



# UNIVERSITÀ DI PARMA

## ARCHIVIO DELLA RICERCA

University of Parma Research Repository

Nanoscale Study of Calcium Handling Remodeling in Right Ventricular Cardiomyocytes Following Pulmonary Hypertension

This is the peer reviewed version of the following article:

*Original*

Nanoscale Study of Calcium Handling Remodeling in Right Ventricular Cardiomyocytes Following Pulmonary Hypertension / Medvedev, R.; Sanchez-Alonso, J. L.; Alvarez-Laviada, A.; Rossi, S.; Dries, E.; Schorn, T.; Abdul-Salam, V. B.; Trayanova, N.; Wojciak-Stothard, B.; Miragoli, M.; Faggian, G.; Gorelik, J.. - In: HYPERTENSION. - ISSN 1524-4563. - 77:2(2021), pp. 605-616. [10.1161/HYPERTENSIONAHA.120.14858]

*Availability:*

This version is available at: 11381/2887682 since: 2022-01-10T12:15:57Z

*Publisher:*

Lippincott Williams and Wilkins

*Published*

DOI:10.1161/HYPERTENSIONAHA.120.14858

*Terms of use:*

openAccess

Anyone can freely access the full text of works made available as "Open Access". Works made available

*Publisher copyright*

(Article begins on next page)

# AUTHOR QUERIES

***Authors please note: Authors are responsible for any page charges as outlined in the acceptance letter or as indicated on the Instructions for Authors (<https://www.ahajournals.org/hyp/revise-accepted-manuscripts>). Unless you have selected open access for your article, or it is otherwise noted on the acceptance letter, page charges are as follows: \$70 per black and white page (print articles only) or \$35 per page (online-only articles only). For each color page (print only), please add \$653/page. If there are any concerns regarding these charges, these should be addressed within 48 hours of receiving the s-proof. Author(s) will be invoiced for all color and page charges post publication. If you have selected open access for your article, please refer to details in the queries below.***

## AUTHOR PLEASE ANSWER ALL QUERIES

- AQ1—Please note only those terms that are used 5 times or more can be abbreviated, except trial names and proteins, which should be expanded at first use but then can be abbreviated throughout regardless of how many times they appear.
- AQ2—Please turn to page 3 of your proof and review the running head, which will appear in the upper right-hand margins of odd-numbered pages. Running heads must be 50 or fewer characters in length, including spaces and punctuation. If your original short title was longer than 50 characters, we may have shortened it. Please modify if necessary (but observe our length guidelines).
- AQ3—Please confirm that all authors are included in the correct order in the byline and that all names are spelled correctly, including special characters, accents, middle initials, and degrees, if applicable. For indexing purposes, confirm author names have been correctly identified as given names (blue), surnames (red), and suffixes (black). Color in the byline will not appear on the final published version. Note that journal style discourages listing honorary degrees (FAHA, FRCP, etc.) in the byline; please delete such degrees from the author byline.
- AQ4—Key words may have been edited to match the US National Library of Medicine's Medical Subject Headings (<http://www.nlm.nih.gov/mesh/MBrowser.html>). If they need modification, please limit the total number of key words to 7.
- AQ5—Please define SR, Di-8-ANEPPS, PKA, CaMKII in text.
- AQ6—Please define SERCA2a in text.
- AQ7—Please note that as per journal style, in the first occurrence of a protein name in the text, protein abbreviation should appear first, followed by its expansion in parentheses. Therefore, please check and add expansion for the protein names within parentheses next to the first use of the protein name in the abstract, text, and in figure legends.
- AQ8—If you have color in your proof, please indicate whether you approve the color charge by returning the color agreement with your proof corrections. The color agreement can

be found at <http://links.lww.com/HYP/A2>. The rate is \$653 per 1 printed color page. If you approve the cost, please sign and return the color letter. If print color is declined, the figures will appear in color online-only. If you have any questions, please contact [HypertensionJournal@wolterskluwer.com](mailto:HypertensionJournal@wolterskluwer.com).

AQ9—Please define AU in Figures 1 and 4. Please note, per style, “N” should be used to indicate total number of units in a study and “n” should indicate the units in a subgroup of the study. Please confirm that these were handled appropriately throughout the article. Example: “Of the patients admitted from the emergency department (N=127), the most frequent admission diagnosis was unstable angina (n=38)”.

AQ10—Please define SICM in text and Figure 4.

AQ11—Please check the clarity of the sentence “While a trend towards a reduction of Cav1.2 protein...”

AQ12—Please confirm that all authors’ institutional affiliations (including city/state/country locations) are correct as shown in the affiliations footnote.

AQ13—Please carefully review any Acknowledgments, Sources of Funding, and/or Disclosures listed at the end of the manuscript (before the References), and confirm that they are accurate and complete for all authors.

# Hypertension

## ORIGINAL ARTICLE

# Nanoscale Study of Calcium Handling Remodeling in Right Ventricular Cardiomyocytes Following Pulmonary Hypertension

Roman Medvedev, Jose L. Sanchez-Alonso, Anita Alvarez-Laviada, Stefano Rossi<sup>1</sup>, Eef Dries<sup>1</sup>, Tilo Schorn<sup>1</sup>, Vahitha B. Abdul-Salam<sup>1</sup>, Natalia Trayanova<sup>1</sup>, Beata Wojciak-Stothard, Michele Miragoli, Giuseppe Faggian<sup>1</sup>, Julia Gorelik<sup>1</sup>

**ABSTRACT:** Pulmonary hypertension is a complex disorder characterized by pulmonary vascular remodeling and right ventricular hypertrophy, leading to right heart failure. The mechanisms underlying this process are not well understood. We hypothesize that the structural remodeling occurring in the cardiomyocytes of the right ventricle affects the cytosolic  $Ca^{2+}$  handling leading to arrhythmias. After 12 days of monocrotaline-induced pulmonary hypertension in rats, epicardial mapping showed electrical remodeling in both ventricles. In myocytes isolated from the hypertensive rats, a combination of high-speed camera and confocal line-scan documented a prolongation of  $Ca^{2+}$  transients along with a higher local  $Ca^{2+}$ -release activity. These  $Ca^{2+}$  transients were less synchronous than in controls, likely due to disorganized transverse-axial tubular system. In fact, following pulmonary hypertension, hypertrophied right ventricular myocytes showed significantly reduced number of transverse tubules and increased number of axial tubules; however, **Stimulation Emission Depletion** microscopy demonstrated that the colocalization of L-type  $Ca^{2+}$  channels and RyR2 (**ryanodine receptor 2**) remained unchanged. Finally, **Stimulation Emission Depletion** microscopy and **super-resolution** scanning patch-clamp analysis uncovered a decrease in the density of active L-type  $Ca^{2+}$  channels in **right ventricular** myocytes with an elevated open probability of the T-tubule anchored channels. This may represent a general mechanism of how nanoscale structural changes at the early stage of pulmonary hypertension impact on the development of the end stage failing phenotype in the right ventricle. (**Hypertension. 2020;77:00-00. DOI: 10.1161/HYPERTENSIONAHA.120.14858.**) • **Data Supplement**

**Key Words:** calcium ■ hypertension ■ myocytes, cardiac ■ phenotype ■ vascular remodeling

**P**ulmonary arterial hypertension (PAH) is a vascular disease induced by increased proliferation of cells in the pulmonary vasculature, leading to progressive narrowing of the vascular lumen and increased workload of the right ventricle (RV).<sup>1</sup> PAH patient survival is largely determined by the ability of the RV to adapt to the pressure overload.<sup>2</sup> In PAH, the RV undergoes structural<sup>3</sup> and electrical remodeling.<sup>4,5</sup> Although the PAH-induced changes in the RV are known, so far there is no specific RV targeted treatment. Although treatments effective for the left ventricular (LV) hypertrophy have been proposed for the RV dysfunction,<sup>1</sup> the differences in the biological origin and working conditions of the 2 chambers<sup>6</sup> raise

questions about the suitability of such approach. A better understanding of the mechanisms of RV remodeling in PAH is clearly needed.

RV hypertrophy has been studied by different groups using a **monocrotaline**-induced model of pulmonary hypertension (PH) in rats.<sup>7-9</sup> This model reproduces several important aspects of the cellular and molecular remodeling linked to human PAH pathogenesis.<sup>10</sup> At the level of  $Ca^{2+}$  handling, some discrepancies have been found, with some groups finding smaller and slower  $Ca^{2+}$  transients<sup>9,11</sup> and others observing an increase in  $Ca^{2+}$  transients and more intensive contractions in myocytes.<sup>7,8</sup> Furthermore, alterations in  $Ca^{2+}$  transients have been

Correspondence to: Julia Gorelik, Department of Cardiovascular Sciences, National Heart and Lung Institute, 4th floor, Imperial Centre for Translational and Experimental Medicine, Hammersmith Campus, Imperial College London, Du Cane Rd, London W12 0NN, United Kingdom. Email jgorelik@ic.ac.uk

The Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.120.14858>.

For Sources of Funding and Disclosures, see page xxx.

© 2020 American Heart Association, Inc.

Hypertension is available at [www.ahajournals.org/journal/hyp](http://www.ahajournals.org/journal/hyp)

## Novelty and Significance

### What Is New?

- We observed that at an early stage of pulmonary hypertension, myocytes of right ventricle develop local nanoscale changes in L-type calcium channel and  $\text{Ca}^{2+}$  spark properties which could encourage dysregulation of  $\text{Ca}^{2+}$  transients which in turn could form a proarrhythmic substrate in the whole heart.

### What Is Relevant?

- We found alterations in transverse and axial tubular network and surface topography of myocytes, leading to changes in local L-type calcium channel activity and  $\text{Ca}^{2+}$  spark properties, which occurred before a significant arrhythmogenic remodeling in the whole heart.

### Summary

Right ventricular myocytes of the heart have a distinct ultrastructure and functional behavior as compared to left ventricle myocytes. Pulmonary hypertension induces membrane remodeling in right ventricular myocytes and alteration of the local properties of L-type calcium channels and  $\text{Ca}^{2+}$  sparks, producing significantly altered  $\text{Ca}^{2+}$  transients and prolonged refractoriness on the whole heart level. Left ventricle at this stage of the disease is mostly preserved in terms of its structure and function, but we cannot exclude its involvement at later stages.

