contribute to t-system remodeling.

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Keywords

fibrosis; t-system; myocardial infarction; remodeling; biomechanics

1. Introduction

Excitation-contraction coupling in cardiac myocytes is the process by which electrical activation triggers mechanical contraction of the sarcomeres (Bers, 2002; Stern, 1992). The process involves opening of sarcolemmal voltage-gated L-type Ca^{2+} channels (LCCs) in response to membrane depolarization, leading to an initial influx of Ca^{2+} , which in turn triggers the opening of ryanodine receptors (RyRs) clustered in the membrane of the sarcoplasmic reticulum (SR). The SR serves as an intracellular Ca^{2+} store. As a result of RyR opening, Ca^{2+} is released from the SR into the cytosol, raising free cytosolic Ca^{2+} levels transiently from 100–200nM at rest to approximately 1 μM . Ca^{2+} then binds to troponin C, inducing a conformational change in the troponin complex and rotation of tropomyosin. This enables actin-myosin interactions, which lead to sarcomere contraction. The contractile force developed by a myocyte depends on the number of contracting sarcomeres and force development in each sarcomere, which is regulated by the concentration of free cytosolic Ca^{2+} during systole. Half-maximal force is developed at about 600nM (Gao et al., 1994; Stern, 1992; Yue et al., 1986). Obviously, efficient and forceful contraction of a myocyte requires a spatio-temporally homogeneous increase in free cytosolic Ca^{2+} by synchronized opening of RyRs. For this purpose cardiomyocytes of humans and most other mammals possess a dense system of tubular membrane invaginations, the transverse tubular system (t-system). The membrane of transverse tubules (t-tubules) comes close to the SR membrane, forming junctions with a distance of only 10–12nm between the sarcolemma and the SR (Forbes and Sperelakis, 1982). These junctions enable immediate and efficient coupling of RyR opening to Ca^{2+} influx through LCCs in response to membrane depolarization. In ventricular myocytes of human, canine, rabbit and other species, most RyRs are found within SR-sarcolemma junctions, but a smaller fraction of RyRs is more distant from the sarcolemma (including the t-system). They are commonly referred to as non-junctional RyRs. The distance between non-junctional RyRs and the sarcolemma ranges up to several micrometers (Dries et al., 2013; Jayasinghe et al., 2009; Torres et al., 2014). The ratio of junctional and non-junctional RyRs depends on species, preparation, method of analysis and the definition of “on-junctional”.

In cardiac diseases, especially in heart failure and myocardial infarction (MI), the t-system undergoes structural remodeling, resulting in alterations of shape and orientation as well as loss of t-tubules (Brette and Orchard, 2003; Ferrantini et al., 2013; Heinzel et al., 2008; Kostin et al., 1998; Louch et al., 2006; Lyon et al., 2009; Wei et al., 2010). These structural changes of the t-system in ventricular myocytes are gradual and differ between species. While t-system remodeling in rodents leads mainly to disorganized orientation and increased axial components (Chen et al., 2012; Wagner et al., 2012; Wei et al., 2010), the predominant remodeling in humans and larger mammals, such as canine or pig, is loss and dilation of t-tubules (Biesmans et al., 2011; Crossman et al., 2017; Kostin et al., 1998; Sachse et al., 2012). We recently described the 3D structure of a sheet-like remodeled t-tubules (t-sheets)

in humans, which is symptomatic for end-stage heart failure (Seidel et al., 2017). It still remains unclear if t-sheets are also present in animal models of heart failure, but most likely not in mice or rats because these species have already been studied extensively. In a porcine model of MI “enlarged, highly branched disordered structures” of the t-system were described in a recent study using three-dimensional electron microscopy (Pinali et al., 2017). These structures may correspond to the “sheet-like” components observed by confocal microscopy in human heart failure. Nevertheless, a common feature of t-system remodeling in all species is a progressive reduction in t-system density.

In animal models, the degree of t-system remodeling and cardiac dysfunction are correlated, for example during the transition from hypertrophy to heart failure (Shah et al., 2014; Wei et al., 2010) and in synchronous versus dyssynchronous heart failure (Sachse et al., 2012). To show similar correlations in patients is more difficult, but recent studies underpin the importance of t-system remodeling as a pathomechanism and potential prognostic factor also in human failing hearts (Crossman et al., 2015; Seidel et al., 2017). At the cellular level, a loss of t-tubules increases the number of non-junctional RyRs, which impairs excitation-contraction coupling because a smaller number of RyR clusters is synchronously activated (Dries et al., 2013; Heinzel et al., 2008; Sachse et al., 2012; Seidel et al., 2017).

Additionally, it was suggested that t-tubules may fail to conduct action potentials. This has similar effects as a structural loss of t-tubules because RyRs in the vicinity of failing t-tubules will not or only inefficiently contribute to excitation-contraction coupling (Crocini et al., 2014). Impaired excitation-contraction in cardiomyocytes then translates to reduced cardiac contractility. This is supported by a study in human failing hearts, showing regional correlation between reduced contractility and t-system remodeling (Crossman et al., 2015). Furthermore, remodeling of t-tubules may facilitate arrhythmias due to altered Ca^{2+} handling (Orchard et al., 2013). The t-system is therefore considered as a potential target of therapy, prevention and diagnosis, especially in heart failure (Manfra et al., 2017).