## Nonstandard Abbreviation and Acronyms

<b>CV</b>	conduction velocity
<b>ERP</b>	effective refractory period
<b>LTCC</b>	L-type $\text{Ca}^{2+}$ channel
<b>LV</b>	left ventricle
<b>PAH</b>	pulmonary arterial hypertension
<b>PH</b>	pulmonary hypertension
<b>Po</b>	open probability
<b>RV</b>	right ventricle
<b>RyR2</b>	ryanodine receptor 2
<b>TATS</b>	transverse-axial tubular system
<b>TTF50</b>	time to reach half-maximal fluorescence

linked to structural changes in the transverse-axial tubular system (TATS).<sup>9,12</sup> Specifically, in PH RV myocytes, it has been shown that the increase in spontaneous  $\text{Ca}^{2+}$  releases could be due to a reduction of SERCA2a pump expression and activity.<sup>7</sup> Interestingly, considering the importance of L-type  $\text{Ca}^{2+}$  channels (LTCC) in the  $\text{Ca}^{2+}$  handling process, no alteration in expression or whole-cell current was reported in PH RV myocytes.<sup>7,13</sup>

These described changes in  $\text{Ca}^{2+}$  signaling could be due to the reorganization in the microdomains. Recently, we reported that microdomain remodeling in the failing human and rat LV myocytes could produce the arrhythmogenic triggers that lead to reentrant arrhythmias.<sup>14</sup> Microdomain organization in ventricular myocytes is maintained by the elaborate organization of TATS, bringing LTCCs close to RyR2 (ryanodine receptor 2) and establishing an excitation-contraction coupling. The loss of TATS in heart failure has been associated with the redistribution of functional LTCCs from T-tubule to sarcolemma membrane<sup>14</sup> and with the uncoupling of

sarcolemmal reticulum from the sarcolemma membrane.<sup>15</sup> The redistribution of the LTCCs is accompanied by an increased open probability,<sup>14,16</sup> likely due to the channel hyperphosphorylation of the channels. Although this is well described in LV myocytes, little is known about the microdomain organization of RV myocytes and their changes during RV failure. We hypothesized that RV myocytes have less complex organization of the excitation-contraction coupling microdomains compared to LV myocytes due to the lower workload of the chamber and that in PH the microdomain remodeling leads to relocation and hyperactivation of LTCCs.

In this study, we evaluated in an early stage of PH, the functional and structural changes in RV and LV myocytes at nanoscale level that could be responsible for the altered cardiac electrophysiology seen in preclinical models of PH. We have identified prominent cytoarchitectural changes at the single-cell level, from the TATS regularity to the surface topography. Our approach allowed us to distinct two subpopulations of LTCC and RyR2 in RV cardiomyocytes; one located close to the T-tubules and one away from them. Hyperactivation of T-tubular LTCC in conjunction with SR RyR2 coupling could be responsible for the high spontaneous  $\text{Ca}^{2+}$  releases, desynchronization of  $\text{Ca}^{2+}$  transients, and appearance of alternans in PH hearts.

## METHODS

The data supporting the results presented in this article is available from the corresponding authors upon reasonable request. Detailed descriptions of animal procedures, cell isolation, and other methodologies are available in the Data Supplement. This work was approved by the Animal Welfare and Ethics Review Board of Imperial College London. It is guided by United Kingdom Home Office guidelines (ASPA 1986 and EU Directive 2010/83). The monocrotaline model of PH was

generated in male Sprague-Dawley rats by 60 mg/kg intraperitoneal injection,<sup>17</sup> with the experiments performed on days 10 to 12 after injection. Epicardial mapping with  $11 \times 11$  multielectrode grid was applied to analyze electrical properties of the tissue.<sup>18</sup> Standard procedures were applied to measure the conduction velocity (CV), excitability, refractoriness, and mean parameters of ECG.<sup>19,20</sup> Cell isolation was performed using a modified Landendorf perfusion protocol.<sup>21</sup> Data analysis was performed using GraphPad prism 6. Normality was assessed by the Kolmogorov-Smirnov test. In cases where data failed the normality test, the nonparametric Kruskal-Wallis ANOVA with Dunn *post hoc* analysis was used instead of the 2-way ANOVA test with Bonferroni *post hoc* comparison. Fisher exact test was used for the occurrence analysis of extrasystole,  $\text{Ca}^{2+}$  transient alternans, and the analysis of LTCC density. All data were expressed as mean  $\pm$  SEM. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Early Stage PH Rat Characterization, RV Hypertrophy With Conduction Abnormalities

PH monocrotaline treated rats showed a significant increase in the mean pulmonary artery pressure within 12 days of monocrotaline injection (Figure 1B), which was accompanied by a higher lung weight (Figure S1B in the Data Supplement) and muscularization (Figure S1C and S1D). RV hypertrophy was indicated by a markedly increased ratio of RV to LV/septum weight in PH rats (Figure 1C), as previously reported in this model.<sup>7B</sup>

The electrophysiological properties of RV and LV control and PH rats were studied by epicardial multiple lead recording. The multielectrode grid and representative isochrone maps measured in RV and LV are presented in Figures S2 and S3A, respectively. PH led to a decrease in CV along epicardial fiber direction longitudinally in both ventricles but only statistically significant in LV tissue ( $P < 0.05$ , Figure S3B). We found no changes in CV across epicardial fiber direction transversally in both RV and LV after PH (Figure S3C). Interestingly, when the CV along epicardial fiber direction longitudinally/CV across epicardial fiber direction transversally was calculated to measure the anisotropy ratio of CV (Figure 1D), it showed a lower value in PH rats and only statistically significant in RV ( $P < 0.05$ ). The refractoriness of the tissue was assessed with the analysis of the effective refractory period (ERP). In control animals, the ERP in RV was significantly shorter than in LV ( $P < 0.01$ , Figure 1E). PH led to a prolongation of ERP both in the RV ( $P < 0.01$ ) and the LV ( $P < 0.05$ ). The basic ECG parameters were also evaluated (Figure S4). We found longer QT intervals in the LV compared with the RV ( $P < 0.001$ ) and the RV in PH showed prolonged QT with respect to that in control RV ( $P < 0.05$ , Figure S4C).

The arrhythmogenicity of the tissue was assessed by the occurrence of extrasystoles. We recorded a

higher number of animals with extrasystoles in the PH group, 5 out of 7 animals, versus 3 out of 7 animals in the control group, but this difference was not statistically significant (Figure 1F).

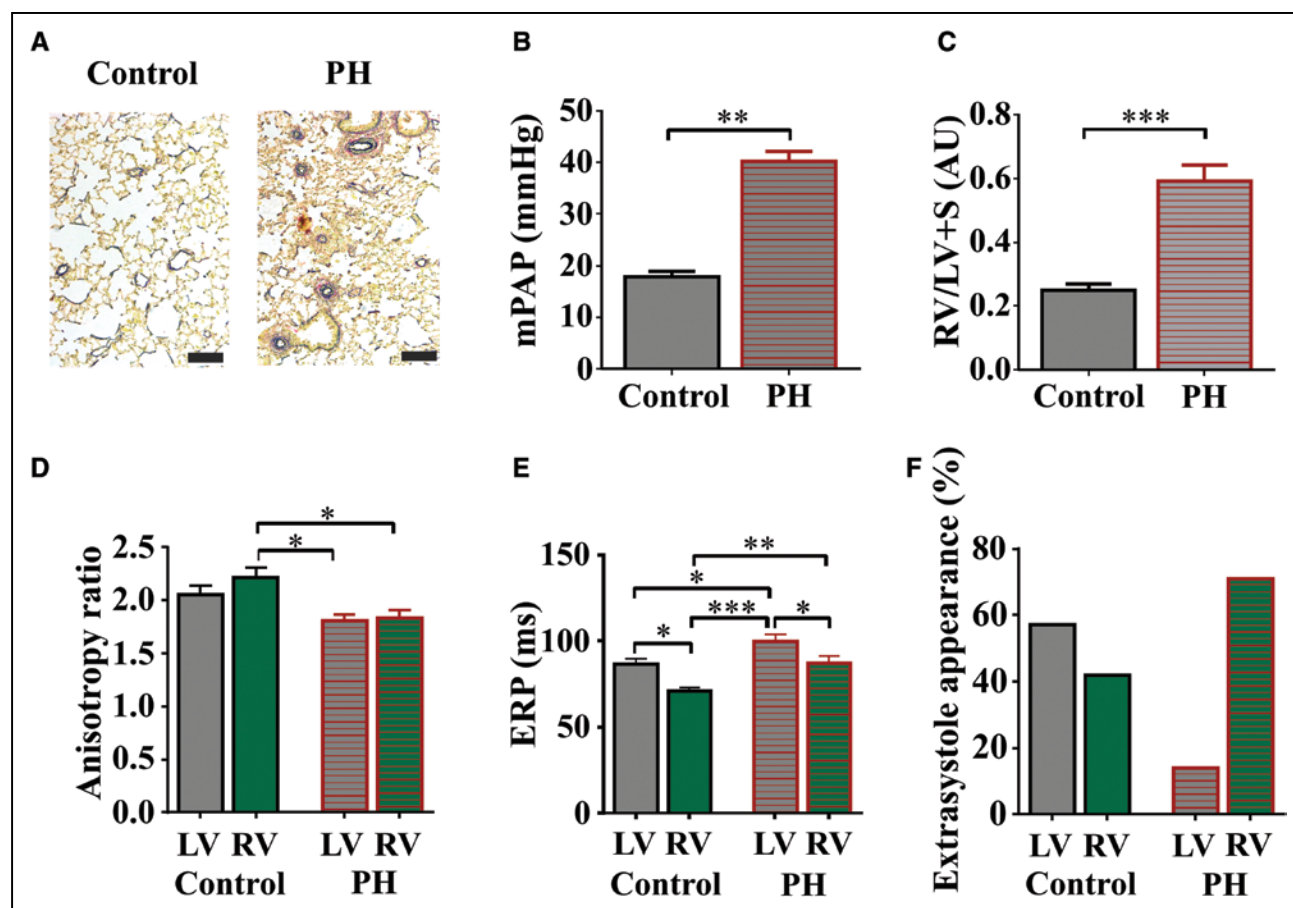
### Increased Local $\text{Ca}^{2+}$ Transients in PH RV Cardiomyocytes Promotes $\text{Ca}^{2+}$ Alternans

To evaluate  $\text{Ca}^{2+}$  handling in PH rat myocytes, we analyzed the spontaneous  $\text{Ca}^{2+}$  waves by optical mapping according to.<sup>22</sup> Two types of waves were examined, local and propagated through the entire cell, as shown in Figure 2A. The frequency of local waves was significantly elevated in PH RV myocytes ( $P < 0.05$ , Figure 2C) without changes in the frequency of propagated waves (Figure S5). Local  $\text{Ca}^{2+}$  releases were then analyzed using the line mode of the confocal microscope.<sup>23</sup> A representative  $\text{Ca}^{2+}$  spark image is shown in Figure 2B. In the control group, RV myocytes showed a similar frequency of  $\text{Ca}^{2+}$  sparks as LV myocytes (Figure 2D), but spark mass was lower in RV myocytes (Figure 2E). In the PH group, RV myocytes exhibited a significant increase in  $\text{Ca}^{2+}$  spark frequency (Figure 2E) and mass (Figure S5B) as compared to the control group. These properties of  $\text{Ca}^{2+}$  sparks were preserved in PH LV myocytes. Elevated rate of  $\text{Ca}^{2+}$  sparks could be associated with elevated SR  $\text{Ca}^{2+}$  load or abnormal SERCA2a  $\text{Ca}^{2+}$  reuptake. However, caffeine-induced  $\text{Ca}^{2+}$  release experiments did not show significant changes in fractional  $\text{Ca}^{2+}$  release and SERCA2a activity (Figure S6).

Abnormal  $\text{Ca}^{2+}$  cycling has been shown to be associated with the appearance of  $\text{Ca}^{2+}$  transients alternans.<sup>24</sup> When single cardiomyocytes were paced at 4 Hz, the proportion of cardiomyocytes showing  $\text{Ca}^{2+}$  transient alternans in PH RV myocytes were significantly higher than in control RV myocytes (Figure 2E).

To examine the effects of the increased spontaneous local  $\text{Ca}^{2+}$  activity in PH RV myocytes, we performed analysis of the electrically evoked  $\text{Ca}^{2+}$  transients (Figures 3A and S7).  $\text{Ca}^{2+}$  transients of significantly slower time to peak were observed in PH RV myocytes (Figure 3B). Next, the spatial variability of time to reach half-maximal fluorescence (TTF50) across the cell was analyzed. Histograms of TTF50 in LV myocytes have a peak around 10 ms, whereas for RV myocytes the peak is lower and the distribution is wider (Figures S8A and S8B). A dys-synchrony index defined as the SD of TTF50,<sup>25</sup> showed a significantly higher value in PH RV myocytes as compared to control RV myocytes (Figure 3C).

We used the approach of Heinzel et al<sup>26</sup> and Biesmans et al<sup>27</sup> to divide  $\text{Ca}^{2+}$  release points into early and late ones according to their TTF50 (Figure S8C). We used 50% of the cumulative histogram as a threshold value for this division (Figure S8D).  $\text{Ca}^{2+}$  sparks occurring in early or delayed areas were analyzed separately. In PH RV myocytes,  $\text{Ca}^{2+}$  spark duration and time to peak in delayed areas were significantly prolonged (Figures 3D



**Figure 1. Characterization of control and pulmonary hypertension (PH) rats.**

**A**, Elastic van Gieson representative staining of peripheral arteries in control and monocrotaline (MCT) treated animals. Scale bar, 100  $\mu$ m. **B**, Mean pulmonary arterial pressure (mPAP) measured in control and MCT treated rats. **C**, Proportion of right ventricle (RV) weight to (LV)+septum (S) weight in control and MCT treated animals (N=7–12 rats, \*\* $P$ <0.01, \*\*\* $P$ <0.001). **D**, Anisotropy ratio averages of conduction velocity (conduction velocity along epicardial fiber direction longitudinally/conduction velocity across epicardial fiber direction transversally). **E**, Effective refractory period (ERP) measures. **F**, The percentage of the RV and LV showing extrasystole appearance (N=7 rats, \* $P$ <0.05, \*\* $P$ <0.01).

and S9G).  $\text{Ca}^{2+}$  sparks from delayed areas had a higher frequency and amplitude in PH RV myocytes but not in PH LV myocytes (Figure S9A through S9F).

### Structural Regularity Is Reduced in PH RV Myocytes

It has been shown previously that the reorganization of membrane structure affects the  $\text{Ca}^{2+}$  handling and synchronization in the LV myocytes.<sup>25,28,29</sup> To study the organization of the sarcolemma membrane, TATS and surface topography of RV and LV myocytes were visualized by Di-8-ANEPPS membrane staining and SICM, respectively. Representative images are shown in Figure 4A. In analyzing the cardiomyocytes shape the length to width ratio was decreased in PH RV myocytes ( $P$ <0.01, Figure 4B). RV myocytes from PH rats had a reduced length (Figure S10A) and increased width (Figure S10B) compared with control ones, whereas LV myocytes became thinner in PH (Figure S10B).

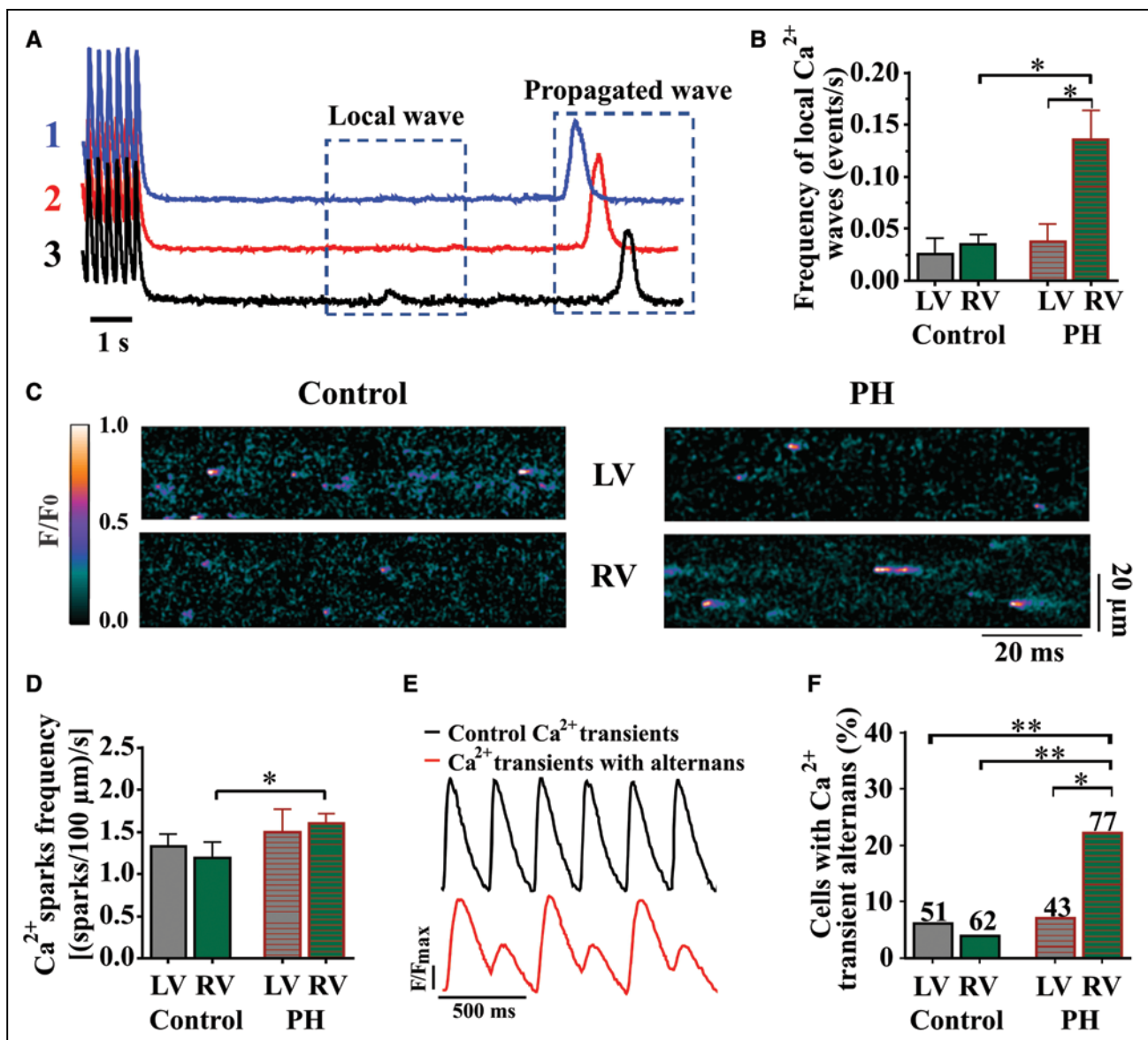
In the analysis of SICM images, we used the z-groove ratio as an index of surface regularity<sup>30</sup> and found a

significant z-groove index reduction in RV myocytes in PH (Figure 4C) as compared to control. However, despite this reduction, the number of T-tubule openings on the surface of PH myocytes was unaffected (Figure S10C).

Although the total density of the TATS was preserved in PH rats (Figure S10D), the average TATS regularity was reduced in PH RV cardiomyocytes (Figure 4D). The directional analysis of TATS<sup>31</sup> showed a rearrangement of tubules from the transversal to the axial direction in PH, which could explain the differences in the regularity (Figure 4E). The average fraction of the axial tubules was significantly higher in PH RV myocytes as compared to control (Figure 4F), whereas the average fraction of transverse tubules was significantly lower (Figure 4F).