While consequences of t-system remodeling in cardiac diseases are well-understood, it remains largely unclear what mechanism underlie the loss and structural changes of t-tubules. Junctophilin-2 downregulation has been related to t-system remodeling in several studies. As a structural protein, junctophilin-2 is thought to bridge the SR and t-tubule membranes, thereby stabilizing junctions and preventing t-tubules from disaggregation (Beavers et al., 2014). Junctophilin is downregulated in cardiac diseases that are accompanied with remodeling and loss of the t-system (Zhang et al., 2013). Junctophilin knockdown caused impaired t-tubule maturation and cardiac hypertrophy in mice (Landstrom et al., 2011; Reynolds et al., 2013). Also, junctophilin overexpression was suggested to normalize excitation-contraction coupling and cardiac function in murine heart failure models (Guo et al., 2014; Reynolds et al., 2016). However, it is still unclear if reduced junctophilin expression leads to t-system remodeling or, vice versa, the loss of t-tubules causes reduced junctophilin protein levels. Some studies suggested that stiffening of the microtubular system and other components of the cytoskeleton are involved directly or indirectly in the remodeling process of t-tubules and junctophilin trafficking (Prins et al., 2016; Zhang et al., 2014). However, what causes junctophilin downregulation or changes in the microtubular system is poorly understood. Possible mechanisms include biomechanical disruption of cytoskeletal proteins and mechanotransduction in response to elevated

mechanical stress (Frisk et al., 2016; Manfra et al., 2017). An integrated mechanism was suggested by a study using genetic mosaics, where junctophilin knockdown in a subset of cardiomyocytes led to t-tubule loss only in the context of cardiac stress (Guo et al., 2017).

In our previous work on animal models of heart failure, we suggested that alterations in mechanical stress directly affect the structure of t-tubules and maintenance of t-system organization (Sachse et al., 2012). In dyssynchronous heart failure, activation and strain profiles of the myocardium exhibit pronounced regional differences. In particular, cells of the left lateral ventricular wall are pronouncedly strained before they are electrically activated and shorten (Prinzen et al., 1999). We proposed that these regional differences are responsible for regional remodeling of the t-system. To explain findings of cellular remodeling in dyssynchronous heart failure, we hypothesized that heterogeneities of subcellular strain lead to subcellular heterogeneity of the t-system (Li et al., 2015). Studies on ventricular tissue from a rat model of myocardial infarction and cultured papillary muscle preparations support these ideas by demonstrating that elevated wall stress is associated with t-system remodeling (Frisk et al., 2016). In our recent work on ventricular tissue from human end-stage heart failure, we extended these concepts by proposing that cardiac fibrosis might cause t-system remodeling (Seidel et al., 2017). This hypothesis is supported by the recent discovery of increased amounts of the extracellular matrix protein type VI collagen within the lumen of dilated t-tubules in human heart failure (Crossman et al., 2017). Thus, although a causal relationship has not been shown, fibrosis appears to be associated with t-system remodeling. Fibrosis is common in many types of heart disease and it is well established that fibrosis is associated with mechanical stiffening of the myocardium (Conrad et al., 1995; Doering et al., 1988). We suggested that altered strain profiles due to fibrosis contribute to changes in t-system structure. Yet, to date, quantitative studies relating local fibrosis to t-system remodeling do not exist.

To support the hypothesis that local fibrosis is spatially associated with t-system remodeling, we investigated the relationship between cardiac fibrosis and t-tubule remodeling in a rabbit model of myocardial infarction. We assessed the dependence of t-system integrity on the amount of extracellular matrix and infarct distance in the infarct border zone and compared these findings to control hearts. Furthermore, we explored structural features of the remodeled t-system in rabbit.

2. Material and Methods

2.1 Animal Model

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. We used 12 male New Zealand White rabbits of 2.5 to 3kg. In 6 randomly selected animals, left-ventricular MI was created by ligation of the left circumflex artery as previously described (Hu et al., 2010; Seidel et al., 2016). Successful ligation was confirmed by ST elevation in ECG recordings and epicardial demarcation of the infarcted area. The remaining 6 animals served as control. Hearts were excised 21d after surgery, fixed by perfusion with 2% paraformaldehyde solution for 10min and stored at 4°C in phosphate-buffered saline (PBS, 137mM NaCl, 0.7mM KCl, 10mM Na₂ HPO₄ and 1.8mM KH₂ PO₄ in deionized water).

2.2 Sample Preparation

Biopsies of 5mm diameter were taken with a punch needle from the MI border zone or matching regions from control hearts. After immersion in 30% sucrose solution, slices of 80µm thickness were obtained by cryosectioning in a depth of 0.4 to 1.6mm below the epicardium as previously described (Seidel et al., 2016). Immediately after sectioning, and after each incubation step during staining, slices were rinsed in PBS.

The slices were labelled for sarcomeric actinin by incubation in anti-alpha-actinin antibody (ab9465, Abcam, Cambridge, UK) at a dilution of 1:200 in blocking solution (5% bovine serum albumin, 5% goat serum and 0.25% Triton X-100 in PBS) for a minimum of 12 h. Goat anti-mouse IgG1 Alexa Fluor 647 (A-21240, Thermo Fisher Scientific, Waltham, MA, USA) was then applied at 1:200 dilution together with 300nM 4',6-Diamidino-2-Phenylindole, diacetate (DAPI, D3571, Thermo Fisher Scientific, Waltham, MA, USA) in blocking solution for a minimum of 12 h. Finally, we applied CF488A-conjugated wheat germ agglutinin (WGA, Biotium, Hayward, CA, USA) at 40 µg/mL in PBS for a minimum of 4h.

Tissue slices were mounted on glass slides using Fluoromount-G (#17984-25, Electron Microscopy Science, Hatfield, PA, USA) and covered with coverslips of thickness 1.5. Samples were left to dry at room temperature for at least 1d before imaging.

2.3 Confocal Microscopic Imaging

To acquire images, we used a Leica SP8 TCS confocal microscope (Leica, Jena, Germany) with a 40× oil immersion lens and a motorized stage. During imaging, we switched between 3 laser lines configured in 3 separate imaging sequences. Laser wavelengths of 405, 488 and 633nm were utilized to excite DAPI, CF 488A and Alexa Fluor 647, respectively. Pixel dwell time was 1.7µs. Tissue slices were completely scanned by two-dimensional large-area tile scans of up to 5×5mm² within a distance of 30µm from the coverslip. Each scan consisted of 400 to 600 individual tiles with 1024×1024 pixels each and a pixel size of 0.2×0.2µm². A 10% overlap of tiles allowed automated registration and stitching using the Leica Application Suite X software package (versions: 1.1.0.12420 and 2.0.2.15022). We also acquired three-dimensional image stacks as described previously (Seidel et al., 2016).