### RV Cardiomyocytes Exhibit Smaller $\text{Ca}_v1.2$ Density

We hypothesized that remodeling of the TATS in PH leads to dissociation of LTCC from RyR2, which could be the cause for the  $\text{Ca}^{2+}$  handling changes mentioned



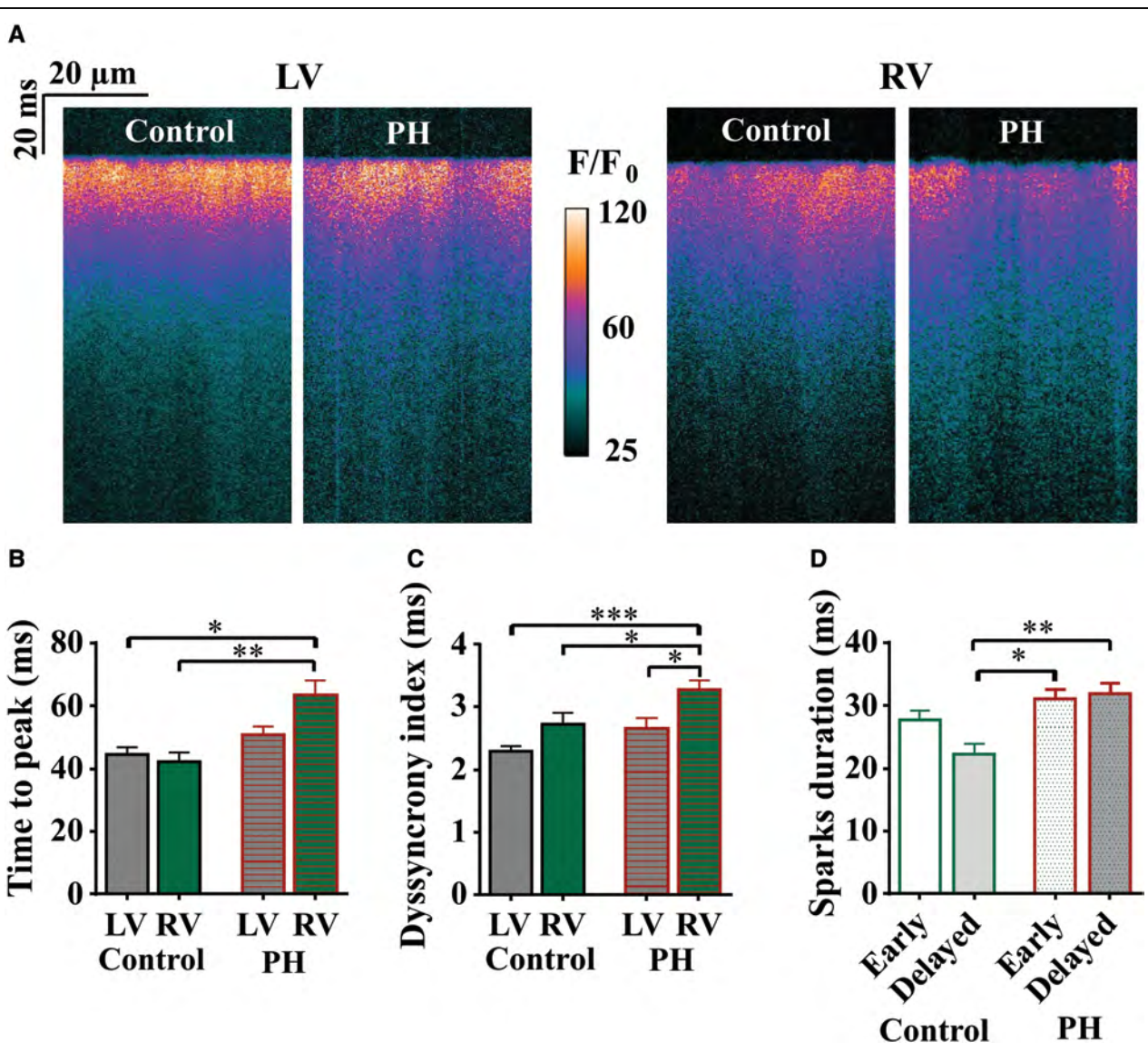
**Figure 2. Pulmonary hypertension (PH)-induced high spontaneous Ca<sup>2+</sup> activity in right ventricular (RV) but not in left ventricular (LV) myocytes.**

**A**, Representative traces of spontaneous Ca<sup>2+</sup> activity measured in isolated ventricular myocytes. Cells were electrically paced at 4 Hz for 1 min to enhance sarcoplasmic reticulum Ca<sup>2+</sup> loading. Local and propagated Ca<sup>2+</sup> waves were recorded during a 16-s rest period after cessation of pacing. On the **left**, optical traces showing changes in [Ca<sup>2+</sup>] from 3 different points (1–3) selected in a single cardiomyocyte, on the **right**. **B**, Average frequency of local Ca<sup>2+</sup> waves (n=25–45 cells, N=5–9 rats, \*P<0.05). **C**, Representative confocal line scans of LV (**top**) and RV (**bottom**) myocytes loaded with Fluo4-AM. Recordings were performed during 2 s rest period after 1 min of pacing. **D**, Average Ca<sup>2+</sup> sparks frequency in control and PH myocytes (n=30–67 cells, N=4–5 rats). **E**, Representative traces of Ca<sup>2+</sup> transients with and without alternans at 4 Hz stimulation. **F**, The percentage of cells showing Ca<sup>2+</sup> transients alternans in control and PH myocytes (n=30–67 cells, N=5–6 rats, \*P<0.05, \*\*P<0.01).

above. It has been shown that in **heart failure**, orphaned RyR2 are the source of increased spontaneous Ca<sup>2+</sup> activity.<sup>32</sup> To determine the amount of LTCC colocalized with RyR2, we performed Stimulation Emission Depletion imaging on fixed myocytes. Figure 5A illustrates representative **Stimulation Emission Depletion** images of RV and LV myocytes stained with antibodies against Ca<sub>v</sub>1.2 (magenta) and RyR2 (cyan). There were no differences in Ca<sub>v</sub>1.2 and RyR2 signal densities between control RV

and LV myocytes (Figure 5B and 5C). PH promoted a reduction of Ca<sub>v</sub>1.2 signal density in RV myocytes, but not in LV ones (Figure 5B). No alterations in signal density of RyR2 staining were found in PH (Figure 5C). These results were supported by Western blot analysis (Figure S11). While a trend towards a reduction of Ca<sub>v</sub>1.2 protein expression in RV tissue after PH (Figure S11B), no changes were observed in the levels of RyR2 protein expression in RV tissue after PH (Figure S11D).





**Figure 3. Pulmonary hypertension (PH)-induced desynchronization of  $\text{Ca}^{2+}$  transients.**

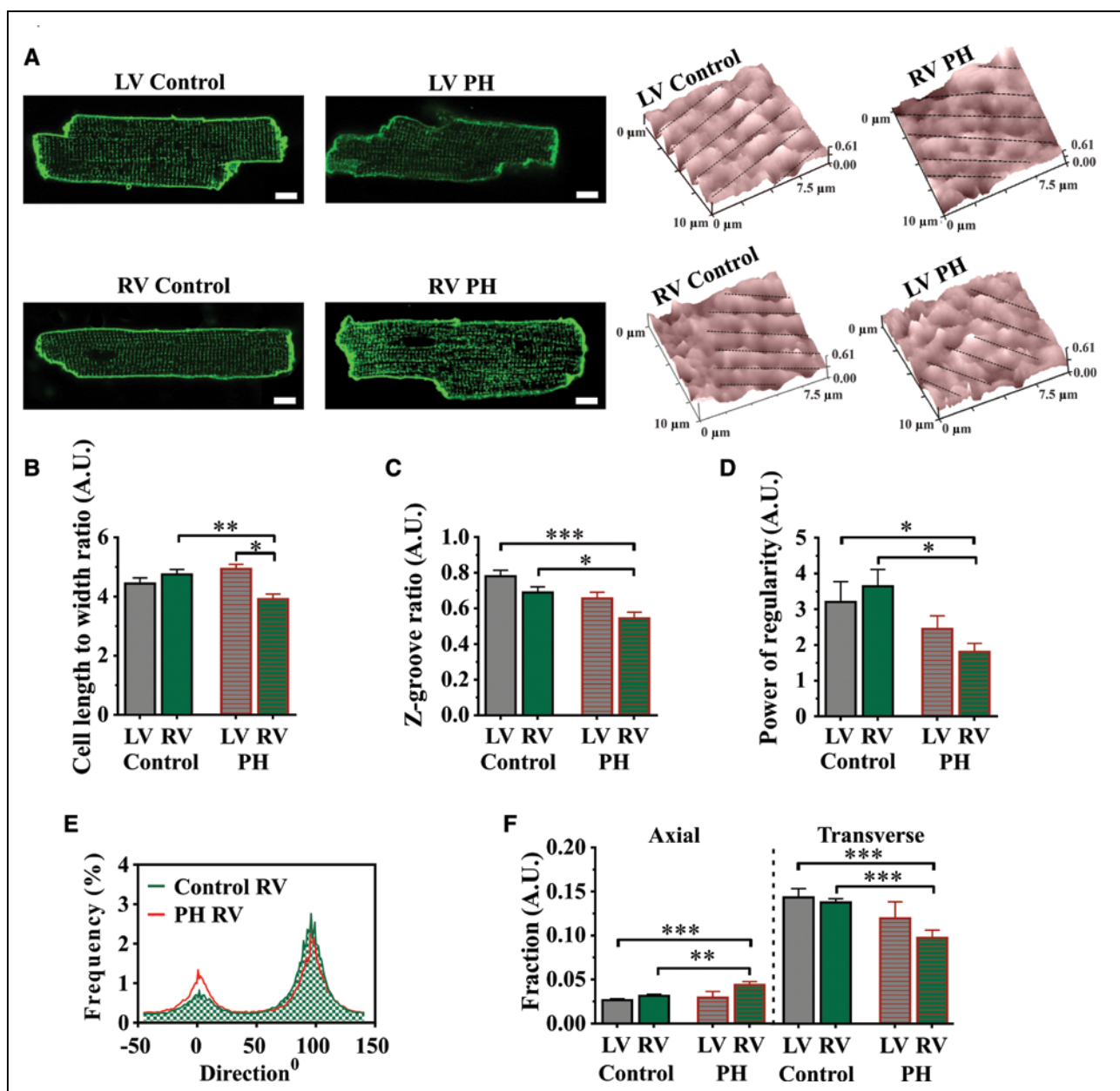
**A**, Representative images of  $\text{Ca}^{2+}$  transients recorded in left ventricular (LV) and right ventricular (RV) myocytes paced at 1 Hz. **B**, Time to peak of the  $\text{Ca}^{2+}$  transients measured in LV and RV myocytes from control and PH rats paced at 1 Hz ( $n=14-20$  cells,  $N=4$  rats,  $*P<0.05$ ,  $**P<0.01$ ). **C**, Average of 'dyssynchrony' index ( $n=24-38$  cells,  $N=4-5$  rats,  $*P<0.05$ ,  $**P<0.05$ ,  $***P<0.001$ ). **D**, Duration of early and delayed  $\text{Ca}^{2+}$  sparks measured in control RV and PH RV myocytes ( $n=34-41$  cells,  $N=4-5$  rats,  $*P<0.05$ ,  $**P<0.01$ ).

Colocalization of  $\text{Ca}_v1.2$  and RyR2 was determined using 2 methods: by defining the percentage of overlap between the 2 stainings and by calculating Manders coefficients.<sup>33,34</sup> The colocalization analysis did not show any significant changes in both parameters: percentages of overlapped  $\text{Ca}_v1.2$  or RyR2 (Figure 5D and 5E) and Manders coefficients (Figure S12).

### Open Probability of L-Type $\text{Ca}^{2+}$ Channels in the T-Tubules of RV Cardiomyocytes Is Increased in PH

Finally, to understand the localization and distribution of functionally active LTCC in RV myocytes in the context of

functional changes in the PH heart, we used super-resolution patch-clamp method (Figure 6). LTCC membrane density is calculated by normalizing the total number of channels observed in TT or crest to the patched area.<sup>35,36</sup> It has been shown that in LV myocytes LTCCs predominantly localize in T-tubules and not in the crest area.<sup>28,37</sup> However, in RV myocytes LTCCs were observed at almost equal density in both locations; only a slightly (not significant) higher channel density was found in T-tubule versus crest microdomains (Figure 6C). Inlet in Figure 6C shows that PH RV myocytes have a reduced density of functional LTCCs; however, no significant changes were found regarding LTCCs in T-tubules or crests. To maximize the chance of recording LTCC current, we applied



**Figure 4. Reorganization of sarcolemma membrane in pulmonary hypertension (PH) rat model.** **A**, Representative images of transverse-axial tubular system (TATS) and SICM scans of left ventricular (LV) and right ventricular (RV) myocytes from control and PH rats; scale bar, 10  $\mu\text{m}$ . **B**, Summary graph of cell length to width ratio ( $n=37-55$  cells,  $N=4$  rats,  $*P<0.05$ ,  $**P<0.01$ ). **C**, Average Z-groove ratio of control and PH myocytes ( $n=23-30$  cells,  $N=3-5$  rats,  $*P<0.05$ ,  $***P<0.001$ ). **D**, Power of TATS regularity measured in RV and LV from control and PH myocytes ( $n=25-40$  cells,  $N=3-5$  rats,  $*P<0.05$ ). **E**, Directionality histograms for the TATS of control and PH RV myocytes and **(F)** summary of the axial tubules and transverse tubules in LV and RV myocytes after PH ( $n=20-73$ ,  $N=4-5$  rats,  $**P<0.01$ ,  $***P<0.001$ ).

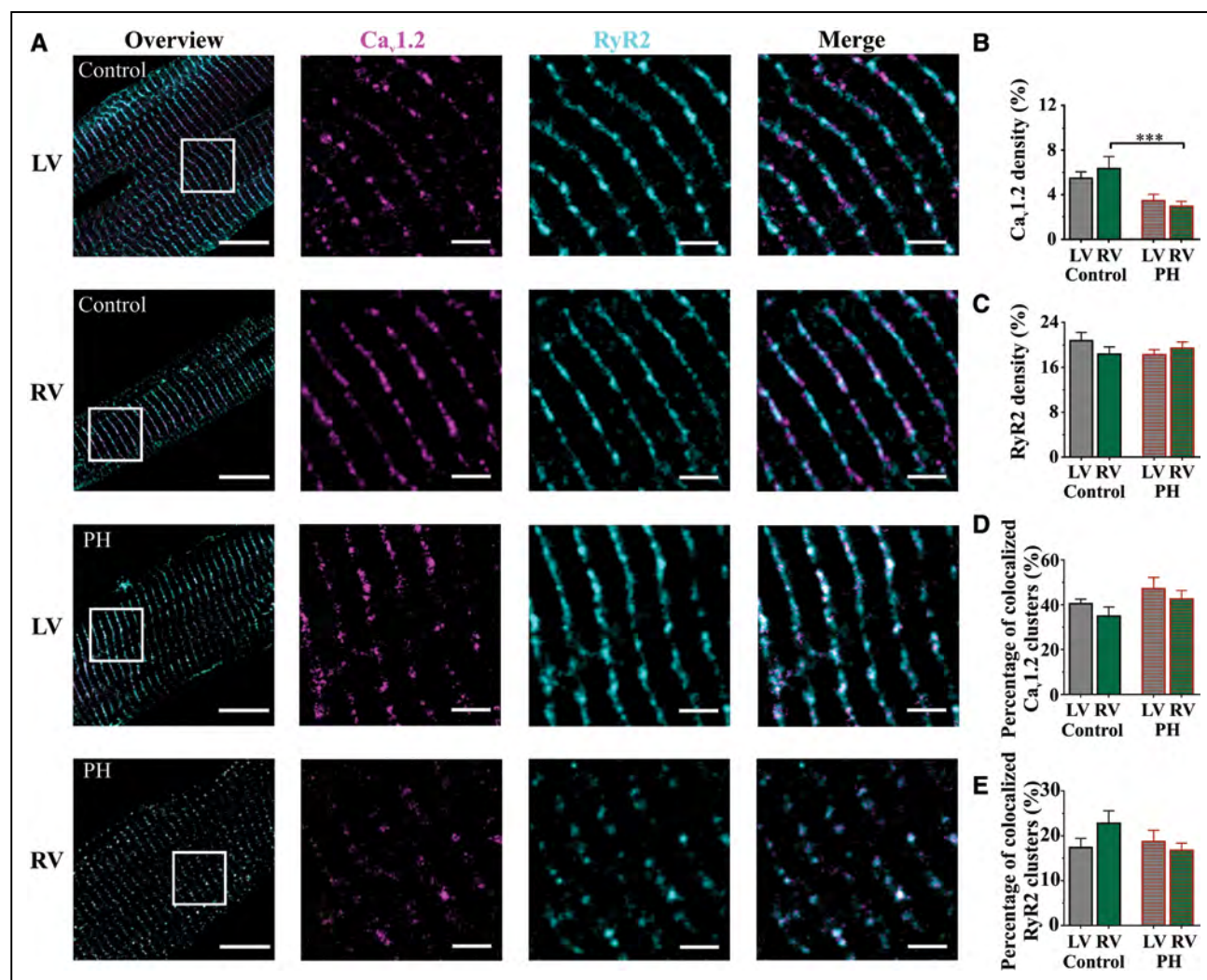
the LTCC agonist BayK8644 in the next set of experiments (Figure 6B). In the presence of BAYK8644 in control myocytes, the average density of LTCC in control and PH myocytes remained equal (Figure 6D).

We then calculated the open probability ( $P_o$ ) of LTCC in all the groups. In control RV myocytes, the  $P_o$  of LTCC was significantly lower in the T-tubules as compared to the crest (Figure 6E). In PH RV myocytes, the LTCCs located in T-tubules exhibited a significantly elevated  $P_o$

in respect to control T-tubules ( $P<0.05$ ), with the crest subpopulation of LTCCs having preserved  $P_o$  after PH. In the presence of BayK8644, the mean LTCC  $P_o$  was preserved (Figure S13).

## DISCUSSION

This study provides evidence of the nanoscale microdomain-related disruptions in  $\text{Ca}^{2+}$  handling that occur



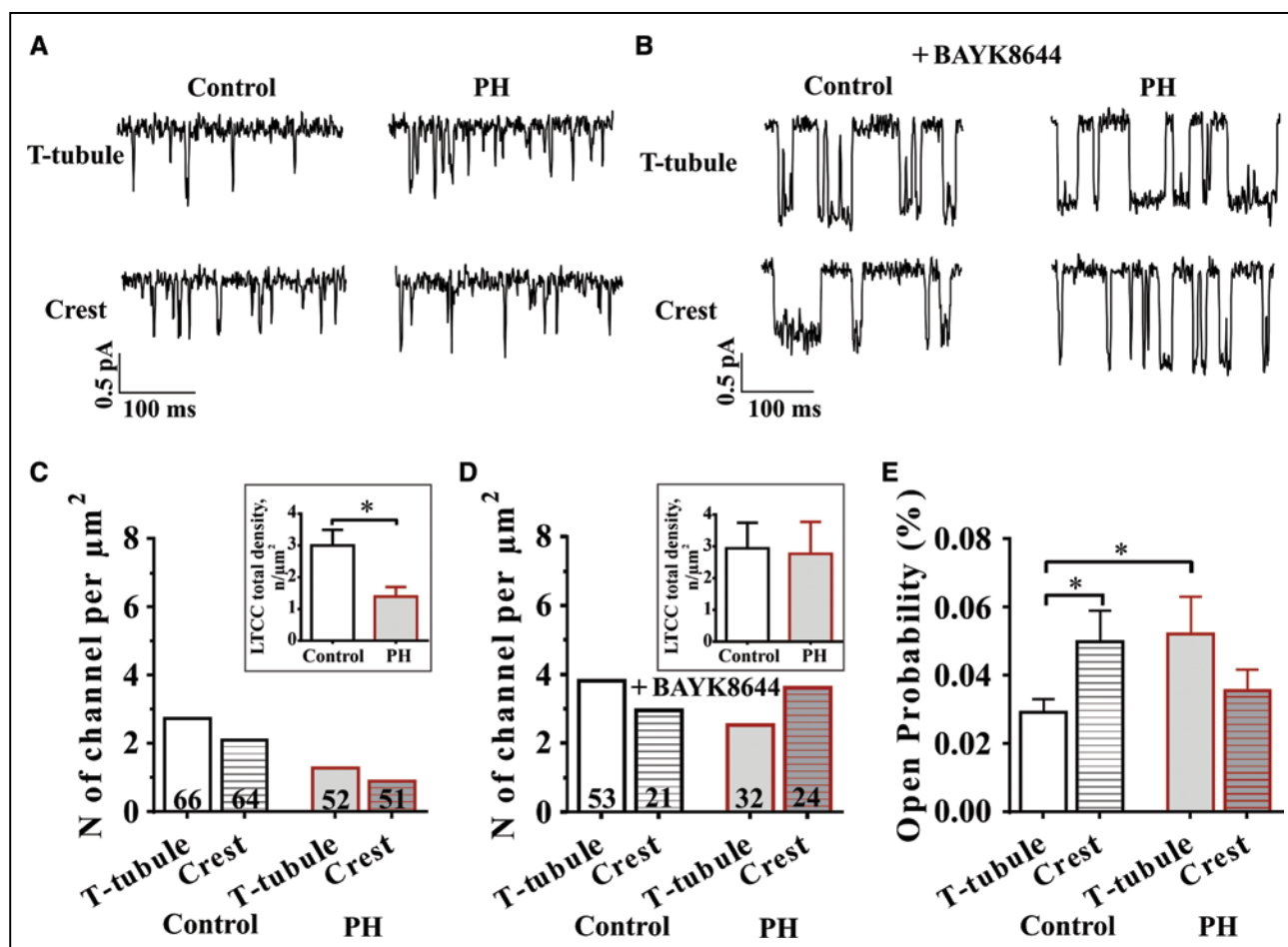
**Figure 5. Colocalization of Cav1.2 and RyR2 (ryanodine receptor 2) in pulmonary hypertension (PH) rat model.**