2.4 Image Analysis

2.4.1 Local Fiber Orientation—Images were divided into subregions of 1024×1024 pixels, i.e. 204×204µm² (Fig. 1). Alpha actinin images of each subregion (Fig. 1A) were transformed into the frequency domain by discrete Fourier transformation (Fig. 1B). Within a wavelength spectrum of 1.8±0.4µm, the maximum magnitude was determined after filtering with a Gaussian mask ($\sigma=2$). The corresponding angle was identified as local fiber orientation, θ_f . The position of the maximum yielded the sarcomere length, SL .

2.4.2 Myocyte Mask—Alpha actinin images of each subregion were segmented by applying a threshold of mode plus one standard deviation (Fig. 1C). Alpha actinin is located in the z-disk of sarcomeres within cardiomyocytes. Using a line-shaped structuring element

of 3 μ m length oriented parallel to ϕ_t , the alpha actinin segmentation was morphologically closed to obtain a cardiomyocyte mask (Fig. 1D).

2.4.3 Quantification of Local Fibrosis—Wheat germ agglutinin (WGA) binds to glycoproteins located in cell membranes and the extracellular matrix (Emde et al., 2014; Seidel et al., 2016). In rabbit and other species, it also binds to membranes of the t-system. WGA images of each subregion (Fig. 1E) were segmented by applying a threshold of mode plus one standard deviation. From the obtained binary image, the myocyte mask was subtracted to exclude cell membranes and the t-system (Fig. 1F). After subtraction, a mean filter with radius 200 μ m was applied to calculate the local fraction of extracellular matrix (f_{ECM}) for each pixel. Border effects were avoided by including pixels from neighboring subregions when applying the mean filter.

2.4.4 Quantification of T-System Integrity—Our analysis of t-system integrity is based on the equivalence of energy E and the integrated energy spectral density in an image (Rayleigh, 1889):

$$E = \int |x(t)|^2 dt = \int |\hat{x}(f)|^2 df \quad (1)$$

where $x(t)$ refers to signal intensity of pixels t in a 2D image and inline $\hat{x}(f)$ refers to the Fourier transform of the image with frequencies f .

To remove the image offset produced by the imaging system and processing, we first subtracted the mean intensity from the WGA image of each subregion. We next applied a Fast Fourier transformation to the resulting image using Matlab (R2016a, Mathworks, Natick, MA). In images of WGA-labeled healthy cardiomyocytes, the t-system results in a regular pattern of intensity peaks in fiber orientation with a spacing corresponding to the sarcomere length. Using the known angle θ_t of fiber orientation and the sarcomere length SL , we defined the domain of the expected t-system peak within $\theta = [\theta_{\min}, \theta_{\max}]$ and $f = [f_{\min}, f_{\max}]$, where $\theta_{\min} = \theta_t - 15^\circ$, $\theta_{\max} = \theta_t + 15^\circ$, $f_{\min} = 1/(SL + 0.2\mu\text{m})$ and $f_{\max} = 1/(SL - 0.2\mu\text{m})$ (Fig. 1H). The energy E_{TT} within this domain was calculated by summing up squared magnitudes in the frequency domain according to the right-hand side of (1):

$$E_{TT} = \sum_{f_{\min}, \theta_{\min}}^{f_{\max}, \theta_{\max}} |\hat{x}(f, \theta)|^2 \quad (2)$$

From the WGA intensity image we subtracted the non-myocyte space using the myocyte mask. Resulting images contained mainly the t-system of cardiomyocytes (Fig. 1G). E_{TT} was then normalized by the sum of squared pixel intensities x to obtain t-tubule integrity, I_{TT} :

$$I_{\text{TT}} = \frac{E_{\text{TT}}}{\sum |x|^2} \quad (3)$$

I_{TT} can be interpreted as the fraction of signal energy within the WGA-labeled myocytes that can be explained by a regular t-tubule pattern (Fig. 1H), which is commonly present in normal myocytes.

2.4.5 Assessing Myocyte Alignment—To measure myocyte alignment with the average fiber orientation of a subregion, θ_f , the orientation of nuclei was determined, following a similar approach as published previously (Huang et al., 2013). DAPI images (Fig. 1I) were first segmented by applying a threshold of mode plus one standard deviation, followed by three iterations of a binary median filter (Fig. 1J). Connected components were identified and their area, oriented bounding boxes and principal components determined using 2nd order image moments. Nuclei with an area less than $20\mu\text{m}^2$ or with a long to short axis ratio less than 2 were excluded. The percentage of nuclei with their main axis oriented within $\theta_f \pm 15^\circ$ was then used as a measure of myocyte alignment (Fig. 1K, L).

2.4.6 Distance from Infarct Scar—Infarcted tissue was defined as tissue containing extracellular matrix and nuclei, but no cardiomyocytes. In addition to visual inspection of the WGA and DAPI signals, the absence of cardiomyocytes was verified by negative alpha actinin staining. Infarct scars were segmented manually by creating a polygonal mask with ImageJ (Schindelin et al., 2012), which was then saved as a binary image. From the binary infarct image, the Euclidean distance transform was calculated to obtain for each pixel the closest distance to the infarct border, d_{scar} .

2.5. Statistical Analysis

Student's t-test was used to compare control and MI groups. The level of significance was set to 0.05. Where necessary, p-values were corrected for multiple comparison according to the Holm-Bonferroni method. Regression analyses and calculation of coefficients of determination (R^2) were performed in Matlab (R2016a, Mathworks, Natick, MA), using linear or mono-exponential fitting models.

3. Results

3.1 Sheet-Like T-System Remodeling in the Rabbit MI Border Zone

We first acquired confocal image stacks from control tissue and the infarct border zone three weeks after MI to investigate t-system structure in normal and remodeled cardiomyocytes (Fig. 2). As expected for the control tissues, we found a dense, regular system of transverse tubules in 2D views (Fig. 2A,B) and three-dimensional reconstructions (Fig. 2C). In inspection of two-dimensional images from MI cardiomyocytes, remodeled t-tubules appeared as longitudinal or axial components in longitudinal sections (Fig. 2D,E, XY views) and as apparently tubular structures in transverse sections (Fig. 2D,E, XZ views). However, three-dimensional reconstructions of extracted t-system components revealed that in the

rabbit MI border zone the t-system is remodeled to sheet-like membrane structures. These “t-sheets” were dilated in longitudinal myocyte direction and still connected to the outer sarcolemma (Fig. 2F), showing high similarity to the phenotype observed in human end-stage heart failure (Seidel et al., 2017). These results support that WGA reliably stains the t-system in normal and remodeled rabbit myocytes.

3.2 Large-Area Tile Scans

We then acquired large-area two-dimensional tile scans from biopsies obtained from 6 control and 6 infarcted hearts. One example from each group is displayed in Fig. 3. Control tissue structure was homogeneous, with parallel alignment of cardiomyocytes, a dense, regular t-system and only small amounts of extracellular matrix (Fig. 3A,C). As expected, alpha actinin signals presented a regular cross-striated pattern within cardiomyocytes and a spacing of approx. $2\mu\text{m}$, corresponding to sarcomere length. Since sarcomeres are only present in myocytes, alpha actinin served also as a marker of cardiomyocytes (Fig. 3B,D). In infarcted tissue, the infarct scar was clearly demarcated and visible by a lack of cardiomyocytes and alpha actinin (Fig. 3E,F). The surrounding border zone presented less homogeneous fiber orientation and an increased amount of extracellular matrix, i.e. fibrosis. Patches of fibrosis were found up to a distance of 3mm from the infarct scar (Fig. 3E). In contrast to the regular t-system in control, t-system structure was severely remodeled in fibrotic regions close to the infarct border (Fig. 3G) or in fibrotic regions more distant from the infarct (Fig. 3K). However, cardiomyocytes in regions without fibrosis exhibited an almost normal t-system (Fig. 3I). The alpha actinin signal appeared regular in the MI border zone and not affected by fibrosis or infarct distance (Fig. 3H,J,L).

3.3 Remodeling of Tissue Structure and T-System after MI

To quantify our observations, we used methods of computational image processing and analysis (Fig. 1). Spectral density analysis of the Fourier-transformed t-system provided a measure of t-tubule integrity (I_{TT} , Fig. 1H). Analysis of the orientation of myocyte nuclei validated the calculated fiber orientation (Fig. 1I–L). We applied these methods to subregions of $204 \times 204 \mu\text{m}$ from large-area scans. Our observations of homogeneous tissue structure in control were confirmed (Fig. 4). Fiber orientation showed only little variation in the shown example, ranging from approx. $30\text{--}45^\circ$ to the horizontal axis (Fig. 4B). Detected sarcomere lengths mainly ranged from 2 to $2.1\mu\text{m}$, indicating that myocytes were in parallel with the imaging plane and not contracted (Fig. 4C). T-tubule integrity (I_{TT}) was homogeneously distributed as well, with values between 0.075 and 0.125 in most subregions (Fig. 4D). This means that up to 12.5% of intracellular intensity in WGA images was attributed to the power spectrum domain defined by $\theta_f \pm 15^\circ$ and $SL \pm 0.2\mu\text{m}$. Thus, the t-system was intact and regular within the whole control sample. As a measure of myocyte alignment with fiber orientation (θ_f) detected by analyzing the alpha actinin spectrum, we used the percentage of nuclei oriented within $\theta_f \pm 15^\circ$. This value was above 75% in most subregions, indicating that within subregions most myocytes were arranged in parallel (Fig. 4E). Control tissue also showed a homogeneous distribution of extracellular matrix. In nearly all subregions f_{ECM} was close to 20% (Fig. 4F). We further found that f_{ECM} did not affect t-tubule integrity ($R^2 = 0.01$) in control tissue (Fig. 4G). Results from analyses of myocyte alignment are presented in Fig. S1.

When looking at quantitative results from the MI sample (Fig. 5) heterogeneity in tissue structure became visible in fiber orientation (Fig. 5B), t-tubule integrity (Fig. 5D) and amount of extracellular matrix (Fig. 5F). Along the infarct border (segmented infarct scar is shown in Fig. S2), fiber orientation changed by approx. 50° . The t-system was irregular adjacent to the infarct scar as indicated from I_{TT} values below 0.01. With increasing infarct distance I_{TT} increased, indicating a transition to normal t-system in regions distant from the infarct. When looking closely at Figs. 5D and 5F, an association between high f_{ECM} and low I_{TT} is revealed, i.e. high fibrosis was associated with low t-tubule integrity. Sarcomere length (Fig. 5C) and myocyte alignment (Fig. 5E) were similar to control.

As in the control example, we correlated I_{TT} with f_{ECM} using a monoexponential model (Fig. 6A). In contrast to control, I_{TT} fell exponentially with increasing f_{ECM} ($R^2 = 0.41$), confirming that the t-system was remodeled in fibrotic, but not in non-fibrotic regions. This correlation was higher than the linear correlation between I_{TT} and infarct distance (Fig. 6B, $R^2 = 0.33$, $p < 0.05$). As expected, f_{ECM} fell with increasing infarct distance, but the correlation was relatively weak (Fig. 6C, $R^2 = 0.27$). Residual analysis of the chosen regression models is shown in Fig. S3.