**A**, Representative Stimulation Emission Depletion confocal images of right ventricular (RV) and left ventricular (LV) myocytes labeled with antibodies against Ca<sub>v</sub>1.2 (magenta) and RyR2 (cyan). Overview scale bars: 10 μm. Magnified area scale bars: 2 μm. **B**, Average density of Ca<sub>v</sub>1.2 signal and **(C)** RyR2 signal from LV and RV myocytes from control and PH rats (n=10–16 cells, N=3–4 rats, \*\*\*P<0.001). **D**, Percentage of Ca<sub>v</sub>1.2 clusters colocalized with RyR2 and **(E)** percentage of RyR2 clusters colocalized with Ca<sub>v</sub>1.2 (n=10–16, N=3–4 rats).

in PH, which could affect electrical conductance in the heart. We conclude that during PH development, the increased workload of RV due to the high pulmonary artery pressure causes structural remodeling of TATS and surface topography in RV myocytes. This structural remodeling promotes local alterations in the Po of LTCC and in the frequency and duration of delayed Ca<sup>2+</sup> sparks, which in turn could be responsible for the desynchronization of Ca<sup>2+</sup> transients and the appearance of Ca<sup>2+</sup> transient alternans observed. The increase in local spontaneous Ca<sup>2+</sup> activity and the appearance of Ca<sup>2+</sup> transient alternans could serve as trigger for arrhythmias in the settings of substantial tissue remodeling.<sup>38</sup> However, our *in vivo* experiments showed that the remodeling is not sufficient to evoke a significant arrhythmic activity, as reported at the more severe stages of disease.<sup>7</sup> In LV myocytes, PH leads to prolongation of Ca<sup>2+</sup> transients but this occurs without changes in TATS organization.

### PH-Induced RV Hypertrophy and Electrical Remodeling

It was previously shown that PH leads to RV hypertrophy formation of arrhythmogenic substrates at the last stages of disease.<sup>7</sup> Here, we studied the preceding changes in electrical remodeling. The increase of CV anisotropic ratio was linked to the generation of unidirectional conduction block and sink to source mismatch.<sup>39</sup> Our data show that that early PH leads to the reduction in the anisotropy ratio in RV but not in LV (Figure 1D). It has also been known that structural remodeling of the tissue influences CV.<sup>40</sup> However, it has been already shown previously that no significant increases in fibrosis, apoptosis, or infiltration of the inflammatory cells occur in the heart tissue after 2 weeks of monocrotaline treatment.<sup>41</sup> However, RV myocytes in PH become significantly hypertrophied (Figure 4B), and redistribution of gap junction occurs<sup>40</sup>



**Figure 6.** Analysis of L-type  $\text{Ca}^{2+}$  channels distribution and function in right ventricular (RV) myocytes after pulmonary hypertension (PH).

**A**, Representative single-channel traces showing L-type  $\text{Ca}^{2+}$  channel (LTCC) current recorded at  $-6.7$  mV in T-tubule and crests of RV myocytes in normal solution and **(B)** in presence of BayK8644. **C**, LTCC density observed in normal solution and **(D)** with the agonist in T-tubule and crests of RV myocytes from control and PH rats. The number in each column represents the total number of successful seals in each group. Insets show total LTCC density analysis from both T-tubules and crests together, from control vs PH RV myocytes. **E**, LTCC open probability at  $-6.7$  mV in control and PH RV myocytes at physiological conditions or with the agonist BayK8644 ( $n=6-19$  channels, 40-100 cells, 6-10 rats,  $*P<0.05$ ).

on the surface of PH RV myocytes; these changes can produce alteration of the passive electrical properties of the ventricle and change the balance between longitudinal and transverse conduction.

We also observed longer ERP in both RV and LV following PH (Figure 1E), which could be related to the prolonged  $\text{Ca}^{2+}$  transient duration (Figure 3B and Figure S7). Increasing the ERP can precipitate torsades de pointes, a type of ventricular tachycardia caused by early or delayed afterdepolarizations.<sup>42</sup> We did not see a significant increase in occurrence of extrasystole in the PH RV as compared to the control RV, so we could assume this stage of the disease is not severe enough for the development of a proarrhythmogenic substrate.

The observed remodeling of LV electrophysiology and cell dimensions could be induced by neurohormonal activation in PH hearts<sup>43</sup> or due to mechanical interdependence of the 2 ventricles.<sup>44</sup>

## PH Increased Spontaneous $\text{Ca}^{2+}$ Activity in RV Myocytes

PH led to an increase in spontaneous local  $\text{Ca}^{2+}$  waves and  $\text{Ca}^{2+}$  sparks frequency in RV but not LV myocytes. Interestingly, the preserved SR  $\text{Ca}^{2+}$  load and SERCA activity in PH myocytes suggest the remodeling happens mostly in RyR2 activity. We observed a significantly slower time to peak of  $\text{Ca}^{2+}$  transients in PH RV myocytes (Figure 3B), with an increased  $\text{Ca}^{2+}$  transient dyssynchronization (Figure 3C). Dyssynchronization of  $\text{Ca}^{2+}$  transients found in PH RV myocytes could be related to the rearrangement of TATS.

We defined the early and delayed  $\text{Ca}^{2+}$  release points of  $\text{Ca}^{2+}$  transients according to their TTF50 (Figure S9). Early release regions of  $\text{Ca}^{2+}$  transients were shown to arise from areas with higher TAT density while delayed ones arose from areas with lower TATS density.<sup>26</sup>

Assigning of  $\text{Ca}^{2+}$  sparks to early or delayed areas was performed according to their position on the scan line. RV myocytes showed a significantly higher frequency of early sparks versus delayed ones (Figure S9). Higher frequency of sparks located close to the T-tubule has been shown in myocytes from different species.<sup>27,45</sup> It has been proposed that the higher local  $\text{Ca}^{2+}$  concentration close to the T-tubule produces  $\text{Ca}^{2+}$  sparks more frequently. PH significantly increased the frequency and prolonged the duration of delayed but not the early  $\text{Ca}^{2+}$  sparks in RV myocytes. This could be due to rearrangement of the TATS microdomains or alterations of RyR2 clustering. Kolstad et al<sup>46</sup> showed that in failing LV myocytes RyR2 undergo significant dispersion and that large RyR2 clusters produce slower  $\text{Ca}^{2+}$  sparks and, consequently, a desynchronization of  $\text{Ca}^{2+}$  transients.

$\text{Ca}^{2+}$  transient alternans observed in PH RV myocytes could be related to the abnormal  $\text{Ca}^{2+}$  release or  $\text{Ca}^{2+}$  reuptake (Figure 2F). As we did not find any reduction in SERCA activity in PH (Figure S6), the appearance of  $\text{Ca}^{2+}$  alternans could be associated with the abnormal RyR2 activity.<sup>47</sup>  $\text{Ca}^{2+}$  transient alternans have been shown to produce action potential duration alternans, reentrant activity, and contractile impairment<sup>24</sup>; however, our *in vivo* data indicates that the remodeling was still not sufficient for the formation of a proarrhythmic substrate (Figure 1).

### TATS Reorganization in PH

TATS organization largely impacts the intracellular  $\text{Ca}^{2+}$  cycling.<sup>25</sup> We observed a reduction of TATS power of regularity in RV PH myocytes due to the lower amount of transverse tubules and a higher number of axial tubules in PH (Figure 4). The recent study by Schobesberger et al<sup>48</sup> documented higher frequency of axial (longitudinal) tubules during hypertrophic state of the myocardial infarction. The role of axial elements in the excitation-contraction coupling is still not fully understood, although recent studies of Asghari et al<sup>49</sup> and Swift et al<sup>50</sup> showed that axial tubules can be fully functional with LTCC coupled to SR and RyR2 clusters. Thus, the increase in the number of axial tubules was proposed to be the first compensatory mechanism in maintaining high working load and secure  $\text{Ca}^{2+}$  influx to support contractions.<sup>50</sup>

Analysis of the membrane topography by SICM showed a reduction in z-groove index in RV but not in LV myocytes in PH (Figure 4C). Studies of LV myocytes from rat and human failing hearts have shown that this reduction is accompanied by an increase of  $\text{Ca}^{2+}$  waves and sparks.<sup>14,28</sup> The disruption of surface topography could be linked with the weakening of sarcomeres and myocardial stretch, which results in opening of stretch-activated channels,<sup>8</sup> and/or altered  $\text{Ca}^{2+}$ -troponin interaction at different sarcomere lengths.<sup>51</sup>

### RV $\text{Ca}_v1.2$ Rearrangement in PH

Our results on PH rats showed that RyR2 protein expression and RyR2 Stimulation Emission Depletion signal densities are unchanged when compared with control (Figure S11 and Figure 5C). In agreement with this, research into heart failure and PH has demonstrated that RyR2 arrangement and expression is preserved in failing myocytes.<sup>8,9</sup> RyR2 activation depends on the  $\text{Ca}^{2+}$  entry through LTCC. Interestingly, we observed the distribution of LTCC between T-tubule and crest in RV is more similar to atrial myocytes than LV myocytes.<sup>14,22,37</sup> Taking into account differences observed in early and delayed  $\text{Ca}^{2+}$  sparks parameters between control RV and LV myocytes, these findings suggest an altered organization of  $\text{Ca}^{2+}$  handling microdomains between both ventricles.