Fig. 7 summarizes results obtained from analyzing biopsy sections of 6 control and 6 infarcted hearts. Commonly, coefficients of determination (R^2) were high for the correlation between t-tubule integrity and extracellular matrix in MI ($R^2 = 0.44 \pm 0.09$), but significantly lower in control ($R^2 = 0.05 \pm 0.02$, $p < 0.01$, Fig. 7A,B). In MI samples the correlation of I_{TT} with distance from the infarct scar was constantly weaker than with fibrosis in MI samples, as seen from a lower coefficient of determination ($R^2 = 0.24 \pm 0.08$, $p < 0.05$). A weak correlation between I_{TT} and myocyte alignment was present in all samples, without detectable differences between control ($R^2 = 0.15 \pm 0.03$) and MI ($R^2 = 0.1 \pm 0.04$), but in MI significantly lower than the correlation with fibrosis ($p < 0.01$). Similarly, we did not find a difference in mean myocyte alignment between control and MI (Fig. 7C) or a correlation of myocyte alignment with either fibrosis or infarct distance (Fig. S4). Thus, the strong association of t-tubule remodeling with fibrosis could not be explained by myocyte disarray or scar distance.

3.4 Gradients of I_{TT} and f_{ECM} in the MI Border Zone

To further dissect the interrelations between scar distance, t-system remodeling and fibrosis, we looked at the change of I_{TT} and f_{ECM} with increasing infarct distance (Fig. 8A,B). Mean f_{ECM} fell from 40% close to the infarct to 22% at a distance of 2.5mm. This value is still slightly above the mean control value ($18 \pm 0.3\%$). The major change occurred within the first 0.5mm, where f_{ECM} fell to 30%, but then fell only slowly with increasing infarct distance. We observed a similar, but opposing gradient when looking at I_{TT} . From very low values of 0.015 close to the infarct, I_{TT} increased mostly within the first 0.5mm and then only slowly until it reached mean values of approx. 0.04 at distances > 2.5 mm. The standard error of mean I_{TT} also increased with infarct distance. Although Fig. 7 shows that f_{ECM} was a better predictor of I_{TT} than infarct distance, we explored if this was also true at higher infarct distances. We first looked at mean I_{TT} at different degrees of fibrosis (Fig. 8C). I_{TT} fell most rapidly when f_{ECM} exceeded 30%. Then we selected only regions with infarct distance less

than 1mm (Fig. 8D) or more than 1mm (Fig. 8E). We found similar distributions within these domains, showing that the correlation between I_{TT} and f_{ECM} was independent of infarct distance. An additional finding supporting this hypothesis is that standard errors were much smaller when plotting I_{TT} against f_{ECM} (Fig. 8C) than infarct distance (Fig. 8B). Furthermore, infarct distance did not have an effect on I_{TT} if f_{ECM} was greater than 30% (Fig. S5). These results collectively suggest that fibrosis predicts t-system remodeling better than infarct distance.

4. Discussion

We utilized large-area scanning confocal microscopy of the rabbit MI border zone to overcome a common scale problem in structural research of the myocardium. Remodeling of tissue structures, for example changes in extracellular matrix, blood vessels and fiber orientation, occurs over the range of millimeters to centimeters, whereas submicrometer resolution is required to analyze structural remodeling at the cellular and subcellular level. It is therefore difficult to relate cellular remodeling to structural changes on larger scales. A recent study used in-situ confocal imaging to assess t-system structure at different distances from the MI border zone in rats (Chen et al., 2012), but without looking at other parameters of tissue remodeling. Another study on rat MI used series of three-dimensional images to reconstruct the border zone and analyze collagen distributions, but the spatial resolution was 1µm in x, y and z-direction, which is too low to visualize the t-system (Rutherford et al., 2012).

Here we presented a set of computational methods to characterize the infarct border zone and normal myocardium in rabbit regarding the distribution of extracellular matrix, t-system structure and myocyte alignment. This allowed us to study the correlation between t-tubule integrity, local fibrosis and infarct distance. We discovered a relationship between local fibrosis and t-system remodeling, which is in agreement with a recently published study reporting increased collagen deposition in dilated t-tubules in human heart failure (Crossman et al., 2017).

4.1 Phenotypes of Remodeled T-System in Different Species

It is known that t-system structure differs in different mammal species. Typically, ventricular cardiomyocytes from larger animals like human, canine, porcine and ovine, but also rabbits, present a t-system of simple topology, comprising regularly arranged t-tubules. In contrast, rodents, which are used in most studies on cardiac disease and t-system remodeling, present a denser network of t-tubules with smaller diameter and a significantly larger fraction of axial, i.e. longitudinal, t-system components. These differences in normal t-system structure have been recognized in several studies (Heinzel et al., 2002; Jayasinghe et al., 2012; Lyon et al., 2009; Pinali et al., 2013; Richards et al., 2011). However, due to differences in normal t-system structure, translation of findings on t-system remodeling from rodents to humans is difficult.

In advanced human heart failure, the t-system remodels to a special phenotype, which was recently recognized as sheet-like t-system components. These “t-sheets” are dilated in axial myocyte direction, but still run transversely (Seidel et al., 2017). In two-dimensional images,

they can easily be mistaken for longitudinally oriented t-tubules (Katriel et al., 2000; Kostin et al., 1998; Seidel et al., 2017). T-system remodeling in failing rodent hearts has been studied extensively, but t-system components as found in human heart failure or in a porcine infarct model (Pinali et al., 2017) have not been identified. In contrast, images of remodeled t-tubules published by others (Driesen et al., 2007) and us (Seidel et al., 2016), including the present study, indicate that t-system remodeling in rabbit resembles the human phenotype. Driesen et al described vacuole-like structures in cardiomyocytes adjacent to the infarct border in rabbit and suggested that “the vacuoles represent plasma membrane invaginations and/or dilatations of t-tubular structures.” Based on our three-dimensional reconstructions (Fig. 2), which revealed startling similarity with those from remodeled human t-system (Seidel et al., 2017), we propose that phenotypes of remodeled t-system are similar in rabbits and humans. Thus, our studies suggest that rabbit models describe human t-system remodeling more accurately than rodent models.

4.2 Association of T-System Remodeling with Fibrosis and Infarct Distance

The MI border zone, roughly defined as the region between the infarct scar and more distant, unaffected myocardium, is hallmarked by ongoing remodeling processes (Sutton and Sharpe, 2000). Remodeling affects cardiomyocytes, e.g. by hypertrophy, necrosis and altered ion channel expression, and other tissue constituents, e.g. fibroblast proliferation and differentiation as well as expansion and stiffening of the extracellular matrix. This remodeling has functional effects on electrical activation and contraction. For instance, electrical activation in the border zone is delayed as a result of altered conduction pathways (Luke and Saffitz, 1991; Peters et al., 1997). This exposes the border zone to abnormal stretch and mechanical stress (Ashikaga et al., 2005), which in turn can induce remodeling of cardiomyocytes and fibroblasts, leading to fibrosis.

While it is common knowledge that fibrosis contributes to this vicious cycle, recent studies also linked remodeling of the t-system to both mechanical (Crossman et al., 2015; Frisk et al., 2016) and electrical alterations (Crocini et al., 2014; Sacconi et al., 2012; Sanchez-Alonso et al., 2016). Electrical alterations include absence of action potential propagation into t-tubules, which may result from increased collagen deposition in remodeled t-tubules (Crossman et al., 2017).

Here we showed that interstitial fibrosis and t-system remodeling accompany each other and follow a similar gradient within the infarct border zone. We found fibrotic patches together with remodeled t-tubules up to several millimeters away from the infarct scar (Fig. 3E), although the gradient of fibrosis and t-tubule remodeling was relatively steep within the first 500µm (Fig. 8). It would be interesting to explore if these gradients change with infarct age. If t-system remodeling expands with fibrosis over time, this would add to progressively reduced contractility often found after MI. One study described a gradient of collagen deposition and myocyte connectivity within a smaller region of only 200–300µm around the infarct scar 2 weeks after MI (Rutherford et al., 2012), whereas infarct age in our study was 3 weeks. However, the extent of border zone remodeling may also depend on species, size of the heart and extent of the infarct.

T-system integrity fell exponentially with increasing amounts of extracellular matrix. Surprisingly, the local amount of extracellular matrix was a better predictor of t-system remodeling than proximity to the infarct scar (Fig. 7), especially when f_{ECM} exceeded 30% (Fig. 8, Fig S5). This suggests that local mechanisms rather than global myocyte stress are responsible for t-system remodeling. An established view on progressive remodeling after MI is that myocyte necrosis results in a sudden increase in load on non-infarcted tissue, which then triggers hypertrophy and structural changes of the extracellular matrix (Pfeffer and Braunwald, 1990; Sutton and Sharpe, 2000). Although this process may eventually lead to heart failure and associated remodeling, it cannot easily explain the presence of structurally normal myocytes next to remodeled myocytes at the same infarct distance (Figs. 3I,K and 5D). It seems more likely that associated fibrotic patches are responsible for the observed myocyte and t-system remodeling at greater infarct distances. Alternatively, fibrosis may indicate altered mechanical load better than infarct distance, although this would mean that strain patterns change on the scale of only 100 to 200 μ m.

It is not fully understood how fibrotic patches distant from the infarct scar develop. Infiltration of inflammatory cells, which then activate fibroblasts was proposed (Kong et al., 2014) as well as microinfarction. Both mechanisms might directly lead to t-system remodeling, but studies linking t-system remodeling to inflammation do not exist. Another possibility is that fibrotic patches change the local environment by increasing tissue stiffness, changing fiber orientation or impeding synchronized electrical activation of myocytes. These conditions would lead to increased mechanical stress because of impaired shortening and relaxation, altered strain patterns during contraction or delayed contraction with initial overstretching.

4.3 Myocyte disarray

Cardiac fibrosis is often accompanied by myocardial disarray (Becker and Caruso, 1982), a condition in which parallel alignment of myocytes is lost. While in most studies this has been assessed by visual inspection of histopathological preparations, it was recently assessed using MRI (Chen et al., 2003; Strijkers et al., 2009). Here, we demonstrated an automated method based on confocal microscopy to measure parallel myocyte alignment: the percentage of nuclei having the same orientation as the mean fiber direction within a subregion. To reduce the influence of non-myocyte and out-of-focus nuclei, nuclei with a long-to-short-axis ratio below two or an area less than 20 μ m² were excluded, respectively.

We used this measure to rule out that our method of assessing t-system remodeling was confounded by poor myocyte alignment in fibrotic regions. We showed that in our samples myocyte disarray correlated only weakly with fibrosis, and even less with proximity to the infarct border. There was a correlation between myocyte alignment and t-tubule integrity (Fig. S4), possibly due to the method used for measuring t-tubule integrity. However, the correlation between t-tubule integrity and fibrosis was significantly higher, which rules out that a methodological artifact was responsible for our findings (Fig. 7).

We did not find a significantly lower degree of cardiomyocyte alignment in MI than in control samples, although myocardial disarray has been described in myocardial infarction (Milei et al., 1985; Whittaker et al., 1989). A possible reason is that disarray may be present

only on a smaller scale. We assessed disarray within subregions of approx. $200 \times 200 \mu\text{m}^2$, which may be too large to detect deviations of small numbers of myocytes from mean fiber orientation. In fact, we observed significantly lower myocyte alignment in MI ($67 \pm 2\%$) than control ($77 \pm 3\%$, $p < 0.05$) after decreasing subregion size to $100 \times 100 \mu\text{m}^2$. Reducing subregion size, however, did not lead to higher correlation between myocyte alignment and t-tubule integrity ($R^2 < 0.05$ in both control and MI).

4.4 Causes of T-System Remodeling: Potential Role of Biomechanics

Our finding that local fibrosis is associated with locally reduced t-system integrity on a scale of only $200 \times 200 \mu\text{m}^2$ suggests that the local microenvironment is involved in tissue and cellular remodeling. It is well established that the local environment is mechanically sensed by cells and can affect cell architecture including the cytoskeleton (Wang et al., 1993). Also, our previous work demonstrated that stretch and shortening of myocytes affect t-system geometry (McNary et al., 2011; McNary et al., 2012). This suggests that forces acting in the cellular environment are transduced to the t-system.