Following PH, we found a reduction of  $\text{Ca}_v1.2$  Stimulation Emission Depletion signal density (Figure 5B) and a reduction in the number of functional LTCCs in RV myocytes (Figure 6C). The analysis of  $\text{Ca}_v1.2$  protein expression from tissue after PH (Figure S11) and the LTCC density in presence of agonist (Figure 6D), however, did not show a significant change. Also, colocalization between RyR2 and  $\text{Ca}_v1.2$  in both RV and LV myocytes was not affected by PH (Figure 5D). This evidence suggests that the expression of  $\text{Ca}_v1.2$  protein remains unaffected at this early stage of the disease, but a functional remodeling of LTCC is already happening. This is supported by other groups that found no changes in the pore-forming  $\text{Ca}_v1.2$  protein expression in the early hypertrophic stage of the disease.<sup>7,13</sup> Although a downregulation of  $\text{Ca}_v1.2$  protein expression has been observed in the late stages of PH,<sup>9,52</sup> in terms of whole-cell  $\text{Ca}^{2+}$  current, which density in PH RV myocytes is preserved,<sup>7</sup> we suggest that the silencing of functional LTCCs is partially compensated by the increase of the  $\text{P}_o$  in T-tubular LTCC. This increased  $\text{P}_o$  of LTCC in T-tubules could be caused by phosphorylation of the channels by PKA or CaMKII.<sup>14,16</sup> In fact, Fowler et al<sup>12</sup> found an increased  $\beta$ -adrenergic stimulation in PH rats, which will activate PKA and consequently, LTCCs. Although this activation could work as a compensatory mechanism to keep the contraction requirements, it could also be the substrate of the increased  $\text{Ca}^{2+}$  activity observed in this work. Overall, our findings underscore the role of nanoscale organization of  $\text{Ca}^{2+}$  handling within myocytes in the pathophysiology of the heart, particularly during development of a disease such as PH.

### Perspectives

Our study shows that during development of PH RV cardiomyocytes undergo structural remodeling that induces functional alterations of intracellular  $\text{Ca}^{2+}$  behavior and could affect the electrical properties of the whole heart. However, the arrhythmic activity in the RV, reported

at the later stages of PH, was not found. This suggests that the changes observed at the cellular level could precede development of arrhythmias. Our findings provide new mechanistic insights into right ventricular dysfunction, which could help identify novel therapeutic approaches for RV failure in PH.

## ARTICLE INFORMATION

Received February 9, 2020; accepted November 20, 2020.

### Affiliations

From the Dipartimento di Cardiocirurgia, Università degli Studi di Verona, Ospedale Borgo Trento, Italy (R.M., G.F.); Department of Cardiovascular Sciences, National Heart and Lung Institute, Imperial College London, United Kingdom (R.M., J.L.S.-A., A.A.-L., E.D., V.B.A.S., B.W.-S., J.G.); Humanitas Clinical and Research Center, Rozzano, Italy (R.M., T.S., M.M.); Dipartimento di Medicina e Chirurgia, Università degli Studi di Parma, Italy (S.R., M.M.); Lab of Experimental Cardiology, University of Leuven, Belgium (E.D.); and Department of Biomedical Engineering and Alliance for Cardiovascular Diagnostic and Treatment Innovation; Johns Hopkins University; Baltimore, MD (N.T.).

### Acknowledgments

We thank Peter O'Gara for myocyte isolation.

### Sources of Funding

This work was supported by British Heart Foundation grants (RG/17/13/33173 to J. Gorelik and PG/15/69/31719 to B. Wojciak-Stothard) and National Institutes of Health grant (ROI-HL 126802 to N. Trayanova and J. Gorelik).

### Disclosures

None.

## REFERENCES

- Handoko ML, de Man FS, Allaart CP, Paulus WJ, Westerhof N, Vonk-Noordegraaf A. Perspectives on novel therapeutic strategies for right heart failure in pulmonary arterial hypertension: lessons from the left heart. *Eur Respir Rev*. 2010;19:72–82. doi: 10.1183/09059180.00007109
- Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. *BMJ*. 2018;360:j5492. doi: 10.1136/bmj.j5492
- Bogaard HJ, Abe K, Vonk-Noordegraaf A, Voelkel NF. The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest*. 2009;135:794–804. doi: 10.1378/chest.08-0492
- Hlaing T, DiMino T, Kowey PR, Yan GX. ECG repolarization waves: their genesis and clinical implications. *Ann Noninvasive Electrocardiol*. 2005;10:211–223. doi: 10.1111/j.1542-474X.2005.05588.x
- Folino AF, Bobbo F, Schiraldi C, Tona F, Romano S, Buja G, Bellotto F. Ventricular arrhythmias and autonomic profile in patients with primary pulmonary hypertension. *Lung*. 2003;181:321–328. doi: 10.1007/s00408-003-1034-x
- Haddad F, Hunt SA, Rosenthal DN, Murphy DJ. Right ventricular function in cardiovascular disease, part I: anatomy, physiology, aging, and functional assessment of the right ventricle. *Circulation*. 2008;117:1436–1448. doi: 10.1161/CIRCULATIONAHA.107.653576
- Benoist D, Stones R, Drinkhill MJ, Benson AP, Yang Z, Cassan C, Gilbert SH, Saint DA, Cazorla O, Steele DS, et al. Cardiac arrhythmia mechanisms in rats with heart failure induced by pulmonary hypertension. *Am J Physiol Heart Circ Physiol*. 2012;302:H2381–H2395. doi: 10.1152/ajpheart.01084.2011
- Sabourin J, Boet A, Rucker-Martin C, Lambert M, Gomez AM, Benitah JP, Perros F, Humbert M, Antigny F. Ca<sup>2+</sup> handling remodeling and STIM1L/Orai1/TRPC1/TRPC4 upregulation in monocrotaline-induced right ventricular hypertrophy. *J Mol Cell Cardiol*. 2018;118:208–224. doi: 10.1016/j.yjmcc.2018.04.003
- Xie YP, Chen B, Sanders P, Guo A, Li Y, Zimmerman K, Wang LC, Weiss RM, Grumbach IM, Anderson ME, et al. Sildenafil prevents and reverses transverse-tubule remodeling and Ca<sup>2+</sup> handling dysfunction in right ventricle failure induced by pulmonary artery hypertension. *Hypertension*. 2012;59:355–362. doi: 10.1161/HYPERTENSIONAHA.111.180968
- Ryan JJ, Huston J, Kutty S, Hatton ND, Bowman L, Tian L, Herr JE, Johri AM, Archer SL. Right ventricular adaptation and failure in pulmonary arterial hypertension. *Can J Cardiol*. 2015;31:391–406. doi: 10.1016/j.cjca.2015.01.023
- Kuramochi T, Honda M, Tanaka K, Enomoto K, Hashimoto M, Morioka S. Calcium transients in single myocytes and membranous ultrastructures during the development of cardiac hypertrophy and heart failure in rats. *Clin Exp Pharmacol Physiol*. 1994;21:1009–1018. doi: 10.1111/j.1440-1681.1994.tb02664.x
- Fowler ED, Drinkhill MJ, Norman R, Pervolaraki E, Stones R, Steer E, Benoist D, Steele DS, Calaghan SC, White E. Beta1-adrenoceptor antagonist, metoprolol attenuates cardiac myocyte Ca<sup>2+</sup> handling dysfunction in rats with pulmonary artery hypertension. *J Mol Cell Cardiol*. 2018;120:74–83. doi: 10.1016/j.yjmcc.2018.05.015
- Rocchetti M, Sala L, Rizzetto R, Staszewsky LI, Alemanni M, Zambelli V, Russo I, Barile L, Cornaghi L, Altomare C, et al. Ranolazine prevents INaL enhancement and blunts myocardial remodeling in a model of pulmonary hypertension. *Cardiovasc Res*. 2014;104:37–48. doi: 10.1093/cvr/cvu188
- Sanchez-Alonso JL, Bhargava A, O'Hara T, Glukhov AV, Schobesberger S, Bhogal N, Sikkell MB, Mansfield C, Korchev YE, Lyon AR, et al. Microdomain-specific modulation of L-type calcium channels leads to triggered ventricular arrhythmia in heart failure. *Circ Res*. 2016;119:944–955. doi: 10.1161/CIRCRESAHA.116.308698
- Zhang C, Chen B, Guo A, Zhu Y, Miller JD, Gao S, Yuan C, Kutschke W, Zimmerman K, Weiss RM, et al. Microtubule-mediated defects in junctional-2 trafficking contribute to myocyte transverse-tubule remodeling and Ca<sup>2+</sup> handling dysfunction in heart failure. *Circulation*. 2014;129:1742–1750. doi: 10.1161/CIRCULATIONAHA.113.008452
- Bryant SM, Kong CH, Watson J, Cannell MB, James AF, Orchard CH. Altered distribution of ICa impairs Ca release at the t-tubules of ventricular myocytes from failing hearts. *J Mol Cell Cardiol*. 2015;86:23–31. doi: 10.1016/j.yjmcc.2015.06.012
- Abdul-Salam VB, Russomanno G, Chien-Nien C, Mahomed AS, Yates LA, Wilkins MR, Zhao L, Gierula M, Dubois O, Schaeper U, et al. CLIC4/Arf6 Pathway. *Circ Res*. 2019;124:52–65. doi: 10.1161/CIRCRESAHA.118.313705
- Savi M, Bocchi L, Rossi S, Frati C, Graiani G, Lagrasta C, Miragoli M, Di Pasquale E, Stirparo GG, Mastrotoaro G, et al. Antiarrhythmic effect of growth factor-supplemented cardiac progenitor cells in chronic infarcted heart. *Am J Physiol Heart Circ Physiol*. 2016;310:H1622–H1648. doi: 10.1152/ajpheart.00035.2015
- Rossi S, Baruffi S, Bertuzzi A, Miragoli M, Corradi D, Maestri R, Alinovi R, Mutti A, Musso E, Sgoifo A, et al. Ventricular activation is impaired in aged rat hearts. *Am J Physiol Heart Circ Physiol*. 2008;295:H2336–H2347. doi: 10.1152/ajpheart.00517.2008
- Carnevali L, Trombini M, Rossi S, Graiani G, Manghi M, Koolhaas JM, Quaini F, Macchi E, Nalivaiko E, Sgoifo A. Structural and electrical myocardial remodeling in a rodent model of depression. *Psychosom Med*. 2013;75:42–51. doi: 10.1097/PSY.0b013e318276cb0d
- Sato M, O'Gara P, Harding SE, Fuller SJ. Enhancement of adenoviral gene transfer to adult rat cardiomyocytes *in vivo* by immobilization and ultrasound treatment of the heart. *Gene Ther*. 2005;12:936–941. doi: 10.1038/sj.gt.3302476
- Glukhov AV, Balycheva M, Sanchez-Alonso JL, Ilkan Z, Alvarez-Laviada A, Bhogal N, Diakonov I, Schobesberger S, Sikkell MB, Bhargava A, et al. Direct evidence for microdomain-specific localization and remodeling of functional L-type calcium channels in rat and human atrial myocytes. *Circulation*. 2015;132:2372–2384. doi: 10.1161/CIRCULATIONAHA.115.018131
- Picht E, Zima AV, Blatter LA, Bers DM. SparkMaster: automated calcium spark analysis with ImageJ. *Am J Physiol Cell Physiol*. 2007;293:C1073–C1081. doi: 10.1152/ajpcell.00586.2006
- Laurita KR, Rosenbaum DS. Cellular mechanisms of arrhythmogenic cardiac alternans. *Prog Biophys Mol Biol*. 2008;97:332–347. doi: 10.1016/j.pbiomolbio.2008.02.014
- Louch WE, Mørk HK, Sexton J, Stromme TA, Laake P, Sjaastad I, Sejersted OM. T-tubule disorganization and reduced synchrony of Ca<sup>2+</sup> release in murine cardiomyocytes following myocardial infarction. *J Physiol*. 2006;574(pt 2):519–533. doi: 10.1113/jphysiol.2006.107227
- Heinzel FR, Bito V, Volders PG, Antoons G, Mubagwa K, Sipido KR. Spatial and temporal inhomogeneities during Ca<sup>2+</sup> release from the sarcoplasmic reticulum in pig ventricular myocytes. *Circ Res*. 2002;91:1023–1030. doi: 10.1161/01.res.0000045940.67060.dd
- Biesmans L, Macquaide N, Heinzel FR, Bito V, Smith GL, Sipido KR. Subcellular heterogeneity of ryanodine receptor properties in ventricular