T-tubules are presumably stabilized by a number of anchoring proteins connecting the t-tubular membrane with the extracellular matrix and the cytoskeleton, for example vinculin, integrins and dystrophin (Kostin et al., 1998). It is easy to imagine that disruption of these anchoring proteins by elevated mechanical stress may destabilize t-tubule structure. Several studies have reported disorganization and altered expression levels of cytoskeletal and anchoring proteins in heart failure (Heling et al., 2000; Schaper et al., 1991). A current view is that in early disease stages, anchoring and cytoskeletal proteins, including microtubules, are upregulated to compensate for elevated mechanical stress. In decompensated stages, the cytoskeleton becomes disorganized (Hein et al., 2000). Similar observations have been made in the infarct border zone (Matsushita et al., 1999). Since heart failure and MI also lead to remodeling of the t-system, there is hence an association between cytoskeletal changes and t-system remodeling. Further evidence for cytoskeletal involvement in t-system remodeling comes from studies of Duchenne muscular dystrophy. Dystrophin, the protein affected in this disease, is an important anchoring protein between the cytoskeleton and extracellular matrix in skeletal and cardiac muscle. Dysfunction of dystrophin leads to increased vulnerability and deformability of the membrane in muscle cells (Garcia-Pelagio et al., 2011). Although skeletal muscles are affected in earlier stages of the disease, patients suffering from Duchenne muscular dystrophy often die from heart failure (Judge et al., 2011). It was recently shown that the t-system is disrupted in a mouse model of Duchenne dystrophy and that microtubules and junctophilin-2 are involved in pathogenesis (Prins et al., 2016). Altogether, these studies suggest that a mismatch between membrane stability and mechanical stress induces t-system remodeling.

In the infarct border zone, biomechanical heterogeneity caused by local fibrosis may lead to heterogeneous stress and abnormal strain within neighboring tissue and cells. This may happen through increased tissue stiffness, which will oppose passive cardiomyocyte relaxation during diastole. Fibrosis will also impede myocyte contraction during systole. It is thus conceivable that due to heterogeneous fibrosis myocytes undergo significant shearing, for instance, if one side of a myocyte is adjacent to a fibrotic region and the other to

normally contracting myocytes. The resulting shear stress could result in mechanical forces on the cytoskeleton that cannot be counterbalanced. It appears plausible that the anchoring between t-tubules and the SR via junctophilin is lost as a result of shear stress or that longitudinal strain dilates t-tubules in axial direction. This would lead to t-tubule loss or changes in t-tubule morphology as observed in MI and heart failure.

Our previous work on dyssynchronous heart failure revealed regional subcellular remodeling (Li et al., 2015), which was heterogeneous within myocytes. We suggested that alterations of mechanical strain profiles cause the remodeling, in particular, intensified strain extrema at cell ends. We here propose a correlated hypothesis to explain the findings in this study, i.e. that local heterogeneity of strain profiles caused by local fibrosis underlies the correlation of fibrosis with t-system remodeling. In our models of synchronous and dyssynchronous heart failure in canine, but also in many other animal models of heart disease without pronounced fibrosis, sheet-like remodeling did not occur. Thus, we suggest that t-sheets are caused by specific strain distributions produced by fibrosis.

4.5 Limitations

A limitation of our study is that we investigated the MI border zone only three weeks after coronary artery ligation. At this point in time, myocardial remodeling processes are not fully completed. Remodeling in humans may progress for years (Pfeffer and Braunwald, 1990; Sutton and Sharpe, 2000; Willems et al., 1994). Furthermore, our analyses were limited to areas less than 3mm from the MI border zones. At greater distances, the correlation between fibrosis and t-system remodeling might be weaker. Another limitation is that we only had two-dimensional information on the infarct scar. Our calculation of infarct border distance may therefore be flawed if biopsy needles were not in parallel with the transmural infarct border. To reduce this error, we took biopsies perpendicular to the epicardium while making sure that the infarct border was visible both on the epi- and endocardial side.

High-resolution analyses of tissue microstructure and its remodeling in large regions required automated methods for thresholding, determining fiber orientation and creating myocyte masks. These methods certainly introduced some variation, for example due to sub-optimal thresholding or misalignment of myocytes with the calculated fiber direction. However, we were still able to detect significant differences between the association of t-system remodeling with infarct distance and fibrosis. Also, we acknowledge that confocal microscopy has only a limited spatial resolution and some of our analyses would profit from higher resolution methods.

Finally, we note that results from rabbit models cannot be readily translated to humans. Nevertheless, we believe that rabbit is an appropriate model for studying t-system structure and its remodeling because of high similarity in t-system morphology between rabbit and human (Jayasinghe et al., 2012). Our finding of t-sheets supports that rabbit models exhibit similar t-system remodeling as human.

While our study supported the hypothesis that local fibrosis is spatially associated with t-system remodeling, we did not investigate mechanisms of the remodeling processes. Potential mechanistic studies include investigating the causal relationship between fibrosis

or biomechanical stress and t-system remodeling, for example by investigating animal models of progressive fibrosis, earlier infarct stages and effects of anti-fibrotic drugs on t-system remodeling.

5. Conclusions

T-system remodeling in the border zone of myocardial infarction in rabbit resembles phenotypes found in end-stage human heart failure and correlates strongly with fibrosis. This suggests a common mechanism that underlies t-system remodeling not only in different species, but also in different cardiac diseases. Since fibrosis was a better predictor of t-system remodeling than proximity to the infarct scar, local mechanisms seem more plausible than global myocyte stress. We suggest that in addition to activation of pro-fibrotic pathways and myocyte necrosis, biomechanical strain profiles caused by local stiffening of the extracellular matrix may lead to t-system remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LCC	L-type Ca^{2+} channel
RyR	Ryanodine receptor
SR	Sarcoplasmic reticulum
T-tubules	Transverse tubules
T-system	Transverse tubular system
WGA	Wheat germ agglutinin
f_{ECM}	Fraction of extracellular matrix
I_{TT}	T-tubule integrity
d_{scar}	distance from infarct scar

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Figure 1. Image processing and analysis applied to subregions of large-area confocal scans
 (A) The raw alpha actinin image was Fourier-transformed to obtain the (B) two-dimensional power spectrum. Fiber direction (θ_f) and sarcomere length (SL) were identified from the local maximum within $f=1/1.4 \mu\text{m}^{-1}$ to $f=1/2.2\mu\text{m}^{-1}$. The alpha actinin image was thresholded to obtain (C) the segmented alpha actinin. Morphological closing in fiber direction then yielded (D) the myocyte mask. The myocyte mask was applied to the (E) WGA image to separate the (F) extracellular and (G) cardiomyocyte WGA signal. The cardiomyocyte signal consisted mainly of the t-system and outer membrane, whereas the extracellular signal comprised collagen, blood vessels and fibroblasts. After thresholding, the extracellular fraction, f_{ECM} , was calculated from the extracellular signal. (H) The power spectral density in the Fourier-transformed myocyte signal was integrated within $\theta_f \pm 15^\circ$ and $SL \pm 0.2\mu\text{m}$ and normalized by total intensity of the myocyte signal to obtain t-tubule integrity (I_{TT}). (I) The DAPI image was thresholded and median-filtered to obtain (J) segmented nuclei. (K) After connected-component identification and principal component analysis, main axis orientation of each nucleus was determined (arrows). (L) Angles between θ_f and nucleus main axis orientations (θ_{nuclei}) were calculated, indicating alignment with θ_f . Scale bar: $40\mu\text{m}$.

Figure 2. Remodeling of t-tubules to t-sheets in rabbit MI border zone

Three-dimensional images obtained by confocal microscopy of WGA-labeled tissue (green) from (A) control and (D) MI border zone. Segmented cells are highlighted in red. (B) and (E) show extracted cells and t-systems from the corresponding cells highlighted in (A) and (D), respectively. The outer sarcolemma is indicated in green, the t-system in red. Three-dimensional reconstructions of (C) normal and (F) remodeled t-system components indicate regular t-tubules in control, but sheet-like components in the MI border zone. Arrows point to the outer sarcolemma. Scale bars: (A) 40 μ m, applies to D, (B) 20 μ m, applies to E, (C) 5 μ m, applies to F.

Figure 3. Large-area tile scans of normal and infarcted cardiac tissue

Confocal scans of left-ventricular biopsies from (A–D) control and (E–L) MI border zone, labeled with WGA (green) and for alpha actinin (red). (A, B) Overview of control tissue. (C, D) Zoom-ins of regions highlighted in A and B, respectively. In control, only a small amount of extracellular matrix is present. T-system and alpha actinin patterns are regular. (E, F) Overview of MI border zone. (G–L) Zoom-ins of regions highlighted in E and F. (G, H) Close to the infarct border, interstitial fibrosis is visible as an increased amount of extracellular matrix. The alpha actinin pattern remains regular, but the t-system is severely remodeled. Distant from the infarct, (I, J) regions without fibrosis exhibit normal cell and tissue structure, but in (K, L) fibrotic regions the t-system is remodeled. Scale bars: (A and

E) 1mm, apply to (B) and (F), respectively; (C) 50 μ m (upper panel) and 20 μ m (lower panel), apply to (D, G–L) upper and lower panels, respectively.

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Figure 4. Representative example of control tissue with analysis

(A) Raw image from labeling with WGA. (B) Local fiber orientation, θ_f , shown as angle in degrees to horizontal axis. (C) Local sarcomere length, SL , in μm . (D) Local t-tubule integrity, I_{TT} . (E) Local myocyte alignment with fiber orientation, displayed as the percentage of nuclei with their main axis within $\theta_f \pm 15^\circ$. (F) Local fraction of extracellular matrix, f_{ECM} . (G) Scatter plot indicating that I_{TT} did not correlate with f_{ECM} . Scale bar: (A) 1mm, applies to (B–F).

Figure 5. Representative example of MI border zone

(A) Raw image from labeling with WGA. (B) Local fiber orientation, θ_f , shown as angle in degrees to horizontal axis. (C) Local sarcomere length, SL , in μm . (D) Local t-tubule integrity, I_{TT} . (E) Local myocyte alignment with fiber orientation, displayed as the percentage of nuclei with their main axis within $\theta_f \pm 15^\circ$. (F) Local fraction of extracellular matrix, f_{ECM} . Scale bar: (A) 1mm, applies to (B–F).

Figure 6. Analysis of MI border zone sample

(A) Scatter plot and monoexponential model, showing that I_T fell exponentially with increasing f_{ECM} . (B) Scatter plot and linear model, showing that I_T correlated with d_{scar} . (C) Scatter plot and linear model indicating that f_{ECM} correlated negatively with d_{scar} .

Figure 7. Summary and statistical analysis of control and MI samples

(A) Coefficients of determination (R^2) obtained from correlating t-tubule integrity, I_{TT} , with the amount of extracellular matrix (f_{ECM}) in a monoexponential model, or with distance from the infarct scar (d_{scar}) and myocyte alignment in linear models. Data points represent individual values from 6 control (circles) and 6 MI samples (crosses). (B) Mean and standard errors of data shown in (A). ** $p < 0.01$ MI vs control (2-sample t-test), # $p < 0.05$ and ## $p < 0.01$ vs f_{ECM} in MI (paired t-test). (C) Mean myocyte alignment with fiber orientation, which did not differ between control and MI.

Figure 8. Fibrosis and t-tubule integrity in MI border zone

(A) Distribution of extracellular matrix (f_{ECM} , mean and standard error) with increasing infarct distance. (B) Distribution of t-tubule integrity (I_{TT} , mean and standard error) with increasing infarct distance. (C) Mean I_{TT} in regions with specified f_{ECM} . (D) Mean I_{TT} in regions with specified f_{ECM} and infarct distance < 1mm. (E) Mean I_{TT} in regions with specified f_{ECM} and infarct distance > 1mm.