- myocytes with low T-tubule density. *PLoS One*. 2011;6:e25100. doi: 10.1371/journal.pone.0025100
28. Lyon AR, MacLeod KT, Zhang Y, Garcia E, Kanda GK, Lab MJ, Korchev YE, Harding SE, Gorelik J. Loss of T-tubules and other changes to surface topography in ventricular myocytes from failing human and rat heart. *Proc Natl Acad Sci U S A*. 2009;106:6854–6859. doi: 10.1073/pnas.0809777106
  29. Litwin SE, Zhang D, Bridge JH. Dyssynchronous Ca(2+) sparks in myocytes from infarcted hearts. *Circ Res*. 2000;87:1040–1047. doi: 10.1161/01.res.87.11.1040
  30. Gorelik J, Yang LQ, Zhang Y, Lab M, Korchev Y, Harding SE. A novel Z-groove index characterizing myocardial surface structure. *Cardiovasc Res*. 2006;72:422–429. doi: 10.1016/j.cardiores.2006.09.009
  31. Wagner E, Lauterbach MA, Kohl T, Westphal V, Williams GS, Steinbrecher JH, Streich JH, Korff B, Tuan HT, Hagen B, et al. Stimulated emission depletion live-cell super-resolution imaging shows proliferative remodeling of T-tubule membrane structures after myocardial infarction. *Circ Res*. 2012;111:402–414. doi: 10.1161/CIRCRESAHA.112.274530
  32. Song LS, Sobie EA, McCulle S, Lederer WJ, Balke CW, Cheng H. Orphaned ryanodine receptors in the failing heart. *Proc Natl Acad Sci U S A*. 2006;103:4305–4310. doi: 10.1073/pnas.0509324103
  33. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. 2006;224(pt 3):213–232. doi: 10.1111/j.1365-2818.2006.01706.x
  34. Gilles JF, Dos Santos M, Boudier T, Bolte S, Heck N, DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis. *Methods*. 2017;115:55–64. doi: 10.1016/j.jymeth.2016.11.016
  35. Novak P, Gorelik J, Vivekananda U, Shevchuk AI, Ermolyuk YS, Bailey RJ, Bushby AJ, Moss GW, Rusakov DA, Klenerman D, et al. Nanoscale-targeted patch-clamp recordings of functional presynaptic ion channels. *Neuron*. 2013;79:1067–1077. doi: 10.1016/j.neuron.2013.07.012
  36. Poulet C, Sanchez-Alonso J, Swiatlowska P, Mouy F, Lucarelli C, Alvarez-Laviada A, Gross P, Terracciano C, Houser S, Gorelik J. Junctophilin-2 tethers T-tubules and recruits functional L-type calcium channels to lipid rafts in adult cardiomyocytes. *Cardiovasc Res*. 2020;cvaa033. doi: 10.1093/cvr/cvaa033
  37. Bhargava A, Lin X, Novak P, Mehta K, Korchev Y, Delmar M, Gorelik J. Super-resolution scanning patch clamp reveals clustering of functional ion channels in adult ventricular myocyte. *Circ Res*. 2013;112:1112–1120. doi: 10.1161/CIRCRESAHA.111.300445
  38. Venetucci LA, Trafford AW, O'Neill SC, Eisner DA. The sarcoplasmic reticulum and arrhythmogenic calcium release. *Cardiovasc Res*. 2008;77:285–292. doi: 10.1093/cvr/cvm009
  39. van Rijen HV, Eckardt D, Degen J, Theis M, Ott T, Willecke K, Jongsma HJ, Opthof T, de Bakker JM. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of connexin43. *Circulation*. 2004;109:1048–1055. doi: 10.1161/01.CIR.0000117402.70689.75
  40. Uzzaman M, Honjo H, Takagishi Y, Emdad L, Magee AI, Severs NJ, Kodama I. Remodeling of gap junctional coupling in hypertrophied right ventricles of rats with monocrotaline-induced pulmonary hypertension. *Circ Res*. 2000;86:871–878. doi: 10.1161/01.res.86.8.871
  41. Deng Y, Wu W, Guo S, Chen Y, Liu C, Gao X, Wei B. Altered mTOR and Beclin-1 mediated autophagic activation during right ventricular remodeling in monocrotaline-induced pulmonary hypertension. *Respir Res*. 2017;18:53. doi: 10.1186/s12931-017-0536-7
  42. Volders PG, Sipido KR, Vos MA, Spätjens RL, Leunissen JD, Carmeliet E, Wellens HJ. Downregulation of delayed rectifier K(+) currents in dogs with chronic complete atrioventricular block and acquired torsades de pointes. *Circulation*. 1999;100:2455–2461. doi: 10.1161/01.cir.100.24.2455
  43. Brunner F. Cardiac endothelin and big endothelin in right-heart hypertrophy due to monocrotaline-induced pulmonary hypertension in rat. *Cardiovasc Res*. 1999;44:197–206. doi: 10.1016/s0008-6363(99)00155-8
  44. Lamberts RR, Vaessen RJ, Westerhof N, Stienen GJ. Right ventricular hypertrophy causes impairment of left ventricular diastolic function in the rat. *Basic Res Cardiol*. 2007;102:19–27. doi: 10.1007/s00395-006-0620-5
  45. Meethal SV, Potter KT, Redon D, Munoz-del-Rio A, Kamp TJ, Valdivia HH, Haworth RA. Structure-function relationships of Ca spark activity in normal and failing cardiac myocytes as revealed by flash photography. *Cell Calcium*. 2007;41:123–134. doi: 10.1016/j.ceca.2006.05.006
  46. Kolstad TR, van den Brink J, Macquaide N, Lunde PK, Frisk M, Aronsen JM, Norden ES, Cataliotti A, Sjaastad I, Sejersted OM, et al. Ryanodine receptor dispersion disrupts Ca<sup>2+</sup> release in failing cardiac myocytes. *Elife*. 2018;7:1–24. doi: 10.7554/eLife.39427
  47. Pieske B, Kockskaemper J. Alternans goes subcellular: a "disease" of the ryanodine receptor? *Circ Res*. 2002;91:553–555. doi: 10.1161/01.res.0000036862.37203.f4
  48. Schobesberger S, Wright P, Tokar S, Bhargava A, Mansfield C, Glukhov AV, Poulet C, Buzuk A, Monzpart A, Sikkil M, et al. T-tubule remodelling disturbs localized  $\beta_2$ -adrenergic signalling in rat ventricular myocytes during the progression of heart failure. *Cardiovasc Res*. 2017;113:770–782. doi: 10.1093/cvr/cvx074
  49. Asghari P, Schulson M, Scriven DR, Martens G, Moore ED. Axial tubules of rat ventricular myocytes form multiple junctions with the sarcoplasmic reticulum. *Biophys J*. 2009;96:4651–4660. doi: 10.1016/j.bpj.2009.02.058
  50. Swift F, Franzini-Armstrong C, Oyehaug L, Enger UH, Andersson KB, Christensen G, Sejersted OM, Louch WE. Extreme sarcoplasmic reticulum volume loss and compensatory T-tubule remodeling after Serca2 knockout. *Proc Natl Acad Sci*. 2012;109:3997–4001. doi: 10.1073/pnas.1120172109
  51. Lab MJ, Allen DG, Orchard CH. The effects of shortening on myoplasmic calcium concentration and on the action potential in mammalian ventricular muscle. *Circ Res*. 1984;55:825–829. doi: 10.1161/01.res.55.6.825
  52. Benoist D, Stones R, Drinkhill M, Bernus O, White E. Arrhythmogenic substrate in hearts of rats with monocrotaline-induced pulmonary hypertension and right ventricular hypertrophy. *Am J Physiol Heart Circ Physiol*. 2011;300:H2230–H2237. doi: 10.1152/ajpheart.01226.2010